

**STUDIES ON BIODEGRADATION OF SUBSTITUTED
PHENOLS BY *ARTHROBACTER CHLOROPHENOLICUS* A6**

A thesis submitted to the Indian Institute of Technology
Guwahati, India, for the award of the degree of

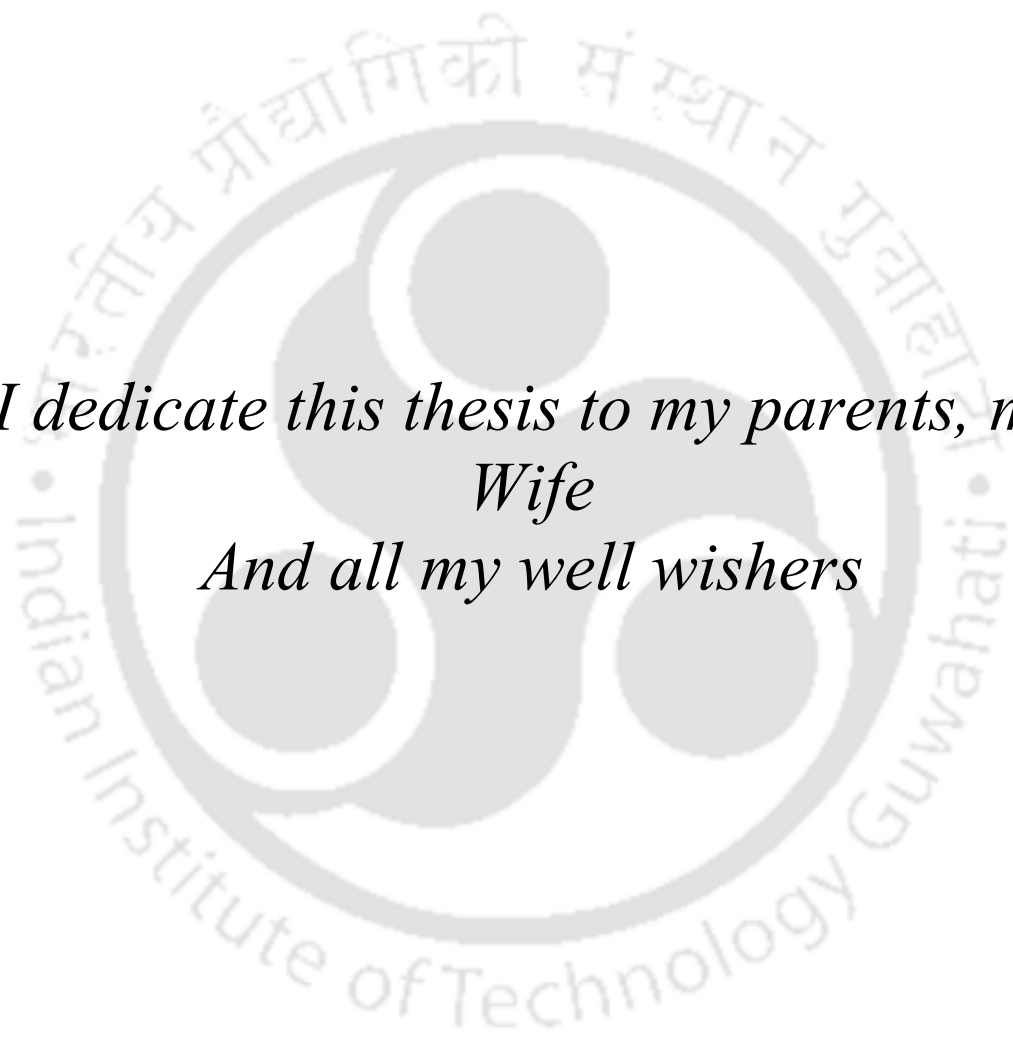
DOCTOR OF PHILOSOPHY

By

NARESH KUMAR SAHOO



**Centre for the Environment
Indian Institute of Technology
Guwahati, INDIA
August, 2011**



*I dedicate this thesis to my parents, my
Wife
And all my well wishers*

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI



Centre for the Environment

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Centre for the Environment, Indian Institute of Technology Guwahati, India, under the supervision of Dr. P. K. Ghosh and Dr. K. Pakshirajan.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date 1st August, 2011.

Naresh Kumar Sahoo

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI



Centre for the Environment

CERTIFICATE

This is to certify that the work presented in this thesis entitled “**Studies on Biodegradation of Substituted Phenols by *Arthrobacter chlorophenolicus* A6**” by **Mr. Naresh Kumar Sahoo** has been carried out under our supervision and guidance. This thesis is being submitted to the Indian Institute of Technology Guwahati for the award of the degree of **Doctor of Philosophy**. Mr. Naresh Kumar Sahoo has worked on the problem in the Centre for the Environment of IIT Guwahati for about four years and six months and this thesis, in our opinion is of the standard required for the award of the degree of Doctor of Philosophy. The results reported in this thesis have not been submitted elsewhere for a degree or diploma.

Dr. Pranab Kumar Ghosh
Associate Professor
Department of Civil Engineering
IIT Guwahati

Dr. Kannan Pakshirajan
Associate Professor
Department of Biotechnology
IIT Guwahati

ACKNOWLEDGEMENTS

Ph. D is again learning process. Throughout these years, I have received much support from my supervisors, parents and friends. It would be difficult to individually acknowledge all those who deserve it, without any serious omissions. However, I will try to express my gratitude to those who supported me in making this thesis possible.

It is with my deepest sense of appreciation that I express my heartiest acknowledgement to my research advisors, Dr. P. K. Ghosh, Department of Civil Engineering and Dr. K. Pakshirajan, Department of Biotechnology for their continuous care, support and encouragement throughout my research work. I must acknowledge the unconditional freedom to think, plan, execute and express, that I was given in every step of my research work, while keeping faith and confidence on my capabilities.

I am thankful to my doctoral committee members, Dr. V. Venkata Dasu, Dr. Pugazhenith, Dr. S. Chakraborty, and Dr. Ajay S. Kalamadhad for their constructive criticism and precious suggestions.

I owe my gratitude to ex-Heads of the Centre for the Environment, IIT Guwahati, Dr. M. Jawed, Dr. M. Ray, Dr. C. Mahanta, and current Head Dr Gopal Das for providing me with the necessary facilities in their respective tenures.

I take this opportunity to express my love and gratitude to my loving parents, my beloved wife and all my family members for being there near to me all through my career.

I wish to acknowledge the ex-staffs of the Centre for the Environment, Monojit da, and present staff Deepmoni, Jyashree, Partha, Rupiwinder and Amjad for their cooperation.

I would express thank to my friends Biswanath da, Shampa didi, Achlesh for their valuable suggestions, Bedabrata, Kasturi, Rachna, Kaushik, Atreyi, Bharti, Aurbind, Aditi, Suresh, Ananda, Biju, Susanta, Samarpita and Bhaskar for providing me moral support whenever I needed.

Last, but not the least I would thank God for the blessings I am always showered with in every step of my life.

Date: 1st August, 2011.

Naresh Kumar Sahoo

CONTENTS

Contents	i
List of Figures	viii
List of Tables	xiii
Abbreviations and Notations	xvi
Abstract	xviii
Chapter1: INTRODUCTION	
1.1 Generalities	1
1.2 Biological Treatment	3
1.3 Bioreactors for Wastewater Treatment.	5
Chapter2: LITERATURE REVIEW	
2.1 Physico-Chemical Properties of Substituted Phenols	7
2.2. Sources and Distribution of Substituted Phenols in the Environment	7
2.2.1 Major Uses	8
2.2.2 Quantity Production	10
2.2.3 Distribution	10
2.3 Toxicity of substituted phenol	13
2.4. Permissible Limit	16
2.5. Major Treatment Techniques for Phenolic Wastewater	16
2.5.1 Adsorption	16
2.5.2 Air Stripping	17
2.5.3 Electrochemical Oxidation	17
2.5.4 Advanced Oxidation Processes	18
2.5.4.1 Ozonation UV	18
2.5.4.2 Ultrasonication	19
2.5.4.3 Solar Photocatalytic Oxidation	19
2.5.5 Pros and Cons of the Above Wastewater Treatment Techniques	20
2.6 Biological Treatment Processes Involving Microbes	21

2.6.1	Anaerobic Biodegradation	22
2.6.2	Biodegradation by Yeast and Fungi	24
2.6.3	Biodegradation of Phenolics by Bacteria	25
2.6.3.1	Aerobic Bacterial Growth on Chlorophenols as a Sole Source of Carbon and Energy	25
2.6.3.2.	Microbial Degradation of Pollutant Mixtures	27
	Diauxic Growth	28
	Simultaneous Degradation of Substrates	29
	Rate deceleration	29
	Rate Acceleration (Aerobic bacterial co- metabolism)	30
2.6.4	Bioremediation of Substituted Phenols by Actinomycetes	31
2.6.4.1	Aerobic 4-chlorophenol degradation by <i>A. chlorophenolicus A6</i>	33
2.6.5	2.6.5.1 Microbial Growth Kinetics and Substrate Inhibition Models for Single Substrate Biodegradation Systems	35
	2.6.5.2 Multi Substrate Biodegradation Systems	41
2.6.6	Bioreactors Systems for Treatment of Wastewater Containing Phenolics Compounds	42
2.6.6.1	Packed Bed Bioreactor (PBR)	45
2.6.6.1. (a)	Fundamental Design Principles	49
	Mixing	50
	Oxygen Transfer	50
	Shear Force	51
2.6.6.2	Biofilm Supporting Materials	52
2.6.6.3	Cell Immobilized by Calcium Alginate Bead Systems for Treatment of Wastewater Containing Phenolic Compounds	52
2.7	Design of Experiments	54
2.7.1	Factorial Design of Experiments	56

2.7.2	Analysis of Variance	56
2.7.3	Student 't' Test	57
CHAPTER3:	OBJECTIVE AND SCOPE	61
	Organization of thesis	62
CHAPTER4:	MATERIALS and METHODS	63
4.1	Materials	63
4.1.1	Glassware, Apparatus and Instruments	63
4.1.2	Chemicals and Reagents	64
4.2	Analytical Methods	65
4.2.1	Chemical oxygen demand (COD)	65
4.2.2	Heavy metals and characterization of petroleum refinery wastewater	65
4.2.3	Substituted phenol analysis (HPLC method)	66
4.2.4	Metabolic intermediate analysis	66
4.2.5	Chloride ions analysis	68
4.2.6	Microscopic methods	68
4.2.7	Biomass concentration	69
4.2.8	Biomass concentration in polyurethane foam	70
4.3	Experimental Methodologies	71
4.3.1	Microorganism and its Maintenance	71
4.3.2	Seed Culture Development	71
4.3.3	Inoculum Preparation	72
4.3.4	Medium Development to Enhance Biodegradation of Substituted Phenol	72
4.3.4.1	Screening of Medium Constituents by 2-Level Factorial Experimental Design	72
4.3.4.2	Optimization of Screened Medium Components Using Central Composite Design (CCD)	74
4.3.5	Optimization of Physical Process Conditions to Enhance Biodegradation of Substituted Phenols	75

4.3.5.1	Experimental Design	76
4.3.6	Biodegradation of Substituted Phenols in Batch Shake Flasks	76
4.3.6.1	Single Substrate System	76
4.3.6.2	Mixed Substrate System	78
4.4	Reactor Configuration and Experimental Set-Up	79
4.4.1	Newly Designed PBR	79
4.4.2	Porosity Determination of Polyurethane Foam	84
4.4.3	Hydrodynamic Experiments in the PBR	85
	Mixing Time Profile	85
	Oxygen Mass Transfer Coefficient Determination	86
	Gas Hold up Determination	87
4.4.4	Abiotic 4-CP, 4-BP and 4-NP Removal in the Newly Designed Packed Bed Bioreactor	87
4.4.5	Biofilm Development	88
4.4.6	Optimization of Dissolved Oxygen Level in the Reactor	88
4.4.7	4-CP, 4-BP and 4-NP Biodegradation Experiments as Single Substrate System in the Newly Designed PBR	89
4.4.8	Stability Studies on the Performance of the Newly Designed PBR	89
4.4.9	Toxicity test	90
4.4.10	Combined removal of 4-CP, 4-BP and 4-NP in PBR	91
4.4.11	Treatment of Refinery Industrial Wastewater	91
	Preliminary Batch Study with Refinery Wastewater	92
	Refinery Wastewater Treatment in PBR	92
4.5.1	Evaluation of <i>A. chlorophenolicus</i> A6 Immobilized with Calcium Alginate Beads Using a Column Reactor	93
4.5.1.1	Preparation of Calcium Alginate Bead Immobilized with <i>A. chlorophenolicus</i> A6 Cells	93
4.5.1.2	Batch Shake Flasks Study for Optimization of pH	94
4.5.1.3	Experimental Set up of Column Reactor Packed	94

	with Calcium Alginate Beads	
	a) Main Reactor	94
	b) Biosupport Material (Calcium alginate beads)	95
	c) Feed Flow Rate Adjustment	96
	d) Air Flow Rate	96
	e) Arrangement for Temperature Control	96
	f) Dissolved Oxygen and pH Measurement	96
	4.5.1.4 Biodegradation of 4-CP in with Cell Immobilized Calcium Alginate Beads Using Column Reactor	97
CHAPTER5:	RESULTS AND DISCUSSION	99
5.1	Screening and Optimization of Media Constituents to Enhance Biodegradation of Substituted Phenols	100
5.1.1	Biomass Growth and Degradation of 4-CP Profile of <i>A. chlorophenolicus A6</i>	100
5.1.2	Screening of Media Constituents Using the 2-Level Factorial Design	100
5.1.3.1	Optimization of Media Constituents Using RSM	103
5.1.3.2	Multiple Response Optimization (Desirability function)	111
5.1.3.3	Validation of the Model	113
5.2	Optimization of Culture Conditions to Enhance Biodegradation of Substituted Phenols	115
5.2.1	Optimization of Culture Conditions Using RSM	115
5.2.2	Multiple Response Optimization (Desirability function)	122
5.2.3	Validation of the Model	123
5.3	Biodegradation of Substituted Phenols in Batch Shake Flasks	125
5.3.1	Single Substrate System	125
5.3.1.1	Biomass Growth and Degradation of Substituted Phenols	125
5.3.1.2	Modelling the Growth Kinetics of <i>A. chlorophenolicus A6</i> in Presence of Substituted	131

	Phenol	
5.3.1.3	Variation of Biomass Yield Coefficient Profile of <i>A. chlorophenolicus</i> A6	138
5.3.2	Culture Growth and Mixed Substrates Degradation Kinetics	143
5.3.2.1	Simultaneous Degradation Profiles of substituted phenols	143
5.3.2.2	Biomass Growth of the Culture in the Mixed Substrate System	147
5.3.2.3	Statistical Analysis of Degradation Results of the Substituted Phenols in Mixed Substrate System	148
5.3.3.2	Analysis of Variance (ANOVA)	150
5.3.2.4	Biomass Yield and Specific Degradation Rate of the Substrates	154
5.4	Biodegradation of Substituted Phenols as Single Substrate by <i>A. chlorophenolicus</i> A6 in the Newly Designed PBR	156
5.4.1	Hydrodynamic Study in the Newly Designed PBR	157
5.4.2	Abiotic Removal of Substituted Phenols by Adsorption and Volatilization in the Newly Designed PBR	160
5.4.3	Optimization of DO concentration	162
5.4.4	Biodegradation of 4-CP, 4-BP, and 4-NP by <i>A. Chlorophenolicus</i> A6 in the PBR	166
	4-CP Biodegradation in PBR	167
	4-BP Biodegradation in PBR	169
	4-NP Biodegradation in PBR	170
5.4.5	Effects of 4-CP, 4-BP and 4-NP Loading Rates on Performance of PBR	174
5.4.6	Biomass Growth in PBR	179
5.4.7	Dissolved oxygen (DO) Concentration Profile in PBR	180
5.4.8	Metabolic Intermediate Analysis	181
5.4.9	Stability Studies on the Performance of the Newly	183

	Designed PBR Under Shock Loading Conditions	
5.4.10	SEM Characterization of PUF	186
5.4.11	Toxicity Removal in PBR	188
5.5	5.5.1 Simultaneously Removal of 4-CP, 4-BP and 4-NP in PBR	190
	5.5.2 Toxicity Removal Efficiency in Mixed Substrate System in the PBR	198
5.6	Treatment of Refinery Industrial Wastewater	198
	5.6.1 Preliminary Batch Study Results	198
	5.6.2 Refinery Wastewater Treatment in PBR	200
	5.6.3 Toxicity Removal Efficiency in Mixed Substrate System in the PBR	205
5.7	Biodegradation of Substituted Phenol Using <i>A. chlorophenolicus</i> A6 Cells Immobilized with Calcium Alginate Beads	205
	5.7.1 Optimization of pH	208
	5.7.2 Biodegradation of Substituted Phenol Using <i>A. chlorophenolicus</i> A6 Cells Immobilized with Calcium Alginate Beads in a Column Reactor	209
5.8	SUMMARY AND CONCLUSIONS	214
5.9	SUGESTION FOR FUTURE WORK	218
5.10	BIBLIOGRAPHY	219
5.11	LIST OF PUBLICATION	267

LIST OF FIGURES

Figure No.	Description	Page No.
2.1	4-chlorophenol degradation pathways.	34
4.1	Calibration curve obtained for substituted phenols estimation using the HPLC (a) 4-CP (b) 4-BP and (c) 4-NP.	67
4.2	Calibration curve biomass optical density (OD _{600nm}) versus mixed liquor suspended solids (MLSS)	69
4.3	Bradford protein assay calibration curve obtained in the present study	70
4.4	Calibration curve for conversion of protein content to mixed liquor suspended solid (MLSS).	71
4.5	Schematic of the experimental set up of the novel PBR with its various components	80
4.6	Photograph of the PBR used in the study.	81
4.7	Perfortaed perspex disc covered with nylon mesh at the top with ceramic gas diffuser assembly.	82
4.8	Schematic of the experimental set up of the column reactor with its various components	95
5.1	Relationship between 4-CP biodegradation and biomass growth profile in unoptimized media of <i>A. chlorophenolicus</i> A6	101
5.2	Main effects plot of the media constituents on (a) 4-CP biodegradation. (b) Specific growth rate of the culture.	102
5.3	(a) Linear plot of predicted versus measured values of 4-CP degradation in the optimization study. (b) Linear plot of predicted versus measured values of specific growth rate of the culture in the optimization study	108
5.4	Contour plot showing relationship between (a) magnesium sulphate and calcium chloride (b) di-potassium phosphate and calcium chloride (c) ammonium nitrate and magnesium sulphate (d) ammonium nitrate and	110

	calcium chloride on 4-CP biodegradation	
5.5	Desirability function plot showing the optimum level of media constituents	113
5.6	Two dimensional response surface contour plots showing the relationship between these factor on 4-CP biodegradation by <i>A.chlorophenolicus</i> A6 (a) pH and temperature (b) temperature and inoculum age (c) agitation and temperature (d) inoculum age and agitation	120
5.7	(a) Linear plot of predicted versus measured values of 4-CP degradation in the optimization study. (b) Linear plot of predicted versus measured values of specific growth rate of the culture in the optimization study	122
5.8	Time profile of <i>A. chlorophenolicus</i> A6 on degradation of (a) 4-CP (b) 4-BP and (c) 4-NP.	127
5.9	Time profile of biomass growth (OD _{600nm}) for different initial substituted phenol concentration (a) 4-CP (b) 4-BP and (c) 4-NP.	130
5.10	Experimental and model predicted specific growth rate of the culture at different substituted phenol concentrations (a) 4-CP (b) 4-BP and (c) 4-NP	133
5.11	Effect of initial substituted phenol concentration on biomass yield (a) 4-CP (b) 4-BP and (c) 4-NP	140
5.12	Effect of initial substituted phenol concentration on proportion of substrate carbon diverted for biomass yield (Y/Y _C) and maintenance energy (Y/Y _E) (a) 4-CP (b) 4-BP and (c) 4-NP.	143
5.13	Biodegradation of 4-CP, 4-BP and 4-NP by <i>A. chlorophenolicus</i> A6 in mixed substrate system for different initial concentration ranges. (a) At low concentration range (75+75+50 mg l ⁻¹ , run-1, and 75+75+100 mg l ⁻¹ , run -5) (b) at high concentration range (125+125+50 mg l ⁻¹ , run 4 and 125+125+100 mg l ⁻¹ , run- 8).	145
5.14	Biomass profile of <i>A. chlorophenolicus</i> A6 when grown in medium containing 4-CP, 4-BP and 4-NP.	147
5.15	Degradation rates of 4-CP, 4-BP and 4-NP mixture obtained at different initial concentration ranges of these substrates	149
5.16(a)	Pareto chart of the standardized effects of the factors on 4-CP biodegradation rate in the mixture study ($\alpha=0.05$).	152

5.16(b)	Pareto chart of the standardized effects of the factors on 4-BP biodegradation rate in the mixture study ($\alpha=0.05$).	153
5.16(c)	Pareto chart of the standardized effects of the factors on 4-NP biodegradation rate in the mixture study ($\alpha=0.05$).	153
5.17	Comparison of hydrodynamic profiles obtained using the newly designed and simple PBRs in the study (a) mixing time profile at superficial flow velocities (U_{GR}) = 0.315 cm s^{-1} . (b) mixing time behavior at varying superficial air flow velocities (c) oxygen mass transfer coefficients (k_{La}) at U_{GR} values of 0.1575 cm s^{-1} and 0.315 cm s^{-1} , (d) overall gas hold-up at different air flow velocities.	159
5.18	Breakthrough curve obtained for determining the adsorption capacity of the biosupport material used in the newly designed PBR on 4-CP, 4-BP and 4-NP (influent concentration = 200 mg l^{-1} , HRT = 10 h).	161
5.19 (a)	Dissolved oxygen profile with respect to substituted phenol removal rate and removal efficiency at a constant loading rate of $910 \text{ mg l}^{-1} \text{ day}^{-1}$ for (a) 4-CP	164
5.19 (b)	Dissolved oxygen profile with respect to substituted phenol removal rate and removal efficiency at a constant loading rate of $910 \text{ mg l}^{-1} \text{ day}^{-1}$ for (b) 4-BP	164
5.19 (c)	Dissolved oxygen profile with respect to substituted phenol removal rate and removal efficiency at a constant loading rate of $910 \text{ mg l}^{-1} \text{ day}^{-1}$ for (b) 4-NP	165
5.20 (a)	Biodegradation of 4-CP and DO profile in the newly designed PBR	168
5.20 (b)	Biodegradation of 4-BP and DO profile in the newly designed PBR.	171
5.20 (c)	Biodegradation of 4-NP and DO profile in the newly designed PBR.	173
5. 21	(a) 4-CP removal efficiency, and (b) 4-CP biodegradation rate in the newly designed PBR at different 4-CP loading rates.	176
5.22	(a) 4-BP removal efficiency, and (b) 4-BP biodegradation rate in the newly designed PBR at different 4-BP loading rates.	177
5.23	(a) 4-NP removal efficiency, and (b) 4-NP biodegradation rate in the newly designedl PBR at different 4-NP loading rates.	178
5.24	Mass spectra of substituted phenol metabolites cleavage pathway obtained by HPLC-MS, Hydroxy quinol (126.04), 4-chloro catechol (143.4) (a) 4-CP and (b) 4-NP.	182

5.25(a)	Substituted phenol removal profile of the newly designed PBR under shock loading conditions operated at varying hydraulic loading rate (a) For 4-CP from 473- 1183 mgL ⁻¹ day ⁻¹ and 1138-2845 mgL ⁻¹ day ⁻¹ (b) For 4-BP from 473- 1183 mgL ⁻¹ day ⁻¹ and 1504-3759 mgL ⁻¹ day ⁻¹ .	184
5.25(c)	Substituted phenol removal profile of the newly designed PBR under shock loading conditions operated at varying hydraulic loading rate (c) For 4-NP from 842-1966 mgL ⁻¹ day ⁻¹ and 2248-4492 mgL ⁻¹ day ⁻¹ .	185
5.26	SEM images of PUF: (a) vesicular structure of PUF before inoculums, (b) bacterial growth on PUF, and (c) magnified picture of (b) showing more clear microbial morphology.	186
5.27	SEM images <i>A. chlorophenolicus</i> A6 growth on MSM in batch shake flask before inoculated in the PUF cube packed in the PBR	187
5.28	Percentage toxicity in the effluent and percentage of substituted phenol removal with percentage of toxicity removal tested on mixed microbial consortia from municipal sludge in the newly designed PBR (a) 4-CP (b) 4-BP (c) 4-NP.	190
5.29	Degradation performance of <i>A. chlorophenolicus</i> A6 using the newly designed PBR treating synthetic wastewater containing mixture of 4-CP, 4-BP and 4-NP at 12.5h HRT. (*S indicates stage of operation).	192
5.30	Substituted phenols removal efficiency and removal rate of <i>A. chlorophenolicus</i> A6 in the PBR at different loading rates, particularly stage 1, 2 and 6. (*RR indicated removal rate and RE indicated removal efficiency).	195
5.31	Effect of mineral salt media (MSM) on COD removal efficiency (%).	200
5.32	Degradation performance of PBR in treating petroleum refinery wastewater containing mixture of 4-CP, 4-BP and 4-NP at 12.5h HRT by <i>A. chlorophenolicus</i> A6. (*S indicates stage of operation)	202
5.33	COD removal performance of PBR in treating petroleum refinery wastewater containing mixture of 4-CP, 4-BP and 4-NP at 12.5h HRT by <i>A. chlorophenolicus</i> A6. (*S indicates stage of operation)	203
5.34	Substituted phenols removal efficiency and biodegradation rate in the PBR at	204

different loading rates. (*RR = Removal rate; RE = Removal efficiency).

- 5.35 Effect of pH on removal of 4-CP by *A. chlorophenolicus* A6 cells 208
immobilized with calcium alginate bead.
- 5.36 4-CP biodegradation profile of cell immobilized calcium alginate beads in 209
the PBR.
- 5.37 (a) Variation of 4-CP removal efficiency and (b) 4-CP biodegradation rate 212
with respect to different 4-CP loading rates in the PBR using cell
immobilized calcium alginate beads.



LIST OF TABLES

Table No.	Description	Page No.
2.1	Physical and chemical properties of the three substituted phenols	8
2.2	Environmental contamination level of substituted phenols estimated at different location of various countries.	12
2.3	Bioreactor system and their performance in treating wastewaters containing substituted phenolics compounds	46
2.4	Process control parameters for monoaromatic degradation using continuous operated bioreactor system.	53
2.5	Performance of cell immobilized alginate bead in different bioreactor system for treatment of various pollutant	55
4.1	Instruments and equipments used in the present investigation	63
4.2	Two level factorial design for screening of media constituents	73
4.3	Central composite design matrix showing used in the optimization study ($\alpha = 2$)	75
4.4	Central composite design matrix showing used in the optimization study ($\alpha = 2$)	77
4.5	2^2 full factorial design with three center point replicates adopted in the mixed substrate biodegradation study	78
4.6	MSM used in the substituted phenols degradation in continuous mode of operation using the PBR	83
4.7	Various dilution of raw refinery wastewater prepared for preliminary batch shake flask study.	92
4.8	Detailed specifications of the reactor and alginate beads used in the study	96

4.9	Operational schedule of the column reactor packed with <i>A. chlorophenolicus</i> A6 cells immobilized with calcium alginate beads.	97
5.1	Student's <i>t</i> -test for 4-CP biodegradation and culture specific growth rate in the screening study	103
5. 2.(a)	ANOVA of 4-CP biodegradation in the media optimization study	104
5.2 (b)	ANOVA of specific growth rate of the culture in the media optimization study	105
5.3	Student's <i>t</i> -test for 4-CP biodegradation and culture specific growth rate in the media optimization study	106
5. 4.(a)	ANOVA of 4-CP biodegradation in the optimization study	117
5.4 (b)	ANOVA of the culture specific growth rate in the optimization study	117
5.5	Result of Student's <i>t</i> -test for 4-CP biodegradation and the culture specific growth rate in the optimization study	118
5.6	performance different microorganism operated in different system and mode of operation for substituted phenol biodegradation reported in literature	128
5.7	Various literature models on biomass growth with substrate inhibition tested in the study	134
5.8	Estimated values of kinetic parameters on substituted phenols degradation using the various models tested in the study (a) 4-CP (b) 4-BP and (c) 4-NP	135
5.9 (a)	ANOVA of 4-CP biodegradation in the mixture experiments	151
5.9 (b)	ANOVA of 4-BP biodegradation in the mixture experiments	151
5.9 (c)	ANOVA of 4-NP biodegradation in the mixture experiments	151
5.10	Student's <i>t</i> test for estimating the coefficients of individual and interaction effects of the substituted phenols and their significance on degradation performance in the mixture study	154
5.11	Estimated biomass yield of the <i>A. chlorophenolicus</i> A6 in the mixture study	155
5.12	Detailed specifications of the reactor	157

5.13	Abiotic removal of substituted phenols by adsorption and volatilization in the newly designed PBR at initial concentration of 200 mg l ⁻¹ and HRT 10h.	161
5.14	Abiotic removal (adsorption and volatilization) of various phenolic pollutants on PUF and activated carbon by various reactor and batch shake flask study.	163
5.15 (a)	Operational schedule of the PBR for 4-CP biodegradation	167
5.15 (b)	Operational schedule of the PBR for 4-BP biodegradation	169
5.16 (c)	Operational schedule of the PBR for 4-NP biodegradation	172
5.16	Performance of <i>A. chlorophenolicus</i> A6 in treating single substrate substituted phenol using the newly designed PBR	174
5.17	Operational schedule and performance of the PBR on biodegradation of mixtures of mono substituted phenols at 12.5 h HRT.	191
5.18	Performance of various bioreactors used on substituted phenols degradation in mixed substrate system	196
5.19	Characteristics of the petroleum refinery industry wastewater used in the study	199
5.20	Operational schedule and performance of the newly designed PBR treating petroleum refinery wastewater spiked with substituted phenol at HRT 12.5h.	201
5.21	Performance of various bioreactor system for treatment of oil refinery wastewater	206
5.22	Toxicity removal profile of in treating various pollutants with different biological system	207
5.23	Operational schedule of the column reactor packed with <i>A. chlorophenolicus</i> A6 immobilized with calcium alginate beads.	210

ABBREVIATIONS AND NOTATIONS

4-CP	4- Chlorophenol
4-BP	4-Bromophenol
4-NP	4- Nitrophenol
PCP	Pentachlorophenol
PUF	Poly Urethane Foam
ANOVA	Analysis Of Variance
CCD	Central composite design
RSM	Response Surface Methodology
COD	Chemical Oxygen Demand (mg l^{-1})
DO	Dissolved Oxygen (mg l^{-1})
MSM	Mineral Salt Media
HRT	Hydraulic Retention Time (h)
PBR	Packed bed Reactor
ILALR	Internal Loop Airlift Bioreactor
SBR	Sequencing Batch Reactor
RBC	Rotating Biological Contractor
FBR	Fluidized Bed Reactor
UASB	Up flow Anaerobic Sludge Blanket Reactor
C_e	Liquid Phase Oxygen Concentration in Equilibrium with Gas Phase (mg l^{-1})
C_L	Concentration of DO in the Liquid

	(mg l ⁻¹)
ϵ_G	Gas hold up (%)
t_{m95}	time to reach 95% homogeneity after a tracer input(s)
kLa	Volumetric Mass Transfer Coefficient (s ⁻¹)
V_{void}	void volume of packed-bed (Litre)
ϵ_e	inter particle porosity
ϵ_p	intra particle porosity
U_{GR}	superficial air velocity (cm s ⁻¹)
ϵ_{tot}	Total bed porosity
K	Constant in Edward and Yano-Koga Models
K_i	Inhibition Constant (mg l ⁻¹)
S	Substrate Concentration at Time (mg l ⁻¹)
S_0	Substrate concentration at time t = 0 (mg l ⁻¹)
X	Biomass Concentration at Time t (mg l ⁻¹)
X_0	X_0 Biomass Concentration at Time t = 0 (mg l ⁻¹)
μ	Specific Substrate growth Rate (h ⁻¹)
μ_{max}	Maximum Specific biomass growth Rate (h ⁻¹)
OD	Optical density
SEM	Scanning electron microscopy

ABSTRACT

The presence of substituted phenols in water, soil and wastewater is of severe public health concern due to their detrimental effect on human and many other living organisms. Their removal from contaminated water and wastewater is necessary for protection of environment and human well being. Substituted phenols especially chloro, bromo and nitro phenols are one of the most widely used chemical compounds in many industries throughout the world. Although several techniques such as photo-decomposition, physical adsorption, solvent extraction, chemical oxidation and electrochemical methods have been tested for the removal of phenol and phenolic compounds from wastewaters, high cost, low efficiency and generation of toxic by-products are some of the limiting factors of these methods. The eco-friendly biodegradation process has gained maximum attention due to its many advantages over the traditional physico-chemical methods.

The main objective of the present investigation was to develop a complete treatment system for substituted phenols bearing wastewater in aerobic process using an actinomycetes strain of *Arthrobacter chlorophenolicus* A6. The substituted phenols investigated in this study were 4-chlorophenol (4-CP), 4-bromophenol (4-BP) and 4-nitrophenol (4-NP) both as single substrate and mixed substrate system. As a first step, optimization of culture media components were carried out employing statistical design of experiments including 2-level factorial design, central composite design and response surface methodology (RSM). The media optimization results revealed 100% of 4-chlorophenol biodegradation efficiency with a maximum specific growth rate of 0.093 h^{-1} at an optimum composition of (g l^{-1}) K_2HPO_4 2.62, KH_2PO_4 0.4, NH_4NO_3 0.58, MgSO_4 0.171, CaCl_2 0.038 and FeCl_3 0.002 in the medium. Similarly the RSM optimized result of physical parameter revealed that at the optimized settings of 7.5 pH, 207 rpm, $29.6\text{ }^\circ\text{C}$ and 39.5 h inoculum age, 100% biodegradation of 4-CP even at a high initial concentration of 300 mg l^{-1} could be achieved within a short span of 18.5 h of culture. The enhancement in the 4-CP biodegradation efficiency was found to be 23% higher than that obtained at the unoptimized settings of the culture conditions. In addition, at the optimized culture conditions the actinomycetes could degrade 4-CP at an

initial concentration as high as 375 mg l⁻¹ within a time period of 40 h, which is so far the best 4-CP degradation concentration in literature in batch shake flask.

Following optimization of culture condition affecting the growth of the microorganism and degradation of the substituted phenol, its growth and degradation kinetics were investigated in batch shake flask. From the experimental results, it was observed that *A. chlorophenolicus* A6 followed substrate inhibition growth kinetics pattern and the data on specific growth rate of the culture due to various initial concentrations of the phenolic compounds were fitted to suitable substrate inhibition models reported in the literature. Among the models tested for the culture growth on 4-CP or 4-BP, Edward model gave the best fit to the experimental data and the model constants were estimated to be maximum specific growth rate ($\mu_{\max}$) = 0.22 h⁻¹, inhibitory constant (K_i) = 105 mg l⁻¹ and half saturation constant (K_s) = 30.83 mg l⁻¹ for cell growth on 4-CP and $\mu_{\max}$ = 0.246 h⁻¹, K_i = 111 mg l⁻¹ and K_s = 30.77 for cell growth on 4-BP. On the other hand, for the culture growth on 4-NP Web-type growth kinetic models was found to be the best fitted and the estimated model parameters were; $\mu_{\max}$ = 0.16 h⁻¹, K_i = 128 mg l⁻¹ and K_s = 20.15 mg l⁻¹. Values of some these estimated model parameters such as specific growth rate ($\mu_{\max}$) and inhibition constant (K_i) were found to be significantly lower as compared with those reported in literatures. Maximum biomass yield of the microorganism was estimated in the range from 0.21 - 0.39 gg⁻¹ for the three substituted phenol tested in the study. However, concentration higher than the inhibitory level viz. (K_i) 105, 111 and 128 mg l⁻¹ for 4-CP, 4-BP, and 4-NP respectively, a considerable decrease in the yield values was observed indicating a strong inhibition effects at these concentration and the energy expended to maintain the cell activity is high. Moreover, at the concentration greater than 125 mg l⁻¹, the relative proportion of the substrate consumed for energy (Y/Y_E) exceeded that for assimilation into cell mass (Y/Y_C) for all these three pollutants, probable due to the requirement of high maintenance energy for overcoming the effect of substrate inhibition at high concentrations of these phenolics. In the mixed substrate system of the phenolic compounds added together in the media, observed that at all its concentration combinations the culture took longer time to degrade 4-CP and 4-BP completely as compared to that for degrading 4-NP. Further, 4-NP was found to be preferentially degraded over the other two pollutants. From statistical analysis of the results from the mixture study, 4-NP degradation was found to be significantly inhibited in

comparison with 4-BP due to the presence of 4-CP. Interaction between the compound on their own degradation were also observed where 4-NP was significantly inhibited by 4-CP.

Biomass growth data were also obtained, which suggested that even though 4-NP was degraded prior to 4-CP and 4-BP, it had a stronger negative impact on the cells than the other two. The maximum yield value obtained in the present study (0.2152 gg^{-1} at 125, 125, and 50 mg l^{-1} of 4-CP, 4-BP and 4-NP respectively) also less when compared with several literatures reported values which ranges from 0.44-0.88 gg^{-1} on biodegradation of phenolic compounds using different microbial culture.

After establishing the culture growth and degradation of substituted phenols in batch shake flasks, experiments were performed in continuous mode of operation to treat synthetic wastewater spiked with these substituted phenol in a newly designed packed bed reactor (PBR). The hydrodynamics conditions (mixing time, oxygen mass transfer coefficient rate and gas hold up) of the newly designed PBR were found superior to those of a simple PBR. Results showed that in the single substrate system best removal efficiencies were achieved for a total pollutant loading rate of $1707 \text{ mg l}^{-1} \text{ day}^{-1}$ (10h HRT with influent 4-CP 600 mg l^{-1}) for 4-CP, $2277 \text{ mg l}^{-1} \text{ day}^{-1}$ (12.5h HRT and with influent 4-BP 1000 mg l^{-1}) for 4-BP and $2787 \text{ mg l}^{-1} \text{ day}^{-1}$ (10 h HRT with influent 4-NP 1000 mg l^{-1}) for 4-NP. In all the experiment the pollutants were completely degraded and their concentrations in the effluents were below the detection limit.

The high performance of the reactor was attributed to high biomass concentration of the system. However, 4-CP degradation performance was comparatively less with that of 4-BP and 4-NP. Analysis of metabolic intermediates during the experiment also revealed accumulation of 4-chlorocatechol and 4-nitrocatechol in the reactor only at very high concentration of the substituted phenols. Substituted phenols 1000 mg l^{-1} and above with very low HRT of less than 10h that resulted in unsteady state performance of the reactor. However, in case of 4-BP no such metabolic intermediate was observed.

Toxicity test performed on a mixed culture from a municipal sludge showed that more than 93 % toxic removal was achieved at the optimum loading rate (reactor shown best removal efficiency) together with very high COD removal efficiency ($\approx 98\%$). Under the

shock loading condition of each of the substituted phenols, the reactor also showed very good ability to regain its original performance within a short time.

Performance of *A. chlorophenolicus* A6 to degrade a mixture of pollutants also evaluated in the newly designed PBR. Complete removal of all the three pollutants was achieved at maximum individual pollutant loading rate of $569 \text{ mg l}^{-1} \text{ day}^{-1}$ and combined loading rate of $1707 \text{ mg l}^{-1} \text{ day}^{-1}$ (250 mg l^{-1} each of the pollutant or combined concentration 750 mg l^{-1}). However, its performance was slightly poor when the combined concentration of the pollutants exceeded 950 mg l^{-1} . Similar to the result obtained in batch shake flask for degrading a mixture of the pollutants, 4-NP was shown to be preferentially degraded by the culture in the bioreactor system. Further, at the individual optimum loading rate of the pollutant ($569 \text{ mg l}^{-1} \text{ day}^{-1}$) more than 97 % toxic removal was achieved when tested on a mixed culture from municipal sludge.

Performance of the newly designed PBR in treating refinery wastewater spiked with substituted phenols revealed complete removal at low concentration combinations of the pollutants (250 mg l^{-1} each) i.e. individual pollutant loading rate of $569 \text{ mg l}^{-1} \text{ day}^{-1}$ /combined loading rate of $1707 \text{ mg l}^{-1} \text{ day}^{-1}$. More than 90% degradation was achieved at 300 mg l^{-1} each of the pollutants. However, the reactor became unstable particularly when operated at high concentration of 450 mg l^{-1} each. The degradation was also confirmed with respective COD removal from the wastewater. At the optimum loading rate of $569 \text{ mg l}^{-1} \text{ day}^{-1}$ each of the pollutant, more than 94.6 % toxic removal was achieved for all the three compounds when tested on a mixed culture from municipal sludge. However, the performance of the PBR on simultaneous removal of substituted phenols from synthetic wastewater was better than that of refinery wastewater. This might be due to the presence of other contaminants including heavy metals, high concentration of chloride ion in the wastewater and their toxic effect on the microorganism.

In order to further extend the scope of the actinomycete in treating these phenolics, experiments were performed to compare its efficiency to degrade the substituted phenols employing an emerging technique viz. cell immobilization using calcium alginate beads under continuous mode of operation in a column reactor. Results revealed that complete removal of 4-CP in the reactor could be achieved at the maximum 4-CP loading rate of $1275.5 \text{ mg l}^{-1} \text{ day}^{-1}$ i.e, at 3 h HRT and 150 mg l^{-1} influent 4-CP concentration. These values of

the pollutant loading rate using cell immobilized calcium alginate bead in the column reactor to degrade 100 mgL⁻¹ of 4-CP concentration was lower than that obtained in the newly designed PBR where completely removal was achieved at pollutant loading rate 1707 mgL⁻¹day⁻¹ and at an influent 4-CP concentration of 600 mgL⁻¹. The high 4-CP degradation efficiency observed in the newly designed PBR was attributed to high biomass in the system (366 mgg⁻¹ of PUF vs 6.5 mgg⁻¹ of alginate bead) and thus better 4-CP biodegradation performance. Overall, the result obtained using the newly designed PBR demonstrated excellent performance of the system in continuous treatment of substituted phenol containing both synthetic and real industrial wastewater.



CHAPTER 1

INTRODUCTION

.....

1.1 Generalities

Generation of gaseous, liquid and solid wastes is an unavoidable consequence of industrial, agricultural and domestic activities. Nevertheless, the environmental impact of these activities must be minimized to ensure sustainable quality of life. While conservation and better utilization of resources would ultimately have the greatest influence on sustainability of the planet reduced generation, improved treatment and utilization of wastes will remain the essential components of an overall strategy for maintenance of environmental quality. One of the most alarming phenomena is the growth of industrial sectors, which discharge a huge amount of wastewater contaminated with toxic organic substances like phenolics. The major industries that discharge phenolic contaminated wastewater include petroleum refineries, petrochemicals, textile, dye manufacturing, phenolics resin and polymers manufacturing, varnish industries, leather, insecticides, pesticides, pharmaceutical and wood preservative etc ([Rhee et al., 2003](#); [Howe et al., 2005](#); [Nuhoglu and Yalcin, 2005](#); [Juang and Tsai, 2006](#); [Hassenklover et al., 2006](#); [Yan et al., 2006](#); [Bai et al., 2007](#); [Uhnáková et al., 2009](#)). The annual industrial production of chlorophenols was estimated at 0.29×10^6 metric tonnes ([Stuart and Woods, 1998](#); [Tirola et al., 2002](#)). The combined global production of all chlorophenols approached 200 million kilograms. More than half consisted of chlorophenols other than pentachlorophenol (PCP), with 2,4- dichlorophenol, 2,4,5-trichlorophenol and 2,3,4,6-tetrachlorophenol predominating ([WHO, 1989](#)). The production volume of bromophenol was estimated to be approximately 2500 tonnes per year in Japan and 9500 tonnes/year worldwide in 2001 ([IUCLID, 2003](#)). Similarly the annual 4-NP

production capacity in USA was 46 million pounds (SRI, 1989). These substituted phenols are also formed in aqueous matrices during pesticide formulation, distribution and field applications (Concetta et al., 2003). In addition, in Japan 4-NP was detected in rain water through the photochemical reaction between benzene and nitrogen monoxide in the atmosphere (EPA, 1980). Similarly chlorophenols are also formed as a by-product when chlorine is used for bleaching of pulp and for disinfection of water (Annachhatre and Gheewala, 1996; Tartakovsky et al., 2001). Bromophenol is also produced during combustion of leaded petrol (Howe et al., 2005; Uhnáková et al., 2009). Furthermore, in nature, several organisms have been described as producers of chlorinated and brominated organic compounds (Ahn et al., 2003; Vetter and Janussen, 2005). Further, the substituted phenols may have the potential to leach through soil and enter groundwater, where they are hardly degradable and present in the environment (Trapido et al., 2000). Salkinoja Salonen (1990) reported chlorophenol (CP) concentrations of a saw mill to be $10,000 \text{ mg CPkg}^{-1}$ soil. Surface waters, ground waters and lake waters in the area of saw mills were also contaminated with the same chlorophenols used in wood preservatives (Valo et al., 1984). There are several reports on high bromophenol concentration on river, sea and estuarine bases; for instance $500\text{-}7000 \text{ }\mu\text{gkg}^{-1}$ and $26\text{-}3690 \text{ }\mu\text{gkg}^{-1}$ of bromophenol was estimated from Norwegian Sea and France Rhone Estuarine respectively (Tolosa et al., 1991; Jensen et al., 1992). Similarly 4-NP concentration detected from industrial effluents is quite high ranging from 10 to $17 \times 10^3 \text{ mg l}^{-1}$ (Tsai and Folsom, 1982; Luthy et al., 1983; Suidan et al., 1983). Substituted phenols are the most important phenolic compounds that are present in wastewater discharged from industries (Tomei et al., 2003). The presence of substituted groups in phenols particularly chloro, bromo and nitro groups increases their toxic effects exerted on the environmental life as well as on the human health. (Crossby, 1981; Uberoi et al., 1997). Humans, who are acutely exposed to substituted phenol by the oral route and consequently suffer from blood pressure, hyperglycemias, cardiovascular stress, damage liver and immuno system (Crosby, 1981; de Wit, 2000; Howe et al., 2005). Furthermore, these pollutants are reported to be carcinogen, teratogen and is highly embryo toxic in nature (Crosby, 1981). Therefore, bromophenol, nitrophenol and chlorophenol are listed as priority pollutant by U.S. EPA and recommended their concentration levels in natural water to below

10 ng l⁻¹ and 1 µg l⁻¹ for 4-NP and 4-CP respectively (Federal Register, 1984; Wild et al., 1993; Erba et al., 2007). Whereas, in Indian standard no report is available on permissible limit of these substituted phenols, however, recommended phenolics substance as phenol to 1 µg l⁻¹ and absolute maximum to 2 µg l⁻¹ (Sprenger et al., 2008). Therefore, removal of these phenolics to sufficiently low levels in wastewater is mandatory. Appropriate strategies of wastewater treatment have to be employed in order to counter balance these growing environmental problems. For the last two decades, rigorous pollution control and legislation in many countries have resulted in an intensive search for new and more efficient water treatment technologies. Important wastewater treatment technologies that have come up in recent times, include but are not limited to flocculation, precipitation, adsorption on granular activated carbon, reverse osmosis, combustion and advanced oxidation process (AOP) viz., photo fenton, photocatalysis and sonication (Chen and Ray, 1998; Ra et al., 2008). However, high cost, low efficiency and generation of toxic by-products are some of the limiting factors of these methods. However, biodegradation seems to be an ultimate solution to the problem (Dolon et al., 1996).

1.2 Biological Treatment

The eco-friendly biodegradation process has gained maximum attention due to its many advantages over the traditional methods (Yan et al., 2006). Although the chloro, bromo and nitro groups enhances the resistance of the aromatic ring against biodegradation (Higson 1992; Spain 1995; Uberoi et al., 1997) and hence only selective microorganisms such as *Flavobacterium*, *Moraxella*, *Nocardia*, *Acaligenes*, *Ochrobactrum sp.*, *Achromobacter piechaudii* TBPZ, *Trametes versicolor*, *Agaricus augustus* *Pseudomonas*, *Rhodococcus* and *Arthrobacter*, can metabolize 4-CP, 4-BP and PNP as a sole source of carbon or/and nitrogen for growth (Spain et al., 1991; Radehaus and Smith, 1992; Bae et al., 1996; Ronen et al., 2005; Ferraroni et al., 2006; Donoso et al., 2008; Yamada et al., 2008). Among these microbial species, actinomycetes secrete both extracellular as well as intra cellular enzymes and have thus shown good potential in degrading these substituted phenols. Furthermore, in actinomycetes the number of residue in the active cleft of the enzymes believed to be responsible for substrate selection are altered mainly at leu80, Asp83, Val107 aminoacids

positions, thus possess broad specificity in nature (Ferraroni et al., 2006) and can interact with wide different toxic substrates that occurs in actual industrial wastewater, along with these substituted phenols. In view of the above, the theme of the research is quest for environmental friendly and economically viable process for treatment of industrial wastewaters containing substituted phenols using an actinomycetes strain of *Arthrobacter chlorophenolicus* A6.

In a biological process, microbial community is confronted with a mixture of substrate composed of toxic and non-toxic compounds. The substrate is consumed by the microorganism through an enzyme-catalyzed reaction. Thereby, the population of community increases. In addition to the presence of a capable microorganism, environmental conditions must also be suitable for the enzyme-catalyzed reactions to proceed at a significant rate. On the other hand, growth in the microbial community depends on the changes in the feed substrate and the physico-chemical conditions (pH, temperature, medium constituents *etc.*) of the process. Moreover, in the contaminated environments a large complex of mixture of compounds and their interaction plays a crucial role on the individual substituted phenol mineralization rate and efficiency.

Regarding the performance of a microbial wastewater treatment system, the basic questions that one may pose are:

- Pure cultures have been extensively studied, but degradation using actinomycetes is scanty although it possesses several advantage over other microbial system.
- Will toxic (inhibitory) compounds be degraded in the presence of non-toxic compounds? If so, to what degree?
- How to improve the culture condition and what are the culture medium constituents and how does it influence degradation of the compounds and growth of the microorganism?
- What should be the optimum loading rate or flow rate through the reactor to achieve complete degradation of the toxic compounds?

By optimizing the physical and chemical culture conditions as well as studying the degradation kinetics of various toxic compounds and growth kinetics of microorganisms, knowledge about the kinetic properties of different biological systems can be gathered and these information may be helpful to understand more complex systems in a step-by-step manner.

The effect of various substrates, on the microbial degradation of organic pollutants, has been the subject of a number of studies ([Juang and Tsai, 2006](#); [Yan et al., 2006](#); [Bai et al., 2007](#)). Although some of these authors, dealing with microbial growth kinetics, emphasized the ecological point of view, they almost totally neglected the facts that (i) microorganisms mostly encounter systems containing mixture of substrates (ii) growth may not be controlled by only a single substrate but by two or more substrates simultaneously (iii) nature and extent of interactions among these substrates on each other. Hence the neglected facts by the other researchers were considered for research in the present thesis work.

1.3 Bioreactors for Wastewater Treatment

In the last few decades, various bioreactor designs have been proposed for wastewater treatment with an aim to ensure increased oxygen transfer rate and minimal power consumption. One of the most promising bioreactors is the fixed film packed bed reactor (PBR). The major advantages of PBR over other bioreactor are it does not need any mechanical agitation and a low energy is sufficient for required aeration in the process. Compared to conventional reactors such as the stirred tanks or bubble columns, shear stress in PBR is relatively constant and mild throughout the reactor. This fact is favorable to microbial growth. Biofilm reactors exhibit high surface area available for microbial growth and offer high volumetric biomass concentration in small reactor volume consequently shows higher metabolic activity when operated under continuous mode of operation ([Israni et al., 2002](#)). In addition, the attached biomass is sometimes claimed to be more resistant to toxicity; offers higher resistance to shock loads and its ability to survive even at low influent substrate concentrations. For example, biofilm cells can be up to 500 times more resistant to antibacterial agents than freely suspended ones ([Pedersen and Arvin, 1995](#)). Furthermore, packed bed bioreactor operating in upflow mode prevents the suspended biomass wash out as well as frequent clogging in support media is less compared to down flow. PBR are also simple to operate, attains a high yield and they can be easily scaled up from a laboratory scale up procedure. Moreover, aerobic biofilm reactors are very compact which enables a rapid start-up, have a high biomass retention capacity and are suitable for use at a low hydraulic retention time (HRT) ([Tchobanoglous, 1998](#); [Langwaldt and Puhakka, 2000](#)). All

these advantages explain why so many researchers have focused their attention on PBR for biodegradation of organic pollutant from contaminated wastewater (Kim et al., 2002; Sheeja and Murugesan, 2002; Tepe and Dursun, 2008; Waul et al., 2008; Gómez-De Jesús et al., 2009; Paca et al., 2009; Akdogan and Pazarlioglu, 2011; Iandolo et al., 2011). The most important disadvantage of a PBR is that the material flows through the reactor. Ideally, in PBR all of the substrate stream flows at different velocities parallel to the reactor axis with no back mixing due to formation of dead pocket in the reactor. All material present at any given reactor cross-section has an unequal residence time. The longitudinal position within the PBR is, therefore, not proportional to the time spent within the reactor; all products emerging with different residence times and all substrate molecules have an unusual opportunity for reaction. In addition, PBR has some other limitations, such as bubble formation and gas channeling leading to the development of liquid-rich and gas-rich regions rather than uniform distribution of oxygen and food throughout the reactor bed (Lee and Pang-Tsui, 1999; Kreft and Wimpenny, 2001). Furthermore, reduction of pressure drop and superficial flow velocity along the height of PBR diminish the growth of microorganisms and thereby degradation of organic pollutants due to lack of availability of sufficient oxygen and/or feed substrate deep inside the reactor bed material, especially when the bed contains a porous but poorly permeable supporting material for the growth of microorganisms (Meyer and Smith, 1985; Radilla, et al., 2005). Therefore, hydrodynamic conditions in a PBR need to be improved to get maximum microbial growth and degradation efficiency. The parameters that characterize the hydrodynamics conditions inside the PBR reactor are the mixing efficiency, dispersion coefficient, mixing time and volumetric mass transfer rate. These parameters are affected by the geometry of the reactor and the operating conditions. The operating conditions that mostly affect hydrodynamics in PBR reactor are the gas flow rates and the liquid flow rates.

This research aims to address the various aspects of substituted phenols, viz. their sources, environmental problems and their removal from industrial wastewaters. In particular, it has contributed to the study and development of an effective system for the biodegradation of substituted phenols employing *Arthrobacter chloroophenolicus* A6. Various physical and chemical process parameters on phenolics biodegradation and growth of the actinomycetes were first screened and optimized under batch conditions. This was

followed by biodegradation in single and multisubstrate systems. A newly designed packed bed reactor (PBR) was fabricated to improve the hydrodynamic conditions for performing experiments on continuous biodegradation of substituted phenols using *A. chlorophenolicus* A6. The substituted phenols performance of the newly designed PBR was also compared with that of the *A. chlorophenolicus* A6 immobilized alginate bead column reactor. The bioreactor system was also evaluated to treat wastewater collected from a petroleum refinery. Overall, the result obtained using the PBR displayed very high performance in continuous treatment of recalcitrant organic substances present in aqueous milieu.



CHAPTER 2

LITERATURE REVIEW

.....

2.1 Physico-Chemical Properties of Substituted Phenols

Chlorophenol and bromophenol are made up by one or more of the chlorine or bromine atoms attached to the benzene ring of phenol. 4-CP and 4-BP are colourless, solid and weakly acidic in nature. The halide substitution makes these compounds stronger acids than phenol. The halogenated phenols are very active to various chemical and biological reactions. Electron donating substituents activate the benzene ring toward electrophilic attack and the strongest activating substituents are the amino ($-\text{NH}_2$) and hydroxyl ($-\text{OH}$) groups. 4-Nitrophenol is a colourless to light yellow solid and weak acid compared to carboxylic acids, but the nitro substitution makes them both stronger acids than phenol. 2-Nitrophenol is volatile in steam, but 4-nitrophenol is not. The nitrophenols can be converted to their water-soluble salts by alkaline hydroxides. The OH-group in these compounds is susceptible to substitution reactions with the formation of ethers and esters. The nitro group can be reduced to the amino group under strong reducing conditions. The nitrophenols may also undergo ring substitution reactions (EPA, 1985). The details about the physical and chemical properties of the three substituted phenol are presented in Table 2.1.

2.2 Sources and Distribution of Substituted Phenols in the Environment

Substituted phenols are characteristic pollutants in wastewater from petroleum refineries, petrochemicals, textile, dye manufacturing, phenolics resin and polymers manufacturing, paper and pulp, varnish industries, explosives, phosphororganic insecticides, pesticides, and wood preservative etc (Rhee et al., 2003; Howe et al., 2005; Nuhoglu and Yalcin, 2005; Juang and Tsai, 2006; Hassenklover et al., 2006; Yan et al., 2006; Bai et al.,

2007; Uhnáková et al., 2009). In general, water contamination by monoaromatics occurs due to improper industrial waste disposal, leakage, spills, accidents during transportation in oil industries (oil refinery, petrochemical companies) and storage tanks (that release petroleum products such as gasoline, diesel fuel, lubricating oil) (Vidali, 2001). Details of the substituted phenols uses, production and its environmental distribution are presented more elaborately as follows.

Table 2.1 Physical and chemical properties of the three substituted phenols (HSDB,1989)

Parameters	4-CP	4-BP	4-NP
Molecular weight	128.56	173	139.11
Melting point	43 - 45°C	64°C	113-114°C
Boiling point	220°C	238°C	287°C
Density	1.22at 45°C	1.84 at 15°C	1.27gcc ⁻¹ at 20°C
Dissociation constant (pKa)	9.3	9.17	7.08-7.18
Solubility (Distilled H ₂ O) at 25°C	27.000 mg l ⁻¹	17.400 mg l ⁻¹	16.000 mg l ⁻¹
Organic solvent soluble in	alcohol, ether, chloroform	Alcohol, ether, chloroform; glacial acetic acid	toluene, ethanol chlorofoam ethyl ether and alkali hydroxide
Partition coefficient	2.39	2.62	1.91
Log octanol/water			
Vapor pressure (mmHg)	0.087 at 25°C	1.17x10 ⁻² at 25°C	0.3x10 ⁻⁴ at 30°C

2.2.1 Major Uses

Chlorophenols have been introduced into the environment through their use as biocides. 4-CP was used as an antiseptic for home, hospital and farm application and also as starting material for making germicides such as 2-benzyl-4-chlorophenol and analgesic viz. acetophenetidin. 2,4-DCP and 2,4,5-TCP were chemical intermediates, especially in the production of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) (WHO, 1989; Solyanikova and Golovleva, 2004).

PCP along with TCP and TeCP isomers were used historically as fungicides in wood preservative formulations (McAllister et al., 1996). PCP was also utilized as an herbicide in rice paddies in Japan (Ohtsubo et al., 1999). In addition, chlorophenols are also used as additives to inhibit microbial growth in a wide array of products, such as adhesives, oils, textiles and pharmaceutical products (WHO, 1989).

Brominated phenolic compounds are widely used in chemical industry, for instance as flame retardants in the production of polymers, or in agriculture as pesticide components (Howe et al., 2005; Hassenklover et al., 2006; Uhnáková et al., 2009). Bromophenols are also used as a precursor to resorcinol (O'Neil, 2006). 2,4,6-TBP is allowed to react with sodium hydroxide to form the sodium salt of tribromophenol in water, which is used as a wood preservative (Chatonnet et al., 2004; WHO, 2005). Further, the solution is very effective in controlling insects, fungi, and bacteria in construction, lumber, plywood timbers, railroad ties, fence posts, utility poles, landscape materials, and foundation materials (WHO, 2005). 2,4,6-TBP is registered as a wood preservative in South America for example, the current pesticide register for Chile reveals that three products based on the sodium tribromophenol salt are approved for use as a fungicide treatment (two manufacturers in Chile and one in Brazil). PBP has been reportedly used as a chemical intermediate for pentabromophenoxy compounds (HSDB, 2003). It has also been reported to have been used as a molluscicide (Clayton and Clayton, 1993). PBP and 2,4-DBP have been used as a reactive intermediate in an epoxy-phenolic polymer. It has been reported that bromophenols can exhibit antimicrobial activity and are possibly of antiseptic importance for wound healing in bottom-living species (Sheikh and Djerassi, 1975). Bromophenols have very low sensory threshold values and have been found to cause “disinfectant” taints at nanogram per kilogram levels in fish products (Whitfield et al., 1988).

4-Nitrophenol (4-NP) is one of the common and important mono-nitrophenols, widely used for industrial production, especially in the synthesis of the aspirin, acetaminophen, paracetamol and manufacture of phosphoorganic pesticides such as methyl parathion and ethyl parathion (HSDB, 1989). It is reduced to 4-aminophenol, then acetylated with acetic anhydride (Ellis, 2002) and some of the amino-phenols are used as dyes and photographic developers. In addition, 4-nitrophenol is used as the precursor for the

preparation of phenetidine and acetophenetidine, indicators, and raw materials for fungicides. Trinitrophenol, for example, is used as a dye manufacture, leather tanning and as an explosive (HSDB, 1989). Further, in peptide synthesis, carboxylate ester derivatives of 4-nitrophenol may serve as activated components for construction of amide moieties. Small amounts of 4-nitrophenol are used as a laboratory reagent (e.g., phosphatase and carboxyesterase determinations) and as a fungicide in military footwear.

2.2.2 Quantity Production

The annual industrial production of chlorophenols was estimated to be 0.29×10^6 metric tonnes (Stuart and Woods, 1998; Tirola et al., 2002). The combined global production of all chlorophenols approached 200 million kilograms. More than half consisted of chlorophenols other than pentachlorophenol (PCP), with 2,4-DCP, 2,4,5-TCP and 2,3,4,6-TCP predominating (WHO, 1989). In another study Ullmann (1985) reported the annual production of chlorophenols was estimated to be 100,000 tonnes heavy chlorophenols (4-5 chlorine atoms), 35-40,000 tonnes light chlorophenols (1-3 chlorine atoms).

2,4,6-TBP is by far the most widely produced brominated phenol. The production volume of 2,4,6-TBP was estimated at approximately 2500 tonnes per year in Japan and 9500 tonnes/year worldwide in 2001 (IUCLID, 2003). In another report the global consumption of bromophenols is about 145 thousand tonnes per year (in 2003; www.bsef.com).

The annual 4-NP production capacity in USA is of 4-NP was 46 million pounds (SRI, 1989). The U.S. demand for 4-nitrophenol, including exports, was 23 million pounds in 1987 and the projected demand is 25 million pounds in 1991. According to TR189 (TRI, 1989), between 1 and 9 million pounds of 2-nitrophenol was produced in the United States in 1989.

2.2.3 Distribution

Chlorophenols are observed as by-products of chlorine bleaching in the pulp and paper industry (Annachhatre and Gheewala, 1996; Tartakovsky et al., 2001). PCP is used as a dip treatment in freshly sawn lumber to prevent sap staining and blue-staining by fungi.

This process usually occurs in open-air basins and can cause pollution of soil and water in the area (Salkinoja-Salonen, 1990). The saw mills were in operation for 30-40 years and all soils were contaminated with chlorophenols. Salkinoja Salonen (1990) reported chlorophenol (CP) concentrations in soil of a saw mill to be 10,000 mg CPkg⁻¹ soil. Due to leaching of chlorophenols through soil permits PCP to enter water systems thus surface waters, ground waters and lake waters in the area of saw mills were also contaminated with the same chlorophenols used in wood preservatives (Valo et al., 1984; Trapido et al., 2000). Table 2.2 presented more detailed the environmental contamination level of 4-CP, 4-BP and 4-NP estimated at different location of various countries. The natural contamination of brominated organic chemicals is most abundant and diverse in the marine environment, where the precursors for their biosynthesis are readily available. Mono-, di-, and tribrominated phenols, in particular, are excreted by marine organisms such as algae, polychaetes, and hemichordates (Whitfield et al., 1999; Flodin and Whitfield, 2000; Ahn et al., 2003; Vetter and Janussen, 2005). Marine sponges are natural sources of brominated organic compounds, including bromoindoles, bromophenols, and bromopyrroles, which may comprise up to 12% of the sponge dry weight (Ahn et al., 2003). Concentrations of 2,4,6-TBP were measured in green macro algae (10–1600 µg/kg), bryozoa (24 and 27 µg/kg wet weight) and hydroid (29 µg/kg wet weight), where as 2,4 DBP in polychaete annelids ranges from 10- 600 µg/kg (Goerke and Weber, 1991; Whitfield et al., 1992; Flodin et al., 1999). In biota likely to form part of human diets, edible portions contain mean 2,4,6-TBP concentrations up to 198 and 2360 µg/kg dry weight in molluscs and crustaceans, respectively, and up to 39 µg/kg dry weight in marine fish (Whitfield et al., 1992; WHO, 2005). Brominated phenols can be formed from the biodegradation of other pollutants, such as brominated benzenes and some brominated diphenyl ethers (Bergman, 1990). Further, brominated anisoles of both biological and anthropogenic origin can be demethylated to some extent under anaerobic conditions to the corresponding brominated phenols. The production and use of 2,4,6-TBP as a reactive flame retardant intermediate or as a wood preservative may result in its release to the environment through various waste streams (HSDB, 2003). 2-BP, 2,4-DBP, 2,6-DBP, and 2,4,6-TBP have all been identified in vehicle emissions of leaded petrol (Müller and Buser, 1986). The raw flue gas from a hazardous waste incinerator fed brominated waste, municipal waste, or peat contained 2,4,6-TBP (Öberg et al., 1987).

Table 2.2 Environmental contamination level of substituted phenols estimated at different location of various countries.

Pollutants	Quantity	Collection site	Remarks	References
Chlorophenol	1000-10,000 mg l ⁻¹	Netherlands (surface waters)	PCP is predominate	Wegman & Hofstee (1979)
Chlorophenols	200-4600 mg kg ⁻¹ dry weight sediment	Netherlands (Sediments of rivers)		Wegman & van der Broek (1983)
Chlorophenols	2,4-DCP at 7.2 - 52.6, PCP at 0.2 - 2.04 mg kg ⁻¹ dry weight	United Kingdom (Sewage sludges)	2,4-DCP is predominate	Wild et al. 1993
Bromophenol	each at 0.4 µg litre ⁻¹	USA (Power plant drawing water from Arkansas River)	Dibromophenols and 2,4,6-TBP,	Grove et al. 1985
Bromophenol	40.3 µg litre ⁻¹	India (River water)	2, 6 DBP	Nomani et al. 1996
Bromophenol	0.9-36 µg litre ⁻¹	Japan River Osaka, Prefecture	2,4,6 TBP	Watanabe et al. 1985
Bromophenol	26-3690 µg/kg	France Rhone Estuarine	2,4,6 TBP	Tolosa et al. 1991
Bromophenol	4-55.6 µg kg ⁻¹	Hong kong	4-BP	Chung et al. 2003
Bromophenol	500-7000 µg kg ⁻¹	Norwegian Sea	2,4,6 TBP	Jensen et al. 1992
Nitrophenol	10 to 17 x 10 ³ mg l ⁻¹	Industrial effluent	4-NP	Suidan et al. 1983, Tsai & Folsom 1982, Luthy et al. 1983

Bromophenols can be formed during the chlorination of natural water and wastewater containing phenol and bromide ions (Sweetman and Simmons, 1980; Watanabe et al., 1984). In power plants using seawater as a source of cooling water, Bean et al. (1983) identified monobrominated phenols, 2,4-DBP, 2,6-DBP, and 2,4,6-TBP. The highest concentration reported was 0.15 µg/litre for 2,4,6-TBP. The formation of both 2-BP and 4-BP has been demonstrated during the treatment of final effluent using peracetic acid

(Booth and Lester, 1995). 4-Bromophenol was identified in automotive emissions in the range of 0.1 to 0.3 $\mu\text{g cm}^{-3}$ (Muller and Buser, 1986). 4-Bromophenol was identified in industrial wastewater samples from photographic industries at 187 $\text{ng } \mu\text{L}^{-1}$ (Bursey and Pellizzari et al. 1982)

4-NP concentration detected in industrial effluents is quite high in the range of 10- 17 $\times 10^3 \text{ mg l}^{-1}$ (Tsai and Folsom, 1982; Luthy et al., 1983; Suidan et al., 1983). 4-NP is also formed in aqueous matrices during pesticide formulation, distribution and field applications (Tomei et al., 2003). In addition, in Japan 4-NP was detected in rain water through the photochemical reaction between benzene and nitrogen monoxide in the atmosphere (EPA, 1980). It is also the intermediate product of some organophosphorus pesticides and nitroaromatic herbicide degradation (Spain and Gibson, 1991; Liu et al., 2007). The incidence of these substituted phenol pollution stresses the necessity of finding methods to remove low-level of these phenolics from the environment

2.3 Toxicity of Substituted Phenol

Substituted phenols are toxic to individual cells, including bacteria, as substituted phenols uncouple mitochondrial oxidative phosphorylation and make cell membranes permeable to protons, resulting in dissipation of transmembrane pH gradients and electrical potentials (McLaughlin and Dilger, 1980; Terada, 1990; Escher et al., 1996). The effect of the uncoupling activity of substituted phenols is that these compounds are lethal to microorganisms over a certain concentration level, and this level varies with both the phenol and microorganism. Furthermore, the extent of toxicity depends upon several factors such as pH, degree of substitution to the aromatic ring and its positions. Under acidic conditions, substituted phenols exist primarily in the toxic molecular (undissociated) form, while at higher pH, the less toxic dissociated form is predominant. The type of the substituents on the phenol will affect the dissociation constant i.e pKa (pH at which an acid compound is 50% dissociated) of the compound. For example, unsubstituted phenol has a pKa of 10.0, whereas it is 9.3 for 4-chlorophenol and as low as 7.18 for 4-nitrophenol. Furthermore, the higher toxicity of the more highly halogenated congeners can be ascribed to the change in lipophilicity nature of the compound which leads to a greater potential for uptake into the

organism. To further complicate matters, phenols have also been found to form dimers which take part in this uncoupling cycle (Escher et al., 1996). Ortho-substituted congeners are generally of lower toxicity than the meta and para substituted compounds, as the close proximity of the ortho-substituted chlorine to the OH group on the molecule appears to 'shield' the OH, which apparently interacts with the active site in aquatic organisms, causing the observed toxic effects (Grimwood and Mascarenhas, 1997). There are several literature reports on toxicity of substituted phenol on microbial cell, as the aromatic ring with its shared resonance electrons is a very stable structure and thus resistant to enzymatic attack. Halogen substituents on the aromatic ring stabilizes the structure further, as the halogens create a steric hindrance to enzymes and also have an electron withdrawing effect (van den Berg, 1990; Copley, 1997; Uberoi and Bhattacharya, 1997).

The exposure of humans to phenolic compounds becomes apparent from the results of a recent study, where some 50 brominated and chlorinated phenols were found in plasma from Swedish blood donors (Hovander et al., 2002). Similarly in another report the mean 2,4,6-TBP concentrations ranging from 0.08 to 26 μgkg^{-1} lipid weight were found in serum samples collected in Norway from 40- to 50-year-old males between 1977 and 1999 and from eight groups of people of differing age and gender during 1998 (WHO, 2005). PCP also absorbs into mammalian skin from soil which is clearly evident that a Rhesus monkey absorbed 24.4% of 17 mg l^{-1} applied PCP from soil (Wester et al., 1993). In mammals, acute exposure toxic doses of chlorophenols leads to elevated body temperature, increased respiratory rate, elevated blood pressure, hyperglycemia and cardiovascular stress, convulsions, coma and finally death (Crosby, 1981), and chronic exposure toxic doses may result in damage to the inner organs (primarily liver) and the bone marrow. Similarly exposure to bromophenols is suspected of having multiple adverse effects on living organisms (de Wit, 2000; Howe et al., 2005). Furthermore, van den Berg (1990) and Meerts et al. (2000) reported exposure of halogenated phenols causes serious endocrine-disrupting effects by competition binding to human transthyretin (one of the thyroid hormone binding transport proteins in plasma of vertebrates) *in vitro* compared with thyroxine (7.1-fold stronger than the natural thyroxine ligand). The investigators suggested that this might have effects on thyroid hormone homeostasis *in vivo*. PCP is a suspected carcinogen, teratogen and is highly embryotoxic (Crosby 1981). Grimwood and Mascarenhas (1997) reviewed the

toxicity data of mono and dichlorophenols on the aquatic life and reported L(E)C50 data ranging from 0.6-19.5, 2.55-29.7 and 5-7 mg l⁻¹ for algae, crustaceans and fish, respectively, indicating moderate to high acute toxicity. Kuiper and Hanstevit (1984) reported long-term exposure of 4-CP and 2,4-DCP concentration at 1.0 mg l⁻¹ was found to cause severe inhibition of growth and biomass of natural phytoplankton communities (mixed species). 2-Bromophenol at doses greater than 1.6 mmolkg⁻¹ caused severe renal necrosis in non-induced rats (Reid, 1973; Rush et al., 1984). The nature of the nephrotoxic metabolite of *ortho*-bromophenol is not known, but an intermediate may be generated in the liver and transported to the kidney. Similarly *p*-bromophenol may uncouple the mitochondrial oxidative phosphorylation causing liver damage acting synergistically with or even independently of other factors such as oxidative stress and lipid peroxidation (Maellaro et al., 1990). The oral rat LD50 value of bromophenol is 1486 mgkg⁻¹ body weight (Fujishima and Fujiwara, 1999). Signs of toxicity during high acute oral exposure of rats to 2,4,6-TBP included hypoactivity, salivation, decreased motor activity, nasal discharge, lacrimation, decreased motor activity, tremors, prostration, chronic convulsions, and death (IRDC, 1974c; Fujishima and Fujiwara, 1999).

Similarly exposure to nitro-aromatic compounds (NACs) particularly nitro-phenol, nitro-benzene etc is suspected to cause multiple disorder in human and animal models. Several literature reports are available on toxicity of these nitro-aromatic compounds (NACs) on human health such as mutagenicity (Mori et al., 1986), immunotoxicity (Burns et al., 1994), reproductive toxicity (Dunnick et al., 1994), and urinary tract tumors (Bruning et al., 1999). Similarly in an animal study chronic exposure of 4-NP by rat for two weeks developed a blood disorder which reduces the ability of the blood to carry oxygen to tissues and organs with similar effects was also observed in human body (U.S. Public Health Service, 1992). Experimental studies have shown that 4-nitrophenol is more harmful than 2-nitrophenol in animals (U.S. Public Health Service, 1992). Weihua et al. (2002) found that the effective concentration affecting 50% (EC50) of the activated sludge was 10.70 mg l⁻¹ for 4-NP. Razo-Flores et al. (1997) reported that nitro-aromatics like 4-NP and NB (nitrobenzene) were clearly very toxic to methanogens, with IC50 values generally ranging from 4.91 to 9.96 mg l⁻¹.

2.4 Permissible Limit

Due the above discussed serious toxic effect of these substituted phenols on human, animal and microbial cells, chlorophenol bromophenol and nitrophenol are listed as priority pollutant by U.S. EPA, which recommended their concentrations in natural water to be below $1\mu\text{g l}^{-1}$ and 10 ng l^{-1} for 4-CP and 4-NP respectively ([Federal Register, 1984](#); [Wild et al., 1993](#); [Erba et al., 2007](#)). In Indian standard, no report is found available on permissible limit of these substituted phenols however, they set a maximum limit for phenolic substance as phenol at range of $1\text{-}2\text{ }\mu\text{g l}^{-1}$ ([Sprenger et al., 2008](#)). Maximum daily effluent concentration of total toxic organics (including nitrophenols) was set to 2.13 mg l^{-1} of wastewater for electroplating plants that discharge less than 10,000 gallons of wastewater per day ([EPA, 1988a](#)). Similarly, the limitation of total toxic organics pollutants in daily effluent discharge from electrical and electronic industries is set at 1.37 mg l^{-1} ([EPA, 1988a](#)).

2.5 Major Treatment Techniques for Phenolic Wastewater

2.5.1 Adsorption

Carbon adsorption is an advanced wastewater treatment method used for the removal of recalcitrant organic compounds as well as residual inorganic compounds such as nitrates, sulfides and heavy metals. This is a separation method in which the contaminants, dissolved in water phase, are transferred to the surface of active carbon, the most commonly used adsorbent, where it is accumulated for subsequent extraction or destruction of the contaminants. The application of adsorption process is not only limited to control of color and odors, removal of organic compounds but also trihalomethanes precursors, removal of chlorine *etc.* Numerous literatures exist reported regarding the treatment of phenolics on activated carbon ([Calleja et al., 1993](#); [Dargaville et al., 1996](#); [Viraraghavan and Alfaro, 1998](#); [Przepiórski, 2006](#); [Vázquez et al., 2007](#)). More particularly chlorophenol and nitrophenol were found to be well adsorbable compounds onto activated carbon, but only in low concentrations ([Chern and Chien, et al., 2003](#); [Hamdaoui and Naffrechoux, 2009](#)).

2.5.2 Air Stripping

Air stripping involves the transfer of volatile organics from liquid phase to the air phase by greatly increasing the air/water contact area. Typical aeration methods include packed towers, diffusers, trays and spray aeration. It is a well established and more widely understood technology than chemical oxidation (Metcalf and Eddy, 2003). If air emissions are not required to be regulated, air stripping is by far the simplest and cheapest solution for the removal of volatile compounds from water. Air stripping has been used in the treatment of trichloroethylene (TCE), dichloromethane (DCM), 1,2-dichloroethylene (DCE), 1,2-dichloroethane (DCA), chlorobenzene (Cl- Bz), and dichloroethyl ether (DCEE). The results showed that they could be removed easily from water solutions except DCEE (Li et al., 2000). However, treatment of phenolic effluents by means of air stripping has not been reported due to its low volatile nature.

2.5.3 Electrochemical Oxidation

The use of electrochemical oxidation for the destruction of organic compounds in aqueous solutions has been tried on bench and pilot plant scale operation (Boudenne et al., 1996; Brillas et al., 1998). Several authors also reported the use of electrochemical oxidation for the destruction of substituted phenols (Borras et al., 2003; Quiroz et al., 2005; Wang et al., 2006; Jiang-tao et al., 2007), but is not used commercially because of its high operating cost. One of the main advantages of the electrochemical processes is that electrons are generated or assimilated by the electrodes. Thus it supplies a clean reactant and does not increase the number of chemical molecules involved in the process. The electrochemical oxidation of organic compounds is thermodynamically favored against the competitive reaction of oxygen production by oxidation of water. However, the kinetics of oxidation of water is much faster than the kinetics of oxidation of the organic compounds, among other reasons because of its higher concentration. The mechanism of the electrochemical processes involves three stages: electrocoagulation, electrofloatation and electrooxidation, and these are depicted by Eqns. (2.1) – (2.3) (Boudenne et al., 1996; Brillas et al., 1998):



Nevertheless, it suffers from certain disadvantages, like:

- Expensive in comparison with other processes and the mechanism in water is rather complex.
- Moreover, the effluent needs to be a conductor and therefore, a salt should be added in the effluent in case it does not have good conductivity.

2.5.4 Advanced Oxidation Processes

The phrase *advanced oxidation processes* (AOP) refers specifically to processes in which oxidation of organic contaminants occurs primarily through reactions with hydroxyl radicals (Glaze et al., 1995). It involves two stages of oxidation: (1) the formation of strong oxidants (e.g., hydroxyl radicals) and (2) the reaction of these oxidants with organic contaminants in water (Alnaizy and Akgerman, 2000). In water treatment applications, AOPs usually refer to a specific subset of processes that involve O_3 , H_2O_2 , and/or UV light. However, often AOPs are also referred to a more general group of processes that also involve semiconductor catalysis, cavitation, E-beam irradiation, and Fenton's reaction (Fox and Dulay, 1993; Legrini et al., 1993). All these processes can produce hydroxyl radicals, which can react with and destroy a wide range of organic contaminants, including phenolics. Although many of the processes noted above have different mechanisms for destroying organic contaminants, in general, the effectiveness of an AOP is proportional to its ability to generate hydroxyl radicals (Fox and Dulay, 1993; Legrini et al., 1993).

2.5.4.1 Ozonation / UV

The O_3 system is one of the AOP for the destruction of organic compounds in wastewater. Basically, aqueous systems saturated with ozone are irradiated with UV light of 253.7 nm. The extinction coefficient of O_3 at 253.7 nm is 3300 Lmolcm^{-1} , much higher than

that of H_2O_2 ($18.6 \text{ L.molcm}^{-1}$). The decay rate of ozone is about a factor of 1000 higher than that of H_2O_2 (Guittonneau et al., 1991). The AOP with UV radiation and ozone is initiated by the photolysis of ozone. The photodecomposition of ozone leads to two hydroxyl radicals, which do not act as they recombine producing hydrogen peroxide, as shown in the following Eqns. (2.4) and (2.5) (Peyton and Glaze, 1988):



Ozonization treatment was reported in many literatures on decolourization of direct dye in textile wastewater and cork processing wastewaters treatment etc (Minhalma et al., 2006; Turhan and Zuhail, 2009). Fernando et al. (2005) reported that the degradation rate constant of photocatalytic ozonation process was higher compared with TiO_2 photocatalysis for phenol, p-chlorophenol, and p-nitrophenol. Photocatalytic ozonation process exhibited the highest mineralization efficiency of phenols. Ozonation and $\text{O}_3/\text{H}_2\text{O}_2$ have been employed for tertiary treatment of pulp and paper mill wastewaters (Salokannel et al., 2007)

2.5.4.2 Ultrasonication

Implosion of cavity bubbles in sonicated water containing dissolved gases results in formation of hydrogen and hydroxyl radicals by fragmentation of water molecules. These radicals in turn combine and generate other oxidative species such as peroxy and super oxide radicals ($\cdot\text{OH}$) as well as hydrogen peroxide; the quantities of each depend on the ambient conditions and the operating parameters. Such $\cdot\text{OH}$ radicals are used for the degradation of the organic compounds (Kidak and Ince, 2006). Several literatures reported the uses of ultrasonication methods for treatment of phenolic compound such as 4-CP, poly aromatic compounds and bisphenol (Hamdaoui et al., 2008; Kwon et al., 2009; Mohapatra et al., 2011).

2.5.4.3 Solar Photocatalytic Oxidation

In the past years, there have been a number of studies and reviews about this process

(Fox and Dulay., 1993; Legrini et al., 1993; Hoffmann et al., 1995; Bahnemann., 2004; Herrmann et al., 2007). Photocatalytic oxidation is based on the use of UV light and a semiconductor. Many catalysts have been tested, although titanium dioxide (TiO_2) in the anatase form seems to possess the most interesting features, such as high stability, good performance and low cost (Fox and Dulay, 1993; Legrini et al., 1993; Hoffmann et al., 1995; Bahnemann., 2004). Matthews (1990) reported that more than 90% of nitro benzene (NB) mineralization was achieved with TiO_2 and sunlight. Minero et al. (1994) studied the photocatalytic degradation of NB on TiO_2 and ZnO and reported its complete mineralization with TiO_2 . Titanium dioxide has become the most used photocatalyst, because it is easily available, chemically robust and durable. It can be used to degrade, *via* photocatalysis, a wide range of organic compounds (Leyva et al., 1998; Robert and Malato, 2002; Hincapié et al., 2005; Herrmann et al., 2007). Photocatalytic degradation of phenolic compounds by employing Degussa P-25® in presence of sunlight has been successfully studied by many researchers (Minero et al., 1994; Curcó et al., 1996). Vione et al. (2005) studied the degradation of phenol and benzoic acid in presence of sunlight with a commercial TiO_2 photocatalyst Wackherr's "Oxyde de titane standard". In his study he observed that the Wackherr's TiO_2 showed a better photocatalytic activity over Degussa P-25® in degrading phenol and benzoic acid. Selvam et al. (2007) indicated that complete degradation of 4-fluorophenol with P25 (photocatalyst) was achieved in 60 min while complete degradation occurred in 90 min in the presence of ZnO. In suspended form, Lachheb et al. (2008) reported that, in comparison to PC 500, P25 was more efficient for the degradation of phenols and poly nitrophenols (4-NP, 2,4-DNP, 2,4,6-TNP) in the presence of either artificial or solar light. The photocatalytic degradation of the tested compounds was shown in the following order: 2,4,6-TNP>2,4-DNP>4-NP>phenol. For PC 500 supported on Ahstrom paper 1048, the order is different: phenol<4-NP<2,4-DNP<2,4,6-TNP. The difference was related to the variation in adsorption behavior. Venkatachalam et al. (2007a) compared the mineralization efficiency of 4-CP using the lamps of wavelength 365 and 254 nm over TiO_2 .

2.5.5 Pros and Cons of the Above Wastewater Treatment Techniques

In adsorption, the pollutant in the wastewater is selectively transferred into the solid phase

(adsorbent) instead of eliminating it from the wastewater. It once again produces a large amount of solid waste, which further requires a safe disposal. Electrochemical oxidation is an uneconomical process with lack of feasibility towards commercialization. The advanced oxidation processes has many drawbacks as listed below:

- No full scale application exists
- Supplement oxidant such as O_3 and H_2O_2 are required to achieve a maximum removal efficiency, which results in increased cost
- Pretreatment is required to avoid fouling of semiconductor catalysts
- If semiconductor catalyst is used as slurry then separation is required
- Rigorous studies are needed to determine the optimum dosage
- Complete degradation of organics is not achieved
- Large amount of toxic intermediates are produced

2.6 Biological Treatment Processes Involving Microbes

Bioremediation is defined as the elimination, attenuation or transformation of polluting or contaminating substances by the use of biological processes (Lynch and Moffat, 2005). Among the various remediation technologies available for treating monoaromatic contaminated water, biological methods or bioremediation processes appear to be a potentially economical, energy efficient and environmentally friendly approach (Zechendorf, 1999; Vidali, 2001; Shim et al., 2002). Further, biological treatment is more attractive method than that of conventional one because of its high potential to degrade phenolics completely that produces innocuous end products and generates minimum secondary waste (Goudar et al., 2000). Biological treatment, generally by means of activated sludge (Wiesmann and Putnaerglis, 1986; Givens et al., 1991) and in adequate conditions has unquestionable advantages for the destruction of organic compounds (Wu et al., 1994). However, as any technology, bioremediation has its own limitations, such as not all materials can be treated, conditions at the treatment site may be restrictive or require costly tailor-made applications, and the time required for treatment may be too long for the method to be useful (Hart, 1996; Zechendorf, 1999). For instance many organic pollutants cannot effectively be eliminated by activated sludge process for effluents with phenols, nitro-aromatic, ether,

aliphatic compounds and textile wastewaters. The restrictions are due to the high toxicity inherent in these wastes. Bioremediation using bacteria takes advantage among other microorganisms to degrade organic compounds due to their ability to evolve degradation pathways for pollutants that are foreign (xenobiotic) in the environment. Some compounds are easily degraded by microorganisms, whereas others persist in nature these are called recalcitrant. In general, xenobiotics, especially halocarbons, tend to be recalcitrant. There are two kinds of microbial processes in the biological treatment of organic compounds: aerobic and anaerobic (Eckenfelder et al., 1989; Wang, 1992).

2.6.1 Anaerobic biodegradation

Chlorinated phenols are readily metabolized by bacteria under anaerobic conditions. Anaerobic conditions favor the reductive dechlorination which results from the displacement of chloro-groups by hydrogen atoms. Reductive dechlorination generally requires the input of electron donating substrates. For example, ferrous iron (Fe^{2+}) recovered from ferric iron (Fe^{3+}) reduction could be completely accounted for by the chlorophenol oxidation, providing strong evidence that the chlorophenol degradation was linked to iron reduction. Only recently, Bae et al. (2002) demonstrated that 2-chlorophenol (2-CP) degradation has been linked to denitrification in an activated sludge. The consumption of nitrate was equivalent to the expected amount based on the stoichiometry of 2-CP denitrification. In the absence of nitrate, 2-CP was not degraded. Enrichment cultures from freshwater sediments degrading 2-CP or 3-CP under methanogenic conditions converted the chlorophenols to methane and CO_2 with temporal accumulation of phenol and benzoate as intermediates (Genthner et al., 1989). Among the most accepted route of anaerobic phenol degradation in which phenol is carboxylated in the para position and subsequently dehydroxylated to yield benzoate as an intermediate product (Londry and Fedorak, 1992; Bisaillon et al., 1993; Letourneau et al., 1995). In fact, 2-CP is sometimes carboxylated and dehydroxylated prior to reductive dechlorination, resulting in the formation of 3-chlorobenzoate as an intermediate (Bisaillon et al., 1993; Becker et al., 1999; Ennik-Maarsen, 1999). All isomers of monochlorinated phenol were degraded by river sediments of the upper Hudson River in New York State under both methanogenic and sulphate reducing conditions (Hagblom et al., 1993). From this

biotransformation process phenol, benzoate and 3-chlorobenzoate were observed as intermediates. Lake sediment samples from the South eastern U.S. were also shown to anaerobically degrade 2,4-DCP, with 4-CP occurring as a major intermediate in the process (Kohring et al., 1989; Zhang and Wiegel, 1990). Similarly, several existing literatures reported the incomplete mineralization and accumulation of their respective toxic metabolic intermediate viz, aminophenols in anaerobic degradation of nitrophenols using methanogenic consortia (Gorontzy et al., 1993; O'Connor and Young, 1993; Donlon et al., 1996; Karim and Gupta, 2003). Further, some anaerobic biodegradation studies of nitrophenols (NPs) have shown that the compounds are not easily biodegraded and are inhibitory to methanogenic microorganisms at high concentration (Shelton and Tiedje, 1984; Battersby and Wilson, 1989; Oren et al., 1991; Uberoi and Bhattacharya, 1997). Similarly, other studies showed that 16 mg l⁻¹ 3-NP and 8 mg l⁻¹ 2,4-NP caused obvious inhibition to methanogenesis (Donlon et al., 1995; Haghighi-Poden and Bhattacharya, 1996). Moreover, substituted phenols biodegradation by anaerobic bacteria species is not a promising method due the following limitations: (i) they could not use these phenolics as a sole source of carbon, needs external carbon sources to serve as electron donor for growth and biosynthesis (Delia et al., 2005). (ii) anaerobic biodegradation of halogenated phenol occurs by reductive dechlorination, however reductive dechlorination are partially or completely inhibited by the presence of other electron acceptors such as sulfate, sulfite and thiosulfite, nitrate, O₂ and CO₂ etc which are usually found in most of the real industrial wastewater accompanied with other toxicant (Madsen and Aamand, 1991; Mohn and Tiedje, 1992; VanBriesen et al., 2004), (iii) simultaneous CPs degradation (reductive dehalogenation) and methane production is not favourable to anaerobic bacteria (Saia et al., 2007) as hydrogen demand increases and consequently occurrence of strong competition for hydrogen takes place between the two processes, (iv) incomplete mineralization usually occurs, as a result of which intermediates of lower substituted phenols such as tetrachlorophenols, trichlorophenols, tribromophenol and other recalcitrant compounds are produced. Therefore, substituted phenols biodegradation by aerobic microbial process is often found most promising and are more popular because of their efficiency and operational simplicity (Ruiz-Ordez et al., 2001).

2.6.2 Biodegradation by Yeast and Fungi

A wide variety of fungi from different taxonomic groups (*Ascomycetes*, *Basidiomycetes*, *Zygomycetes*, *Deuteromycetes*) were shown to have the capacity to remove substituted phenols (Fernando et al., 1990; Seiglemurandi et al., 1991; Benoitguyod et al., 1994). Phenol-degrading strains of the fungus *Penicillium* and a phenol-degrading yeast *Candida maltosa*, oxidized monochlorinated phenols (Polnisch et al., 1992; Hofrichter et al., 1994; Marr et al., 1996). 4-Chlorophenol was converted to 4-chlorocatechol where as 3-chlorophenol was converted to chlorohydroquinone, 4-chlorocatechol and 5-chlorocatechol as well as chloropyrogallol. The 4-chlorocatechol was oxidized and dehalogenated to 4-carboxymethylenebut-2-ene-4-olide; whereas, 3-chlorocatechol was oxidized to 2-chloromuconic acid (Polnisch et al., 1992; Hofrichter et al., 1994). Similarly, the wood-decaying white-rot basidiomycete fungus *Phanerochaete chrysosporium* has been shown to degrade nitroaromatic compounds (Fernando et al., 1990). Lignolytic enzymes from fungus such as LiP and MnP were shown to oxidize PCP to tetrachloro-1,4- benzoquinone (TeCBQ) (Hammel and Tardone, 1988; Reddy and Gold, 2000) or 2,4,6-TCP to 2,6- dichloro-1,4- benzoquinone (TCBQ) (Hammel and Tardone, 1988; Reddy et al., 1998), releasing the chlorines in the para position as chloride. However, substituted phenols biodegradation by anaerobic yeast and fungi is not a promising method due to the following limitations: they could not use these phenolics as a sole source of carbon, needs easy source of carbon for biomass growth such as glucose, peptone, yeast extract (Qiu, et al., 2007), low efficiency and incomplete mineralization of substituted phenols, for example, the brown-rot fungi, *Gloeophyllum striatum* and *Gloeophyllum trabeum*, degraded 2,4-dichlorophenol and pentachlorophenol only up to 54% and 27%, respectively, based on [14C]-labeled substrate converted to $^{14}\text{CO}_2$ (Fahr et al., 1999). Similarly, mineralization of 2,4,6-[14C]-TCP (Reddy et al., 1998), 2,4,5- [14C]-TCP (Joshi and Gold, 1993) and 2,4-[14C]-DCP (Valli and Gold, 1991) to $^{14}\text{CO}_2$ by 58, 61 and 50%, respectively, was observed. In another report, up to 62-70% mineralization of [14C]-PCP to $^{14}\text{CO}_2$ was achieved by the white-rot fungi (Alleman et al., 1995; Aiken and Logan, 1996; Mileski et al., 1988; Lin et al., 1990). Incomplete mineralization and accumulation of toxic metabolic intermediate product are also obtained. For instance, during degradation of chlorinated phenol, by fungal species, chlorinated

hydroquinone intermediates are occasionally subject to methylation, leading to the formation of chlorinated 1,4-dimethoxybenzenes or chlorinated 4- methoxyphenols (Valli and Gold, 1991; Reddy et al., 1998; Reddy and Gold, 2000). The fungal conversion of PCP to recalcitrant toxicant like pentachloroanisole is well established in several literatures (Okeke et al., 1997; Tuomela et al., 1999; Chung et al., 2001; Rigot and Matsumura, 2002). Also the conversion of pentaclorophenol, 3,4- and 2,4-dichlorophenols to their respective non-transformable secondary toxic byproducts such as anisoles has been reported (Mcbain et al., 1995; Coulter et al., 1993; Deschler et al., 1998). Hofrichter et al. (1993) reported a *Penicillium* strain co-metabolically transformed 4-, 3- and 2-nitrophenols, forming nitrocatechols as intermediate product which was found accumulated. There are also several literatures available on inhibition of fungal growth and enzyme system at lower concentration of substituted phenols: Alleman et al. (1992) reported that white-rot fungi failed to grow at PCP concentrations higher than 5 mg l⁻¹. In the case of *P. chrysosporium*, however, the hydroxylamino metabolites directly inhibit lignin peroxidase (Bumpus and Tatarko, 1994; Michels and Gottschalk, 1994). Similarly, in a thorough investigation, Michels and Gottschalk, (1994) have confirmed that trinitoluene (TNT) is a pronounced inhibitor of lignin mineralization by this fungus. Thus it seems, in general, bioremediation of substituted phenols by fungi is not an encouraging technique.

2.6.3 Biodegradation of Phenolics by Bacteria

2.6.3.1 Aerobic bacterial growth on chlorophenols as a sole source of carbon and energy

Bioremediation of phenolics compounds by pure culture aerobic bacteria is one of the most potential methods; however, the presence of aromatic ring with its shared resonance electrons build very stable structure and thus offers resistance to enzymatic attack. Halogen substituents on the aromatic ring stabilizes the structure further, as the halogens create a steric hindrance to enzymes and also have an electron withdrawing effect (van den Berg, 1990; Copley, 1997; Uberoi and Bhattacharya, 1997). Therefore, only selective microorganisms such as *Flavobacterium*, *Moraxella*, *Nocardia*, *Acaligenes*, *Ochrobactrum* sp., *Achromobacter piechaudii* TBPZ, *Trametes versicolor*, *Agaricus augustus*

Pseudomonas, *Rhodococcus* and *Arthrobacter*, can metabolize 4-CP, 4-BP and PNP as a sole source of carbon or/and nitrogen for growth (Spain et al., 1991; Radehaus and Smith, 1992; Bae et al., 1996; Ronen et al., 2005; Bhushan et al., 2000; Ferraroni et al., 2006; Donoso et al., 2008; Yamada et al., 2008). There is extensive evidence that chlorophenols are mineralized by bacteria that utilize the compounds as sole source of carbon and energy. The evidence is based on the stoichiometric release of inorganic chloride (Hollender et al., 2000; Solyanikova and Golovleva, 2004; Yang et al., 2005), the concomitant production of biomass linked to chlorophenol utilization (Rutgers et al., 1997; Banerjee et al., 2001; Kumar et al., 2005) or the conversion of [^{14}C]-labeled chlorophenols to $^{14}\text{CO}_2$ (Apajalahti and Salkinoja-Salonen, 1986; Haggblom et al., 1988b).

The main strategies used to degrade chlorophenols by aerobic bacteria utilizing these compounds as sole source of carbon and energy are discussed briefly as follows. In *Shingomonas chlorophenolica* ATCC 39723 (formerly *Flavobacterium* sp), the hydroxylation is carried out by PCP 4-monooxygenase (PcpB) (Tirola et al., 2002). pcpB monooxygenase requires molecular oxygen (Xun et al., 1992a; Thakur et al., 2002). The tetra-chloro hydroquinone (TeCHQ) formed in the PCP degradation process via hydroxylation by PCP 4-monooxygenase (PcpB) is sequentially dechlorinated in two steps to 2,6-dichloro-1,4-hydroquinone (2,6-DCHQ) by a reductive dehalogenase, known as PcpC (Xun et al., 1992b; Orser et al., 1993). Finally 2,6-DCHQ is oxidized by 2,6-DCHQ 1,2-dioxygenase (PcpA) which requires O_2 and results in the formation 2-chloromaleylacetate as well as the liberation of one of the chloro groups as chloride (Ohtsubo et al., 1999; Xu et al., 1999; Xun et al., 1999). Similarly, the monooxygenase from *Ralstonia eutropha* strain JMP134 (*pJP4*) catalyzes successive dechlorination reactions of 2,4,6-TCP to 2,6-dichlorohydroquinone and then to 6-chlorohydroxyquinol prior to ring cleavage by a hydroxyquinol dioxygenase (Matus et al., 2003). A unique property of the monooxygenase is that the second dechlorination reaction does not require O_2 , instead the dechlorination of 2,6-dichlorohydroquinone is catalyzed by a hydrolytic activity associated with the monooxygenase (Xun and Webster, 2004). Similar monooxygenases are also found in strains of other PCP-degrading aerobic bacteria (Orser and Lange, 1994; Thakur et al., 2002). Therefore, the major enzyme responsible for chlorophenol degradation by aerobic bacteria is PCP-4-monooxygenase (McCarthy et al., 1997) which is active towards various halogenated

phenols like 2,3,5,6 TeCP, 2, 6 TCP and 2,3 DCP, pentafluorophenol, triiodophenol, tribromophenol, dibromophenol (Xun et al., 1992a, and 1992b). The enzyme also catalyzes the release of an amino group as hydroxylamine, nitro group as nitrite, cyano group as cyanide, iodine as iodide from the para position of substituted phenols (Xun et al., 1992b). Therefore PCP-4 monooxygenase of aerobic bacteria play a vital role in substituted phenols as well as several other toxicant biodegradation usually present in industrial wastewaters. The high efficient (CPs) degrading *Pseudomonas* species pcpB gene is shown to be homologous with pcpB gene of actinomycetes named as *Arthrobacter* ATTC 33790 (Ferraroni et al., 2006). Thus actinomycetes are believed to be as potent as aerobic bacteria in substituted phenols biodegradation.

2.6.3.2 Microbial degradation of pollutant mixtures

The ultimate goal of biodegradation research is often application of the process for bioremediation of contaminants in the environment. However, laboratory studies of biodegradation are usually performed with a single pollutant, whereas a polluted site often is contaminated with more than one compound. In addition to substituted phenols being toxic when present as single compound, two differently substituted phenols may have synergistic deleterious effects on microbial cell by uncoupling the mitochondrial oxidative phosphorylation (Escher et al., 2001). This mixture effect is due to formation of a dimer between a phenol and a phenolate ion and is most likely to arise when the two phenols have widely differing pKa-values, which varies from 7.18 -9.3 for 4-CP and 4-NP respectively (Escher et al., 2001). Because of this, mixtures of differently substituted phenols may have a more serious toxic effect than estimated by the effect of each single compound. Some cases, their interaction plays a crucial role on the individual substituted phenol mineralization rate and efficiency. Pollutants that may be present as mixtures are for example BTEX compounds, pesticides (e.g. benzene derivatives, organophosphorous compounds and organometals) and chemicals used for wood preservation such as chlorobenzenes, chlorophenols, bromophenol, nitrophenol and polyaromatic hydrocarbons (PAHs) (Swoboda-Colberg, 1995). Moreover, additional carbon-containing compounds can serve as potential growth substrates and complicate degradation of pollutants. For example, in a soil

environment, carbon sources may become available via hydrolysis of naturally occurring polymeric material such as cellulose, lignin, chitin, proteins, nucleic acids and waxes (Egli, 1995). Numerous studies have shown that mixed growth substrates often affect each other's degradation (Kovárová-Kovar and Egli, 1998). The effects vary both with the microorganism involved and the growth substrates and are therefore difficult to predict. Thus, substrate interactions need to be carefully considered in order to optimize the efficacy of bioremediation. In the following sections, different patterns of microbial growth in mixed substrate system are discussed.

Diauxic growth

In diauxic growth, one growth compound or its metabolites represses the synthesis of the enzymes required for degradation of a second substrate, delaying metabolism of the second substrate until the first is exhausted. Diauxic growth was previously thought to be the norm for degradation of mixed substrates (Kovárová-Kovar and Egli, 1998). The most famous example is the *lac* operon in *Escherichia coli*, which encodes enzymes for lactose metabolism. Expression of the genes in the *lac* operon is repressed in the presence of glucose, and hence glucose is degraded before lactose in a mixture of the two growth compounds. Diauxic is typically accompanied by a biphasic growth curve, where the cell density reaches a plateau when the first compound is exhausted. During this time, the genes for degradation of the second compound are induced and growth eventually recommences. Diauxic growth may also occur in pollutant biodegradation, as the presence of organic acids and carbohydrates has been shown to repress enzymes for catabolism of pollutants. For example, a number of organic acids (such as succinate) and carbohydrates (such as glucose) repress transcription of the genes for phenol degradation in *Pseudomonas putida* H (Müller et al., 1996). In another study, the presence of benzoate completely abolished phenol degradation by *Ralstonia eutropha* (Ampe et al., 1998). Moreover, the presence of two or more pollutants may also cause diauxic growth. This was seen during growth of *Comamonas testosteroni* JH5 on a mixture of 4-methylphenol and 4-chlorophenol (Hollender et al., 1994), and by *Pseudomonas fluorescens* PC18 on a mixture of phenol and methylphenol (Heinaru et al., 2001).

Simultaneous degradation of substrates

Two compounds that are incompatible at high concentrations are often degraded simultaneously at low concentrations (Kovárová-Kovar and Egli, 1998). This may be an adaptation to life in nutrient-limited environments such as soil, where the available growth substrates are often innumerable of carbon sources, each present at a very low concentration. In fact, carbon starvation itself has been shown to induce expression of a number of catabolic enzymes, presumably so that the cells are ready if these compounds become available (Egli, 1995; Kovárová-Kovar and Egli, 1998). Two compounds may also be degraded simultaneously at high concentrations, and this is often accompanied by an increased maximum specific growth rate as compared to growth on each compound by itself (Kovárová-Kovar and Egli, 1998). Such an increased growth rate may be due to the induction of two separate degradation pathways at the same time, thus increasing the flux of carbon into the central metabolism of the cell. The degradation of two pollutants simultaneously by a single microorganism has been reported in many literatures; for example, during degradation of a mixture of phenol and 4-methylphenol by *Pseudomonas mendocina* PCI (Heinaru et al., 2001) and degradation of toluene and benzene by *Pseudomonas putida* F1 (Reardon et al., 2000).

Rate deceleration

In addition to causing diauxic growth, the presence of several growth substrates may also affect the rate of degradation of the individual compounds. Even if an inhibitory effect does not lead to diauxic growth, degradation of one or several compounds may be decelerated in a mixture. For example, ethanol concentrations over a certain threshold were shown to have a negative effect on the degradation of benzene by *Pseudomonas putida* F1 (Lovanh et al., 2002), and phenanthrene degradation by two *Pseudomonas* species was subject to competitive inhibition by several other PAHs (Stringfellow and Aitken, 1995).

Rate acceleration (Aerobic bacterial co-metabolism)

This enhancement of removal of a pollutant may be due to the greater biomass formed because of the additional carbon source available, or one compound may induce enzymes beneficial for degradation of the second compound (Alexander, 1999). More particularly, primary substrates are expected to induce either dioxygenases or monooxygenases facilitating the co-oxidation of the co-metabolized substituted phenols. However, primary substrates such as sugar, which do not require oxygenases for metabolism, have also been implicated in the co-metabolism of 2-CP (Wang and Loh, 1999), 2,4-DCP (Beltrame et al., 1982) and PCP (Banerji and Bajpai, 1994; Yang et al., 2005). Sugars as primary substrate may support co-metabolism by generating NADH required by oxygenases (Wang and Loh, 1999). The presence of one compound may also enhance the degradation of another, as in the enhanced mineralization of carbazole in groundwater when fluorene was present (Millette et al., 1995). In another study 3-CP degradation by *Alcaligenes sp. A7-2* which was made possible by the addition of phenol (Menke and Rehm, 1992). Similarly, in several studies the analogue substrate like phenol has been utilized by various bacterial strains as the primary substrate to support the co-metabolism of 2-CP (Cobos-Vasconcelos et al., 2006; Loh and Wu, 2006), 4-CP (Kim et al., 2002; Cobos-Vasconcelos et al., 2006; Loh and Wu, 2006) and 2,4-DCP (Cobos-Vasconcelos et al., 2006). 2,4,6-Trichlorophenol served as a growth substrate of either *Azobacter sp.*, *Streptomyces rochei* or *Pseudomonas pickettii* to support the co-oxidation of a large number of other chlorophenols (Li et al., 1991; Golovleva et al., 1992; Kiyohara et al., 1992). Benzoate-induced cells of *Rhodococcus erythropolis M1* co-metabolized 2-CP, 4-CP and 2,4-DCP (Goswami et al., 2002). Cassidy et al. (1999) observed degradation of nitrophenols by *Sphingomonas sp. strain UG30* after induction of hydroquinone dioxygenase by pentachlorophenol. Pentachlorophenol-induced enzyme transformed benzenetriol formed during p-nitrophenol degradation. On the basis of the structural analogue the other phenolic compounds are frequently used as growth substrate for mononitrophenols degradation (Cho et al., 2000; Wan et al., 2007). Similarly, there are several literature reports on co-metabolic transformation of nitroaromatics using benzoic acids, peptone, yeast extract, urea etc (Qiu et al., 2007; Danuta et al., 2011). These results

suggest the feasibility of the use of chemicals such as phenols, benzoic acid, peptone, yeast extract, and other chlorophenols as co-substrates in field decontamination processes.

2.6.4 Biodegradation of substituted phenols by actinomycetes

The most commonly reported substituted phenols degrading actinomycetes (high GC content aerobic bacteria) species belong to the group of *Flavobacterium*, *Moraxella*, *Nocardia*, *Acaligenes*, *Pseudomonas*, *Rhodococcus* and *Arthrobacter*. Furthermore, these substituted phenol degrading actinomycetes are aerobic and are more efficient at degrading toxic compounds as they grow faster than anaerobe and usually achieve complete mineralization of toxic organic compounds rather than transformation as in case of anaerobic bacteria (Kim et al., 2002). The salient feature of the actinomycetes is due to their ability to synthesize wide variety of enzymes mainly monooxygenase, dehalogenase, catechol 1,2 dioxygenase, 1,2 hydroquinol dioxygenase and membrane bound enzyme like cytochrome P₄₅₀ etc which are responsible for the substituted phenols biodegradation. Actinomycetes produce both membrane bound and cytoplasmic enzymes that enhance biodegradation rate of substituted phenols as compared to low guanine and cytosine (DNA nucleotide bases, GC) content aerobic and anaerobic bacteria which usually do not produce membrane bound enzymes. Actinomycetes like *Rhodococcus*, *Mycobacterium* species may employ the cytochrome P₄₅₀ for PCP dechlorination and hydroxylation rather than only PcpB monooxygenase enzymes used by aerobic bacteria of *Sphingomonads* species (Uotila et al., 1991). Although the rate of growth of actinomycetes is slow single inoculums continue to mineralize substituted phenols for several months (Radehaus and Schmidt, 1992). In actinomycetes, the number of residue in the active cleft of the enzymes believed to be responsible for substrate selection are altered mainly at leu80, Asp83, Val 107 aminoacids positions, subsequently posses broad specificity nature and can interact with wide different toxic substrate (Ferraroni et al., 2006) usually present in industrial wastewater. There are several reports on single actinomycetes strains capable of degrading wide different types of toxicant in wastewater containing chlorophenols. *Rhodococcus erythropolis* CCM 2595 (ATCC 11048) is shown to degrade phenol, pyrocatechol, resorcinol, p-nitrophenol, p-chlorophenol, hydroquinone and hydroxybenzoate, respectively, or as respective binary

mixtures with phenol (Čejková et al., 2005). *Rhodococcus chlorophenolicus* PCP-1 and other *Rhodococcus* sp. exhibited the ability to degrade various chlorinated phenol, chlorinated guaiacols, chlorinated syringols and o-methylate chlorinated p-hydroquinones (Hagglblom et al., 1988a, 1988b, 1988c). *Arthrobacter chlorophenolicus* A6 could also degrade other substituted phenols (4-nitro-, 4-bromo-, 4-iodo-, and 4-floro-, phenol) via the hydroquinone pathway (Wasteberg et al., 2000). It has been observed that some actinomycetes stains are capable to degrade chlorophenol both under aerobic and anaerobic condition; *R. chlorophenolicus* PCP-1 can degrade PCP anaerobically in the presence of iodosobenzene at a rate similar to aerobic degradation of PCP (Uotila et al., 1992). The growth rate of *Pseudomonas* sp. was found to be decreased from 0.09 h^{-1} to 0.05 h^{-1} when the PCP concentration was increased from 40 mg l^{-1} to 150 mg l^{-1} (Radehaus and Schmidt, 1992), whereas, in actinomycetes which develop hyphae and form large micro colonies, due to which inner cells are protected, shows favourable for degradation and can tolerate even up to higher concentration of substituted phenols. Thus actinomycetes can be taken as an important tool for substituted phenols biodegradation. However, there are few literature reports on performance of actinomycetes strain on substituted phenol biodegradation. Golovleva et al. (1991–1992) reported that *Streptomyces rochei* 303 immobilized in polycaporamide fibres can degrade combined mixture of 2,4,6-TCP, 2,4-DCP, 2,6-DCP up to a concentration of 205 mg l^{-1} , 143 mg l^{-1} , 61 mg l^{-1} respectively using these as sole sources of carbon and energy. *Arthrobacter* sp. ATCC 33790 mineralized PCP at high influent concentrations of 525 mg l^{-1} and 349 mg l^{-1} when operated in chemostat and fixed film bioreactor respectively (Stanlake and Finn, 1982; Edgehill, 1994). Goswami et al. (2002) reported that benzoate induced *R. erythropolis* M1 completely mineralized 2-CP, 4-CP, and 2,4 -DCP at an influent concentration 300, 100, 50 mg l^{-1} respectively. *Arthrobacter ureafaciens* CPR706 could degrade 4-CP via new pathway, in which the chloro substituent was eliminated in the first step and hydroquinone was produced as a transient intermediate (Bae et al., 1996). Strain CPR 706 exhibited much higher substrate tolerance and chlorophenol degradation rate than other bacterial strains that degraded p-chlorophenol by hydroxylation at the second carbon position to form chlorocatechol (Bae et al., 1996). These aspects provide a strong support in favour of actinomycetes for substituted phenol biodegradation. Thus, actinomycetes are found

to be one of the most preferred and efficient candidates for substituted phenol biodegradation for wastewater treatment.

2.6.4.1 Aerobic 4-chlorophenol degradation by *A. chlorophenolicus* A6

In general, 4-CP biodegradation is usually accomplished by oxidation of the substrate to 4-chlorocatechol, followed by *ortho* cleavage of the aromatic ring [Figure 2.1 (a)] (Chih-Jen et al., 1996; Farrell and Quilty, 2002; Park and Kim, 2003). Some microorganisms process 4-chlorocatechol by *meta* cleavage, although it is less common [Figure 2.1 (a)] (Hollender et al., 1994; Bae et al., 1996b; Farrell and Quilty, 1999). After ring cleavage, the chlorine atom is removed and the carbon skeleton is transformed into products that are assimilated into the central metabolism of the cell [Figure 2.1 (a)]. There are a few microorganisms that do not degrade 4-chlorophenol via cleavage of 4-chlorocatechol. *Arthrobacter ureafaciens* CPR706 (Bae et al., 1996a) was found to transform 4-chlorophenol to hydroquinone (1,4-dihydroxybenzene), but the pathway is yet unclear. Similarly, *Nocardioides* sp. NSP41 (Cho et al., 1998) also produced hydroquinone from 4-chlorophenol, but the entire pathway was not elucidated in that strain either. Nordin et al. (2005) reported that using *A. chlorophenolicus* A6, 4-chlorocatechol was produced from 4-chlorophenol, but the compound was not the ring cleavage substrate. Instead, 4-chlorocatechol was further transformed into hydroxyquinol, which was subsequently cleaved to yield maleylacetate [Figure 2.1(b)]. Also, a second pathway branch was found, where 4-chlorophenol was transformed into hydroquinone which in turn was hydroxylated to yield hydroxyquinol [Figure 2.1 (b)]. Although hydroxyquinol and its chlorinated derivatives are commonly ring-cleavage intermediates in degradation of more highly chlorinated phenols (Häggblom and Valo, 1995; Padilla et al., 2000; Uotila et al., 1995; Zaborina et al., 1998), Nordin et al., (2005) reported degradation of 4-CP via hydroxyquinol before ring cleavage by *A. chlorophenolicus* A6. Hydroxyquinol has also been found to be the ring cleavage substrate in degradation of 4-nitrophenol (Jain et al., 1994) and 4-aminophenol (Takenaka et al., 2003). Nordin et al. (2005) predicted several enzymes involved in the 4-chlorophenol degradation pathway in *A. chlorophenolicus* A6 such as monooxygenase for the initial transformations of 4-CP,

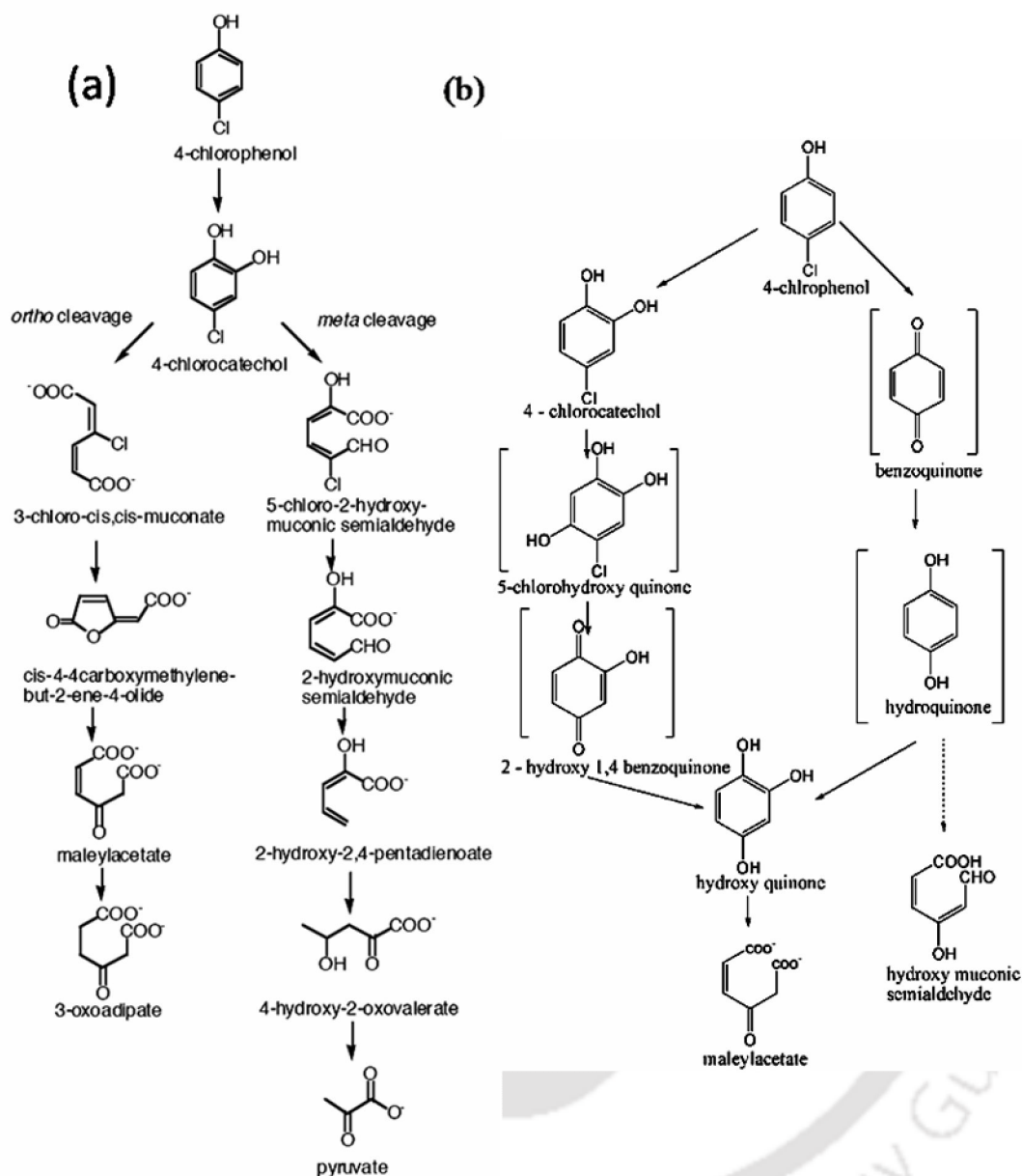


Figure 2.1 4-chlorophenol degradation pathways. **(a)** Conventional pathways (The University of Minnesota Biocatalysis/Biodegradation Database, [Ellis and Wackett, 1997](#)). **(b)** Proposed 4-CP degradation pathway in *A. chlorophenolicus* A6 ([Nordin et al., 2005](#)). The transformation from 5-chlorohydroxy quinone to 2-hydroxy 1,4 benzoquinone is theoretically due to reductive dechlorination. The presence of maleylacetate is inferred from genetic and biochemical evidence. Brackets indicate hypothetical intermediates supported, but not confirmed.

an enzyme for reductive dechlorination of 5-chlorohydroxyquinol, a hydroxyquinol dioxygenase and finally a maleylacetate reductase for funneling the product of ring cleavage into the central metabolism of the cell. Further, they reported genes and open reading frames encoding these types of enzymes in the *cph* gene cluster in *A. chlorophenolicus* A6. The cluster was found to harbor two putative monooxygenases, two hydroxyquinol 1,2-dioxygenases, two putative maleylacetate reductases and two putative regulatory genes. Nordin et al. (2005) reported that the mutant strain, of *A. chlorophenolicus* A6 called T99 (where one of the hydroxyquinol 1,2-dioxygenase genes was disrupted by a transposon), grew poorly on 4-chlorophenol, showing that the hydroxyquinol 1,2-dioxygenase gene (*cphA-I*) was vital for effective growth on the compound. They also reported that same enzyme system was used for degradation of 4-CP, 4-BP and PNP degradation. However, they found that mutant T99 grew well on unsubstituted phenol, showing that another or an additional enzyme system is responsible for metabolism of this compound. Similar existence of two pathway branches was also reported in literature for the transformation of toluene in *Burkholderia cepacia* JS150 (Johnson and Olsen, 1997) and *Rhodococcus rhodocrous* OFS (Vanderberg et al., 2000). The existence of multi pathway branches leading from 4-chlorophenol to hydroxyquinol in *A. chlorophenolicus* A6 may reflect the fact that two monooxygenases appears to be encoded by the *cph* gene cluster (Nordin et al., 2005). Possibly, the monooxygenases could be responsible for the transformations in one pathway branch each. Alternatively, one monooxygenase could perform *ortho*-hydroxylations in both branches, and the other enzyme *para*-hydroxylations

2.6.5.1 Microbial Growth Kinetics and Substrate Inhibition Models for Single Substrate Biodegradation Systems

For any bacteria to grow appreciably, the amount of substrate must be sufficiently high relative to the number of cells to permit several or many doublings in the initial population. If the bacterial cell density is high relative to the substrate concentration, little or no increase in the cells is possible. Therefore, it is apparent that the extent of bacterial growth depends on the initial substrate concentration. In order to describe the kinetics of substrate degradation by microbes, several kinetic models such as growth-associated models

(logarithmic, logistic and Monod with growth), non-growth associated models (zero order, first order and Monod based) and three-half order models have been reported in the literature (Schmidt et al., 1985; Alexander, 1999). Very recently, Mahanty et al. (2008) in their study on biodegradation of pyrene by *Mycobacterium frederiksbergense* observed that an three-half-order kinetic model provided the best fit to the entire degradation profiles with coefficient of determination (R^2) value >0.99 . The mathematical forms of these models are shown below.

Non-growth associated	{	Zero order	$S = S_0 - k_0 t$	$S_0 \gg K_S$	(2.6)
		First order	$S = S_0 \exp(-k_1 t)$	$S_0 \ll K_S$	(2.7)
		Monod with no growth	$K_s \ln \frac{S}{S_0} + S - S_0 = -k_2 t$ $(k_2 = \mu_{\max} X_0)$	$S_0 \sim K_S$	(2.8)

Growth associated	{	Logarithmic	$S = S_0 + X_0 [1 - \exp(\mu_{\max} t)]$	$S_0 \gg K_S$	(2.9)
		Logistic	$S = \frac{S_0 + X_0}{1 + \frac{X_0}{S_0} [\exp(K(S_0 + X_0)t)]}$ $\left(K = \frac{\mu_{\max}}{K_S}\right)$	$S_0 \ll K_S$	(2.10)
		Monod with growth	$K_s \ln \frac{S}{S_0} = (S_0 + X_0 + K_s) \ln \frac{X}{X_0} - (S_0 + X_0) \mu_{\max} t$	$S_0 \sim K_S$	(2.11)

Three-half-order model	{	$\frac{1}{t} [\ln(S_0 - (S_0 - S + k_0 t) + k_0 t) / S_0] = -k_1 - \frac{k_2 t}{2}$	(2.12)
---------------------------	---	---	--------

where, μ is the specific growth rate (h^{-1}), $\mu_{\max}$ is the maximum specific growth rate (h^{-1}), K_S is the half-saturation constant (mg l^{-1}), X and X_0 are the biomass concentrations (mg l^{-1}) at times 0 and t (h) respectively; S_0 is the initial substrate concentration (mg l^{-1}), S is substrate concentration at time t (mg l^{-1}) and k_0 , k_1 and k_2 are zero, first (h^{-1}) and second (h^{-2}) order rate constants, respectively. To establish the effect of substrate concentration on growth of microbial culture, specific growth rate of cultures at different substrate concentrations is calculated as per the following relationship:

$$\mu = \frac{1}{X} \frac{dx}{dt} \quad (2.13)$$

where, μ is the specific growth rate (h^{-1}), X is the biomass concentration (mg l^{-1}). Inhibition on the growth of cultures can be modeled using suitable substrate inhibition models described in literature (Kumar et al., 2005; Nuhoglu and Yalcin, 2005; Okpokwasili and Nweke, 2005). Usually, the microbial growth can be represented by a simple Monod equation (Monod, 1949):

$$\mu = \frac{\mu_{\max} S}{K_S + S} \quad (2.14)$$

where, S is the limiting substrate concentration (mg l^{-1}), $\mu_{\max}$ is the maximum specific growth rate (h^{-1}), K_S is the half saturation constant (mg l^{-1}). However, Eqn. (2.14) is not capable of explaining growth inhibition of microorganisms at higher substrate concentrations. In such a case, Edward, Andrews, Web, and Haldane models are normally used to represent growth kinetics of a culture. The Haldane model (Kumar et al., 2005) has the following form:

$$\mu = \frac{\mu_{\max} S}{K_S + S + \frac{S^2}{K_i}} \quad (2.15)$$

Where, K_i is the inhibition coefficient (mg l^{-1}).

Yano and Koga (1969) proposed a model based on a theoretical study on the dynamic behavior of single vessel continuous bioreactor. In their study, growth inhibition occurred at high concentration of rate limiting substrate *e.g.*, the acetic acid fermentation from ethanol, the gluconic acid fermentation from glucose, the tannase fermentation with tannic acid as the sole source of carbon. The model form is given in Eqn. 2.16 (Yano and Koga, 1969):

$$\mu = \frac{\mu_{\max}}{(K_s / S) + 1 + \sum_{j=1}^n (S / K_j)^j} \quad (2.16)$$

where, K_j is a positive constant. Similarly, a kinetic model (Edward model) was proposed (Eqn. 2.17), which is the modified form of Haldane model (Mulchandani and Luong, 1989):

$$\mu = \frac{\mu_{\max} S}{S + K_s + (S^2 / K_i)(1 + S / K)} \quad (2.17)$$

where, K_i is the substrate inhibition constant (mg l^{-1}) and K is a positive constant.

The model proposed by Luong (1987) as represented in Eqn. (2.18), appeared to be useful for representing the kinetics of substrate inhibition. Although the proposed model is of generalized Monod type, it accounts for substrate stimulation at both its low and high concentrations. The model has the capability to predict the values of S_m , the maximum substrate concentration, above which the growth is completely inhibited (Luong, 1987).

$$\mu = \frac{\mu_{\max} S}{K_s + S} \left[1 - \frac{S}{S_m} \right]^n \quad (2.18)$$

Several authors, as stated earlier in this chapter, have successfully applied most of these models to adequately describe single substrate degradation and microbial growth in their studies. Few important findings reported in the literature are discussed in this section. Kumaran and Paruchuri (1996) studied the kinetics of biotransformation of phenols using pure microbial cultures and mixture of microbial consortium. They estimated the bio-kinetic parameters by fitting the experimental data obtained from a wastewater treatment plant to Monod and Haldane models. Monterio et al. (2000) studied phenol biodegradation by *Pseudomonas putida* 548 in a batch reactor by varying the initial phenol concentration from 1 to 100 mg l^{-1} and observed that the length of the lag phase occurring before the exponential growth phase increases linearly with initial phenol concentration. The maximum specific growth rate ($\mu_{\max}$) of *Pseudomonas putida* was higher than that observed in mixed cultures (0.436 h^{-1}). Banerjee et al. (2001) evaluated phenol degradation using both free and calcium alginate immobilized cells of *Pseudomonas putida* in batch shake flasks. The free cell growth was carried out with an initial phenol concentration of 2.6 – 10.6 mmol l^{-1} , while the growth of immobilized cells were carried out with an initial phenol concentration of 2.4 – 20 g l^{-1} . They also observed that the cell growth was inhibited at high phenol concentration (above

5.31 mmol l^{-1}). Haldane model was employed to evaluate the bio-kinetic parameters and the estimated value of μ_{max} was found to be 0.06 h $^{-1}$, which is very low for a shake flask study. Vinod and Reddy (2005) carried out experiments to study the biodegradation of phenolic wastewater using microorganisms in a fluidized-bed bioreactor (FBR). They performed the experiments at various conditions of wastewater flow rate, dissolved oxygen (DO) concentration and inlet phenol concentration. The wastewater with feed phenol concentration as high as 1254 mg l^{-1} was found successfully biodegraded to 50 mg l^{-1} in the fluidized-bed bioreactor, whereas feed with concentration up to 1034 mg l^{-1} could be completely biodegraded to zero mg l^{-1} . They also evaluated the biokinetic parameters for the growth of the cells in batch experiments. Rodriguez et al. (2006) studied the biodegradation of phenol by an acclimatized activated sludge. They found the Haldane model inadequate to describe the microbial growth, and hence adopted a new two-step modeling technique. In the first step, phenol was considered to be degraded by a fraction of the total biomass, having one or several metabolic intermediate products. They also assumed that the intermediates are degraded during the second step by another portion of biomass. The proposed model is capable of describing both phenol degradation and biomass growth profiles at various initial conditions with only one set of model parameters. Aerobic batch degradation of phenol using immobilized *Pseudomonas putida* was studied by Hannaford and Kuek (1999). In their study, they changed the alginate concentrations between 2 and 4%, which had little effect on the degradation rate of phenol. They observed that when the initial phenol concentration was increased, degradation rate was not significantly affected until levels higher than 1200 mg l^{-1} . When the ratio of total bead volume to the volume of medium was increased, degradation rate increased too progressively, however the quantum of increase was small.

Although the growth and degradation kinetic of bromophenol is not yet reported in literature a few reports are available for nitrophenol and degradation of chlorophenol in aerobic and anaerobic environments has been studied extensively. High growth rates were observed for aerobic bacteria specialized in the utilization of chlorophenols as a carbon and energy source. Growth rates of more than 2 d $^{-1}$ were commonly observed for 2,4-DCP, 2,4,6-TCP and PCP (Golovleva et al., 1992; Radehaus and Schmidt, 1992; Hu et al., 1994; Rutgers et al., 1996). Cell yields during aerobic growth on chlorinated phenols decreased with increasing chlorine number. This was partly due to the molecular weight of chlorine (which

is not incorporated into biomass) and partly because of lower carbon conversion efficiencies. For instance, aerobic cell yields with DCPs and TCPs were between 0.132 and 0.421 g dwt biomass per gram g^{-1} chlorophenol metabolized (Stanlake and Finn 1982; Chudoba et al., 1989; Rutgers et al., 1997), whereas, the values ranged from 0.054 and 0.190 g dwt biomass g^{-1} chlorophenol metabolized for PCP (Edgehill and Finn, 1983a; Hu et al., 1994; Rutgers et al., 1997; Karamanev and Samson, 1998). The specific activities of aerobic bacteria utilizing chlorophenols as growth substrates mostly ranged between 29 and 851 mg compound biotransformed g^{-1} dwt biomass day^{-1} (Melin et al., 1998a; Lora et al., 2000; Kargi and Eker, 2004; Sahinkaya and Dilek, 2005; Snyder et al., 2006). On the contrary, using anaerobic microorganisms (reductive dechlorination of chlorophenols) the specific activities was observed between 0.37 and 12.9 mg chlorophenol biotransformed g^{-1} dwt biomass day^{-1} (Wu et al., 1993; Ning et al., 1997; Mohn et al., 1999; Guiot et al., 2002; Ye et al., 2004). Remarkably, high specific activities are noted for aerobic chlorophenols microorganism than anaerobic process. Half-velocity coefficients are generally low during chlorophenol and nitrophenol biodegradation indicating a high affinity (Juang and Tsai, 2006). Most microorganisms had K_s or K_m values between 0.01-11.7 mg l^{-1} and 13-35 mg l^{-1} for chlorophenol and nitrophenol respectively (Tyler and Finn, 1974; Kumaran and Paruchuri, 1997; Melin et al., 1998a; Karamanev and Samson, 1998; Ray et al., 1999; Mannisto et al., 2001; Bhatti et al., 2002; Kumar et al., 2005). On the contrary, a higher K_s value of 112 mg l^{-1} was reported by Kargi and Eker (2004) for co-metabolism by an aerobic bacterium. Microorganisms which were previously enriched in bioreactors treating low concentrations of chlorinated phenols in groundwater displayed remarkable affinities as judged from half-velocity coefficients as low as 0.014–0.016 mg l^{-1} under both aerobic (Melin et al., 1997) and anaerobic conditions (Magar et al., 1999). The maximum specific growth rate ($\mu_{\max}$) of aerobic bacteria utilizing chlorophenol, nitrophenol, cresol and phenol as growth substrates mostly ranged between 0.15 and 0.55 h^{-1} (Livingston and Chase, 1989; Kumaran and Paruchuri, 1997; Sahinkaya and Dilek, 2005; Salehi et al., 2010) indicating that the substrate is readily utilized by the respective microorganism for their growth. The value of the inhibition constant (K_i) signify the degree of resistance of the microorganism to toxic effects to the pollutants and in general, a larger K_i value reveals that the biomass is highly resistant to inhibition by its substrate. The values of inhibition constant (K_i) mostly ranged between

145- 516 mg l⁻¹ for 4-CP, 4-NP, phenol, cresol biodegradation by mixed culture, *C. tropicalis*, *Acinetobacter calcoaceticus*, *Pseudomonas species* (Livingston and Chase, 1989; Kumaran and Paruchuri, 1997; Sahinkaya and Dilek, 2005) indicating a high tolerance of these microorganisms towards the substrate.

2.6.5.2 Multi Substrate Biodegradation Systems

Mixtures of organic chemicals are highly prevalent in wastewaters from industrial sites. Occurrence of the environmental contaminants in mixtures is a crucial problem because the removal or degradation of one component can be inhibited by other compounds in the mixture. Moreover, different conditions may be required to treat different compounds within the same wastewater sample (Reardon et al., 2000). Currently, quantitative information on the rates and patterns of microbial degradation of substrate mixtures is scarce. Reardon et al. (2000) studied biodegradation kinetics of benzene, toluene and phenol, both in single and mixed substrate systems using *Pseudomonas putida F1*. They reported the kinetics of the growth of the culture and developed mathematical models to describe their results. In the tested concentration range, toluene and benzene biodegradation kinetics were well described by the Monod model. Although a small degree of substrate inhibition was noted, the Monod model was able to characterize phenol biodegradation. Of the models tested, Sum Kinetics with Interaction Parameters (SKIP) model provided the best description of the paired substrate results. This model, with parameters determined from one and two substrate systems provided an excellent prediction of the biodegradation kinetics for the three substrate system. Gallego et al. (2003) studied degradation and detoxification of a mixture of persistent compounds (2-chlorophenol, phenol and *m*-cresol) by using pure and mixed indigenous cultures in aerobic reactors. Biodegradation assays were performed in batch and continuous flow reactors. In their study, individual compounds were degraded by pure bacterial cultures within 27 h. The mixture of 2-chlorophenol (100 mg l⁻¹), phenol (50 mg l⁻¹) and *m*-cresol (50 mg l⁻¹) was degraded by mixed bacterial culture under batch conditions within 36 h. Bai et al. (2007) studied biodegradation of phenol and *m*-cresol as mixed substrates using *Alcaligenes faecalis*. They observed strong interaction effect between the two during the culture growth on the degradation of the substrates. Both *m*-cresol and phenol inhibited the degradations of

each other. The overall culture growth rate was, however, due to the co-action between phenol and *m*-cresol. In addition, the cell growth and substrate degradation kinetics of phenol and *m*-cresol, both as single and mixed substrates, were investigated over a wide range of their initial concentrations (phenol: 10 – 1400 mg l⁻¹ and *m*-cresol: 5 – 200 mg l⁻¹). The single-substrate kinetics was well described using the Haldane-type kinetic models.

2.6.6 Bioreactors Systems for Treatment of Wastewater Containing Phenolics Compounds

Most biological reactor systems can be classified into two main groups viz. suspended biomass and immobilized biomass system. Stirred tank, air-lift and bubble column reactors are mainly for the former case. Whereas, immobilized bioreactor can be classified further into fixed film systems, such as packed-bed bioreactors, membrane bioreactor and rotating biological contactors and the second one where the effluent is passed through particle-based reactors, such as fluidized bed bioreactors, air lift bioreactor where the biofilm is attached onto a moving carrier. Particle based systems allow better mass transfer of pollutants and oxygen and are generally very efficient but packed-bed bioreactors have also been successfully tested for various organic pollutants (Guieysse et al., 2000; Soares et al., 2003). Packed bed reactors are also easier and cheaper to operate because it is not necessary to suspend the carriers. Implementation of a novel bioreactor to remove such highly toxic phenolics has been studied recently by some researchers (Quan et al., 2004; Ryan, et al., 2005; Vinod and Reddy, 2005; Jajuee et al., 2007). There are several reports on treatment of substituted phenolics waste water using different bioreactor systems and some of these are discussed further.

Biodegradation of 2,4 - dichlorophenol (2, 4 -DCP) and phenol in an internal loop airlift bioreactor immobilized with *Achromobacter* sp. was performed by Quan et al. (2004). The bioreactor was packed with honeycomb-like ceramic as carrier to immobilize microorganism *Achromobacter* sp., capable of degrading 2,4 - DCP. They operated the reactor under continuous mode of operation. Effects of phenol on 2,4 - DCP removal were also investigated under fed-batch and continuous operations. Their results showed that the pure strain could easily be immobilized on the carrier and proliferated using 2,4 -DCP as the

sole source of carbon. Moreover, in the process of fed-batch operation, removal rate of 2, 4 - DCP decreased with the increase in run number, while that of phenol was just the contrary. Their results also showed that with the increase of phenol loading rates, the removal efficiency of 2, 4 - DCP declined from 100 to 87.9%, while that of phenol remained at about 99.6%. Presence of phenol inhibited the biodegradation of 2, 4 - DCP and caused the major carbon source shift from 2, 4 -DCP to phenol. Jajuee et al. (2007) studied the biodegradation performance and the rate of oxygen transfer in a pilot-scale novel airlift immobilized bioreactor of 10 L working volume. They inoculated the reactor with an acclimatized population of contaminant degrading microorganisms. Immobilizing the microorganisms on a non-woven polyester textile, they developed the active biofilm and thereby obtained biodegradation rates of $81 \text{ mg l}^{-1} \text{ h}^{-1}$ and $40 \text{ mg l}^{-1} \text{ h}^{-1}$ for *p*-xylene and naphthalene respectively. Monod kinetic model was found to be suitable for correlating their experimental data, obtained during the course of batch and continuous operations. Sahinkaya and Dilek (2007) studied the biodegradation kinetics of 2,4-DCP by (a) culture (Culture M) acclimatized to mixture of 4-chlorophenol (4-CP) and 2,4-DCP and (b) culture (Culture 4) acclimated to 4-CP only. The study was carried out in aerobic batch reactors. Also, pure strains isolated from mixed cultures were tested for their ability towards the biodegradation of 2,4-DCP. They observed a linear relationship between time required for complete degradation and initial 2,4-DCP concentrations for both mixed cultures. It was also observed that the Haldane equation can be used to predict specific degradation rate with value of determination coefficient (R^2) greater than 0.99 as a function of initial 2,4-DCP concentrations; and, it also adequately described 2,4-DCP concentration profiles during its biodegradation. Several studies were conducted in fluidized bed reactors to evaluate the treatment performance of chlorophenols contaminated groundwater at lower concentration (Valo et al., 1990; Jarvinen et al., 1994; Puhakka et al., 1995b; Melin et al., 1998a, b). Loh and Ranganathan (2005) performed the biotransformation of 4-CP in presence of phenol in an External-loop Fluidized Bed Airlift Bioreactor (EFBAB). The authors reported the advantages of a 4 L external-loop inversed fluidized bed airlift bioreactor.

They tested the bioreactor for batch co-metabolic biotransformation of 4-CP in presence of phenol at various concentration ratios of phenol and 4-CP (ranging from 600 mg l⁻¹ phenol and 200 mg l⁻¹ 4-CP to 1600 mg l⁻¹ phenol and 200 mg l⁻¹ 4-CP) at 9% expanded polystyrene beads loading and 2.8% (10 g) granulated activated carbon loading. The bioreactor system achieved successful biotransformation of 4-CP and phenol. Tomei et al. (2004) reported biodegradation kinetics of 4-NP at an influent concentration of 300-400 mg l⁻¹ in a lab-scale sequencing batch reactor and the kinetic test data was found well correlated by the Haldane equation, with maximum removal rate in the range of 3.3–8.4 mg 4-NP mg VSS⁻¹ d⁻¹ depending on the substrate concentration. Go'mez-DeJesu's et al. (2009) reported *Burkholderia kururiensis* and *Stenotrophomonas* sp. microbial consortium capable to degrade 139 mg l⁻¹ of influent 2,4,6-TCP at volumetric loading rate of 46.83 mg l⁻¹ d⁻¹ in a packed bed biofilm reactor. This consortium removed nearly 99% of 2,4,6-TCP. Mineralization and dehalogenation of 2,4,6-TCP was evidenced by high COD removal efficiencies (≈93%) and by the stoichiometric release of chloride ions from the halogenated compound (≈89%). Rezouga et al. (2009) reported 99% removal of 4-NP in an aerobic stirred tank bioreactor from an influent 4-NP concentration of 528.73 mg l⁻¹, which is equivalent to 1490 mg l⁻¹ d⁻¹ of 4-NP loading rate. An influent 4-NP concentration more than 528.73 mg l⁻¹ reduced the reactor performance significantly. Similarly Tomei et al. (2008) reported complete removal of 4-NP at an initial concentration of 450 mg l⁻¹ using a two-phase sequencing batch system even at a higher removal rate of 3 mg 4-NP mg VSS⁻¹ day⁻¹. Table 2.3 presents some of the bioreactor studies and their performance in treating substituted phenols contaminated wastewater operated under continuous mode. Quan et al. (2004) performed a study using an internal loop airlift reactor immobilized with *Achromobacter* sp to degrade phenol and 2,4 dichlorophenol. They obtained a removal efficiency of about 99.6% at a hydraulic retention time (HRT) of 8 h for a maximum phenol concentration of 200 mg l⁻¹. Carvalho et al. (2001) studied the biodegradation of wastewater containing 4-CP using a mixed culture in a GAC-fluidized-bed bioreactor (GAC-FBR). Experiments were conducted at 20-50 mg l⁻¹ of 4-CP with an HRT of 0.28 h but; they achieved a 4-CP removal efficiency varied from 69-100%. However a fluidized bed bioreactor, airlift bioreactor has a complicated operational protocol and is thus difficult in adopting it to real wastewater treatment. Zilouei et al. (2006) studied the kinetics of biodegradation of mixture of 2-CP, 4-CP, 2,4- DCP and 2,4,6- TCP in a

packed bed reactor. The reactor was operated under continuous mode. They obtained a removal efficiency of about 99.7% at a HRT of 2.5 h for a maximum chlorophenol concentration of 107 mg^{-1} .

Similar studies were carried out on PBR by other researchers; however, their studies had the following limitations:

- In maximum cases they adopted pollutants with lower concentration as a single substrate, instead of mixed substrate with higher concentration.
- They used conventional bioreactor without consideration of the limiting factors.
- They did not improve the hydrodynamic condition of conventional PBR
- They did not evaluate the reactor stability towards shock loading of pollutants, which helps to study the stability of reactor towards higher pollutant concentrations.
- They did not operate the reactor in continuous mode, similar to that of a real wastewater treatment plant.

Therefore all these unexplored research directions were extensively studied and reported in the present thesis.

2.6.6.1 Packed bed bioreactor (PBR)

The most important characteristic of a PBR is the material flows through the reactor as a plug; therefore, they are also called plug flow reactors (PFR). Ideally, all of the substrate stream flows at the same velocity, parallel to the reactor axis with no back mixing. All material present at any given reactor cross-section has an identical residence time. The longitudinal position within the PBR is, therefore, proportional to the time spent within the reactor; all product emerging with the same residence time and all substrate molecule having an equal opportunity for reaction. The conversion efficiency of a PBR, with respect to its length, behaves in a manner similar to that of a well -stirred batch reactor with respect to its reaction time.

Table 2.3 Bioreactor system and their performance in treating wastewaters containing substituted phenolics compounds

Waste Details	Influent Conc. (mgL ⁻¹)	Effluent Conc. (mgL ⁻¹)	Removal efficiency (%)	Bioreactors	Microorganisms	HRT (h)	References
4-NP	450	BDL	100	Two-phase (SBR)	Mixed culture		Tomei et al., 2008
4-NP, 2,4-DNP, aniline	50 to 180	1-2	99	Sequencing batch reactor (SBR)	TM7, <i>Proteobacteria</i> , and <i>Proteobacteria</i>	12h	Xing-yu et al., 2007
PCP	21	BDL.	100	Anaerobic(HAIB) Fixed film reactor PUF	<i>Methanosarcina</i> and <i>Methanosaeta</i>	-	Saia et al., 2007
4-CP, 2,4DCP	220 & 110	0.05	100	Batch & SBR Reactor	<i>Pseudomonas stutzeri</i>	24 h	Sahinkaya et al., 2006
4-CP 2,4DCP peptone	220 & 110	BDL int2nd stage	>98	2 stage RBC	<i>Pseudomonas stutzeri</i>	16.8h	Sahinkaya et al., 2006
4-CP and Phenol,	200 4-CP & 600 phenol	BDL	-	(EFBAB) GAC fluidized bed	<i>Pseudomonas putida</i> ATCC 49451	10–15 h	Loh et al., 2006
Mixture 2-CP, 4CP, 2,4 DCP2,4, 6- TCP	107	0.3	99.7	PBR	Mixed culture	2.5	Zilouei et al., 2006

PCP	Loading rate of 12–40 mg m ⁻³ d ⁻¹	BDL	99.9	Membrane bioreactor acrylic sheet Hollow fiber polyethylene membrane	Mixed culture	12 h	Visvanathana et al., 2005
4-NP	300-400	BDL	100	Sequencing batch reactor	Mixed culture	16h	Tomei et al., 2004
2,4-DCP & phenol	49.96 mg l ⁻¹	-	94–99	Honeycomb-like ceramic column packed air-lift bioreactor.	<i>Achromobacte</i> rsp.	36 h	Xiangchun et al., 2003
2,4,6-TCP, PCP	19 µmol ⁻¹ TCP	-	95	Fluidized-bed reactor.	<i>Pseudomonas testosteroni</i> CPW301. <i>P. solanacearu</i> m TCP114	-	Langwaldt et al., 2002
4-CP	20	BDL	100	PBR	Mixed culture	2.8h	Kim et al., 2002
4-CP	20-50	0-14	69-100	GAC-Fluidized-bed reactor.	Mixed culture	0.28	Carvalho et al., 2001
2NP, 4-NP, and 2,4 DNP	2-30	-	90-99	UASB	Mixed culture	12-30	Karim and Gupta, 2001
2,4,6-TCP, , 2,3,4,6-Te CP & PCP	16.8 TCP, 28.8 TeCP, 9.1 PCP Total CP 54.7mg l ⁻¹	0.04	99.9	Batch-fed Air-lift percolator	Mixed culture	64 days	Langwaldt J et al., 1998
PCP	0.727	0.006	99.79	(FBR). Porous Celite R-633 diatomaceous earth	Oligotrophic enriched culture	4.45h	Melin et al., 1997

PBR is one such biofilm reactor that has gained much popularity and one of the most promising bioreactors systems in wastewater treatment due to its several advantages over other bioreactors. For example, PBR does not need any mechanical agitation and low energy is sufficient for required aeration in the process. Compared to conventional reactors such as the stirred tanks or bubble columns, shear stress in PBR is relatively constant and mild throughout the reactor. This fact is favorable to microbial growth. Biofilm reactors exhibit high surface area available for microbial growth and offer high volumetric biomass concentration in small reactor volume consequently shows higher metabolic activity when operated with continuous processes ([Israni et al., 2002](#); [Nicolella et al., 2000](#)). Furthermore, the attached biomass is sometimes claimed to be more resistant to toxicity; offers higher resistance to shock loads and able to survive even at low influent substrate concentrations. For example, biofilm cells can be 500 times more resistant to antibacterial agents than freely suspended ones ([Pedersen and Arvin, 1995](#)). In addition, packed bed bioreactor operating in upflow mode prevents the suspended biomass wash out as well as frequent clogging in support media is less compared to down flow. PBR are also simple to operate, attains a high yield and they can be easily scaled up from a laboratory scale up procedure. Moreover, if aerobic biofilm reactors are very compact, enable a rapid start-up, have a high biomass retention capacity and are suitable for use at a low hydraulic retention time (HRT) ([Tchobanoglous, 1998](#); [Langwaldt and Puhakka, 2000](#)). All these advantages explain why so many researchers have focused their attention on PBR on biodegradation of organic pollutant from contaminated wastewater ([Sheeja and Murugesan, 2002](#); [Kim et al., 2002](#); [Tepe and Dursun, 2008](#); [Waul et al., 2008](#); [Gómez-De Jesús et al., 2009](#); [Paca et al., 2009](#); [Akdogan and Pazarlioglu, 2011](#); [Iandolo et al., 2011](#)).

However, when a PBR is operated in an upflow mode with the supply of oxygen from the bottom of the reactor, bubble formation and gas channeling occurs leading to the development of liquid-rich and gas-rich regions rather than uniform distribution of oxygen and food throughout the reactor bed ([Lee and Pang-Tsui, 1999](#); [Kreft and Wimpenny, 2001](#)). Further, at low Reynolds number the flow rate is proportional to the pressure drop across the PBR. This pressure drop is, in turn, generally found to be proportional to the bed height, the linear flow velocity and dynamic viscosity of the substrate stream and bed porosity but inversely proportional to the cross-sectional area of the immobilized cell pellets ([Radilla et](#)

al., 2005; Meyer and Smith, 1985). Thus the formation of gas-liquid channels in the reactor bed due to excessive pressure drop may be another reason. Therefore, reduction of pressure drops consequently diminishes the gas-liquid superficial flow velocity in the reactor inhibits the growth of microorganisms and therefore degradation of organic pollutants due to lack of sufficient oxygen and/or food deep inside the reactor bed material, especially when the bed contains a porous but poorly permeable supporting material for the growth of microorganisms (Meyer and Smith, 1985; Radilla, et al., 2005). Heterogeneity could be an important disadvantage, i.e. concentration and cell gradients over the fixed bed height and within the biofilm attached to support, or the strongly heterogeneous kLa coefficients observed along PBR vertical axis (Pe´rez et al., 2006). In order to reduce the pressure drop excessive increases in gas-liquid flow rate may distort compressible or physically weak particles, which may lead to biomass erosion and consequently affect the reactor performance.

It is clear that the above researchers focused on treating wastewater in an biofilm reactor (PBR) by employing microbes, they, however, neglected the design aspect of PBR to overcome some of the major limitations cited above, which could yield a complete treatment of phenolics even at higher concentration in shorter time period.

2.6.6.1. (a) Fundamental Design Principles

The design and selection of each type of bioreactors is unique but there are some fundamental principles. In general, the main criteria for designing bioreactor should consider adequate oxygen transfer, low shear stress and adequate mixing (Wang and Zhong, 2006; Zhou et al., 2010). In considering the bioreactor design and selection, nutrients must be effectively provided to the cells and waste products must be removed. Cell growth and degradation kinetics should be assessed so that an optimal environmental condition can be defined and an operational mode can be determined. Transport phenomena should be studied in order to define the criteria for bioreactor design and scale-up. Operating parameters, such as temperature, pH, dissolved oxygen and substrate concentrations should be easy to control and monitor. In addition, the bioreactor should be as simple and inexpensive as possible and it should easily operate free of unnecessary contamination with microorganisms.

Mixing

Mixing is a physical operation which reduces non uniformity in fluid by eliminating gradient concentration of the nutrient, gases, temperature and to prevent the accumulation of toxic metabolites (Bailey and Ollis, 1986). Thus mixing time is a critical parameter to be studied and evaluated. Mixing time (t_{m95}) is time to reach 95% homogeneity after a tracer input in a mixture. Therefore, smaller the mixing time resulted a better and quicker distribution plus dispersion of oxygen, food and temperature etc to the microorganism for enhanced growth and degradation of the compounds. The fluid hydrodynamics, fluid rheology, power input, and vessel size can all influence the mixing conditions. In large-scale bioreactors, poor mixing often leads to undesirable concentration gradients and a decrease in mass transfer efficiency. In microbial cell cultures, which are typical shear sensitive biological systems, mixing could be enhanced by increasing aeration rates or agitation speed in bioreactors, but it has to balance with the increase of shear stress because shear force caused by intense aeration or agitation can bring damage to living cells and occurrence of cell erosion.

Oxygen Transfer

Most nutrients required for cellular growth and metabolism are highly soluble in water, however, oxygen transfer often becomes a limiting step to the optimal performance of biological systems and also for scale-up because oxygen is only sparingly soluble in aqueous solutions. When the supply of oxygen is limited, both cell growth and degradation performance of packed bed reactor can be severely affected (Gomez-DeJesus et al., 2009). For example, it was reported that ceasing aeration in the medium during penicillin fermentation for just a few minutes seriously impacted the ability of the cells to produce the antibiotic (Nielsen and Villadsen 1994). Similar result are also reported in many literature that the oxygen utilization by cell include synthesis of enzymes responsible for utilization of substrate, cell maintenance energy, as well electron acceptor for growth and metabolism of the microorganism (Gomez-DeJesus et al., 2009; Costura and Alvarez, 2000).

Shear Force

Microorganisms, cells are more sensitive to shear force. Shear has been mainly considered in the technical literature as a destructive element. A couple of parameters are related to the shear-induced cell death, such as shear stress, shear time, power dissipation and the growth phase of the cells (Zhong, 2010). As aforementioned, sparging can also cause the shear damage, which can happen at different locations in bioreactor; bubble generation zone at the sparger; the rising zone through the bulk liquid; and the surface of the suspension (either be covered with foam layer or free of foam). The mechanism of cell damage at bubble disengagement zone is as follows; cells attach to the bubble surface (Jordan et al., 1994), and then bubble bursts at the suspension surface, which causes high shear (Papoutsakis, 1991). There have been abundant reports on shear damage suffered by animal cells grown in suspension, especially damaged by disengaging bubbles (Chalmers and Bavarian, 1991; Jobses et al., 1991; Cherry and Hull, 1992; Chisti, 2001). Furthermore, in simple conventional bioreactor system aeration at single port with high flow rate creates high shear stress that hampers the biomass immobilization process as well as cause cell erosion from the supporting materials. However, fragmented approach of aeration (separation of the aeration zone) can reduce the shearing stress (Fassnacht and Portner, 1999). This is well supported by literature that cross flow design by multiple points along the height in packed beds reduces pressure drop enabling the use of practical flow rates and even flow distribution across wide packed bed (Ajmera et al., 2002). To overcome the problem of the rising air bubbles, aeration using membranes in which the supply of oxygen is diffusion controlled can be employed (Kretzmer, 2002). Therefore, for shear-sensitive cultures, the shear stress intensity can be minimized by uses of the perspex disc covered by nylon wire mesh together with ceramic gas diffuser reduces the bubble formation and gas channelling effect by discharging the air through the very narrow pores of the diffuser with a larger surface area. Wang and Zhong (1996) successfully designed a novel centrifugal impeller bioreactor (CIB) for shear-sensitive biological systems including animal and plant cell cultures.

2.6.6.2 Biofilm supporting materials

Biofilm reactors have been constructed using different packing materials, such as GAC, polyurethane, kaolin, polystyrene, wood chips, soil sand, ceramic saddles, polyvinyl chloride and polyethylene (PE) (Guerin, 2002; Pruden et al., 2003; Kermanshahi pour et al., 2005; Ohlen et al., 2005). Activated carbons are special supports, since they are also able to adsorb pollutants (Zhao et al., 1999; Guerin, 2002; Pruden et al., 2003). For the successful application of any material as support in fixed film stationary bed reactors, the following general requirements should be fulfilled: availability of the material in big quantities, low cost, inert behavior (mechanically and microbiologically stable), low pressure drop and provide sufficient surface area per unit volume for biomass immobilization and growth. Polyurethane foam is found to satisfy most of the above mention criteria and thus many researchers have used it as biosupport materials. For example, polyurethane foam enhanced the ability of *R. chlorophenolicus* PCP-1 cells to maintain viability and degrade PCP in liquid culture at high concentrations (Briglia et al., 1990). O'Reilly and Crawford (1989) demonstrated the ability of polyurethane foam immobilized *Flavobacterium* sp. ATCC 39723 cells to degrade 300 mg l⁻¹ PCP, with 70-80% mineralization. In the presence of polyurethane foam, lag phases were also decreased. Polyurethane-immobilized *Flavobacterium* sp. ATCC 39723 cells degraded up to 700 mg l⁻¹ PCP in liquid culture (Hu et al., 1994). Table 2.4 represent some of the control strategy for effective wastewater treatment using bioreactor systems.

2.6.6.3 Cell Immobilized by Calcium Alginate Bead Systems for Treatment of Wastewater Containing Phenolics Compounds

The entrapment of cells in alginate bead is a promising method for microbial degradation of toxic substances due the following potential advantages. It provides high cell concentrations, cell can reuse, and eliminates the costly processes of cell recovery and cell recycle. Immobilized cells avoid washout of cells, even under conditions of negligible cell growth, while the volumetric reaction rates and overall productivity compare favourably with those of suspension cultures.

Table 2.4 Process control parameters for monoaromatic degradation using continuous operated bioreactor system.

Parameters	Description/importance
Temperature	In general, rate of biological reaction in mesophilic conditions increases by a factor of 2 for each 10 °C rise in temperature. However, an increased temperature induces an enhanced volatilization rate of monoaromatic hydrocarbons.
Hydraulic retention time (HRT) = V/Q	Flexible handling of influent flow rates is required for effective control.
Influent quality and flow direction (such as monoaromatic concentration, pH, EC, TSS, COD, alkalinity, salinity, inhibitors, etc.)	Variations in influent quality affect the efficiency of bioreactors; selection of flow direction (upflow or down flow) could influence mixing and degree of contamination removal. Volatilization rates of VOC compounds may be accelerated by mixing and thermal energy and inhibited by dissolved chemicals such as organic compounds, salts, etc.
Flow regime	A bioreactor may be set up as a completely mixed stirred tank or a plug-flow bioreactor. Flow regime affects the efficiency of bioreactors.
Biomass	Potential problems arise by the attachment and detachment (sloughing) of biomass; biomass can build up and large oxygen demands associated with aerobic bio-treatment process may occur; potential problems with foaming (also dependent on type of chemicals in influent), important factors for biomass control in the biological bioreactor are mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS). On the other hand quantities and qualities of biomass applied as starter affect start-up and efficiency of bioreactors.
Nutrient requirements	Varies with contaminants; provides a macronutrient (such as N, P) and micronutrient (such as trace element) on the basis of TOC in contaminated water and elemental biomass composition
pH	pH control is critical and contaminated water may not have adequate buffer capacity.
OLR = QS/V	Organic loading rate can determine the capacity of bioreactor for organic pollutant removal.
F/M = QS/VX	Ratio of food to microorganism is important and influential on the efficiency of biological system.
Electron acceptor	Aerobic condition: O_2 ; anaerobic condition: compounds such as NO_3^- and SO_4^{2-}
Packing materials	Surface area, porosity and nature of the packing material affect the efficiency of biofilm reactors. Also, evaluation of adsorption and desorption capacity of media and bioregenerability of packing must be considered in biofilm reactors.
Gas control	Gas exiting from bioreactors must be controlled. In aerobic

	bioreactors, diffusion and stripping of volatile organic compounds by aeration must be considered. In anaerobic conditions monoaromatic compounds can be evaporated and stripped by the biogas produced.
Recycle ratio (QR/Q)	Recycle flow may be necessary for feed dilution or biomass recycling.

From Tchobanoglous (1998); Langwaldt and Puhakka (2000); Guerin (2002); Farhadian et al. (2006).

Further cell immobilization method is not toxic to the cells, and is inert, inexpensive, and practical (Tanaka et al., 1984; Shuler and Kargi, 1992; Chen et al., 1993; Aksu and Bu'lbül, 1999). Alginate immobilization of *Flavobacterium* sp. ATCC 39723 permitted degradation of PCP at concentrations as high as 150 mg l⁻¹ in batch reactors. Concerns about the use of alginate included its low mechanical strength, incompatibility with anions such as phosphate and susceptibility to biodegradation (O'Reilly and Crawford 1989). Table 2.5 presents some of the cell immobilized calcium alginate beads and their performance in treating substituted phenols contaminated wastewater operated under continuous mode.

2.7 Design of Experiments

Design of experiments is a statistical design technique, which involves changing the levels of influential variables simultaneously, as compared to varying only one variable and its level at a time in conventional experimentation. The statistical interpretation of the results are in the form of analysis of variance (ANOVA), student's *t*-test, P-value, *F*-value etc. which give better understanding of the factor effects and their interaction on a given response. Statistical designs of experiments are necessary for systematic investigations requiring only a low number of experiments, and to interpret results in a meaningful manner (Montgomery, 2004). The factorial experimental design methodology involves changing all variables from one experiment to the next.

Table 2.5 Performance of cell immobilized alginate bead in different bioreactor system for treatment of various pollutant

Pollutant treated	Influent Phenolics Conc. (mg l ⁻¹)	Phenolics Removal efficiency (%)	Degradation rate	Bioreactors	Micro-organisms	HRT (h)	References
4-Aminobenzenesulfonate	200–400	At 300 mg l ⁻¹ – 100%, 400 mg l ⁻¹ 82.5%	3 days at each concentration	Packed bed reactor	<i>Agrobacterium sp. strain PNS-1</i>	Semi-continuous mode 12 h each day	Singh et al., 2006
Phenol	100	-	3.672 mg g ⁻¹ day ⁻¹	Packed bed reactor	<i>Ralstonia eutropha</i>	Flow rate 0.52 ml min ⁻¹	Tepe and Dursun, 2008
phenol	100	-	1400 mg l ⁻¹ day ⁻¹	Bubble column bioreactor	<i>P. putida</i>	-	Mordocco et al., 1999
Phenol	1000	-	2100 mg l ⁻¹ day ⁻¹	Column reactors	<i>Rhodococcus DCB-p0610.</i>	-	Pai et al., 1995
Phenol	250-2000	-	1440-2880 mg l ⁻¹ day ⁻¹		Activated sludge	-	Esparza et al., 2006
Pyrene	1000	-	250 mg l ⁻¹ day ⁻¹	Fermentor (Stirred tank reactor)	<i>Mycobacterium frederiksbergense</i>	10 day	Sarma and Pakshirajan, 2011
phenol	2000	> 97%	-	Packed bed reactor	<i>Candida tropicalis (NCIM 3556)</i>	2 h	Varma and Gaikwad, 2010
Acetamide, Propionamide, and Acrylamide	100 mM + 100 mM +10 mM	97, 100 and 90%, respectively	-	Plug flow reactor (five-stage)	<i>Rhodococcus rhodochrous NHB-2</i>	4.167 h	Chand et al., 2004

The reason for this is that variables can influence each other, and the ideal value for one of them can depend on the values of the others. This interaction between variables is a frequent phenomenon.

2.7.1 Factorial Design of Experiments

The 2^k design is particularly useful in the early stages of experimental work, when there are many factors likely to be investigated. It provides the smallest number of runs with which ' k ' factors can be studied in a complete design. Because there are only two levels for each factor, it is assumed that the response is approximately linear over the range of the factors level chosen. The statistical model for a 2^k design would include k main effects, $(K/2)$ two factor interactions, $(K/3)$ three factor interaction and so on and one k -factor interaction. That means, for 2^k design, the complete model would contain k^2-1 effects. The levels of the factors may arbitrarily be called "low" and "high" and denoted by "-" and "+" respectively. For example, in 2^2 design, two factors viz. A and B at two levels are chosen. The experimental design consists of treatment combinations denoted by (1) "a", "b", "c", "ab". A suitable number of runs carried out to replicate the levels of variables at their center point (0) provide an estimate of the residual error associated with the experiments and also the curvature of the response. Three degrees of freedom is associated with the four treatment combination in 2^2 design, which in turn consists of two degrees of freedom associated with main effects of A and B, one degree of freedom associated with interaction effect between A and B (Montgomery, 2004).

2.7.2 Analysis of Variance

Analysis of variance (ANOVA) is a collection of statistical models, and their associated procedures, in which the observed variance is partitioned into components due to different explanatory variables. In general terms, ANOVA explains any variation in the statistically derived model and significance of the model parameters. The model parameters, usually indicated in ANOVA, are the main effects, interaction effects and error terms, and

their significance in the model is represented by Fischer ' F ' and associated P values. The other items in ANOVA table are degrees of freedom (df), sum of squares (SS) and mean squares (MS). The MS value of a model term in an ANOVA table is obtained by dividing SS over df and its F value is obtained by dividing MS due to the model term by MS due to error. Normally, larger F and lower P values of a model term in ANOVA indicate good significance of the term over others. The fundamental technique is a partitioning of the total sum of squares into components related to the effects used in the model.

$$SS_{Total} = SS_{error} + SS_{Treatment} \quad (2.19)$$

The number of degrees of freedom (df) can be partitioned in a similar way and it specifies the chi-square distribution, which describes the associated sums of squares.

$$df_{Total} = df_{error} + df_{Treatment} \quad (2.20)$$

2.7.3 Student ' t ' Test

A t -test is any statistical hypothesis test in which the test statistic has a Student's t distribution if the null hypothesis is true. It is applied when sample sizes are small enough that using an assumption of normality and the associated z -test leads to incorrect inference. The Student t -distribution in probability and statistics is a probability distribution that arises in the problem of estimating the mean of a normally distributed population when the sample size is small. It is the basis of the popular Student t -tests for the statistical significance of difference between two sample means and for confidence intervals for difference between two population means. The Student t -distribution is a special case of the generalized hyperbolic distribution. A test of the null hypothesis is that the means of two normally distributed populations are equal. Given two data sets, each characterized by its mean, standard deviation and number of data points, one can use some kind of ' t ' test to determine whether the means are distinct, provided that the underlying distributions can be assumed to be normal. All such tests are usually called Student t tests, though strictly speaking that name should only be used if the variances of the two populations are also assumed to be equal. There are different versions of the t test depending on whether the two samples are:

1. Independent of each other (e.g., individuals randomly assigned into two groups)

2. Paired, so that each member of one sample has a unique relationship with a particular member of the other sample (*e.g.*, the same people measured before and after an intervention). If the calculated P-value is below the threshold chosen for statistical significance (usually, the 0.05 level), then the null hypothesis, which usually states that the two groups do not differ, is rejected in favor of an alternative hypothesis, which typically states that the groups do differ.

1. A test of whether the mean of a normally distributed population has a value specified in a null hypothesis.

2. A test of whether the slope of a regression line differs significantly from 0. Once a *t* value is determined, its corresponding P value can also be found using a table of values from Student's *t*-distribution.

Factorial design of experiments has been highly adopted in biosorption studies and medium optimizations (Sen and Swaminathan, 2004; Carmona et al., 2005). Peternele et al., (1999) studied the uptake of cadmium and lead, using a 2^3 factorial design. Three operational factors were analyzed: temperature, ionic strength and pH. Similarly, Barros et al. (2003) studied the effects of pH, heavy metal and biomass concentration on biosorption of cadmium from oil field waters using *Aspergillus niger*. The biosorption process was modeled based on 2^3 factorial design. The authors had chosen biomass concentration, as the most important factor in their study. For degradation studies, Kargi and Eker (2005) investigated the 2,4-dichlorophenol (2,4-DCP) and toxicity removal from synthetic wastewater employing a continuous mode operated Rotating Perforated Tubes Biofilm Reactor (RTBR). A Box–Wilson statistical experiment design method was used to investigate the effects of major operating parameters such as type of feed chemical oxygen demand (COD) and feed 2,4 dichlorophenol concentrations on COD, 2,4-DCP removals. Niccolai et al. (2003) performed medium optimization for the production of constitutive recombinant *Helicobacter pylori* Neutrophil Activating Protein (NAP) in *Escherichia coli*. They adopted Central Composite Design (CCD) composed from the superimposition of two-level factorial design. Sen and Swaminathan (2004) evaluated the statistical factorial design based response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production. Carmona et al. (2005) studied biosorption of chromium using factorial experimental design. The removal of Cr^{3+} and Cr^{6+} was studied, separately, using the

factorial design 2^3 . The three factors considered were pH, temperature, and metal concentration at two markedly different levels. The results were analyzed statistically using the Student's *t*-test, analysis of variance and F-test to define the most important process variables affecting the metal removal efficiency. Recently, many researchers have applied statistical experimental designs for medium optimization to enhance the production formation using various cultures (Imandi et al., 2008; Reddy et al., 2008; Wang et al., 2008a; Wang et al., 2008b). In all the above studies, the authors have utilized a factorial design of experiments and observed that the statistical approach yielded a better and meaningful interpretation of the results. The factorial design approach for substituted phenol biodegradation studies is, however, scant.

It is clear that the above researchers focused on treating wastewater using bioreactor system by employing different microbial culture; however, they neglected the process involving an actinomycetes, which could yield a complete treatment of phenolics even at higher concentration in shorter time. Immobilized cell culture, instead of suspended the culture on a medium, which yields simplicity of operation and better biomass growth with higher toxicity tolerance show comparable performance with the suspension culture. In addition, it is feasible to adopt such systems for real wastewater treatment. The researchers have also failed to evaluate the reactor stability towards shock organic loading conditions, which helps to study the stability of reactor towards higher pollutant concentration. Also, continuous operation of the reactor, similar to that of real wastewater treatment plant, has not been largely dealt with. Further they ignore the major limitations of immobilized cell culture (PBR) such as bubble formation, gas-liquid channeling effect, reduction of pressure drop and poor oxygen mass transfer etc.

Therefore all these unexplored research directions were extensively studied and reported in the present thesis. Hence considering the major limitation of PBR and its designed principle the present thesis is focused to develop a newly designed PBR and study its effectiveness in treating synthetic wastewater containing substituted phenols as single and mixture of substrates employing the *A. chlorophenolicus* A6. In addition the performance of the newly designed bioreactor was also evaluated using a petroleum refinery wastewater supplemented with substituted phenols in continuous mode of operation. It is also intended to

compare the results of biodegradation performance of substituted phenols with that of simple PBR packed with cell immobilized calcium alginate bed.



CHAPTER 3

OBJECTIVE AND SCOPE

.....

Based on an extensive literature review on remediation of phenolic compounds from contaminated environment, the present study focused on biodegradation of substituted phenols viz., 4- chlorophenol (4-CP), 4- bromophenol (4-BP) and 4- nitrophenol (4-NP) both in single and mixed substrate conditions, by *Arthrobacter chlorophenolicus* A6. This research contributes to the study and development of a new degradation system that employs a newly designed packed bed bioreactor using an actinomycetes strain of *Arthrobacter chlorophenolicus* A6 for the treatment of recalcitrant organic substances present in aqueous milieu. To achieve the above objective, following investigations were performed.

- Optimization of media components and physical process parameters to enhance the rate and efficiency of substituted phenols biodegradation
- Batch shake flask studies on the culture growth and biodegradation of individual phenolic compounds in single substrate system
- Evaluation of substrate inhibition patterns and estimation of kinetic parameters involved in biodegradation of phenolics in batch shake flasks
- Simultaneous degradation of mixtures containing chloro, bromo and nitro phenols (multisubstrate system) in batch shake flasks
- Design and evaluation of a newly designed packed bed bioreactor (PBR) with immobilized *A. chlorophenolicus* A6 in treating 4-CP, 4-BP and 4-NP both individually and in mixtures under continuous mode of operation
- Stability study of the newly designed PBR under shock-loading conditions of the phenolics
- Assessing the comparative performance of the newly designed PBR packed with cell immobilized polyurethane foam (PUF) and with simple PBR packed with cell immobilized calcium alginate beads

- Examining the efficiency of the *A. chlorophenolicus* A6 to treat real industrial petroleum refinery wastewater supplemented with substituted phenols in the newly designed PBR

Organization of the Thesis

The presentation of the work has been divided into five chapters. The current **Chapter 1** presents a general introduction of the present work. While literature that supports the present work is presented in **Chapter 2**; objective and scope of the study is presented in **Chapter 3**. Details of the materials and methods adopted in the present study are described in **Chapter 4**. It elaborates the details of the newly designed packed bed bioreactor and description of cell immobilized with calcium alginate bead PBR system. It also provides technical information about the analytical methods adopted in the present work. **Chapter 5** contains the results and discussions which start with optimization of media components and culture conditions. This chapter addresses the efficiency of the biological system in treating phenolics, both in single and mixed substrate systems in batch shakes flasks. This chapter emphasizes the performance of newly designed PBR in treating the phenolics in single and mixed substrate systems under continuous mode of operation. It also elaborates the performance of *A. chlorophenolicus* A6 immobilized in calcium alginate beads in simple PBR. Finally it presents and discusses the efficiency of the *A. chlorophenolicus* A6 to treat actual industrial petroleum refinery wastewater supplemented with substituted phenols in the newly designed PBR. **Chapter 6** draws summary and appropriate conclusion based on the previous results and discussion and also provides some useful recommendations for future research in the relevant field.

CHAPTER 4

MATERIALS AND METHODS

.....

4.1 Materials

In this section various materials and equipments used and various methodologies exercised in the present investigation are described.

4.1.1 Glassware, Apparatus and Instruments

All glass wares used in this study were kept immersed overnight in chromic acid solution (2.25 gm $K_2Cr_2O_7$ and 20 ml concentrated H_2SO_4 in 1litre of distilled water) followed by washing with tap water and then distilled water. For drying cleaned glassware were kept in oven at $110^\circ C$ for hours before being used in the experiment.

Various instruments used in the present study are listed in Table 4.1

Table 4.1 Instruments and equipments used in the present investigation

Instruments/ Equipments	Parameters tested/ measured	Model/Manufacturer Specification
High performance liquid chromatography	4-CP, 4-BP and 4-NP	Varian Prostar 210
Liquid chromatography /Mass Spectroscopy	Metabolic intermediate such as 4-chlorocatechol, 4-nitrocatechol etc	WATERS LC-MS/MS system, Q-Tof Premier
Ion Chromatograph	Chloride ion	(Metrohm 792 AG, Herisau, Switzerland)
Scanning electron microscopy	Picture of microorganism immobilized in PUF	Make: LEO, Model: 1430 VP, U.S.A
Atomic Absorption spectrophotometer	Sr, Ni, Pb	Varian, USA, Model No.Spectra AA 240FS
UV-visible spectrophotometer	Biomass concentration. Protein concentration, toxicity removal etc	Model lambda-45, Perkin Elmer, USA
Eppendroff	To separate biomass and other	Sigma 1-15, Germany

Centrifuge	suspended solids from solution	
Centrifuge	To separate biomass and other suspended solids from solution	Remi C-24-BL Mumbai, India
Chemical oxygen demand digester	COD	HACH, DRB-200, USA
Autoclave	Sterilization	Equitron-7407PAD India
Digital pH meter	pH	Orion 3 star –QY-14478, Thermo Scientific, Singapore
Dissolved oxygen meter	DO	Orion 3 star –QY-14478, Thermo Scientific, Singapore
Electronic balance	weight	Sartorius, BT-224S Max. 220 g. d = 0.01mg
Oven	MLSS, Drying other materials	Tonco-PLT-125, India
Peristaltic Pump	Feeding into PBR and column reactor	Miclin India limited PP10
Incubator shaker	For optimization of media components, culture condition and bio-kinetic study	Daihan Lab tech, LSI-100SR Korea
Temperature controlled and circulation water bath	To maintain constant temperature of the reactor	LUADA
Multi-parameter water quality measurement kit	Conductivity, redox potential, TDS, NH ₄ , NO ₃ etc	Model 6920-M Yellow Springs, Ohio-USA
Air compressor	To provide air to reactor for microbial growth	Rocker- oil free 224, Tarson
Laminar Air flow hood	To transfer pure culture in aseptic manner	Clean air system, CAH -1800 Chennai, India
Water purification system	To provide distilled water and Millipore water	Sartorius –AG, arium -611uv, 61315. Germany
Circulating temperature control Water bath	To control reactor temperature by circulating temperature control hot water	Lauda-RA series, AL2HA-RA8, China
BOD incubator	To prepare slant culture in agar media	Delux model- IK-120 Delhi, India

4.1.2 Chemicals and Reagents

All the chemicals used were either of analytical grade (AR) or laboratory grade (LR). 4-chlorophenol, 4-bromophenol and 4- nitrophenol used in the study were of analytical grade and obtained from Hi-media (India) and Sigma Aldrich USA. HPLC grade methanol and acetic acid were used as solvent for HPLC (99.5% purity). 4-chlorocatechol and 4-

nitrocatechol used for metabolic intermediate analysis were analytical grade (99% purity) purchased from Sigma Aldrich USA. Low viscosity alginate was purchased from Loba Chemie, India.

4.2 Analytical Methods

In this section, different analytical techniques and methodologies used for the present investigation have been described. In general, standard techniques as detailed in standard methods (APHA, 1998) has been followed unless otherwise specified. pH was measured using a digital pH meter (Orion 3 star –QY-14478, Thermo Scientific, Singapore) with a sensitivity of 0.01 with temperature correction facility. The instrument was calibrated periodically with standard buffer solutions.

4.2.1 Chemical oxygen demand (COD)

Chemical oxygen demand (COD) of samples was measured by closed refluxed methods as per the Standard Methods (APHA, 1998). Digestion was carried out using a COD thermo reactor (HACH, DRB-200, USA) fitted with temperature controller and timer. Refluxing was done for 2 h at a temperature of 150°C. Digested samples were cooled down to room temperature and titrated against 0.010M ferrous ammonium sulphate (FAS). The FAS solution was standardized daily against standard $K_2Cr_2O_7$ digestion solution.

4.2.2 Heavy metals and characterization of petroleum refinery wastewater

Concentrations of heavy metals, such as strontium, lead and Nickel etc, in the petroleum refinery wastewater were measured using atomic absorption spectrometer (AAS) (Varian, USA, Model No. Spectra A A 240 FS) at their respective absorbance maxima. Conductivity, turbidity, ammonia, nitrate etc were performed using a multi-parameter water quality measurement kit (Model 6920-M Yellow Springs, Ohio-USA).

4.2.3 Substituted phenol analysis (HPLC method)

About 300 μl of sample was filtered through sterile syringe filter (PTFE filter media of 13 mm diameter, pore size 0.22 μm , Pall, USA) to remove biomass or any other particulate matters. Concentration of 4-CP, 4-BP and 4-NP were estimated using reverse phase HPLC (Varian Prostar 210) equipped with an Onsphere C-18 column (Varian) using methanol-water and acetic acid (50:49.1 v/v) as the mobile phase. The retention time of 4-CP, 4-BP and 4-NP were found to be 5.41, 6.42 and 3.21 min respectively at the flow rate of 0.4 ml min^{-1} . The substituted phenol concentrations were measured in the HPLC equipped with a UV-Vis detector at wavelength 280 nm. The standard curves for estimating 4-CP 4-BP and 4-NP in this HPLC analysis are shown in Figure 4.1 (a) (b) and (c).

For determination of substituted phenols the HPLC used in the present study has following specification

Detector: Perkin – Varian UV-Detector 325

Detection: UV-Absorption = 280 nm

HPLC-pump: Varian Prostar 210 pump model 210series

Flow rate: 0.4 ml min^{-1}

Column: C-18 column (Onsphere C-18 column)

Mobile-phase: Methanol: water: acetic acid (50:49:1)

Temperature: Room temperature.

4.2.4 Metabolic intermediate analysis

For identification of 4-CP, 4-BP and 4-NP biodegraded intermediates, the samples were analyzed using LC/MS (WATERS LC-MS/MS system, Q-Tof Premier). A capillary ACQUITY UPLC® BEH shield RP 181.7 μm C-18 column (10 $\times$ 50mm length) was used for separation of product intermediates. The mobile phase was a mixture of acetonitrile–water–formic acid (1:1:0.1 v/v) filtered through Millipore syringe filter of 0.22 μm (Pall USA).

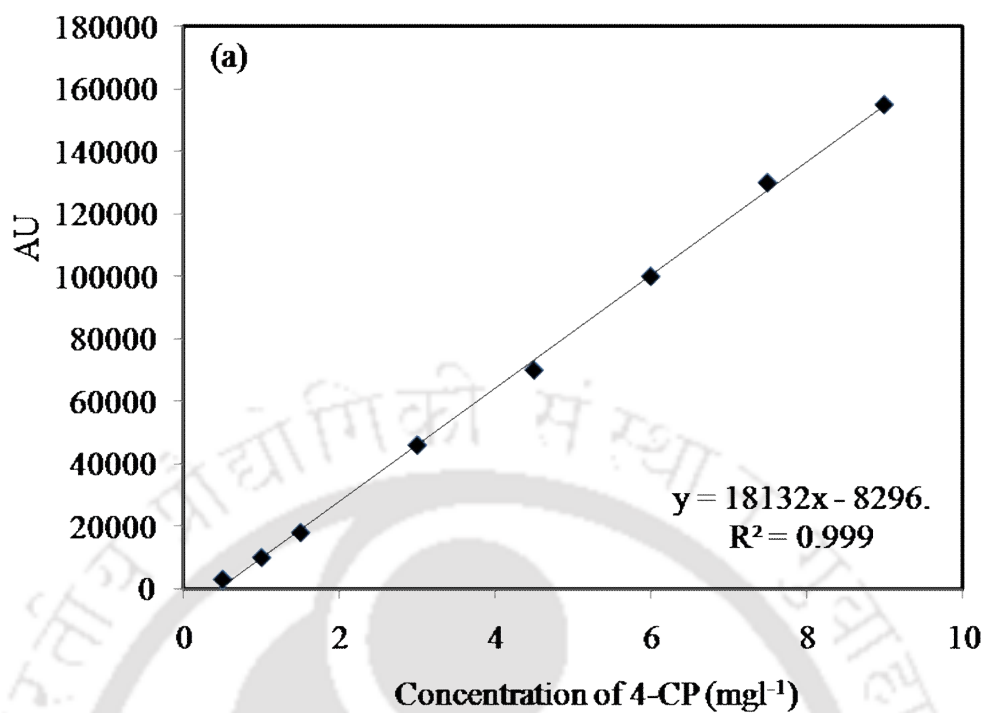


Figure 4.1 Calibration curve obtained for substituted phenols estimation using the HPLC (a) 4-CP.

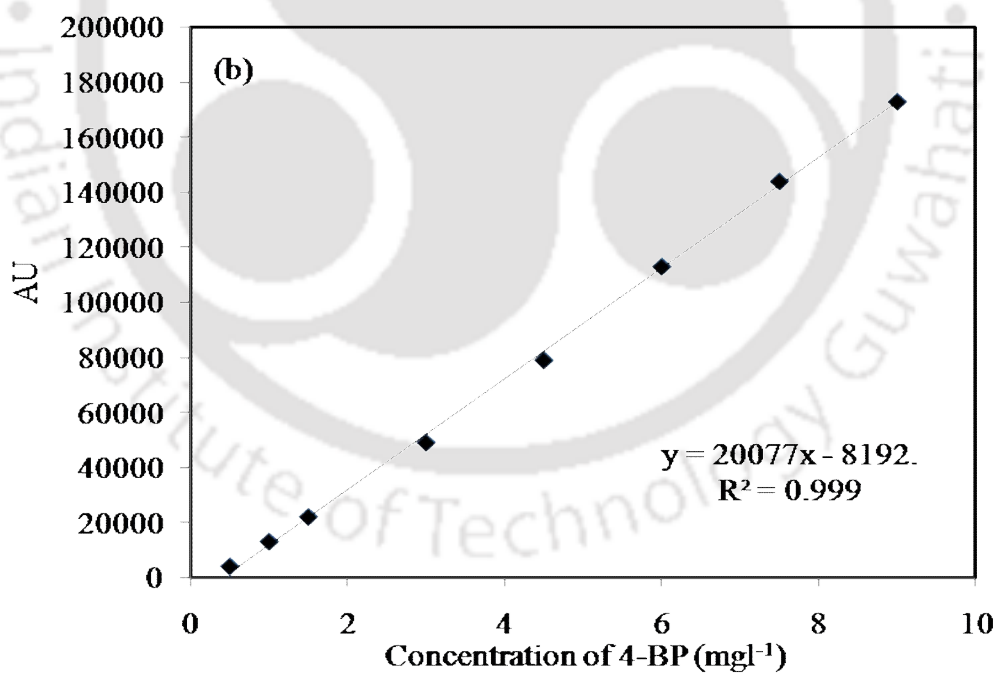


Figure 4.1 Calibration curve obtained for substituted phenols estimation using the HPLC (a) 4-BP.

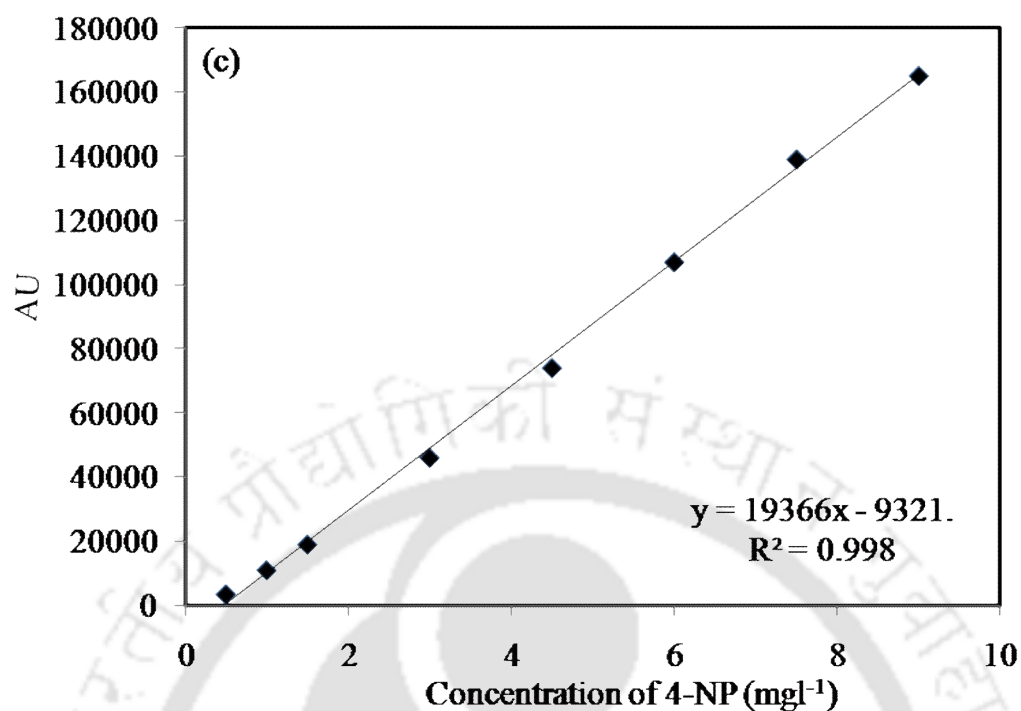


Figure 4.1 Calibration curve obtained for substituted phenols estimation using the HPLC (c) 4-NP.

4.2.5 Chloride ions analysis

Chloride ions were measured using a Ion Chromatograph (Metrohm AG 792, Herisau, Switzerland) equipped with a Dual 3 column (250mm×4 mm), a RP guard column, and a conductivity detector. Samples taken during the experiments were centrifuged at 8000 rpm for 10 min and were filtered through a C-18 reverse-phase cartridge and then through 0.45µm filter for analysis. NaOH (5mM) served as the eluent and sulphuric acid (2.0mM) as the regenerant in the chromatogram analysis.

4.2.6 Microscopic methods

Fragments of polyurethane foam (PUF) containing the microbial bio-film were sampled at various sampling points of the packed bed reactor and cut into small thin pieces. The sample was mounted on aluminum stubs and then coated with gold using sputter coater

(Edward, UK). The stubs were then introduced into the specimen chamber of LEO 1430vp scanning electron microscope for scanning.

4.2.7 Biomass concentration

Quantification of *A. chlorophenolicus* A6 biomass in liquid culture samples was done by measuring its optical density at wavelength 600 nm (OD_{600}) using a UV-visible spectrophotometer (Model lambda-45, Perkin Elmer, USA). The absorbance values were expressed as dry cell weight using a calibration curve plotted between the biomass optical density (OD) versus mixed liquor suspended solids (MLSS) of the sample as shown in Figure 4.2.

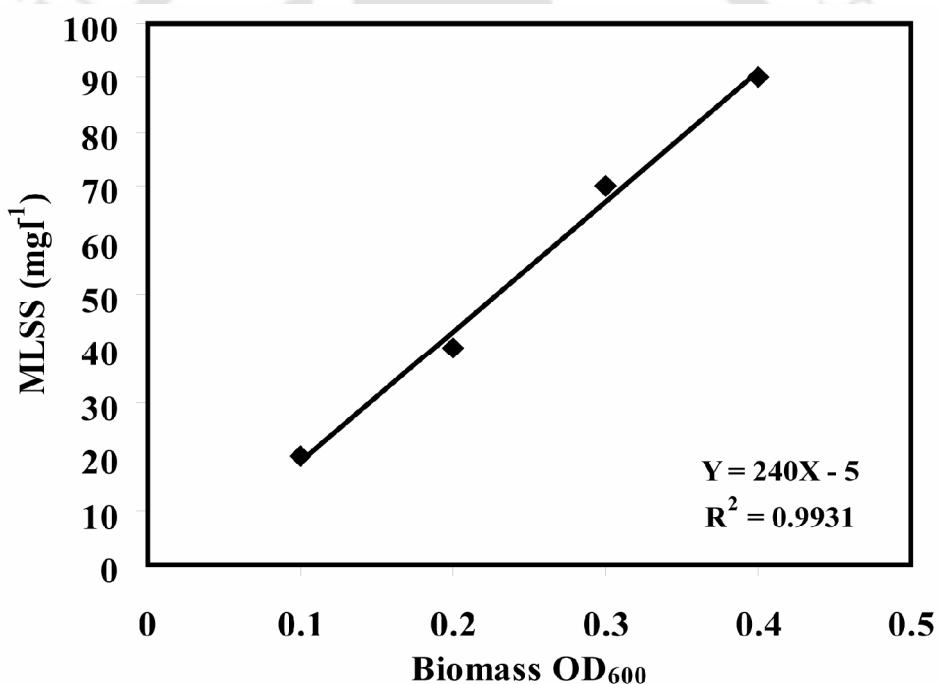


Figure 4.2 Calibration curve biomass optical density (OD_{600}) versus mixed liquor suspended solids (MLSS).

For determination of biomass concentration as dry weight, 10 ml of samples at different optical density ranging from 0.1-0.8 OD_{600} were centrifuged for 10 min at 10000 g in ambient temperature (Remi C-24-BL Mumbai, India) in a previously weighed 15 ml centrifuge tube (Tarson, India). Obtained pellets were dried at 105 °C to constant weight and

its final weight was measured. One unit of absorbance was found equivalent to 235 mg l⁻¹ of MLSS.

4.2.8 Biomass concentration in polyurethane foam (PUF)

Quantification of *A. chlorophenolicus* A6 biomass immobilized in PUF cubes was measured in term of its protein content following Bradford method of protein assay (1976). About 1 g of PUF was dried at 105°C for 12 h and soaked in 1M NaOH solution, which was then digested at 90 °C for 10 min. After cooling, the Bradford protein assay was performed. The calibration curve for Bradford protein assay was given in Figure 4.3

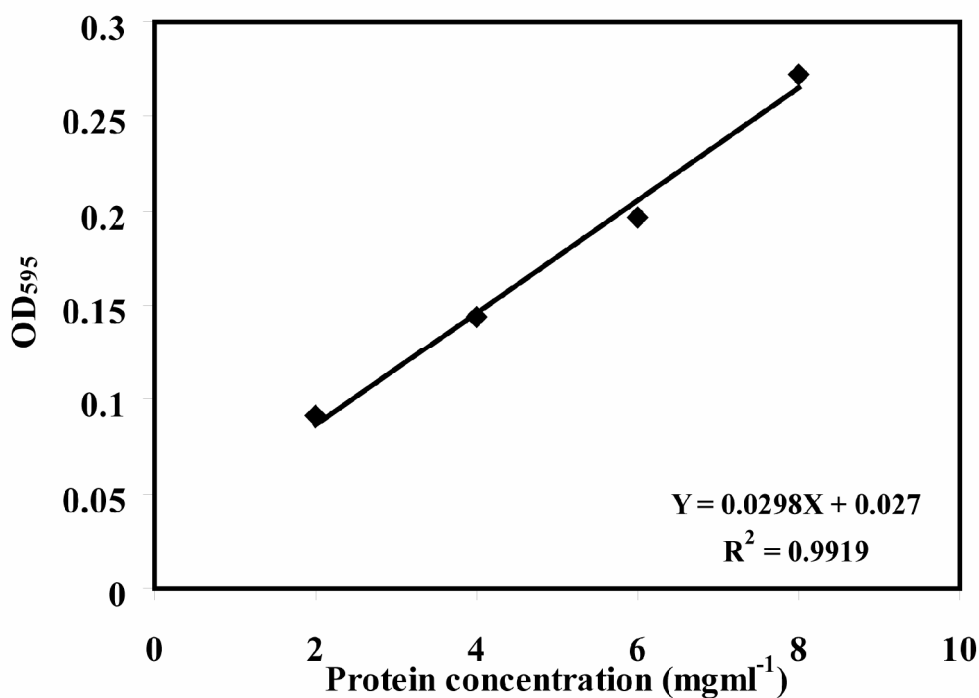


Figure 4.3 Bradford protein assay calibration curve obtained in the present study.

The protein concentration of the PUF was converted to biomass concentration by comparing the earlier standard curve plotted between known biomass MLSS versus its respective protein content as shown in Figure 4.4.

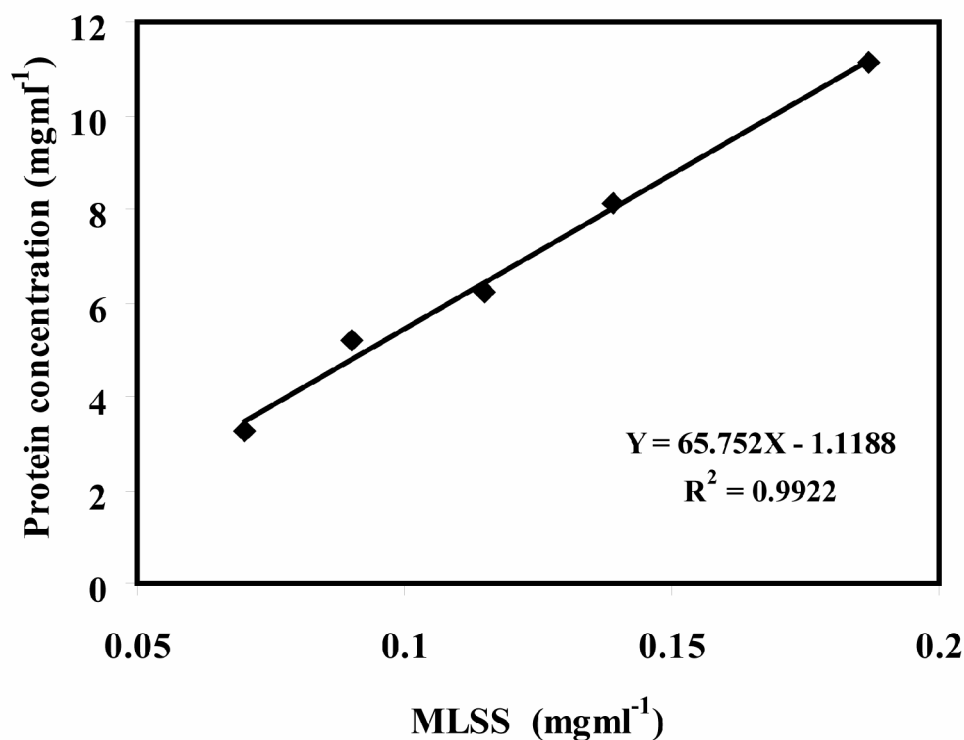


Figure 4.4 Calibration curve for conversion of protein content to mixed liquor suspended solid (MLSS).

4.3 Experimental Methodologies

4.3.1 Microorganism and its Maintenance

The microorganism *Arthrobacter chlorophenolicus* A6 used in the study was kindly gifted by Professor J.K. Jansson, Department of Biochemistry, Stockholm University, Sweden. The culture was maintained on slants containing mineral salt media as described by Westerberg et al. (2002) with 3 gml⁻¹ yeast extract and 20 gml⁻¹ agar, pH 7.4.

4.3.2 Seed Culture Development

Media used for developing seed culture of the microorganism for use in biodegradation experiments contained mineral salt media (MSM) having the composition (g l⁻¹): K₂HPO₄ 2.1, KH₂PO₄ 0.4, NH₄NO₃ 0.6, MgSO₄ 0.2, CaCl₂ 0.023, FeCl₃ 0.002,

(Alexandar and Lustigman, 1996) with 1gml^{-1} yeast extract and 4-CP at a concentration of 150 mg l^{-1} . The seed culture medium (100 ml) taken in a 250 ml Erlenmeyer flask was inoculated with a loop full of the culture freshly grown on agar slants and incubated for 48 h at 28°C and 180 rpm.

4.3.3 Inoculum Preparation

Seed culture cells obtained as before were harvested at late exponential phase (48h) by centrifugation (6000g, 20 min at 22°C), washed in $1\times$ phosphate buffer saline (10X PBS gml^{-1} ; 14.4 Na_2HPO_4 , 2.4 K_2HPO_4 , 8.0 NaCl , 2.0 KCl) pH 7.4 (Jernberg and Jansson, 2002), added to the 250 ml Erlenmeyer flasks with 100 ml of MSM containing 300 mg l^{-1} 4-CP as the sole source of carbon and energy. The flasks were incubated for overnight in an orbital incubator shaker and were re-grown at 28°C , 180 rpm. Obtained cells were re-centrifuged and washed with PBS buffer as before, and subsequently used as inoculum in biodegradation experiments to give an initial inoculum concentration of 0.1 OD_{600} .

4.3.4 Medium Development to Enhance Biodegradation of Substituted Phenol

4.3.4.1 Screening of medium constituents by 2-level factorial experimental design

For selecting the most significant medium components affecting 4-CP degradation and specific growth of the culture, a variety of inorganic salts and nitrogen source were screened using the 2-level factorial design of experiments. The screened media components were subsequently used for optimization of their respective levels. The experimental design for screening consisted of an adequately chosen fraction ($1/4$) of the full factorial design (2^6), which was powerful enough for screening of independent variables with reasonable resource and time. The media constituents chosen for screening study were K_2HPO_4 , KH_2PO_4 , NH_4NO_3 , MgSO_4 , CaCl_2 and FeCl_3 . The range and levels of the constituents used in the study are presented in Table 4.2, which also shows the experiment combinations adopted. Thus, a total of 20 runs (16 runs as per the design and 4 runs at the center point of each constituent) were performed (Table 4.2).

Table 4.2 Two level factorial design for screening of media constituents

Run order No	K ₂ HPO ₄	KH ₂ PO ₄	NH ₄ NO ₃	MgSO ₄	CaCl ₂	FeCl ₃
1	3.1(1)	0.2(-1)	0.31(-1)	0.11(-1)	0.033(1)	0.001(-1)
2	3.1(1)	0.6(1)	0.31(-1)	0.31(1)	0.013(-1)	0.001(-1)
3	1.1(-1)	0.2(-1)	0.91(1)	0.31(1)	0.033(1)	0.001(-1)
4	3.1(1)	0.6(1)	0.91(1)	0.31(1)	0.033(1)	0.003(1)
5	3.1(1)	0.6(1)	0.91(1)	0.11(-1)	0.033(1)	0.001(-1)
6	3.1(1)	0.2(-1)	0.91(1)	0.11(-1)	0.013(-1)	0.003(1)
7	3.1(1)	0.2(-1)	0.91(1)	0.31(1)	0.013(-1)	0.001(-1)
8	2.1(0)	0.4(0)	0.61(0)	0.21(0)	0.023(0)	0.002(0)
9	2.1(0)	0.4(0)	0.61(0)	0.21(0)	0.023(0)	0.002(0)
10	2.1(1)	0.2(-1)	0.31(-1)	0.31(1)	0.033(1)	0.003(1)
11	1.1(-1)	0.6(1)	0.31(-1)	0.31(1)	0.033(1)	0.001(-1)
12	2.1(0)	0.4(0)	0.61(0)	0.21(0)	0.023(0)	0.002(0)
13	2.1(0)	0.4(0)	0.61(0)	0.21(0)	0.023(0)	0.002(0)
14	1.1(-1)	0.6(1)	0.91(1)	0.31(1)	0.013(-1)	0.003(1)
15	1.1(-1)	0.2(-1)	0.31(-1)	0.31(1)	0.013(-1)	0.003(1)
16	1.1(-1)	0.2(-1)	0.31(-1)	0.11(-1)	0.013(-1)	0.001(-1)
17	1.1(-1)	0.6(1)	0.91(1)	0.11(-1)	0.013(-1)	0.001(-1)
18	1.1(-1)	0.6(1)	0.31(-1)	0.11(-1)	0.033(1)	0.003(1)
19	3.1(1)	0.6(1)	0.31(-1)	0.11(-1)	0.013(-1)	0.003(1)
20	1.1(-1)	0.2(-1)	0.91(1)	0.11(-1)	0.033(1)	0.003(1)

Uncoded values (g l⁻¹) are given without bracket where as coded values are in bracket

The principal effects of each variable on 4-CP degradation and culture specific growth rate were estimated as the difference between both averages of measurements made at the higher level and at the lower level. The effect of each variable was determined using the following equation:

$$E_{(xi)} = \frac{2(\sum M_{i+} - M_{i-})}{N} \quad (4.1)$$

E_{xi} is the concentration effect of the tested variable. M_{i+} and M_{i-} are the 4-CP degradation activity and culture specific growth rate from the trials in which the concerned variable (X_i) was present at high and low levels respectively, in different N number of trials. In Table 4.2 the levels -1, 0 and +1 of the media constituents were chosen in such a way that center point (0) values first represented the factor levels used conventionally for growing *A. chlorophenolicus* A6 (Westerberg et al., 2000); and, accordingly the other levels of the variables were determined using the following relationship:

$$x_i = \frac{U_i - U_0}{\Delta U} \quad (4.2)$$

where X_i is the coded level (-1, 0, +1) of any independent variable, U_i is the uncoded/actual level of the independent variable, U_0 is the uncoded level of the independent variable at its centre point and ΔU is the step change. The significance of each variable was determined via Student's t-test.

In all these experiments inoculum was prepared as described in section 4.3.3 and added to 250 ml biodegradation flasks containing 100 ml of media along with 300 mg l⁻¹ 4-CP and varying media components as per the experimental design with an initial inoculum concentration of 0.1 OD₆₀₀. Following inoculations the flasks were incubated in orbital shaking incubator at 28°C and 180 rpm. Growth of *A. chlorophenolicus* A6 in the flasks was determined by taking samples at regular interval of time and 4-CP degradation was monitored by analyzing its concentration in the culture supernatant after centrifuging at 6000×g for 10 min. Statistical analysis of the results obtained were performed using the MINITAB, Release 15.1, PA, USA.

4.3.4.2 Optimization of screened medium components using central composite design (CCD)

Following the result of the most significant medium components affecting 4-CP degradation by the culture, their levels were optimized using response surface methodology (Box and Hunter, 1957). The screened media components were K₂HPO₄, NH₄NO₃, MgSO₄ and CaCl₂ and according to CCD, the total number of treatment combinations was 2^k + 2k + n₀, i.e. 31 = 2⁴ + 2·4 + 7, where 'k' is the number of independent variables and n₀ the number of replicates at the center point (Araujo et al., 1996). Table 4.3 shows the range and levels of these four factors: twenty four experiments were augmented with seven replications at the center points to evaluate error in the experiments. The levels of the statistically non-significant medium components (as reflected from the 2-level factorial design analysis) were kept at their mid values (center point, 0) in the experiments (Table 4.3) viz., KH₂PO₄ (0.4 g l⁻¹), FeCl₃ (0.002 g l⁻¹). The inoculum preparation and addition as discussed in section 4.3.3.

In addition, the experimental condition, and statistical analysis were the same as noted in the screening study (4.3.4.1).

Table 4. 3 Central composite design matrix showing used in the optimization study ($\alpha=2$).

Run order No	Variables			
	KH_2PO_4 gl^{-1}	NH_4NO_3 gl^{-1}	MgSO_4 gl^{-1}	CaCl_2 gl^{-1}
1	1.1(-1)	0.91(+1)	0.31(+1)	0.013(-1)
2	3.1(+1)	0.31(-1)	0.31(+1)	0.033(+1)
3	2.1(0)	0.61(0)	0.21(0)	0.003(- α)
4	3.1(+1)	0.91(+1)	0.31(+1)	0.033(+1)
5	3.1(+1)	0.31(-1)	0.11(-1)	0.013(-1)
6	2.1(0)	0.61(0)	0.21(0)	0.023(0)
7	3.1(+1)	0.91(+1)	0.11(-1)	0.013(-1)
8	2.1(0)	1.21(+ α)	0.21(0)	0.023(0)
9	1.1(-1)	0.91(+1)	0.31(+1)	0.033(+1)
10	2.1(0)	0.61(0)	0.41(+ α)	0.023(0)
11	2.1(0)	0.61(0)	0.21(0)	0.023(0)
12	2.1(0)	0.61(0)	0.21(0)	0.023(0)
13	1.1(-1)	0.91(+1)	0.11(-1)	0.033(+1)
14	3.1(+1)	0.31(-1)	0.11(-1)	0.033(+1)
15	0.1(- α)	0.61(0)	0.21(0)	0.023(0)
16	3.1(+1)	0.91(+1)	0.11(-1)	0.033(+1)
17	3.1(+1)	0.91(+1)	0.31(+1)	0.013(-1)
18	2.1(0)	0.01(- α)	0.21(0)	0.023(0)
19	2.1(0)	0.61(0)	0.21(0)	0.023(0)
20	3.1(+1)	0.31(-1)	0.31(+1)	0.013(-1)
21	2.1(0)	0.61(0)	0.21(0)	0.023(0)
22	2.1(0)	0.61(0)	0.21(0)	0.023(0)
23	1.1(-1)	0.31(-1)	0.11(-1)	0.033(+1)
24	2.1(0)	0.61(0)	0.01(- α)	0.023(0)
25	4.1(+ α)	0.61(0)	0.21(0)	0.023(0)
26	1.1(-1)	0.91(+1)	0.11(-1)	0.013(-1)
27	2.1(0)	0.61(0)	0.21(0)	0.043(+ α)
28	1.1(-1)	0.31(-1)	0.11(-1)	0.013(-1)
29	2.1(0)	0.61(0)	0.21(0)	0.023(0)
30	1.1(-1)	0.31(-1)	0.31(+1)	0.013(-1)
31	1.1(-1)	0.31(-1)	0.31(+1)	0.033(+1)

Uncoded values (g l^{-1}) are given without bracket where as coded values are in bracket.

4.3.5 Optimization of Physical Process Conditions to Enhance Biodegradation of Substituted Phenols

4.3.5.1 Experimental design

The physical process parameters chosen for this study were pH, agitation (rpm), temperature (°C) and inoculum age (h). Employing CCD, the total number of treatment combinations was $2^k + 2k + n_0$, i.e. $31 = 2^4 + 2 \cdot 4 + 7$, where 'k' is the number of independent variables and n_0 the number of replicates at the center point (Araujo et al., 1996). Table 4. 4 shows the range and levels of these four factors: twenty four experiments were augmented with seven replications at the center points to evaluate the error. The name of the variables, symbol and their actual and coded level in the experimental design is shown in design Table 4.4. In all these experiment the inoculum was prepared as described in above section 3.2.3 at different time interval as per the CCD experimental design and was added to 250 ml biodegradation flask containing 100 ml of earlier optimized mineral salt media with varying initial pH as per the experimental design containing initial 4-CP concentration of 300 mg l^{-1} so as to give an initial inoculum concentration of 0.1 OD₆₀₀. Then these biodegradation flasks were incubated at different culture conditions (agitation speed and temperature) in a rotating shaker incubator as per the CCD design. Growth of *A. chlorophenolicus* A6 in the flasks, degradation of 4-CP and statistical analysis were the same as noted in the screening study (4.3.4.1). The data obtained from CCD on 4-CP degradation percentage and culture specific growth rate were subjected to analysis of variance (ANOVA) and Student's t-test.

4.3.6 Biodegradation of Substituted Phenols in Batch Shake Flasks

4.3.6.1 Single substrate system

Batch biodegradation experiments in shake flasks using the *A. chlorophenolicus* A6 were carried out in 250 ml Erlenmeyer flasks with 100 ml of optimized MSM containing different initial concentration of substituted phenol (s) as the sole source of carbon and energy, and incubated in an orbital shaker at 30 °C and 210 rpm. Single substrate degradation experiments were carried out with an initial concentration range varied from 25 – 350 mg l^{-1} for both 4-CP and 4-BP: whereas, for 4-NP it was in the range varied from 12.5- 225 mg l^{-1}

Table 4.4 Central composite design matrix showing used in the optimization study ($\alpha = 2$).

Run order No	Variables			
	pH	Agitation (rpm)	Temp (°C)	Inoculum age (h)
1	7.4 (0)	180(0)	28(0)	72(+ α)
2	7.0 (-1)	210(+1)	32(+1)	60(+1)
3	7.0(-1)	150(-1)	32(+1)	60(+1)
4	7.4 (0)	180(0)	28(0)	24(- α)
5	8.2 (+ α)	180(0)	28(0)	48(0)
6	7.8(+1)	150(-1)	24(-1)	60(+1)
7	7.4(0)	180(0)	28(0)	48(0)
8	7.4(0)	180(0)	28(0)	48(0)
9	7.0 (-1)	210(+1)	32(+1)	36(-1)
10	7.4 (0)	180(0)	28(0)	48(0)
11	6.6 (- α)	180(0)	28(0)	48(0)
12	7.4(0)	120((- α))	28(0)	48(0)
13	7.4(0)	240(+ α)	28(0)	48(0)
14	7.8(+1)	150(-1)	32(+1)	60(+1)
15	7.8(+1)	150(-1)	24(-1)	36(-1)
16	7.8(+1)	210(+1)	24(-1)	36(-1)
17	7.0(-1)	210(+1)	24(-1)	60(+1)
18	7.0(-1)	150(-1)	24(-1)	60(+1)
19	7.0(-1)	150(-1)	24(-1)	36(-1)
20	7.8(+1)	210(+1)	32(+1)	60(+1)
21	7.8(+1)	150(-1)	32(+1)	36(-1)
22	7.8(+1)	210(+1)	24(-1)	60(+1)
23	7.4(0)	180(0)	20(- α)	48(0)
24	7.4(0)	180(0)	28(0)	48(0)
25	7.4(0)	180(0)	28(0)	48(0)
26	7.0(-1)	210(+1)	24(-1)	36(-1)
27	7.8(+1)	210(+1)	32(+1)	36(-1)
28	7.4(0)	180(0)	28(0)	48(0)
29	7.0(-1)	150(-1)	32(+1)	36(-1)
30	7.4(0)	180(0)	36(+ α)	48(0)
31	7.4(0)	180(0)	28(0)	48(0)

Meas.* and Pred.* indicate measured and predicted responses

In all the three experiments, an initial biomass concentration of 0.1 OD_{600 nm} was added as the inoculum. The culture flasks were incubated in an orbital shaker incubator at 30 °C and 210 rpm. Samples were collected at regular intervals and analyzed for biomass and residual concentration of the compounds in the medium. Each experiment was performed in triplicate.

4.3.6.2 Mixed substrate system

Mixed substrate degradation study containing 4-CP, 4-BP and 4-NP as the substrates were planned as per 2^2 factorial design of experiments. Different concentration ranges of these substrates ($75\text{--}125\text{ mg l}^{-1}$ for both 4-CP and 4-BP whereas $50\text{--}100\text{ mg l}^{-1}$ in case of 4-NP) were chosen in this study. Table 4.5 presents various combinations of the initial concentrations of phenolics, where -1 and $+1$ indicate respective low and high concentration levels and 0 indicates center point or middle level of these three factors. Three center point replicates were included in the design to check for experimental error. Inoculum for this experiment was prepared as before (section 4.3.3) and a freshly grown 4-CP acclimatized biomass ($0.1\text{ OD}_{600\text{nm}}$) was added to the biodegradation flasks containing 100 ml of optimized MSM. Samples were collected during the experiments and analyzed for biomass and phenolics concentrations. Results of biomass growth and substrate degradation in the study were statistically analyzed in the form of analysis of variance (ANOVA) and Students ' t ' test using the statistical software MINITAB (Version 12.2 PA, USA).

Table 4.5 2^2 full factorial design with three center point replicates adopted in the mixed substrate biodegradation study

Expt. Run No.	Factors and their levels		
	4-CP	4-BP	4-NP
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+
9	0	0	0
10	0	0	0
11	0	0	0

4.4 Reactor Configuration and Experimental Set-Up

In this section, description on various experimental set-ups, reactor specification, feeding and operating conditions are given in detail.

4.4.1 Newly Designed PBR

A laboratory scale PBR was fabricated with a transparent perspex cylinders with internal cross sectional area of 130 cm^2 and height 60 cm. Schematic diagram of the PBR is shown in Figure 4.5 and the actual photograph of the experimental set-up of the PBR system is given in Figure 4.6.

The following are the main components of the newly designed PBR:

a) Main reactor

The main reactor was made up of perspex tube with an internal and external diameter of 11.4 cm and 12 cm respectively. Total length of the reactor was 60 cm. Working volume of the reactor was kept as 4L. Polyurethane foam (PUF) was used as supporting material for microbial growth. For sampling as well as for aeration, ports were provided at every 10 cm length along the height of the reactor.

b) Feed and aeration distribution system

For uniform aeration and efficient distribution of feed up to deep inside the cell immobilized PUF the PBR was fabricated by employing cross flow design, uses of circular perforated perspex disc wrapped with nylon mesh together with ceramic gas diffuser along the height of the reactor. The purpose of cross flow design and uses of perforated disc wrapped with nylon mesh, and ceramic gas diffuser along the height was given below: It is clearly evident from literature that cross flow design by multiple points along the height in packed beds reduces pressure drop enabling the use of actual flow rates and even flow distribution across packed bed (Ajmera et al., 2002).

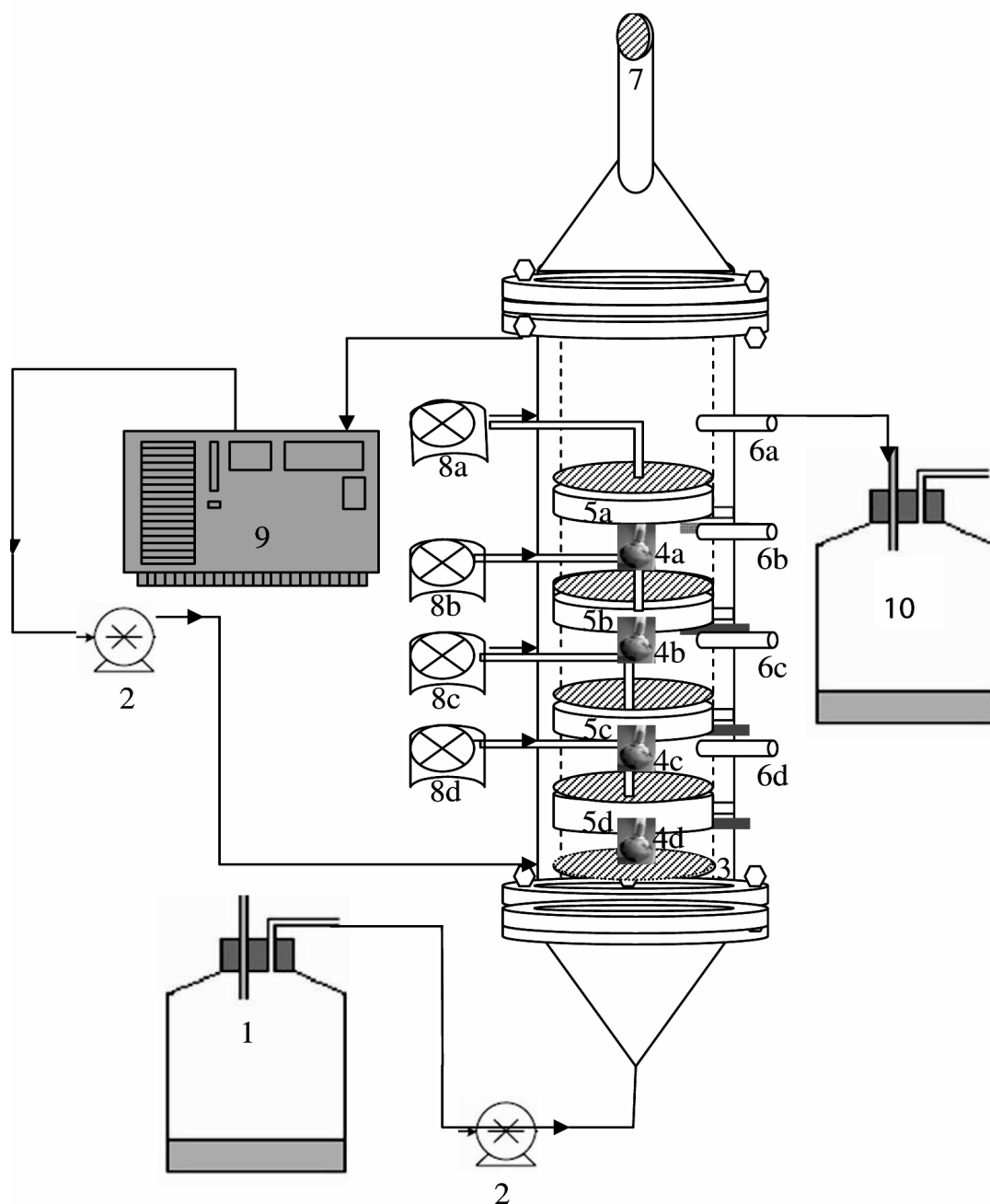


Figure 4.5 Schematic of the experimental set up of the novel PBR with its various components: (1) Influent tank, (2) peristaltic pump, (3) wire gauze, (4) a, b, c and d, ceramic gas diffuser, (5) a, b, c and d, perforated perspex disc with its top wrapped with nylon mesh, (6) a, b, c and d sampling port, (7) gas outlet, (8) a, b, c and d aeration pumps, (9) water bath, and (10) effluent reservoir.

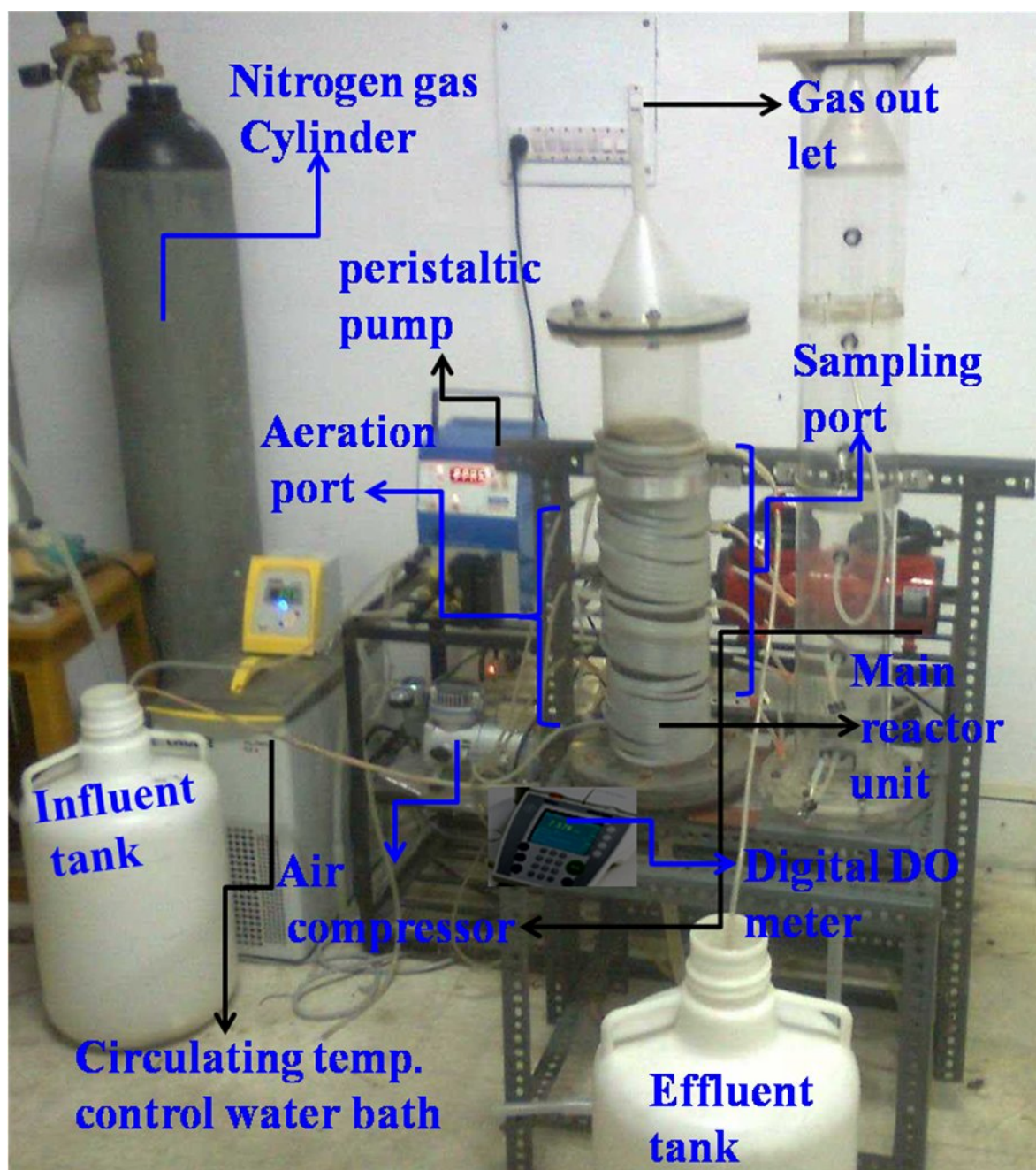


Figure 4.6 Photograph of the PBR used in the study.

Uses of cross flow design:

Furthermore, aeration at single port with high flow rate creates high shear stress that hampers the biomass immobilization process as well as can cause cell erosion from the

supporting materials. However, fragmented approach of aeration (cross flow design/separation of the aeration zone) can reduce the shearing stress (Fassnacht and Pörtner, 1999). Therefore, the PBR used in the present study was devised by adopting cross flow design with fragmented approach of aeration to minimize the pressure drop as well as shear stress on microorganism.

Uses of circular perforated perspex disc wrapped with nylon mesh, and ceramic gas diffuser along the height:

A circular perforated perspex disc was designed to have about 70% openings through a number of holes of diameter 5 mm. A nylon mesh with mesh size 0.25 mm was wrapped over the top of the disc (Fig. 4.7). Four numbers of such discs wrapped with nylon mesh were placed at 4 different places (each 10 cm height) along height of the reactor. Ceramic gas diffusers were placed on the bottom of the each disc.

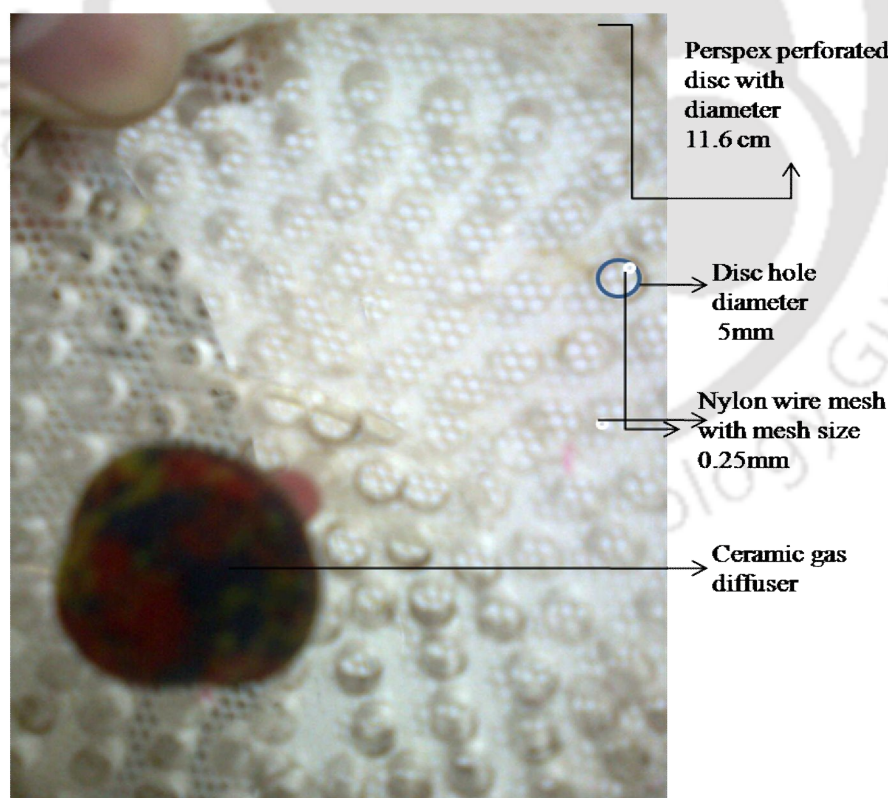


Figure 4.7 Perforated perspex disc covered with nylon mesh at the top with ceramic gas diffuser assembly.

This arrangement was made to improve of mass transfer and distribution of dissolved oxygen together with nutrients even deep inside the PUF (Meyer and Smith, 1985; Radilla et al., 2005). Such arrangement can further reduce in pressure drop, which is a common phenomenon in conventional (Radilla et al., 2005; Meyer and Smith, 1985). Furthermore, uses of the perspex disc covered with nylon mesh together with ceramic gas diffusers helps in minimizing large bubble formation and thus reduce gas liquid channelling.

c) Bio-support material (PUF)

Inert PUF was cut into sizes of approximately $2.5 \text{ cm} \times 1.5 \text{ cm} \times 1.5 \text{ cm}$ washed with de-ionized water, autoclaved (20 min, 120°C), rewashed, and dried overnight at 70°C in a hot air oven before being used as bio-support material in the newly designed PBR. About 100 gm of oven dried PUF were placed in the PBR manually, more or less uniformly and homogeneously in layers starting from bottom in between weir gauge and to the perforated discs inside the reactor.

d) Feed flow rate adjustment

The reactor was fed with synthetic wastewater containing 4-CP, 4-BP and/or 4-NP at controlled flow rate using a peristaltic pump (Model: pp10, Miclins India). Flow rate was maintained for hydraulic retention time (HRT) of 24, 18, 12.5, 10 and 7.5 hours were 2.88, 4.32, 6.48, 8.64 and 11.52 litre day⁻¹ respectively.

Table 4.6 MSM used in the substituted phenols degradation in continuous mode of operation using the PBR

Sl. No	Name of the chemical	Concentration (gl ⁻¹)
1	K ₂ HPO ₄	2.6
2	KH ₂ PO ₄	0.4
3	NH ₄ NO ₃	0.58
4	MgSO ₄	0.17
5	CaCl ₂	0.038
6	FeCl ₃	0.002

e) Feed composition

Optimized mineral salt media (MSM) (section 5.1.3.2) spiked with required amount of substituted phenols was used as feed for the microorganisms in the newly designed PBR. The composition of the optimized MSM is given in Table 4.6.

f) Air flow rate adjustment

Oxygen in the form of air was supplied through an air compressor (Rocker-440 Tarson, India) at varying air flow rates. The aeration rate was monitored using a digital flow meter (Flow-tech engineers, Mumbai, India).

g) Arrangement for temperature control

The temperature inside the reactor was maintained at $29 \pm 1^\circ\text{C}$ by circulating warm water from a thermostat installed temperature controlled water bath (LAUDA, RA-8 Germany) through silicon pipes wrapped over the reactor throughout its length.

h) Dissolved oxygen and pH measurement

Dissolved oxygen concentration was measured using a digital DO meter (Orion 3 star –QY-14478, Thermo Scientific, Singapor) with a sensitivity of 0.01. pH was measured using a digital pH meter (Orion 3 star –QY-14478, Thermo Scientific, Singapore) with a sensitivity of 0.01 with temperature correction facility. The instrument was calibrated periodically with standard buffer solutions

4.4.2 Porosity Determination of Polyurethane Foam

Porosity and void volume of PUF was determined according to the method described by Hodge and Devanny (1995) as follow: PUF material density analysis was evaluated by weighing a sample of known volume. The porosity of samples was estimated by determining

the sample to volume ratio. The volume of the sample was determined with a graduated cylinder (1L). The sample was weighed with an analytical balance before water was added to fill the void space volume. Air bubbles were dislodged by periodically tapping the cylinder. The saturated sample weight was then determined and percent porosity was calculated from the following relationship.

$$\%porosity = \frac{\text{Void space volume}}{\text{volume of the sample}} \quad 4.3$$

$$\%porosity = \frac{\left(\text{weight of sample + cylinder + water} \right) - \left(\text{weight of sample + cylinder} \right)}{\frac{\text{density of water}}{\text{volume of sample}}} \quad 4.4$$

4.4.3 Hydrodynamic Experiments in the PBR

Hydrodynamic experiments were performed with the PBR under two different aeration systems viz., aeration only through the bottom aeration port and aeration through all the 4 ports to achieve cross flow aeration. The reactor operated under the first condition (aeration through single port) was designated as “simple PBR”, whereas, the reactor operated under the second condition (aeration through all the 4 ports) was designated as “newly designed PBR”. In the first case, no perforated circular disks were used in the reactor whereas, in the latter case 4 nos. of perforated circular discs wrapped with nylon mesh at the top were placed just above the ceramic porous stones through which oxygen (air) was diffused into the reactor content by supplying compressed air from an air compressor.

Mixing Time Profile

Mixing time in both the conditions of the reactor was determined using a tracer technique that measured pH change in a pre-homogenized solution after an acid pulse (Gomez-DeJesus et al., 2009). The simple PBR was first filled up with 0.15 N NaCl solution before an aliquot of 6N HCl (2.0ml) was injected at the bottom of the reactor. Then aeration

was done at four different superficial gas flow rates of (cm s^{-1}); 0.078, 0.157, 0.236, 0.315. The effluent pH was then measured at different time intervals using a pH meter (Sartorius, PA 20, USA). The experiment was repeated for newly designed PBR after the acid retained by the support material in the reactor was washed out by draining and rinsing the bed three times with distilled water. The transitory changes in the relative values of pH ($\Delta pH[t]$), were estimated using the following relation:

$$\Delta pH[t] = \frac{pH[t] - pH_0}{pH_0 - pH_\infty} \quad (4.5)$$

Where $pH[t]$ represents pH at time $[t]$, pH_0 is initial pH and pH_∞ represent pH at infinitive time or saturated pH value.

By plotting $\Delta pH[t]$ data, t_{m95} (i.e. time to reach 95% homogeneity after a tracer input (s)) was estimated for each value of U_{GR} (superficial air velocity, cms^{-1}) in the reactor.

Oxygen Mass Transfer Coefficient Determination

The oxygen mass transfer coefficients in both the operating conditions of the reactor (i.e., simple PBR and newly designed PBR) were determined by classical dynamic gassing-in method under abiotic condition (i.e. before inoculation of microorganism) (Taguchi and Humphrey, 1996). In this method, the PBR was filled up with 0.15 N NaCl solutions and then the dissolved oxygen was removed by purging nitrogen gas. The initial DO was measured (Model: HQ 40d HACH, USA) and oxygen was supplied in the form of air through the bottom aeration port only (simple PBR condition), to raise the DO level in the reactor. Effluent DO concentration was measured at different time intervals until DO level saturated in the reactor. The experiment was performed at two different superficial air flow rates of 0.157 cms^{-1} and 0.315 cms^{-1} . The experiment was repeated by supplying air through all the four aeration ports to determine the oxygen mass transfer coefficient in the newly designed PBR. Oxygen supply rates into the reactor in the above two conditions (simple and newly designed PBR) were, however, maintained the same. The oxygen balance in the reactor was expressed in its integrated form as given below by assuming negligible resistance due to the DO sensor:

$$\ln(C_S - C_L) = -k_L a t + \ln(C_S - C_{L0}) \quad (4.6)$$

Where, C_S is the saturated DO concentration, C_L and C_{L0} are the bulk DO concentration at any time t and $t = 0$, respectively. The values of $k_L a$ in each experiment were obtained from the slope of $\ln(C_S - C_L)$ vs t .

Gas Holdup Determination

The overall gas holdup (ε_G) in both the conditioned of reactor run (i.e., simple PBR and newly designed PBR) was measured by volume expansion method (Vasconcelos et al., 2003) using the following expression:

$$\varepsilon_G = \frac{\Delta V}{\Delta V + V_{LS}} \quad (4.7)$$

where V_{LS} represents the liquid volume in both the packed bed system. ΔV is the volume expansion after gas dispersion, calculated from the average liquid level change and the cross-section area of the reactor. In order to calculate ΔV , a millimetric scale was fixed to the external glass column, the liquid level at each gas flow rate was recorded. The experiment was performed in triplicate and average liquid height at each gas flow rate was used to estimate the parameter.

4.4.4 Abiotic 4-CP, 4-BP and 4-NP Removal in the Newly Designed Packed Bed Bioreactor

As mentioned earlier, PUF was used as supporting material in the pack bed reactor (PBR). There are several reports on removal of various pollutants through adsorption on PUF (Hu et al., 1994; Zheng et al., 2009). Therefore, experiment was carried out to estimate the abiotic loss of 4-CP, 4-BP and 4-NP in the reactor due to adsorption onto bio-support material, and volatilization with air supplied into the reactor. In order to estimate abiotic 4-CP, 4-BP and 4-NP removal, the newly designed bioreactor was operated without aeration by supplying synthetic wastewater containing 200 mg l^{-1} of the respective substituted phenols as

single substrate system in the earlier optimized mineral salt medium (Table 4.6). The HRT of the reactor was maintained at an arbitrary value of 10 h for each of the pollutants.

When both effluent and influent substituted phenol concentrations were the same, the reactor was aerated through all the four ports to estimate these substituted phenol loss due to volatilization.

4.4.5 Biofilm Development

The inoculum was prepared as described in section 4.3.3. The active cells grown in MSM were centrifuged (5000g, 20 min at 22°C), washed with 1× phosphate buffer saline (pH 7.4), and suspended in four liter of optimized MSM (Table 4.6) containing 300 mgL⁻¹ of 4-CP to give a cell suspension of 0.75 OD₆₀₀. The cell suspension was inoculated by pumping into the reactor packed with PUF pieces by means of a peristaltic pump (Model: pp10, Miclins India). Any biomass washed out of the reactor along with the effluent was recycled back. The reactor was continuously fed with the above mentioned MSM spiked with 4-CP concentrations varying between 200 and 600 mgL⁻¹ and was operated for about one month at different flow rates to maintain different HRT of 18h - 12.5h, until a steady state performance was achieved. To confirm the biomass in the PBR, a few pieces of PUF (biosupport material) were sampled for SEM analysis as mentioned earlier.

4.4.6 Optimization of Dissolved Oxygen Level in the Reactor

Dissolved Oxygen concentration can affect the synthesis of enzymes, growth rates of the microorganism, and on the regulation of gene expression (Costura and Alvarez, 2000). In the present study, after achieving steady state result performance of the reactor operated at different DO level was monitored for its optimization. Experiment for determination of optimum DO concentration in the reactor was performed by feeding with 400 mgL⁻¹ (loading rate of 910 mgL⁻¹day⁻¹) of 4-CP and 4-BP; and 600 mgL⁻¹ of 4-NP (loading rate of 1264 mgL⁻¹day⁻¹) and operated at 12.5 h HRT. The DO concentration was maintained between 2 and 7 mgL⁻¹ by supplying air at different rate between 2 and 10 L min⁻¹. At each DO level the experiment was carried out until a steady state condition was achieved.

4.4.7 4-CP, 4-BP and 4-NP biodegradation Experiments as Single Substrate System in the Newly Designed PBR

The performance of the newly designed PBR on 4-CP, 4-BP and 4-NP biodegradation as single substrate was studied at their respective optimum DO level. The temperature of the reactor was maintained at $29 \pm 1^\circ\text{C}$ (section 5.2.2). The reactor was initially operated with synthetic wastewater prepared by spiking substituted phenols in optimized MSM at pH 7.5 (section 5.2.2) as done before and at lower loading rate as described before near about one month to achieve steady state performance (4.4.15). Then the reactor was run at the above mentioned conditions with varying concentrations of 4-CP ($400\text{--}1000\text{ mg l}^{-1}$) as the sole source of carbon and energy. The wastewater was fed into the reactor at varying flow rates with the help of a peristaltic pump to maintain different HRT between 24 h and 7.5 h. Samples were taken at regular time interval and analyzed for substituted phenol concentration, COD, metabolic intermediate, percentage of toxicity and chloride concentration. After the PBR performance for 4-CP degradation was evaluated, the reactor performance was evaluated for 4-BP followed by 4-NP with varying concentrations of 4-BP ($400\text{--}1200\text{ mg l}^{-1}$) and 4-NP ($600\text{--}1400\text{ mg l}^{-1}$). In all the above cases no external carbon source was added.

4.4.8 Stability Studies on the Performance of the Newly Designed PBR

The key problems of any industrial wastewater treatment involves irregular organic loading due to change in process conditions. In order to address this problem, experiment was carried out by subjecting the newly designed PBR to operation leading to intermittent and shock loading conditions. Shock loading experiments were performed at two conditions by sudden increase in these pollutants loading rate at lower ranges from $473\text{--}946\text{ mg l}^{-1}\text{day}^{-1}$, and at higher ranges from $1137\text{--}3007\text{ mg l}^{-1}\text{day}^{-1}$ for 4-CP. Similarly, sudden change was made for 4-BP from $473\text{--}1183\text{ mg l}^{-1}\text{day}^{-1}$, and from $1138\text{--}3759\text{ mg l}^{-1}\text{day}^{-1}$, whereas, for 4-

NP from 842- 1966 $\text{mg l}^{-1}\text{day}^{-1}$ and 2247-4492 $\text{mg l}^{-1}\text{day}^{-1}$. Samples were taken at regular time interval and analyzed for substituted phenol concentration.

4.4.9 Toxicity Test

Resazurin reduction method was used (Eker and Kargi, 2007; Strotmann et al., 1993) to determine the percentage toxicity of the influent as well as effluents of newly designed PBR while treating all the three the pollutants as single substrate, mixed substrate and petroleum wastewater spiked with the mixture of these pollutants. In single substrate system the toxicity test was performed at highest pollutant feeding concentration viz. 1000, 1200 and 1400 mg l^{-1} of 4-CP, 4-BP and 4-NP at different HRTs between 24h and 7.5 h during the operation period of the reactor. On the other hand, in mixed substrate system both in synthetic wastewater and refinery wastewater spiked with these mixtures of pollutants, the tests were conducted at the highest pollutant concentration where complete removal was achieved. Mixed microbial consortia, collected from a local municipal wastewater treatment plant were used as test organisms. The test organisms (mixed microbial consortia) were cultivated on nutrient broth and were transferred to a new medium everyday to keep the sludge age constant. Five ml of the test culture was centrifuged and re-suspended in distilled water resulting in an optical density of about 0.4 at 625 nm. The centrifuged cells obtained after draining the supernatant in the test tube were used in toxicity determination using appropriate controls. 50 mg resazurin and 10 ml phosphate buffer were dissolved in 100 ml distilled water to prepare the resazurin solution. A 3.75 g of nutrient broth (Merck, India) was dissolved in 10 ml of distilled water and used as the growth media in the test tubes. Reagent control solution contained 0.275 ml of growth medium, 9.525 ml distilled water and 0.2 ml resazurin solution. Cell control solution contained 0.2 ml growth medium, 9.6 ml distilled water, 0.2 ml resazurin solution (plus cell) and test solution contained 0.2 ml growth medium, 9.6 ml centrifuged wastewater sample (influent or effluent) and 0.2 ml resazurin solution (plus cell). The test tubes were incubated for 20 min at room temperature before adding resazurin solution and its colour was monitored until cell solution turned pink. After about 30 min, 50 μl of HgCl_2 solution (10 mg ml^{-1}) was added to the cell control and the test tubes to stop the reaction following centrifugation for 5 min. In the presence of active

bacterial culture with dehydrogenase enzyme activity, resazurin changes colour from blue to pink forming the reduced compound resorufin. Inactive bacteria do not cause any change in resazurin colour and remain blue. A spectrometer was used to determine the colour change at a wavelength of 610 nm. Following equations were used to calculate % toxicity and % toxicity removal:

$$\%Toxicity = \left[\frac{A - B}{C - B} \right] \quad (4.8)$$

where, A = optical density (OD) of the test tubes containing influent or effluent wastewater sample from the PBR; B = OD for the cell tube (cells +water with no 4-CP); and C= OD for the control tube (only water).

$$E = 1 - \left[\frac{\%Toxicity_e}{\%Toxicity_i} \right] \quad (4.9)$$

where, % Toxicity_i and % Toxicity_e are the percentage toxicities of the influent and the effluent, with respect to the control.

4.4.10 Combined Removal of 4-CP, 4-BP and 4-NP in PBR

In order to evaluate the performance of the PBR on combined removal of 4-CP, 4-BP and 4-NP, synthetically prepared wastewater was spiked with all the above mentioned 3 substituted phenols with varying concentration between 250 - 450 mg l⁻¹ and was operated at a constant HRT of 12.5 h. For choosing combination of concentrations levels of these three compounds, a 2³ full factorial design of experiments with three compounds as factors at two different levels was applied (Montgomery, 2004). All other culture conditions including media constituents were kept similar to that for single mono- substituted phenol degradation (section 4.4.1.7). Samples were collected with regular interval of time from the outlet of the PBR and analyzed for substituted phenols concentration, COD removal and effluent toxicity removal. As before the results presented are average of three values obtained from the analysis of these samples.

4.4.11 Treatment of Refinery Industrial Wastewater

To evaluate the performance of the PBR on removal of phenol and substituted phenols, a refinery wastewater was collected from a petroleum refinery complex located at Guwahati, India. The wastewater was collected from inlet point to the treatment plant. The wastewater was initially characterized for its phenol content, chemical oxygen demand (COD), pH, Dissolved Oxygen (DO), heavy metals (nickel, strontium, lead) along with other parameters. Mono- substituted phenols with different concentrations were spiked with the collected refinery wastewater to make the wastewater contaminated with these pollutants.

Preliminary batch study with refinery wastewater

Before evaluation of efficiency of PBR, preliminary batch experiments were carried out in a series of 250 ml shake flasks with raw and diluted refinery wastewater. Dilution was done with optimized MSM (Table 4.7) and/or with distilled water. In all this flasks, the refinery wastewater was spiked with 75 mg l^{-1} each of the three substituted phenols. The inoculum volume and its preparation along with the culture conditions were maintained similar to that of single mono- substituted phenol degradation study in batch mode (section 4.4.1.7).

Table 4.7 Various dilution of raw refinery wastewater prepared for preliminary batch shake flask study.

Sl. No	Dilution factors	Raw refinery wastewater Added (ml)	MSM Added (ml)	Distilled water added (ml)
1	No dilution	100	0	0
2	1/10 th dilution	90	10	0
3	1/5 th dilution	80	20	0
4	1/2 nd dilution	50	50	0
5	1/2 nd dilution	50	0	50

Refinery wastewater treatment in PBR

Based on the preliminary batch study results, refinery wastewater was mixed with equal volume of MSM and the pH was adjusted to 7.5, before being fed into the PBR. The concentration ranges of individual substituted phenols were chosen from $250 - 350 \text{ mg l}^{-1}$ and

the PBR was operated at 12.5h HRT. All other operating conditions were kept similar to that during single substituted phenol degradation experiment in the PBR (section 4.3.6.1).

Effluents samples were collected at regular intervals of time, centrifuged at $6000 \times g$ for 10 min before being analyzed for residual substituted phenols concentrations, COD, and toxicity removal.

4.5.1 Evaluation of *A. chlorophenolicus* A6 Immobilized with Calcium Alginate Beads Using a Column Reactor

Upflow column reactor was used to assess the performance of *A. chlorophenolicus* A6 cells immobilized calcium alginate beads for degradation of substituted phenols in continuous mode of operation. The reactor performance was evaluated 4-CP as single substrate system. This section mainly deals with preparation of cell immobilized beads, preliminary batch shake flask study, detailed description of the column reactor and experimental methodologies used.

4.5.1.1 Preparation of Calcium Alginate Bead Immobilized with *A. chlorophenolicus* A6 Cells

The centrifuged and PBS washed *A. chlorophenolicus* A6 cells were prepared as described earlier (section 4.3.3). The cells were then re-suspended in a previously sterilized 3 % sodium alginate solution to obtain final cell concentration of 6.5 mgg^{-1} of bead. The alginate-cell suspensions were then added drop wise through a needle in to a sterilized and continuously agitated 7.5% CaCl_2 solutions using a peristaltic pump. The drops of sodium alginate solution containing micro-organism were gelled into uniform spherical shaped granules of 1.8–1.9 mm diameter, upon contact with calcium chloride solutions. In order to improve the stability, the beads were again stored in sterilized 7.5% calcium chloride solution at 4°C for about 6 h. The calcium alginate beads were then washed with Millipore water.

4.5.1.2 Batch Shake Flasks Study for Optimization of pH

Optimization of pH was carried out in a series of 250 ml Erlenmeyer flasks with 100 ml of above mentioned optimized MSM (Table 4.6) containing 300 mg l⁻¹ of 4-CP. The pH of the culture media in different flasks was adjusted to different values between 6 and 7.5 by using HCl or NaOH solution. After that the culture media were sterilized by autoclaving at 15 lb pressure and 121°C for 20 min. Then to each flask 6.5 gm of previously prepared cell immobilized alginate beads were added and incubated for 21 h in an incubator shaker at 30 °C and at an agitation speed of 210 rpm. Samples were collected at regular interval of time and analyzed for 4-CP concentration.

4.5.1.3 Experimental Set up of Column Reactor Packed with Calcium Alginate Beads

A laboratory scale simple PBR was fabricated with a transparent perspex cylinder with internal diameter of 2.54 cm (cross sectional area of 6.5 cm²) and height 45 cm. The reactor was operated at different operating and feeding conditions. Schematic diagram of the laboratory scale experimental set-up of column reactor is shown in Figure 4.8.

The following are the main components of the column reactor:

a) Main reactor

The main reactor was made up of perspex tube with an internal and external diameter of 2.54 cm and 3 cm respectively. Total length of the reactor was 45 cm. Working volume of the reactor was kept as 126.5 ml and the reactor was packed with cell immobilized calcium alginate beads. For sampling four numbers ports were provided along the height of the reactor.

b) Biosupport material (Calcium alginate beads)

A. chlorophenolicus A6 cell immobilized calcium alginate bead of 180-190 mm diameter were prepared as discussed above. About 86.5 gm of these cell immobilized calcium alginate beads were packed in the column reactor manually.

c) Feed flow rate adjustment

The reactor was fed with synthetic wastewater containing 4-CP at controlled flow rate using a peristaltic pump (Model: pp10, Miclins India). Flow rate was maintained for empty bed contact time (EBCT) of 8, 4, 3 and 2 hours.

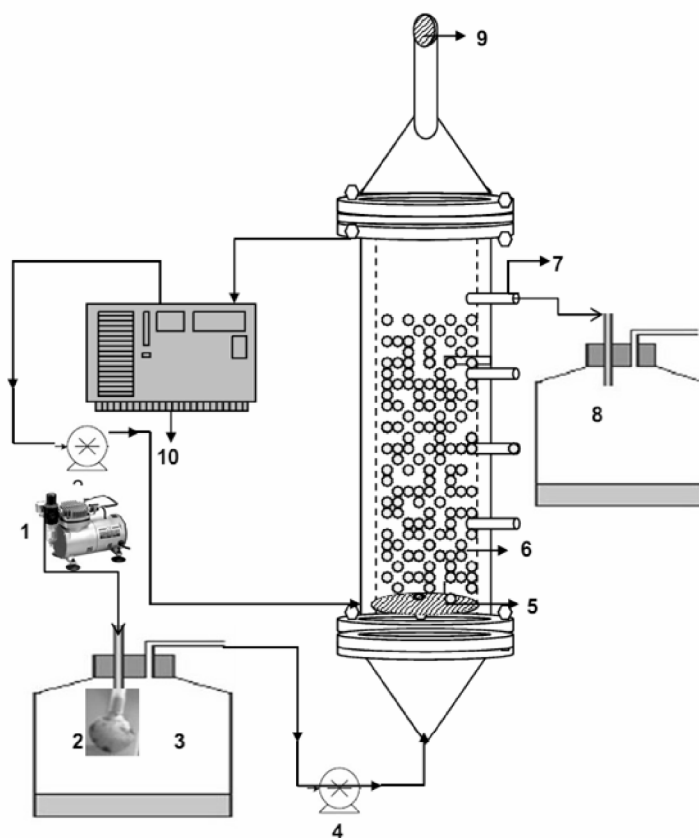


Figure 4.8 Schematic of the experimental set up of the column reactor with its various components: (1) Air compressor, (2) ceramic gas diffuser, (3) Influent tank, (4) peristaltic pump, (5) wire gauze, (6) Cell immobilized alginate beads, (7) Sampling ports, (8) effluent reservoir, (9) gas outlet, (10) water bath.

Prior to the biodegradation experiments, the values of EBCT were determined by varying the feed flow rate into the column reactor using a variable speed peristaltic pump (Model: pp10, Miclins India) and the same values were maintained during the biodegradation experiments, as well.

d) Air flow rate

Aeration from air compressor (Rocker 440 Tarson, India) was provided in the reactor influent tank through ceramic diffusers and DO level was maintained between 3.5-4 mg l⁻¹.

e) Arrangement for temperature control

The temperature inside the reactor was maintained at 29±1°C by circulating warm water from a thermostat installed temperature controlled water bath (LAUDA, RA-8 Germany) through silicon pipes wrapped over the reactor throughout its length.

f) Dissolved oxygen and pH measurement

Dissolved oxygen concentration was measured using a digital DO meter (Orion 3 star-QY-14478, Thermo Scientific, Singapore) with a sensitivity of 0.01. pH was measured using a digital pH meter (Orion 3 star-QY-14478, Thermo Scientific, Singapore) with a sensitivity of 0.01 with temperature correction facility. The instrument was calibrated periodically with standard buffer solutions. Detailed of the reactor specification and calcium alginate beads used in the study is presented in Table 4.8.

4.5.1.4 Biodegradation of 4-CP in with cell immobilized calcium alginate beads using column reactor

The reactor was fed with synthetic wastewater contained optimized MSM (Table 4.6) and was operated in up flow mode. Table 4.9 shows details about the design matrix employed in the study along with the operating conditions. Based on the results of the

previous 4-CP biodegradation study in the newly designed PBR, the DO concentration was maintained between 3.5 - 4.2 mg l⁻¹ (section 5.4). Samples were collected at regular interval of time and were centrifuged at 8000 rpm for 10 min and the supernatant was used to determine 4-CP concentration in the treated effluent.

Table 4.8 Detailed specifications of the reactor and alginate beads used in the study

Total reactor volume	160 ml
Liquid working volume	135 ml
Packed-bed volume, V_{PB} (cm ³)	126ml
Ca-alginate bead size	180-190 mm
Na-alginate	3%
CaCl ₂	7.5%
Biomass conc.	6.5mgg ⁻¹
Adsorption capacity of beads without biomass immobilization	0.45 mgg ⁻¹ beads

Table 4.9 Operational schedule of the column reactor packed with *A. chlorophenolicus* A6 cells immobilized with calcium alginate beads.

Operation stages	Operation period (day)	EBCT (h)	Influent conc. 4-CP (mg l ⁻¹)
1	0-4	8	100
	5-7	4	100
	8-11	3	100
	12-15	2	100
	16-19	8	150
2	20-23	4	150
	24-27	3	150
	28-31	2	150
3	32-35	8	200
	36-38	4	200
	39-43	3	200
	44-47	2	200
4	48-52	8	250
	53-56	4	250
	57-60	3	250
	61-65	2	250

CHAPTER 5

RESULTS AND DISCUSSION

Substituted phenols are the most important phenolic compounds that are present in wastewater discharged from many industries (Tomei et al. 2003). The presence of substituted groups particularly in chloro, bromo and nitro in phenol increases their toxic effects exerted on the environmental life as well as on the human health owing to their carcinogenic and recalcitrant properties (Crossby, 1981; Uberoi and Bhattacharya). Therefore, efficiency treatment of wastewaters contaminated with such pollutants is highly essential prior to their discharge into the environment. Substituted phenols are widely used in many industries and believed to be the most toxic and recalcitrant nature due its shared resonance electrons in the aromatic structure is a very stable and thus resistant to enzymatic attack. Halogen substituents on the aromatic ring stabilizes the structure further, as the halogens create a steric hindrance to enzymes and also have an electron withdrawing effect (van den Berg, 1990; Copley, 1997; Uberoi and Bhattacharya, 1997). Furthermore, among the halo-substituents chloro group believed to be strong electron withdrawing capacity (higher electro negativity) and makes the attack on aromatic ring more difficult (Pitter, 1985). Further, in the degradation process of chlorophenols accumulation of chloride ion (Cl^-) occurs and thus leading to enhance the toxicity to the microorganism (Stanlake and Fin, 1982). In addition, the liberation of chloride ion (Cl^-) may also produce hydrochloric acid that consequently drops down the pH of the culture media. In the present study, due to the higher toxicity nature of 4-CP and it's widely applications in many industries hence 4-CP is chosen as the model compound for chemical and physical process parameters optimization by the *A. chlorophenolicus* A6.

5.1 Screening and Optimization of Media Constituents to Enhance Biodegradation of Substituted Phenols

It is reported that among the aerobic chlorophenol degrading microorganisms *A. chlorophenolicus* A6 degrade the compound by a novel route via hydroxyquinol pathway with reductive dechlorination being one of the key intermediate steps in the process (Nordin et al., 2005). However, constituents such as sulphates (MgSO_4) and nitrates (NH_4NO_3) that are usually added in the media for culture growth may hinder the reductive dechlorination step of chlorophenol degradation. Further, medium constituent like CaCl_2 and FeCl_3 as well as pollutant like chlorophenols it self liberated chloride ion (Cl^-) during the degradation process and thus leading to enhance the toxicity to the microorganism (Stanlake and Fin, 1982). Therefore, using such conventional media constituents and that too at unoptimized levels seem dubious for growth and degradation of 4-CP by the culture. Hence, there is a need to screen and optimize the media components for enhancing the biodegradation of 4-CP by *A. chlorophenolicus* A6.

5.1.1 Biomass Growth and Degradation of 4-CP Profile of *A. chlorophenolicus* A6

Figure 5.1 depicts the growth and 4-CP degradation profile shown by *A. chlorophenolicus* A6 using the unoptimized media containing 300 mg l^{-1} of the phenolics, which clearly reveals that 4-CP degradation by the actinomycetes is a growth associated process. Hence, based on this preliminary observation, both the culture specific growth rate and 4-CP degradation efficiency were considered for screening and optimizing the medium constituents as well as physical process paramters in this study.

5.1.2 Screening of Media Constituents Using the Two Level Factorial Design

All the six media constituents of K_2HPO_4 , KH_2PO_4 , NH_4NO_3 , MgSO_4 , CaCl_2 , and FeCl_3 in this screening study were investigated for their effects on 4-CP biodegradation and

specific growth rate of *A. chlorophenolicus* A6 taking 90% as confident level with alfa value 0.1.

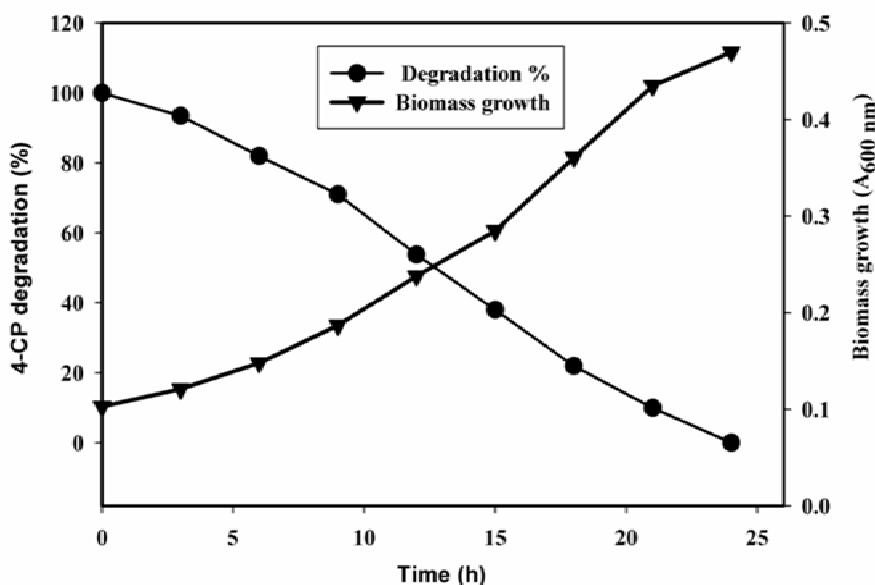


Figure 5.1 Relationship between 4-CP biodegradation and biomass growth profile in unoptimized media of *A. chlorophenolicus* A6.

The main effect plot presented in Figure 5. 2 (a) and (b) indicated that, among these six factors all except FeCl_3 and KH_2PO_4 showed significant effects on 4-CP biodegradation efficiency as well as on the culture specific growth rate; while the effect of calcium chloride was positive, magnesium sulfate and ammonium nitrate displayed negative effect on both 4-CP biodegradation and the culture specific growth rate. On the other hand, although dipotassium hydrogen phosphate exhibited negative effect on 4-CP biodegradation activity, it showed slightly positive effect on specific growth rate of the culture. Also, it was revealed that magnesium sulfate had a large negative effect on both 4-CP biodegradation and specific growth rate; compared to this medium constituent, ammonium nitrate showed only a slightly negative effect on the culture specific growth rate, but a large negative effect on 4-CP

biodegradation activity. The other two constituents KH_2PO_4 and FeCl_3 had no significant effect on both the responses

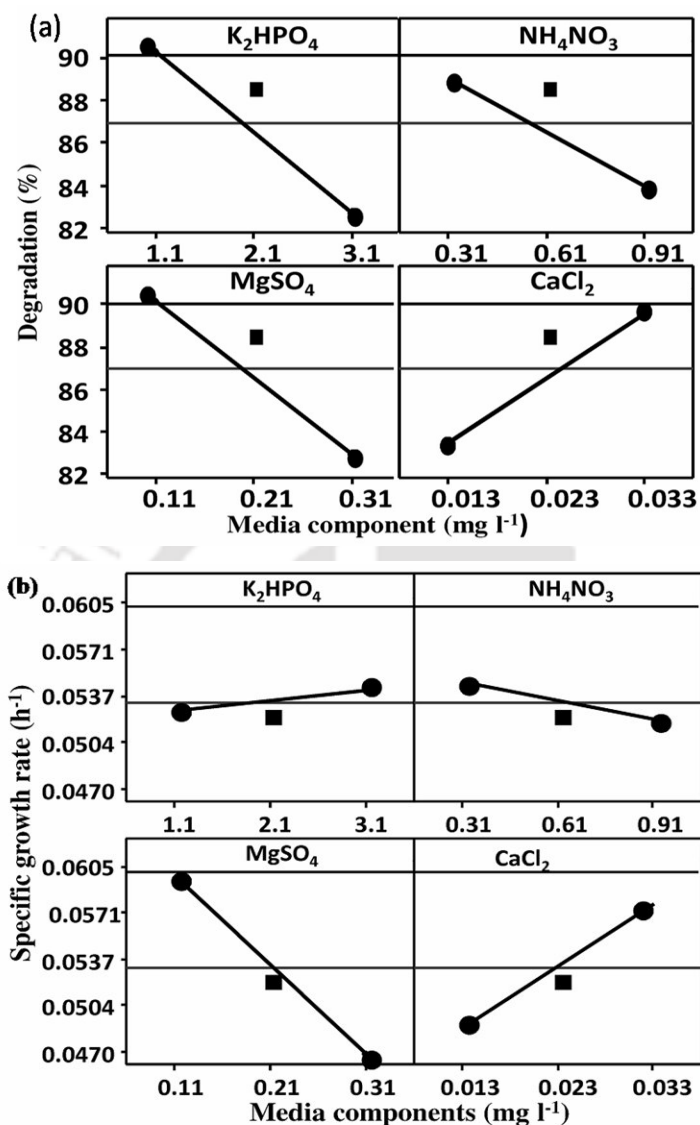


Figure 5. 2 Main effects plot of the media constituents on (a) 4-CP biodegradation. (b) Specific growth rate of the culture.

In order to assess the significance of each term in the models for 4-CP degradation and specific growth rate, student's *t*-test was performed, and the results are presented in Table 5.1 Generally a large *t* value associated with a low *P* value indicates a high significance of the corresponding model term.

Table 5. 1 Student's *t*-test for 4-CP biodegradation and culture specific growth rate in the screening study

Term	4-CP biodegradation		Culture Specific growth rate	
	<i>T</i>	<i>P</i>	<i>t</i>	<i>P</i>
Constant	49.67	0.000	15.56	0.001
K ₂ HPO ₄	- 2.74	0.071	1.46	0.241*
KH ₂ PO ₄	0.94	0.416*	0.73	0.521*
NH ₄ NO ₃	-3.68	0.035	-0.76	0.50*
MgSO ₄	-3.43	0.041	-2.71	0.073
CaCl ₂	3.09	0.054	2.24	0.110*
FeCl ₃	0.18	0.871*	0.30	0.783*

* Insignificant values

In this screening study, ammonium nitrate was therefore found to have a maximum effect on 4-CP biodegradation activity (*P* = 0.035) followed by magnesium sulfate (*P* = 0.041) and calcium chloride (*P* = 0.054); however, on the culture specific growth rate magnesium sulfate (0.073) had maximum significant effect followed by calcium chloride. Dipotassium hydrogen phosphate was found to have a slightly significant effect on both 4-CP biodegradation and specific growth rate. All the other factors did not show any significant effect on 4-CP biodegradation as well as on the culture specific growth rate. Hence, based on the student's *t*-test the four medium constituents' dipotassium phosphate, ammonium nitrate, magnesium sulfate and calcium chloride were chosen for further optimization of their levels.

5.1.3.1 Optimization of Media Constituents Using RSM

For maximizing 4-CP biodegradation efficiency and the culture specific growth rate, the levels of the four screened medium constituents {K₂HPO₄, NH₄NO₃, MgSO₄ and CaCl₂} were varied using the central composite design of experiment.

The data obtained from the CCD on 4-CP degradation percentage and culture specific growth rate were subjected to analysis of variance (ANOVA). The experimental results were fitted to the following second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i,j} \beta_{ij} X_i X_j \quad (5.1)$$

where Y is the predicted response, k is the number of factor variables. X_i and X_j are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the i^{th} quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient.

The statistical significance of the model terms in the model equation were valuated through the Fisher's F -test. The quality of the fit of polynomial model was expressed by the coefficient of determination R^2 , and its statistical significance was checked by the F statistics. A F -test that is larger than the critical value from the F distribution, using the appropriate confidence level and degrees of freedom (supports rejecting the null hypothesis that the means are equal), whereas P -value determines the appropriateness of rejecting the null hypothesis. Table 5. 2. (a) and (b) presents ANOVA of 4-CP biodegradation and specific growth rate of culture obtained in this optimization study. The Fisher's F value (5.34) for 4-CP biodegradation in the model owing to regression were found to be higher than the critical F value ($F_{0.05, 14, 6} = 2.54$). The large F value indicates that most of the variations in the response could be explained by the regression model equation for 4-CP biodegradation in the study. Generally, a large F value with a corresponding small P -value indicates a high significance of the respective coefficient (Tanyildizi et al., 2005). The associated P value is used to judge whether F is large enough to indicate statistical significance or not.

Table 5. 2. (a) ANOVA of 4-CP biodegradation in the media optimization study

Source	Df	SS	MS	F	P	R^2
Regression	14	1106.9	79.06	5.34	0.001	82.38
Linear	4	573.99	89.45	6.05	0.004	
Square	4	460.54	115.1	7.78	0.001	
Interaction	6	72.45	12.07	0.82	0.573	
Error	6	4.18	0.697			
Total	30	1343.6				

Table 5. 2. (b) ANOVA of specific growth rate of the culture in the media optimization study

Source	Df	SS x10 ⁻³	MS x10 ⁻³	F	P	R ²
Regression	14	0.085	0.006	12.74	0.00	91.77
Linear	4	0.046	0.006	12.32	0.00	
Square	4	0.031	0.008	16.11	0.00	
Interaction	6	0.008	0.001	2.92	0.04	
Error	6	0.002	0.000			
Total	30	0.093				

df, degrees of freedom; SS, sum of squares; MS, mean sum of squares. F Fisher's F value (calculated by dividing the MS owing to the model by that due to error), P probability of incorrectly rejecting the null hypothesis when it is actually true

The linear and square terms of both the regression models for 4-CP biodegradation and specific growth rate were found to be highly significant ($P < 0.004$). In the present study the model F -value of 12.74 for specific growth rate indicates that the regression model could explain most of the variation in the response, which was also found to be true for the model due to 4-CP degradation. These findings confirmed that the second-order polynomial models for 4-CP biodegradation and specific growth rate were adequate in predicting both the responses (4-CP biodegradation and culture specific growth rate). These regression model equations are presented below.

$$Y_1 \quad f(X) = 23.5 + 12.1X_1 + 48.4X_3 - 164.1X_4 + 1697.2X_5 - 1.4X_1^2 - 32.8X_3^2 - 267.1X_4^2 - 16159.5X_5^2 - 3.3X_1X_3 - 5.0X_1X_4 - 51.9X_1X_5 - 8.0X_3X_4 - 77.9X_3X_5 - 1713.8X_4X_5 \quad 5.2$$

$$Y_2 \quad f(X) = 0.01367 + 0.00385X_1 + 0.01084X_3 - 0.04646X_4 + 0.35677X_5 - 0.00053X_1^2 - 0.00686X_3^2 + 0.07798X_4^2 - 3.17262X_5^2 - 0.00121X_1X_3 - 0.0288X_1X_4 - 0.01250X_1X_5 - 0.00250X_3X_4 - 0.00417X_3X_5 - 0.53750X_4X_5 \quad 5.3$$

Where Y_1 = 4-CP biodegradation, Y_2 = specific growth rate, X_1 is K_2HPO_4 , X_3 is NH_4NO_3 , X_4 is $MgSO_4$ and X_5 is $CaCl_2$.

Further to determine the significance of the regression coefficients in the two models, the results were subjected to a Student's *t*-test as before and are presented in Table 5.3. It could be seen that the regression coefficients of linear terms for MgSO₄, CaCl₂ in the model for 4-CP biodegradation were found to be highly significant ($P < 0.003$).

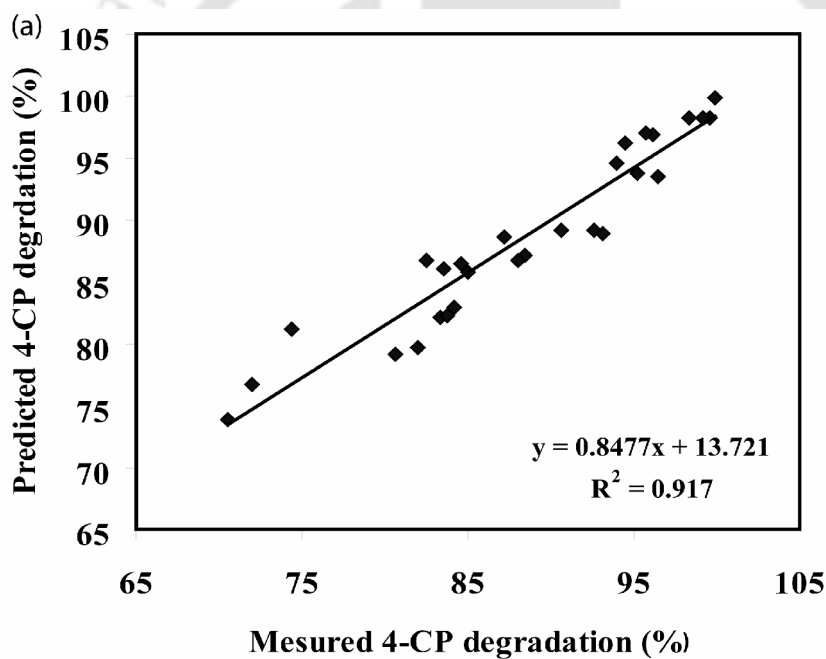
Table 5. 3 Student's *t*-test for 4-CP biodegradation and culture specific growth rate in the media optimization study

Term	4-CP biodegradation		Culture specific growth rate	
	t	p	t	p
Constant	1.825	0.087	5.893	0.000
K ₂ HPO ₄ (X ₁)	-2.558	0.021	4.512	0.000
NH ₄ NO ₃ (X ₃)	3.081	0.007	-3.834	0.001
MgSO ₄ (X ₄)	-3.457	0.003	-5.440	0.000
CaCl ₂ (X ₅)	3.498	0.003	4.087	0.001
X ₁ ²	-1.948	0.069	-4.094	0.001
X ₃ ²	-4.100	0.001	-4.770	0.000
X ₄ ²	3.713	0.002	-6.026	0.000
X ₅ ²	-2.246	0.039	-2.452	0.026
X ₁ x X ₃	-1.018	0.324	-2.095	0.052
X ₁ x X ₄	-0.521	0.609	-1.662	0.116
X ₁ x X ₅	-0.539	0.597	0.723	0.480
X ₃ x X ₄	0.251	0.805	0.434	0.670
X ₃ x X ₅	-0.243	0.811	-0.072	0.943
X ₄ x X ₅	-1.782	0.094	-3.107	0.007

For the specific growth rate, the model coefficients of all the linear terms of the four factors were highly significant ($P < 0.001$). Also the significance of all the coefficients of the quadratic terms in the culture specific growth rate model was found to be high ($P < 0.03$); but, for 4-CP biodegradation only NH₄NO₃ and MgSO₄ showed high significance ($P < 0.002$). As observed earlier in ANOVA of culture specific growth rate, the regression coefficient terms for interaction between MgSO₄ and CaCl₂ were found to be highly important ($P = 0.007$). Other interactions effects between the factors did not play any considerable role ($P > 0.3$) on 4-CP biodegradation as well as on specific growth rate of the culture.

It should be noted here that such observations on significance of interaction effects between the variables would have been lost if the experiments were carried out by conventional methods (Ravikumar et al., 2005). Figure 5.3 (a) and (b) represent linear plots of measured versus predicted values of 4-CP degradation and culture specific growth rate, respectively, which clearly reveals that both the experimental and predicted values were in close agreement with each other

In order to illustrate the main and interactive effects of the independent variables on the dependent ones, the fitted polynomial equation was then represented in the form of two dimensional response surface contour plots. The contour plots were constructed by plotting the response against any two of the four independent variables and by maintaining the other variables at their middle (0) levels. Figure 5.4 is a typical response surface plot shown to illustrate the interaction effect between MgSO_4 and CaCl_2 .



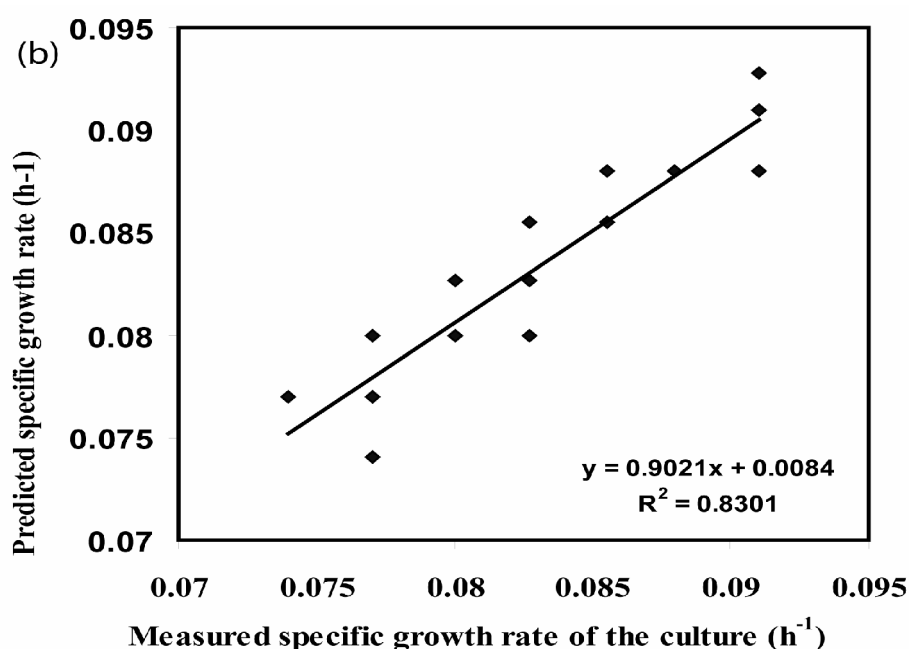
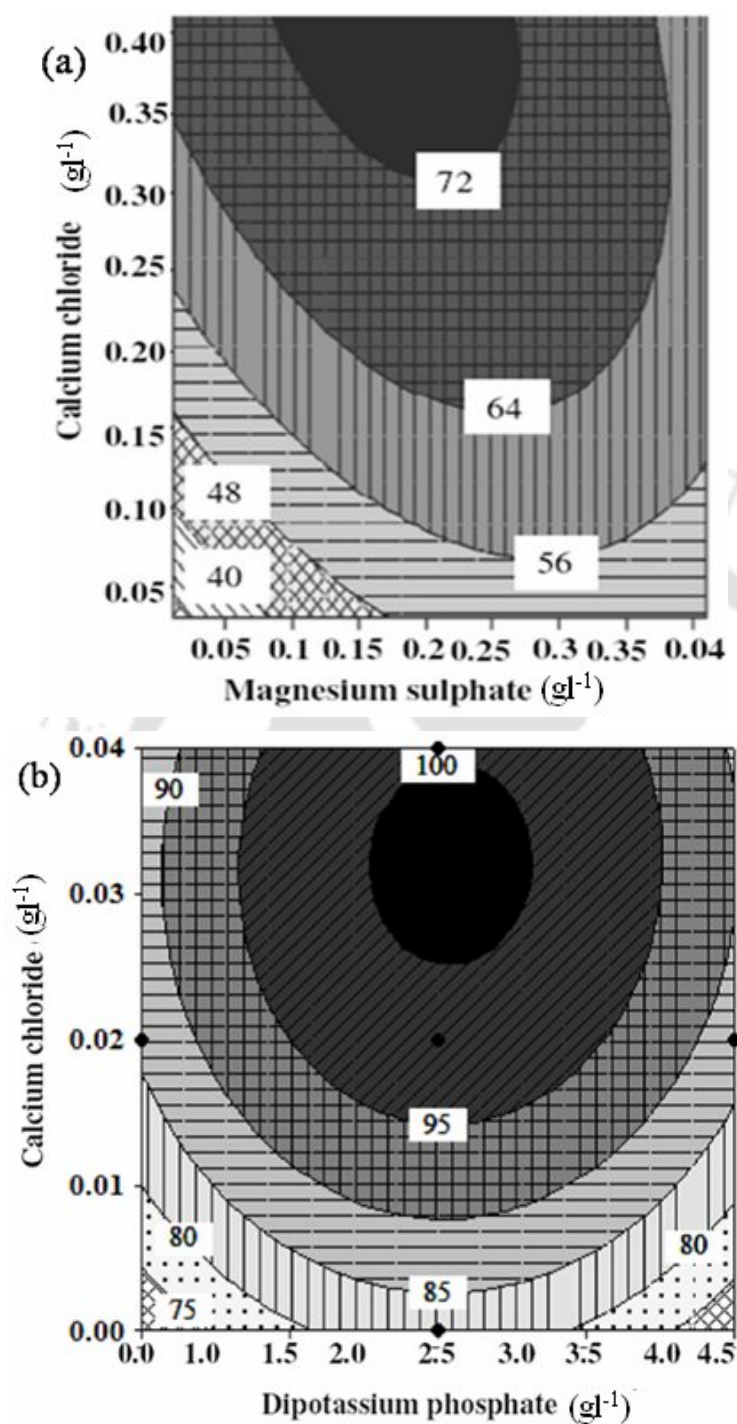


Figure 5.3 (a) Linear plot of predicted versus measured values of 4-CP degradation in the optimization study. **(b)** Linear plot of predicted versus measured values of specific growth rate of the culture in the optimization study.

In general, the contours in such plots help in proper identification of the type of interactions between test variables, and the surface confined in the smallest curve of the contour diagram can also be used to predict maximum response in the process. Hence, from the given plot depicted in [Figure 5.4](#), the corresponding coordinates in the region of the contour diagram gave the optimum values of the respective factors. Also, the response surface contour plots of mutual interaction between the variables between MgSO_4 and CaCl_2 [[Figure 5.4 \(d\)](#)] was found to be elliptical indicating significant interactions between the two. However, response surface contours drawn between other factors were circular indicating non-significant nature of their interactions.



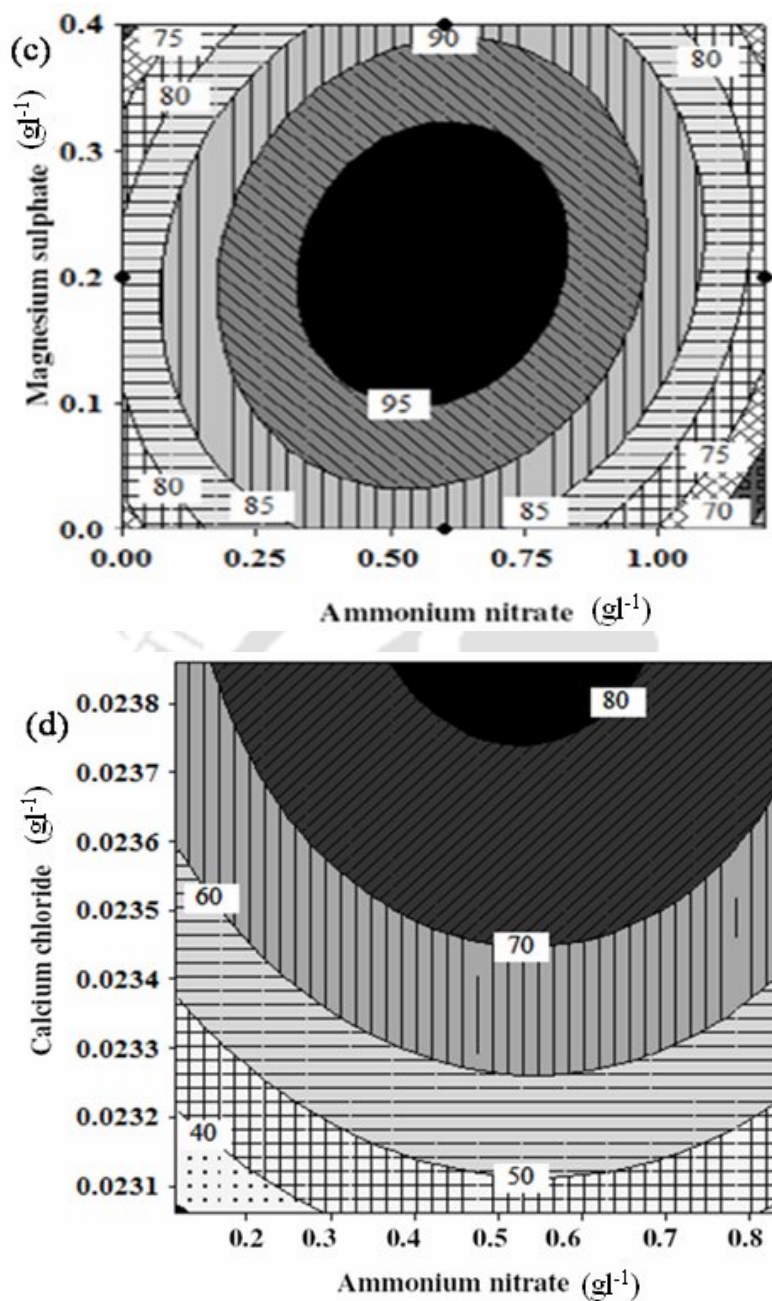


Figure 5.4 Contour plot showing relationship between (a) magnesium sulphate and calcium chloride (b) di-potassium phosphate and calcium chloride (c) ammonium nitrate and magnesium sulphate (d) ammonium nitrate and calcium chloride on 4-CP biodegradation

5.1.3.2 Multiple response optimization (Desirability function)

Further, the levels of the medium constituents were optimized using desirability function in such a way that both the responses 4-CP biodegradation and specific growth rate by the culture were at maximum. Desirability function is an analysis in which a number of responses (output variables) are measured simultaneously for each setting of a group of parameters (input variables). In systems having a large number of input variables and responses, the single response analysis has serious limitations as the optimum conditions for one response may not be suitable or practical for other responses and thus the meaning of optimum becomes unrealistic. The optimal conditions evaluated by this analysis are sometimes called 'near' optimal for all responses. The optimization methodology based on the individual desirability using a desirability function (also called utility transfer function) evaluates how the settings optimize a single response. The composite desirability evaluates how the settings optimize a set of responses overall. Desirability has a range of zero to more than one. One represents the ideal case which indicating the fact that the function increases linearly towards the desired target values of the responses and greater than one indicating the fact that the function increases exponentially towards the desired target values of the responses (Derringer and Suich 1980; Jahani et al. 2008). On contrary zero indicates that one or more responses are outside their acceptable limits and the target response can not be achieved. Optimal settings for input variables were determined by maximizing the composite desirability. These values are combined to determine the composite or overall desirability of the multi-response system. An optimal solution occurs where composite desirability reaches its maximum. Therefore, in order to achieve simultaneously both the responses at their maximum level (4-CP biodegradation and culture specific growth rate) by the culture, following equation was solved (Harrington 1965; Derringer and Suich 1980):

$$d_i(\hat{y}_i) = \begin{cases} \left(\frac{\hat{y}_i - L_i}{T_i - L_i}\right)^{r_i} & \hat{y}_i < T_i \\ 1 & L_i \leq \hat{y}_i \leq T_i \\ 0 & \hat{y}_i > T_i \end{cases} \quad (5.4)$$

where $d_i(\hat{y}_i)$ is desirability function of a response, L , and T are the lower and target values of response measured from experimental data, and in the present study, while L for the two responses (4-CP degradation and specific growth rate) were 70.02 % 0.074 h⁻¹ respectively, T values were set at 100 % and 0.0928 h⁻¹, respectively. $\hat{y}_i$ is the value of a response predicted by the second order polynomial equations generalized before; r is the weight of desirability function of a response. In this study, both the responses were given equal weight. The overall desirability function D in turn is computed as shown below:

$$D = \{\prod (d_i^{w_i})\}^{1/W} \quad (5.5)$$

where $d_i(\hat{y}_i)$ is individual desirability for the i^{th} response, w_i = importance of the i^{th} response, and $W = \sum w_i$, in this study the importance was taken to be one for each of the responses. For solving the desirability function in this study, the statistical software package MINITAB® Release 15.1, PA, USA, was used. The desirability function plots in this multiple response optimization method are depicted in Figure 5.5. The figure reveals that that the overall desirability function for 4-CP biodegradation and specific growth rate activity were close to 1 indicating the fact that the desirability function increases linearly towards the desired target values of the two responses (Derringer and Suich, 1980; Jahani et al., 2008). In addition, individual desirability values of the two responses were calculated; while the value for specific growth rate was computed to be 1 with a maximum predicted response of 0.0928 h⁻¹, the value for 4-CP biodegradation was also found to be nearly 1 (0.99) with maximum predicted value of 100 % (99.77). Thus, using the desirability function method for optimizing both the responses, optimum values of the media components were estimated as follows: dipotassium hydrogen phosphate, 2.62 g l⁻¹; ammonium nitrate, 0.58 g l⁻¹; magnesium sulfate, 0.17 g l⁻¹ and calcium chloride, 0.038 g l⁻¹.

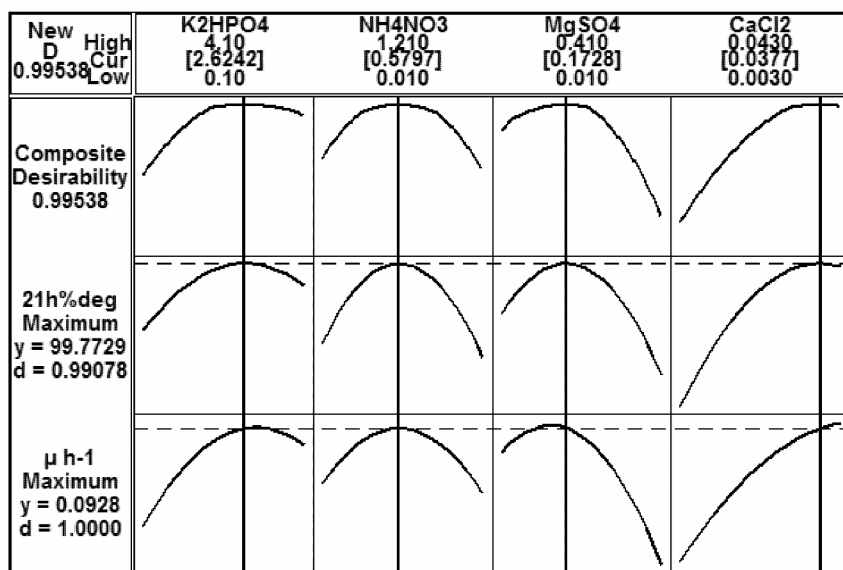


Figure 5.5 Desirability function plot showing the optimum level of media constituents.

5.1.3.3 Validation of the model

To validate the model, experiments were carried out in 250 ml of Erlenmeyer flask with 100 ml of optimal levels of the media constituents as predicted by the CCD results containing 300 mg l⁻¹ of 4-CP. Under the optimized medium composition (g l⁻¹): K₂HPO₄ 2.62, KH₂PO₄ 0.4, NH₄NO₃ 0.58, MgSO₄ 0.17, CaCl₂ 0.038, FeCl₃ 0.002, experiments were carried out for 4-CP degradation and culture specific growth rate. Results revealed that the corresponding % of 4-CP degradation and specific growth rate were found to be 99.98% and 0.0925 h⁻¹ respectively, which perfectly matched with the predicted values of 100% of 4-CP degradation and 0.0928 h⁻¹ of specific growth rate of the culture. The validation study was performed in triplicate and results reported are average with standard deviation ±2.6%. The microorganism could also degrade a higher 4-CP concentration of 350 mg l⁻¹ within 30 h of culture, which was earlier reported to be inhibitory to the microorganism growth and its degradation (Westberg et al., 2002).

It is well known that ammonium nitrate, magnesium sulphate, calcium chloride and dipotassium hydrogen phosphate are important as either an essential component of microbial

cell growth or an inhibitory component at and beyond certain concentration. In this study ammonium nitrate and magnesium sulphate was found to be optimum at 0.58 g l^{-1} and 0.173 g l^{-1} respectively. This drop down of these compounds from the initial value at the optimum setting may be attributed due to the accumulation of sulphate and nitrate ions, thus leading to inhibition of the reductive dechlorination step in 4-CP biodegradation pathway by the microorganisms as noted earlier by Nordin et al. (2005). Other authors are also reported nearly same optimum concentration of nitrate for degradation of various pollutants such as chlorobenzenes and polychlorinated biphenyl (PCB), i.e. at concentrations in the range of 10 -16 mM inhibited reductive dechlorination (Morris et al., 1992; Rhee et al., 1993). Similarly the optimum sulphate ion concentration obtained in the present study is also well matched with other authors reported for degradation of polychlorinated biphenyl (May et al., 1992), Aroclor 1242 (Rhee et al., 1993), nona-and octa-chlorobiphenyls (Kuipers et al., 1999) and chloroethene (Heimann et al., 2005). Calcium chloride is yet another important media component for growth and degradation of chlorophenols by microorganisms, and the optimum calcium chloride concentration in this study was found to be increased from 0.023 g l^{-1} to 0.038 g l^{-1} . Similar observations on enhancement in 2.5 fold higher dextransucrase enzyme productions due to addition of 0.005% of CaCl_2 in culture medium was reported as compared to the medium without calcium ions by Qader et al. (2008). However, any further increase in the calcium chloride concentration did not improve the growth and 4-CP degrading ability by the actinomycetes in the present study, probably due to the accumulation of chloride ion (Cl^-) and thus leading to enhance the toxicity to the microorganism (Stanlake and Fin, 1982). In addition, the accumulation of chloride ion (Cl^-) may also produce hydrochloric acid that consequently drops down the pH of the culture media. Di-potassium hydrogen phosphate is an important source of phosphate to microorganism reported to have a strong impact on microbial degradation of aromatic compounds and plays an equivalent or larger role than nutrient availability in the degradation media. In the present study it is observed that the culture K_2HPO_4 concentration when increased to an optimum level of 2.62 g l^{-1} an enhancement in 4-CP degradation could be achieved.

And this enhancement of 4-CP degradation at higher K_2HPO_4 concentration in the optimized media might be due to a strong buffering action of K_2HPO_4 which provided better resistance to change in pH during the growth and degradation process of the culture compared with unoptimized medium constituents. This finding is well correlated with experimental observation that, the initial pH dropped down from 7.4 to a final value of 6.9 in case of unoptimized medium constituents; whereas, in case of the optimized medium constituents, the drop down of pH was found to be comparatively less (7.15). However, it is well understood that any further rise in this optimum K_2HPO_4 concentration increases the phosphate ionic strength in the medium which inhibits the reductive dechlorination step in the 4-CP degradation pathway by the actinomycetes (Gunasekara et al., 2005). Although both K_2HPO_4 and KH_2PO_4 are essential components of phosphate buffer system as well as an important source of phosphate to microorganism, the optimum value of KH_2PO_4 was found to less than K_2HPO_4 . This observation may be due to the fact that since KH_2PO_4 produces higher H^+ ion concentration than K_2HPO_4 it may result in a decreased pH even below 6.9, which may hinders the growth and 4-CP degradation by the actinomycetes. This is also strongly supported by literature that higher concentrations of KH_2PO_4 inhibit dechlorination of herbicide thiobencarb and chlorothalonil (Gunasekara et al., 2005).

All these results suggested that the present screening and optimization study adopting non-conventional statistical design technique proved helpful not only in enhancing the efficiency of the *A. chlorophenolicus* A6 to degrade 4-CP but also in overcoming the toxic effect of the phenolic compounds.

5.2 Optimization of Culture Conditions to Enhance Biodegradation of Substituted Phenols

5.2.1 Optimization of Culture Conditions Using RSM

As it is well established that *A. chlorophenolicus* A6 utilizes both aerobic and as well as reductive dechlorination metabolic pathway for 4-CP degradation (Nordin et al., 2005),

however, reductive dechlorination usually inhibited by higher concentration of dissolved oxygen (Mohn and Tiedje, 1992; VanBriesen et al., 2004). Therefore it is essential to optimize the culture conditions, particularly the agitation speed as a higher value of the parameter affects dissolved oxygen concentration in the culture media, which may hinder reductive dechlorination step in effective degradation of the compound (Mohn and Tiedje, 1992; VanBriesen et al., 2004) and a lower value is detrimental to the microorganism growth (Sharma and Pant 2001). It has also been reported that the *A. chlorophenolicus* A6 strain can degrade 4-CP over a wide range of temperature from 5 to 28°C; however, the degradation rate at 28 °C is found to be higher than at 5°C, while viability of the microorganisms at 5 °C after complete degradation of 4-CP is better than at 28 °C (Backman and Jansson 2004). In the present study it is also observed that during the course of 4-CP degradation, pH of the culture medium drops down from its initial value of 7.4 to a final value of 6.9. Inoculum age of microorganisms is another parameter that has been reported to play a vital role in secretion of enzymes responsible for biodegradation of such xenobiotic compounds (Van and Ward 2001). Hence, optimization of the culture conditions agitation speed, temperature, pH, inoculum age, can plausibly enhance in efficiency of 4-CP biodegradation by *A. chlorophenolicus* A6.

For maximizing 4-CP biodegradation efficiency and the culture specific growth rate, the levels of the four important variables i.e. pH, agitation (rpm), temperature (°C) and inoculum age (h) were varied using the central composite design of experiment, and the results were analyzed in the form of analysis of variance (ANOVA) as before. Table 5.4. (a) and (b) presents the results of ANOVA of 4-CP biodegradation and specific growth rate of the culture, respectively. The Fisher's F value (8.2) for 4-CP biodegradation in the model owing to regression is found to be higher than the critical F value ($F_{0.05} 14, 6 = 2.54$). (Table 5.4.a); the large F value indicates that most of the variations in the response could be explained by the regression model equation for 4-CP biodegradation in the study. The linear and square terms of both the regression models for 4-CP biodegradation and specific growth rate were found to be highly significant at P less than 0.02. In the present study the model F -values of 8.2 and 7.48 for 4-CP biodegradation and culture specific growth rate, respectively, indicate that the respective regression models could explain most of the variation in the

responses. These findings confirmed that the second-order polynomial models for 4-CP biodegradation and specific growth rate were adequate in predicting both the responses.

Table 5. 4. (a) ANOVA of 4-CP biodegradation in the optimization study.

Source	df	SS	Adj MS	F	P	R ²
Regression	14	14559.8	1039.98	8.20	0.000	94.48
Linear	4	3022.4	592.98	4.68	0.011	
Square	4	8496.2	2124.04	16.76	0.000	
Interaction	6	3041.2	506.87	4.00	0.012	
Lack-of-fit	10	48.463	4.8463	0.3241	0.134	
Error	6	89.7	14.95			
Total	30	16587.9				

Table 5. 4. (b) ANOVA of the culture specific growth rate in the optimization study

Source	df	SS $\times 10^{-3}$	MS $\times 10^{-3}$	F	P	R ²
Regression	14	14.31	1.022	7.48	0.000	86.8
Linear	4	2.99	0.567	4.15	0.017	
Square	4	8.437	2.109	15.43	0.000	
Interaction	6	2.884	0.481	3.52	0.021	
Lack-of-fit	10	0.0525	0.0525	0.375	0.118	
Error	6	0.085	0.014			
Total	30	0.016498				

df, degrees of freedom; SS, sum of squares; MS, mean sum of squares. F Fisher's F value (calculated by dividing the MS owing to the model by that due to error), P probability of incorrectly rejecting the null hypothesis when it is actually true.

These regression model equations are presented below.

$$Y_1 \quad f(x) = -2874.87 + 490.65X_1 + 1.14X_2 + 54.46X_3 + 12.66X_4 - 34.23X_1^2 \quad 5.6$$

$$+ 0.00X_2^2 - 1.01X_3^2 - 0.04X_4^2 + 0.03X_1X_2 + 0.62X_1X_3 + 0.00X_1X_4 +$$

$$0.01X_2X_3 - 0.04X_2X_4 - 0.11X_3X_4$$

$$Y_2 \quad f(x) = -2.78 + 0.46X_1 + 0.00139X_2 + 0.05674X_3 + 0.01098X_4 - 0.03190X_1^2 \quad 5.7$$

$$- 0.00X_2^2 - 0.001104X_3^2 - 0.00004X_4^2 + 0.00002X_1X_2 + 0.00048X_1X_3 +$$

$$0.00003X_1X_4 + 0.00001X_2X_3 - 0.00003X_2X_4 - 0.00011X_3X_4$$

Where Y_1 = 4CP biodegradation, Y_2 = specific growth rate, X_1 is pH, X_2 is agitation (rpm), X_3 temperature (°C) and X_4 is inoculum age (h).

Further, to determine significance of the regression coefficients in the two models, the results were subjected to student's *t*-test and are presented in [Table 5.5](#).

Table 5.5 Result of Student's *t*-test for 4-CP biodegradation and the culture specific growth rate in the optimization study.

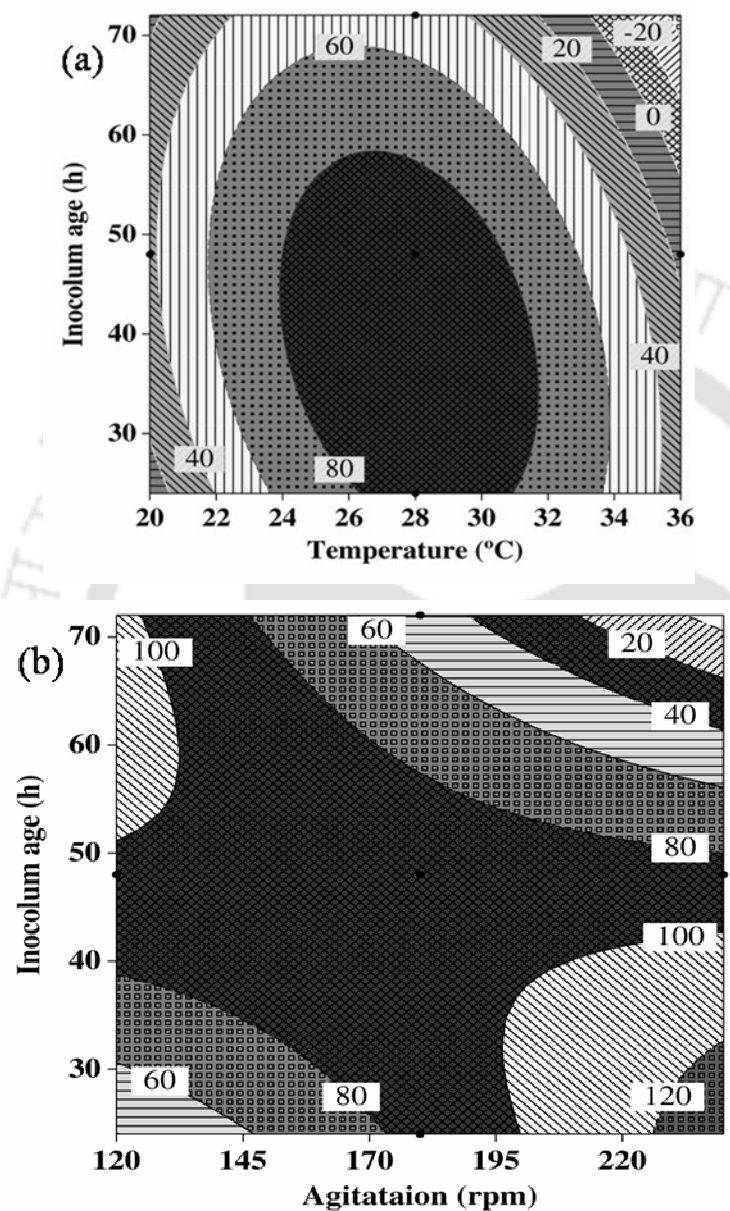
Term	4-CP degradation		Specific growth rate	
	t	P	t	P
Constant	-3.083	0.007	-2.872	0.011
pH (X_1)	2.367	0.031	2.141	0.048
Agitation (rpm) (X_2)	0.549	0.590	0.643	0.529
Temperature ($^{\circ}\text{C}$) (X_3)	3.446	0.003	3.457	0.003
Inoculum age (h) (X_4)	2.507	0.023	2.093	0.053
X_1^2	-2.602	0.019	-2.335	0.033
X_2^2	0.026	0.979	-0.219	0.829
X_3^2	-7.668	0.000	-7.600	0.000
X_4^2	-2.860	0.011	-1.900	0.076
$X_1 \times X_2$	0.132	0.896	0.068	0.946
$X_1 \times X_3$	0.354	0.728	0.261	0.798
$X_1 \times X_4$	0.001	0.999	0.056	0.956
$X_2 \times X_3$	0.322	0.751	0.368	0.718
$X_2 \times X_4$	-4.497	0.004	-4.208	0.005
$X_3 \times X_4$	-1.877	0.079	-1.783	0.094

From [Table 5.5](#), it could be seen that the regression coefficients of linear and quadratic terms for temperature in both the models for 4-CP biodegradation and specific growth rate were found to be highly significant ($P < 0.003$) whereas the coefficient due to pH and inoculum age indicated less significance on the responses. On the other hand, agitation did not show any significance ($P > 0.5$). From the Student *t*-test of 4-CP biodegradation and specific growth rate, the regression coefficient terms for interaction between agitation and inoculum age were found to be highly important ($P < 0.005$); however, interaction effects between temperature and inoculum age revealed slightly less significance. Other coefficient terms in the models did not seem to be of considerable significance ($P > 0.3$) on 4-CP biodegradation as well as on specific growth rate of the culture.

In order to illustrate the main and interactive effects of the independent variables on the dependent ones, the fitted polynomial equation was then represented in the form of two dimensional response surface contour plots as before ([section 5.1.3.1](#)).

The contour plots were constructed by plotting the response against any two of the four independent variables and by maintaining the other variables at their middle (0) levels.

Figure 5.6 represent the typical response surface contour plot between these factors.



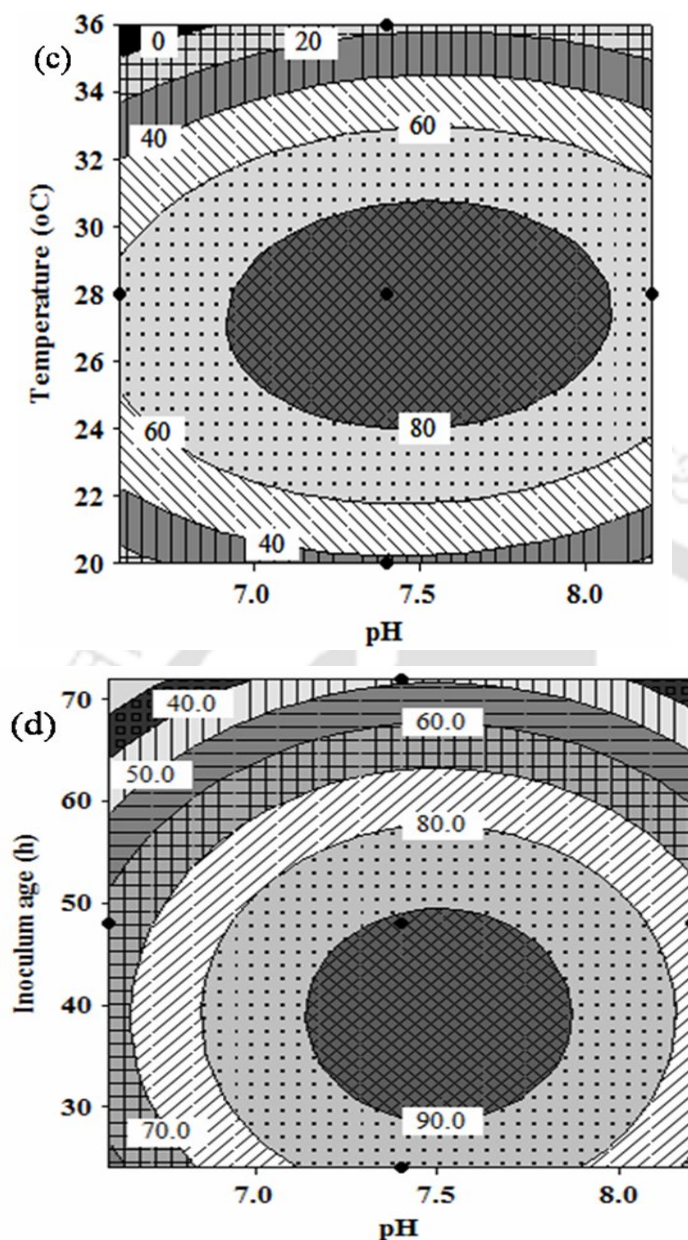
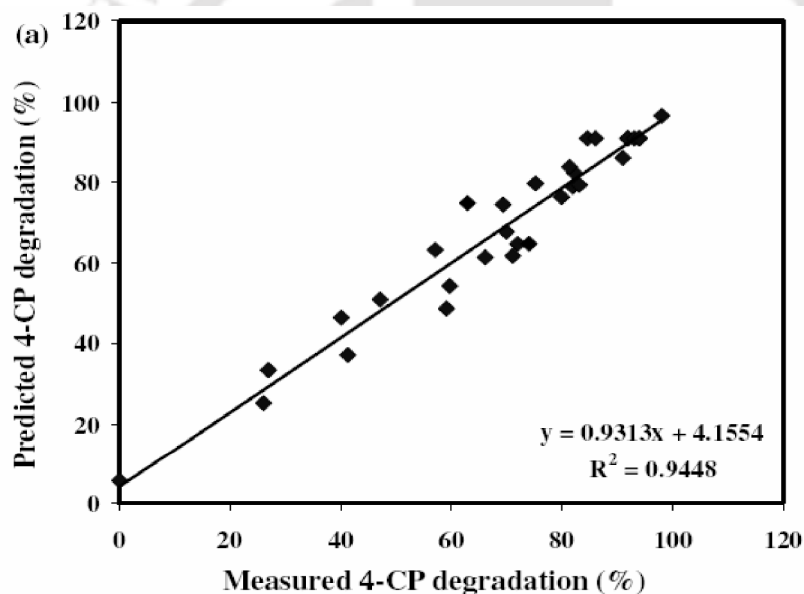


Figure 5.6 Two dimensional response surface contour plots showing the relation ship between these factor on 4-CP biodegradation by *A.chlorophenolicus* A6 (a) pH and temperature (b) temperature and inoculum age (c) agitation and temperature (d) inoculum age and agitation

Hence, from the given plot in Figure 5.6 (a), the corresponding coordinates in the region of the contour diagram gave the optimum values of the respective factors. Also, the response surface contour plots of mutual interaction between the variables inoculum age and temperature (Figure 5.6 (a)) was found to be elliptical indicating significant interaction between the two. In Figure 5.6. (b), which is a typical saddle point contour plot, the optimum values were obtained at the point of intersection of the lines that are formed by joining the locus (Murthy et al., 2000). Besides the two contour plots showing interaction between the variables, response surface contours drawn between other factors were circular as shown in Figure 5.6 (c) and (d) indicating insignificant nature of their interactions.

Figure 5.7 (a) and (b) represent linear plots of measured versus predicted values of 4-CP degradation and culture specific growth rate, respectively, which clearly reveals that both the experimental and predicted values were in close agreement with each other.



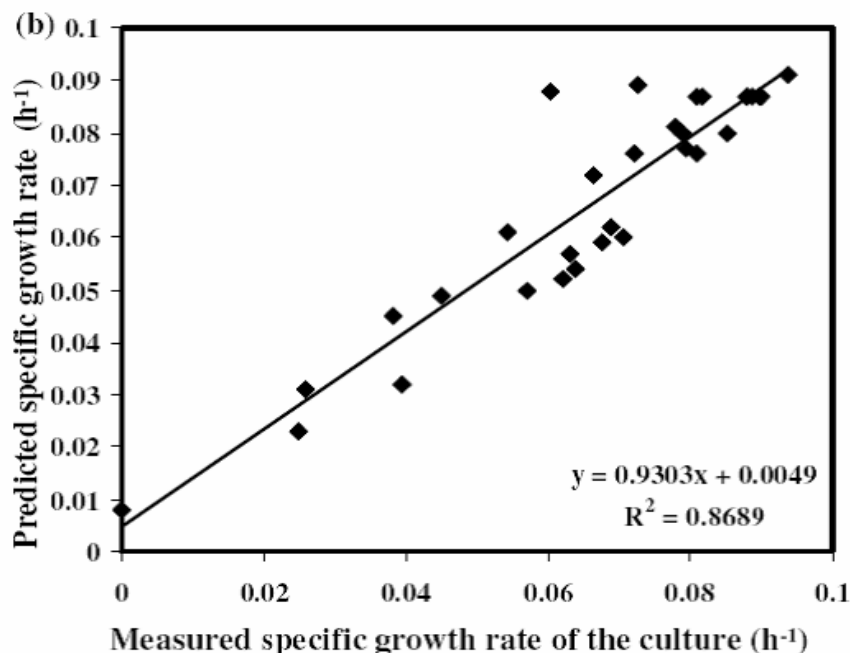


Figure 5.7 (a) Linear plot of predicted versus measured values of 4-CP degradation in the optimization study. **(b)** Linear plot of predicted versus measured values of specific growth rate of the culture in the optimization study

5.2.2 Multiple response optimization (Desirability function)

In order to determine the optimal levels of each variable for maximizing both 4-CP biodegradation and the culture specific growth rate, the method of desirability function was applied as detailed given in [section 5.13.2](#). Taking Lower value (L) for the two responses (4-CP degradation and specific growth rate) were 0.0 % 0.0 h^{-1} respectively, and target value (T) were set at 100 % and 0.094 h^{-1} , respectively

The overall desirability functions for 4-CP biodegradation and specific growth rate were close to 1 indicating the fact that the function increases linearly towards the desired target values of the two responses ([Derringer and Suich, 1980](#); [Jahani et al., 2008](#)).

In addition, individual desirability values of the two responses were calculated; while the value for specific growth rate was computed to be 1 with a maximum predicted response of 0.0943 h^{-1} , the value for 4-CP biodegradation was also found to be 1 with maximum predicted value of 102.66 %. Thus, using the desirability function method for optimizing both the responses (discussed earlier) optimum values of the culture conditions were estimated to be: pH 7.5; agitation 207 rpm; temperature 29.6°C and inoculum age 39.5 h.

5.2.3 Validation of the model

To validate the model, experiments were carried out in 250 ml of Erlenmeyer flask with 100 ml of optimal levels of the media constituents with pH 7.5 containing 300 mg l^{-1} of 4-CP. Under the model predicted optimized culture condition i.e pH 7.5; agitation 207 rpm; temperature 29.6°C and inoculum age 39.5 h experiments were carried out for 4-CP degradation and culture specific growth rate. Results revealed that the corresponding % of 4-CP degradation and specific growth rate were found to be 99.9% and 0.095 h^{-1} respectively within 18.5h of its culture, which is also perfectly matched with the predicted values of 100% of 4-CP degradation and 0.0943 h^{-1} of specific growth rate of the culture. The validation study was performed in triplicate and results reported are average with standard deviation $\pm 2.6\%$. The microorganism could also degrade a higher 4-CP concentration of 375 mg l^{-1} within 40 h of culture, which was earlier reported to be inhibitory to the microorganism growth and its degradation (Westerberg et al., 2000).

Agitation speed is important for maintaining homogenous chemical and physical conditions, dispersion of dissolved oxygen into smaller bubbles for increased interfacial area and oxygen mass transfer rate, and all these factors play a crucial role in enhancing both substrate utilization and growth of microbial cultures (Sharma and Pant, 2001). In this study the agitation speed was found to be optimum at 207 rpm, which is in agreement with the literature reports on the effect of agitation on microbial growth and degradation of xenobiotics (Sharma and Pant, 2001; Khleifat, 2006).

However, any further increase in the agitation rate to 250 rpm did not improve the 4-CP degradation ability by the actinomycetes in the present study, which might be attributed to induced shear stress on the cells leading to the cell loss or reduced biomass concentration (Hoq et al., 1995). Moreover, since this particular microorganism utilizes both aerobic and as well as reductive dechlorination metabolic pathway for 4-CP degradation (Nordin et al., 2005), however, reductive dechlorination usually inhibited by higher concentration of dissolved oxygen (Mohn and Tiedje, 1992; VanBriesen et al., 2004). Thus higher agitation speeds may hinder the reductive dechlorination step in effective degradation of the compound. Mesophilic temperature range is reported to have a strong impact on microbial degradation of aromatic compounds and plays an equivalent or larger role than nutrient availability in the degradation media (Margesin and Schinner, 1997). In the present study it was observed that an optimum culture temperature of 29.58 °C was required for complete degradation of 4-CP. Similar observations on biodegradation of phenol, endosulfan and crude oil due to temperature effect are reported by other authors, as well (Bandyopadhyay et al., 1998; Sharma and Pant, 2001; Hussain et al., 2007). In the current study, at temperature above 36 °C ceased both the growth and 4-CP degradation rate by the actinomycetes probably due to inhibition of multi-enzyme complex system of the cell (Bandyopadhyay et al., 1998). pH is another important factor for growth and degradation of chlorophenols by microorganisms, and the optimum pH in this study was found to be 7.5. It is known that at acidic pH values chlorophenols remain in its unionized and more toxic form (Penttinen, 1995; Leeuwen and Vermeire, 2007). Therefore an optimum pH of 7.5 in the study for better degradation of the compound and growth of the actinomycetes is quite likely. However, pH values above 7.5 inhibited the growth and chlorophenol degradation due to its negative effect on the activities of phenol oxidase and peroxidase enzymes (Tabatabai, 1994; Sinsabaugh et al., 2008). Similar finding on phenol biodegradation was reported by Khleifat (2006). Since the biodegradation media used in the present study contained only 4-CP as the sole source of carbon and energy, inoculum age plays a direct role on the rate and extent of lag phase of the actinomycetes. From the optimization results, inoculum age was found to be optimum at 39.5h, a value which is well supported in literature for phenol degradation (Bandyopadhyay et al., 1998).

At the optimum settings of the culture conditions, the organism was capable of completely degrading (100%) 4-CP for an initial concentration of 300 mg l^{-1} within 18.5h of its culture. The time required to degrade 4-CP was also found to be much less (by 5.5 h) compared to that required at the unoptimized culture conditions reported earlier by [Westberg et al. \(2000\)](#). Furthermore, the 4-CP degradation efficiency obtained at the optimum levels of the culture conditions was observed to be 22.99% higher than that obtained using the unoptimized culture conditions. Overall, the results of the study clearly showed very good enhancement in the 4-CP biodegradation efficiency by *A. chlorophenolicus* A6 by optimizing the culture conditions employing the non-conventional statistical based design technique.

5.3 Biodegradation of Substituted Phenols in Batch Shake Flasks

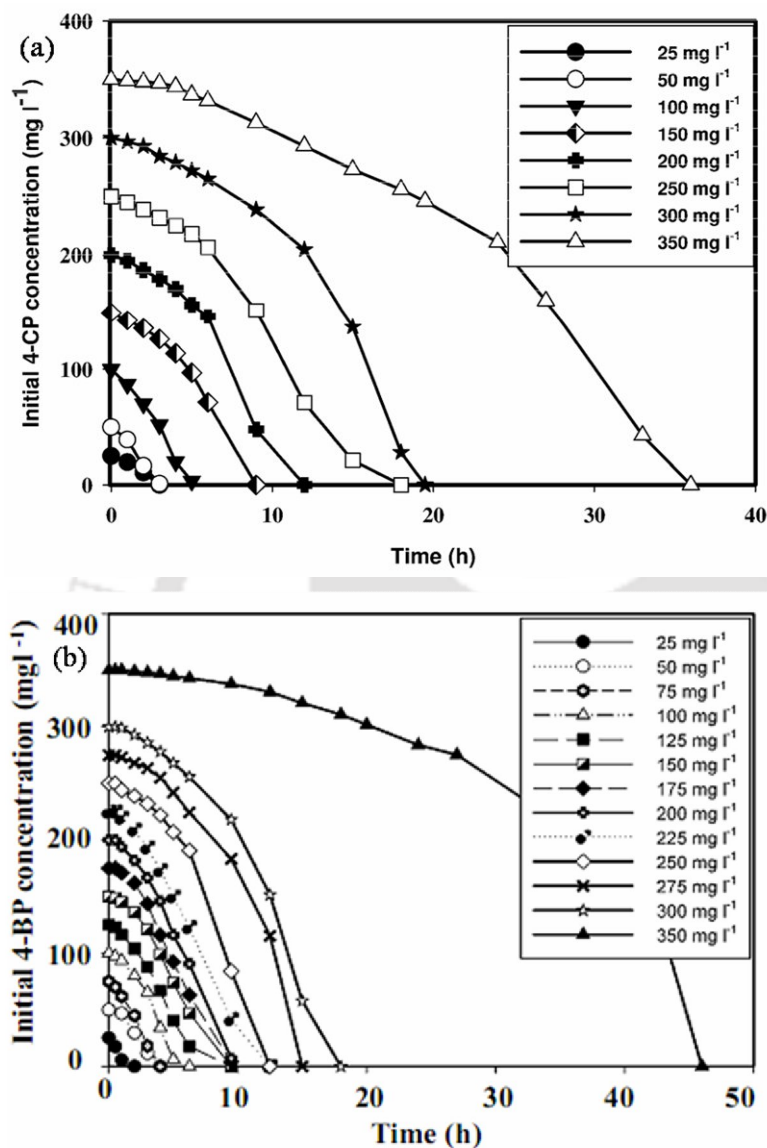
Knowledge of microbial growth and substrate utilization kinetics is important for the purpose of prediction of effluent quality by biological treatment processes ([Ellis et al., 1996a, b](#); [Grady et al., 1996](#); [Ellis and Anselm, 1999](#)). Biokinetic parameters also help in optimizing the operational conditions to meet the discharge requirements ([Ellis and Anselm, 1999](#)). Therefore, in the present thesis batch degradation kinetics of these substituted phenols by *A. chlorophenolicus* A6 was investigated using the above optimized media constituents and culture conditions.

5.3.1 Single substrate system

5.3.1.1 Biomass growth and degradation of substituted phenols

[Figure 5. 8. \(a\), \(b\) and \(c\)](#) shows the time profile of 4-CP, 4-BP and 4-NP degradation by the *A. chlorophenolicus* A6 at its various initial concentrations in the medium, which clearly reveals that the time taken by the microorganism to degrade the compound was dependent upon its initial concentration. For instance, to degrade 100, 75 and 125 mg l^{-1} of 4-CP, 4-BP and 4-NP the culture took about 6h, 6h and 9h respectively but for 350, 350 and 225 mg l^{-1} it took a higher time of 36h, 46h, 30h respectively for complete degradation of

these compounds. The results also showed that a maximum degradation rate was achieved at 100, 75 and 125 mg l^{-1} of 4-CP, 4-BP and 4-NP, which indicated a strong influence of these substituted phenol concentration on its degradation rate (Hao et al., 2002).



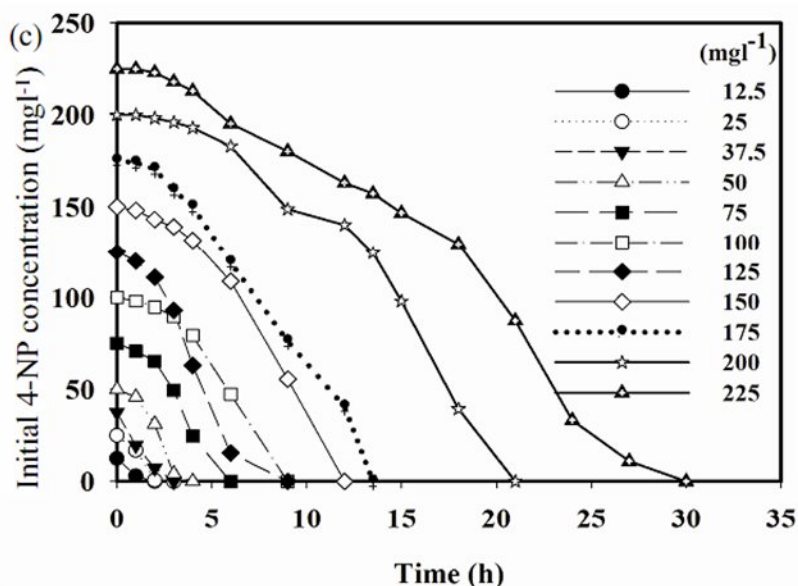


Figure 5.8 Time profile of *A. chlorophenolicus* A6 on degradation of (a) 4-CP (b) 4-BP and (c) 4-NP.

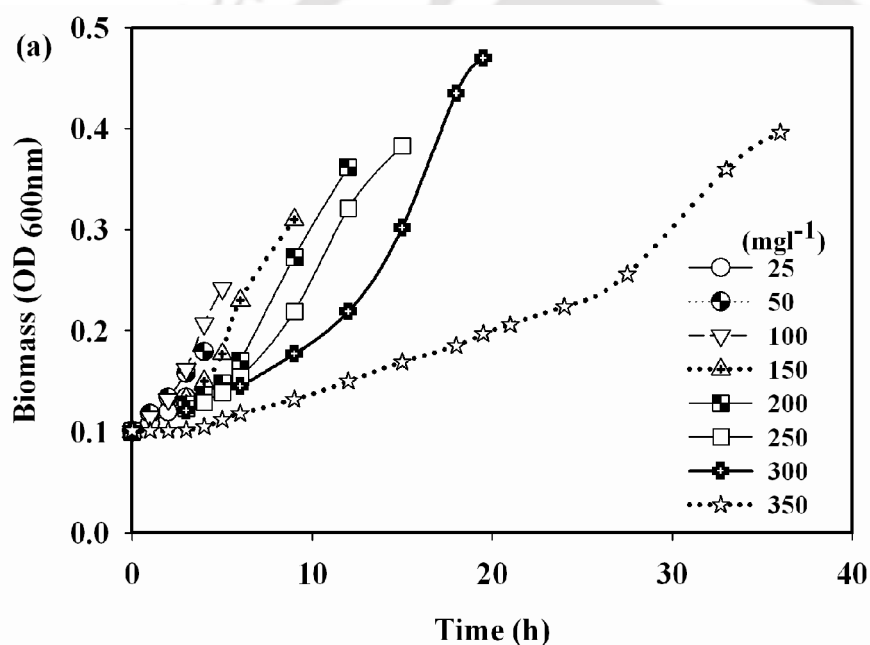
It is also observed that towards the end of the substrate consumption curve (Figure 5.8. (a), (b) and (c)) a region of relatively less rate of substrate removal existed in each concentration of the phenolics. Possible explanations towards this phenomenon may be given based on a fall in pH (from 7.5 to 7.1) and depletion of oxygen in the culture medium (Yang and Humphrey 1975, Lallai and Mura 1989, Blanch and Clark 1996). The 4-CP degradation performance achieved in the present study using the *A. chlorophenolicus* A6 is found superior to several literatures reported on chlorophenols biodegradation as shown in Table 5.6. To the best of our knowledge, the substituted degradation by *A. chlorophenolicus* A6 is found to be one highest biodegradation performance at a concentration as high as 350 mg l⁻¹ for 4-CP and 4-BP. The maximum 4-NP concentration studied so far in the literature for its degradation in a bioreactor system is only 320 mg l⁻¹ (Tomei et al., 2003), whereas in batch shake flasks it is 400 mg l⁻¹; however, in both these cases a very high initial MLSS of 1.5 g ml⁻¹ was used in comparison with only 20 mg l⁻¹ used in the present study (Cho et al., 2000).

Further Similarly specific degradation rate by *A.chlorophenolicus* A6 obtained in the present study is found to be very high i.e 0.829h^{-1} for an initial 4-NP concentration of 225 mg l^{-1} in comparison with literature reported value of only 0.009h^{-1} using *Nocardioides* sp. NSP41(Cho et al., 2000). Thus the present study also revealed the potential of *A. chlorophenolicus* A6 culture in treating wastewaters containing highly recalcitrant compound such as nitrophenol.

Table 5.6 performance different microorganism operated in different system and mode of operation for substituted phenol biodegradation reported in literature

Pollutants	Concentration (mg l^{-1})	% degradation	Mode of system	Microorganism	References
chlorophenols	4-CP, 220	100	Batch and SBR	<i>Pseudomonas putida</i> ATCC 49451	Sahinkaya et al., 2006
	4-CP, 20	100	PBR	Mixed culture	Kim et al., 2002
	4-CP, 100	100	Batch shake flask	<i>Rhodococcus erythropolis</i> M1	Goswami, et al., 2002
	4-CP, 20-50	69-100	GAC-FBR	Mixed culture	Carvalho et al., 2001
	2,4,6-TCP, 46.96	94-99	Airlift bioreactor	<i>Achromobacter</i> sp	Xiangchun et al., 2003
Bromophenol	TBP, 173	85%	Batch shake flask	<i>Trametes versicolor</i>	Uhnáková et al., 2009
	276.8	60	-		Entezari and Petrier (2003)
Nitrophenol	2NP, 4-NP, and 2,4 DNP, 2-30	90-99	UASB	Mixed culture	Karim and Gupta, 2001
	4-NP, 300-400	100	SBR	Mixed culture	Tomei et al., 2004
	4-NP, 2,4-DNP, aniline 50 to 180	99	SBR	<i>Bacterioidetes</i> , Candidate division TM7,	Xing-yu et al., 2007

Similar to time taken by the culture to degrade these substituted phenols at its various initial concentrations, the culture followed a similar pattern for its growth. This is illustrated in Figure 5. 9. (a) (b) and (c), where biomass growth (OD 600 nm) of the culture is plotted against time for various initial phenolics concentrations in the media. It could be seen from Figure 5. 9. (a), (b) and (c), that 4-CP and 4-BP concentration between 25 mg l^{-1} - 300 mg l^{-1} and 4-NP concentration between 12.5 - 175 mg l^{-1} did not show any significant repression on the biomass output without any lag phase. However, at concentration higher than the above, a prominent lag phase in its the growth was evident and correlated well with the delay in utilization of these compounds by the culture. Thus, it could be inferred that 4-CP, 4-BP at concentration above 300 mg l^{-1} exerted a significant toxic effect on the culture growth where as for 4-NP it is 175 mg l^{-1} .



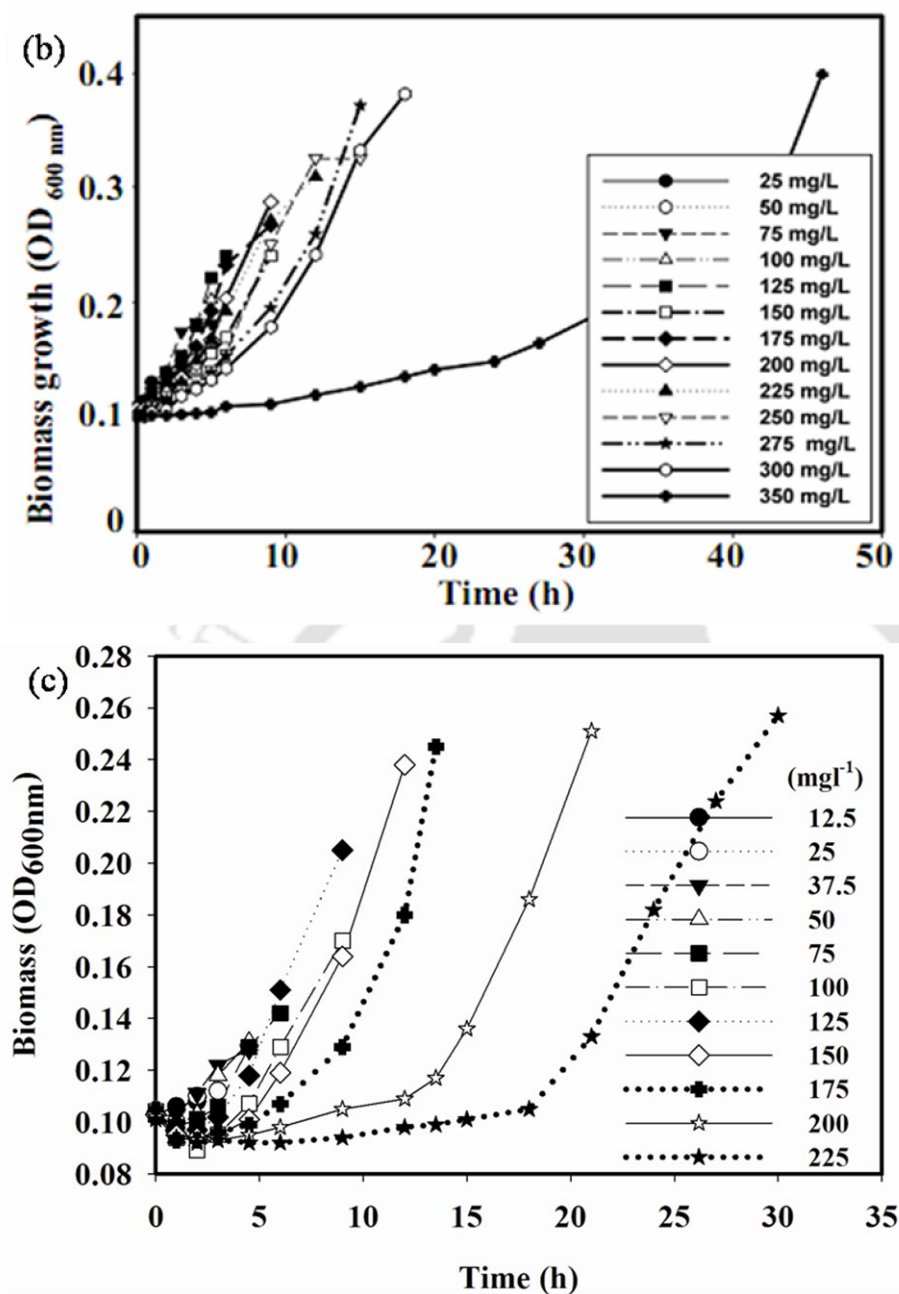


Figure 5.9 Time profile of biomass growth (OD_{600nm}) for different initial substituted phenol concentration (a) 4-CP (b) 4-BP and (c) 4-NP.

A typical growth curve shows a decline in cell population following complete consumption of substrate. During this declining phase some part of the cell population becomes food for the rest of the cell population. This part of the growth curve in a batch reactor can be modeled using the following equation (Kumar et al., 2005).

$$\frac{dx}{dt} = -k_d X \quad (5.8)$$

where X is biomass concentration (mg l^{-1}) at time t (h) and k_d is decay constant, whose value correlates with biomass growth rate but inversely and is also independent of substrate initial concentration. Thus, the batch growth experiment with 300 mg l^{-1} initial 4-CP, 4-BP and 225 mg l^{-1} of 4-NP concentration in the present study was continued further for another 4 days even after complete consumption of the substrate and decay constant value was estimated from negative slope of the curve obtained by plotting $\ln(\text{optical density})$ versus time. The k_d value of 0.0185 h^{-1} , 0.013 h^{-1} and 0.0132 h^{-1} obtained for 4-CP, 4-BP and 4-NP respectively was found to be very much in agreement with several literature reported values, which ranges from 0.1 to 0.6 day^{-1} (Kappeler and Gujer, 1992; Henze et al., 2002). The larger decay coefficient for 4-CP obtained in the present study may be due the production of toxic chloride ion (Stanlake and Fin, 1982) leading to formation of hydrochloric acid and drop in pH.

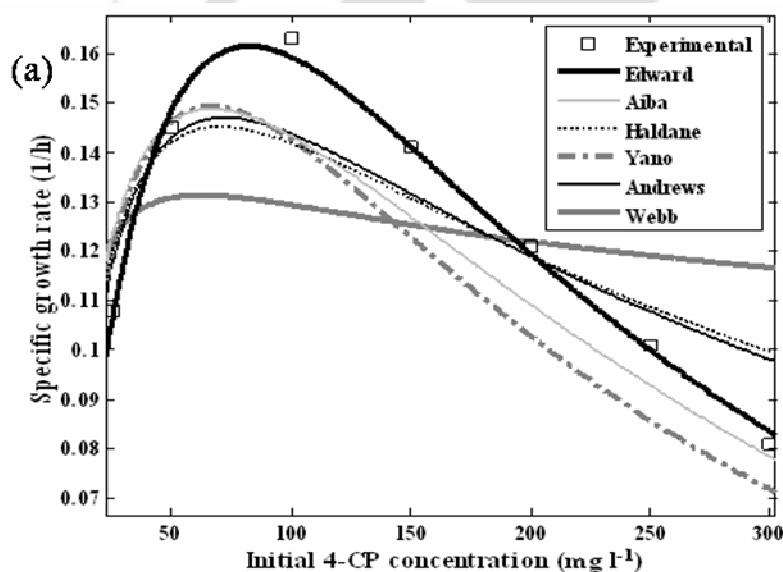
5.3.1.2 Modeling the growth kinetics of *A. chlorophenolicus* A6 in presence of substituted phenol

In order to establish the effect of substituted phenol concentration on growth of *A. chlorophenolicus* A6, specific growth rates of the culture at different pollutant concentrations were calculated as per the following relationship:

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad (5.9)$$

where X is biomass concentration (mg l^{-1}) at time t (h) and μ is the specific growth rate (h^{-1}) (Kovari and Elgim, 1998).

For each batch culture with a certain initial substituted phenol concentration, the specific growth rate was estimated by performing a linear least squares regression on the semilogarithmic plot of biomass concentration over cultivation time in the exponential growth phase. Figure 5. 10. (a), (b) and (c) depicts the variation of specific growth rate of the culture with the initial concentrations of these pollutants, which shows that the culture specific growth rate increased gradually to a maximum values of 0.161 h^{-1} , 0.17 h^{-1} and 0.118 h^{-1} with the initial 4-CP, 4-BP and 4-NP content up to 100, 75 and 125 mg l^{-1} respectively. On the other hand culture specific growth rate decreased to 0.08 h^{-1} when initial concentration was increased from 75 to 300 mg l^{-1} for 4-CP and 4-BP. In case of for 4-NP, drop in specific growth rate was relatively low (0.114 h^{-1}) possible due to lower initial concentration of the compound used in the study ($12.5\text{-}175 \text{ mg l}^{-1}$). Thus, the results clearly indicated inhibitory effect of 4-CP, 4-BP and 4-NP at concentrations above 100, 75 and 125 mg l^{-1} respectively on the culture growth.



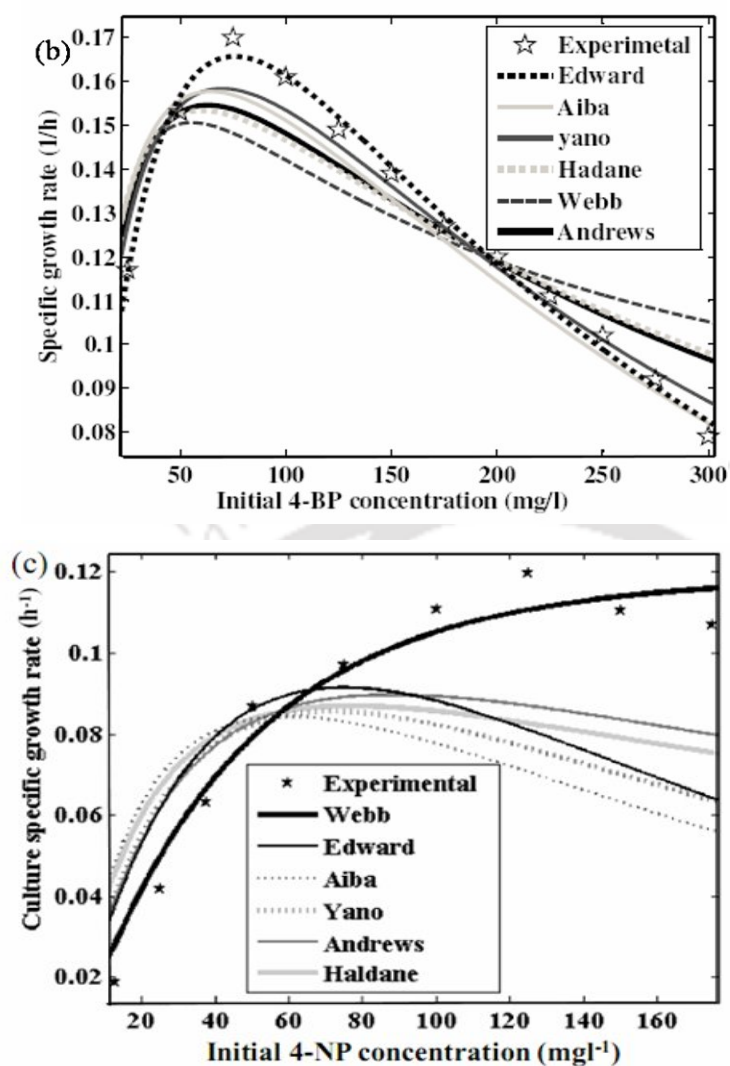


Figure 5.10 Experimental and model predicted specific growth rate of the culture at different substituted phenol concentrations (a) 4-CP (b) 4-BP and (c) 4-NP

This type of growth behavior by *A. chlorophenolicus* A6 due to higher concentration of these substituted phenol demonstrated substrate inhibition pattern that has been studied by other authors, as well, for other substrates using different microorganisms (Yan et al., 2006; Bai et al., 2007).

In order to predict the patterns degradation of these substituted phenols and culture growth in the system, kinetics of these two phenomena were analyzed by fitting the data to substrate inhibition models found in the literature. Table 5.7 presents the model equations tested in the study. For solving the model equations nonlinear regression method was applied using MATLAB 7.0. The aforementioned Figure 5. 10. (a), (b) and (c) also shows the model predicted specific growth rate along with the experimentally obtained values.

Table 5. 7 Various literature models on biomass growth with substrate inhibition tested in the study

Author(s)	Model	References
Edward	$\mu_g = \mu_{\max} [\exp(-S/K_i) - \exp(-S/K_s)]$	Edward (1970)
Aiba et al.	$\mu_g = \mu_{\max} S [\exp(-S/K_i)] / (S + K_s)$	Aiba et al. (1968)
Yano et al.	$\mu_g = \mu_{\max} S / (S + K_s + S^2 / K_i (1 + S/K))$	Yano et al. (1966)
Andrews	$\mu_g = \mu_{\max} / (1 + K_s/S + S/K_i)$	Andrews (1968)
Haldane	$\mu_g = \mu_{\max} S / (S + S^2 / K_i + K_s + SK_s / K_i)$	Haldane (1965)
Webb et al.	$\mu_g = \mu_{\max} S (1 + S/K) / (S + K_s + S^2 / K_i)$	Webb et al. (1963)

$\mu_{\max}$, maximum specific growth rate; K_s , half saturation constant ; K_i , substrate inhibition constant; K , Yano constant; S , Substrate concentration, μ_g , predicted specific growth rate.

From these figures it could be seen that among the six models tested, for 4-CP and 4-BP Edward model gave the best fit to the experimental data with more accuracy (RMSE = 0.003 and $R^2 = 0.99$) and the model constants estimated to be maximum specific growth rate ($\mu_{\max}$) = 0.22 h^{-1} , inhibitory constant (K_i) = 105 mg l^{-1} and half saturation constant (K_s) = 30.83 mg l^{-1} for cell growth on 4-CP, in case of 4-BP these parameters were: $\mu_{\max} = 0.246 \text{ h}^{-1}$, $K_i = 111 \text{ mg l}^{-1}$ and $K_s = 30.77 \text{ mg l}^{-1}$. On the other hand, for 4-NP Web-type growth kinetic models was found to be best fitted (RMSE = 0.006 and $R^2 = 0.97$) and with model constant value of $\mu_{\max} = 0.16 \text{ h}^{-1}$, $K_i = 128 \text{ mg l}^{-1}$ and $K_s = 20.15 \text{ mg l}^{-1}$. Values of the kinetics parameters estimated from these six models are presented in Table 5. 8. (a), (b) and (c) along with root mean square (RMSE) error between model predicted and experimental specific growth rate of the culture.

All the models adopted in this study have generally been used in the literature to describe substrate inhibition on growth of microbial cultures. Therefore, it is more likely that the models fitted the experimental data obtained in the present study to a reasonable level of accuracy. However, some models showed slight deviation in the values of bio-kinetic constants, such as $\mu_{\max}$, K_s and K_i , probably due to their differences in the origin of development. For example, Edward model mainly concerns with the effect of a metabolite that may be formed during degradation on the growth of microbial culture, where as Haldane model is valid only at constant biomass yield (Wang and Loh, 1999). Furthermore, discrepancy of model fitting and lower half saturation constant obtained for 4-NP may be due the different chemical nature and properties of the pollutants, viz, 4-CP and 4-BP are halogenated substituted phenol expressing similar behavior to the microbial metabolic process and its permeability to lipid membrane of the microorganism

Table 5. 8 Estimated values of kinetic parameters on substituted phenols degradation using the various models tested in the study (a) 4-CP (b) 4-BP and (c) 4-NP

(a)

Model	Estimated value of parameters for 4-CP				RMSE	R^2
	$\mu_{\max}$ (h ⁻¹)	K_s (mg/L)	K_i (mg/L)	K (mg/L)		
Edward	0.22	30.83	275	-	0.0031	0.99
Aiba et al.	0.25	21.3	275	-	0.0161	0.78
Haldane.	0.26	21.91	224.2	-	0.0149	0.77
Yano	0.26	24.53	290	250	0.0160	0.68
Andrews	0.26	25.2	204.9	-	0.0134	0.81
Webb	0.16	6.8	150	250	0.026	0.30

(b)

Model	Estimated value of parameters for 4-BP				RMSE	R^2
	$\mu_{\max}$ (h ⁻¹)	K_s (mg/L)	K_i (mg/L)	K (mg/L)		
Edward	0.246	30.77	111.1	-	0.0030	0.99
Aiba	0.26	19.6	275	-	0.0080	0.91
Haldane.	0.27	18.25	200.1	-	0.0120	0.83
Yano	0.254	23.97	275.1	399.9	0.0065	0.95
Andrews	0.26	21.53	185.3	-	0.0106	0.87
Webb	0.29	20.14	92.06	400	0.016	0.72

(c)

Model	Estimated value of parameters for 4-NP				RMSE	R ²
	$\mu_{\max}$ (h ⁻¹)	K _s (mg l ⁻¹)	K _i (mg l ⁻¹)	K (mg l ⁻¹)		
Edward	0.24	38.83	205	-	0.028	0.37
Aiba	0.10	30.88	61	-	0.036	0.21
Haldane	0.27	34.91	164.2	-	0.026	0.45
Yano	0.10	44.53	177	300	0.030	0.25
Andrews	0.17	47.2	134.9	-	0.0201	0.62
Webb	0.16	20.15	128	100	0.0062	0.97

. For example, halogen substituents on the aromatic ring stabilizes its shared resonance electrons structure, as the halogens create a steric hindrance to enzymes and also have an electron withdrawing effect (van den Berg, 1990; Copley, 1997; Uberoi and Bhattacharya, 1997). Thus, in the present study halo-substituents phenols enhance the toxicity effect on microbial cells leading to lower affinity to cell (higher K_s value). On the other hand, the differing pKa values of the compounds would affect their K_s value. 4-NP has a pKa of 7.1, whereas it is 9.3 for 4-CP and 9.17 for 4-BP. This means that at pH 7.2 – 7.5 of the culture media typically used in these experiments, about 55% of the 4-NP was present in its dissociated form, i.e. as a phenolate ion, whereas in case of 4-CP and 4-BP it is less than 2%. This may be the possible reason for higher affinity of 4-NP (lower K_s value) to the *A. chlorophenolicus* A6 cells over these halo substituted phenols. Although predicted values using the Edward model equation correlated well with the experimental data with R² = 0.99, the fit was valid for 4-CP and 4-BP concentration only upto 300 mg l⁻¹; above this concentration the overall prediction accuracy of the model was slightly poor. Similarly for 4-NP the Webb type model was found valid only upto 175 mg l⁻¹. Analogous discrepancy in model prediction of experimental specific growth rate of microorganisms has been noted in the literature (Hao et al. 2002).

An attempt was made to compare the model parameter values obtained in the present study with the literature. Although large numbers of relevant kinetic studies on phenol are found available in literature, however, very few study are reported on 4-CP and 4-NP degradation kinetics and no report on 4-BP degradation were found using pure as well as mixed culture.

Half saturation constant (K_s) in the model is a measure of affinity between biomass and substrate (Juang and Tsai, 2006) and the value (30.77-30.83 mg l⁻¹) estimated in the present study for 4-BP and 4-CP is found to correlate well with those found in literature on phenol and p-cresol degradation by mixed and pure cultures (Kumaran and Paruchuri, 1997; Kumar et al., 2005). The model parameter value is also found to be greater than the K_s value obtained by Monteiro et al. (2000), Juang and Tsai (2006), Sahinkaya and Dilek (2007) for degradation of cresol, phenol and chlorophenol by *Candida tropicalis*, *pseudomonas putida* and mixed culture respectively, which indicates a lower affinity of *A. chlorophenolicus* A6 towards the substrate. Furthermore, half saturation constant (K_s) for 4-NP was estimated to be nearly half of that obtained for 4-CP and 4-BP (20.15 mg l⁻¹) which correlates well with values found in literature on 4-NP and phenol degradation using mixed cultures (Pawlowsky and Howell, 1973; Abuhmed et al., 2005). The model parameter value is also found to be greater than the K_s value of 6.19 and 13.33 mg l⁻¹ obtained by Bhatti et al. (2002), Ray et al. (1999) and Monterio et al. (2000) for degradation of phenol and 4-NP using a *P.putida* DSM 548 and mixed culture which indicates a lower affinity of *A. chlorophenolicus* A6 towards the substrate. The value of maximum specific growth rate (μ_{max} = 0.22, 0.246 and 0.16 h⁻¹ for 4-CP, 4-BP and 4-NP respectively) estimated from the best fitted model, correlated well with the value found in literature on 4-CP and 4-NP degradation by mixed culture and pure culture such as *Ralstonia eutropha* (Sahinkaya and Dilek, 2005; Salehi et al., 2010). However, in the present study the maximum specific growth rate of *A. chlorophenolicus* A6 was found significantly low when compared with several literature values. For example, with compare to the present study maximum specific growth rate of *C. tropicalis*, *Acinetobacter calcoaceticus* and *Pseudomonas species* was found to be considerably higher values (0.48 h⁻¹, 2.78 h⁻¹, 0.418 h⁻¹, 0.456 h⁻¹, 0.542 h⁻¹) for biodegradation of 4-NP, cresol and phenol (Acuna-Arguelles et al. 2003; Livingston and Chase 1989 ; Kumaran and Paruchuri, 1997) thus, indicating the substrates are poorly utilized by *A. chlorophenolicus* A6 for its growth. The smaller μ_{max} value may also be due to low initial inoculum size used in the experiments.

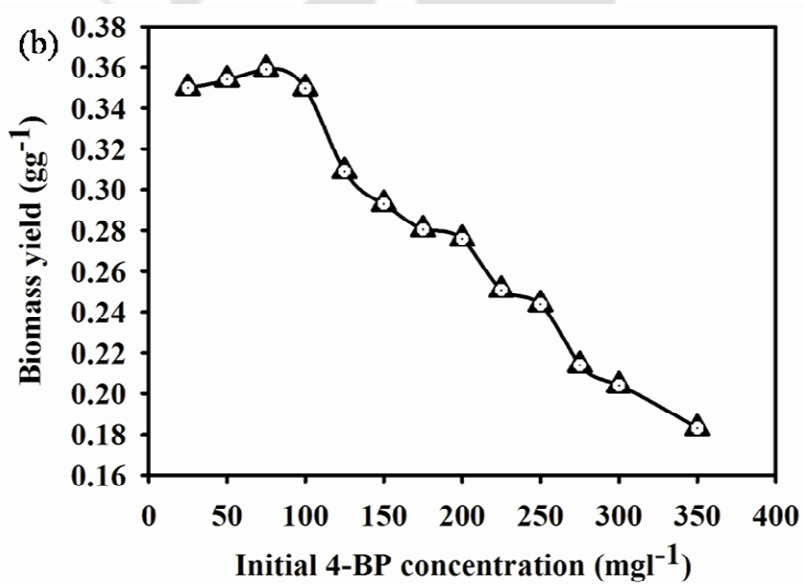
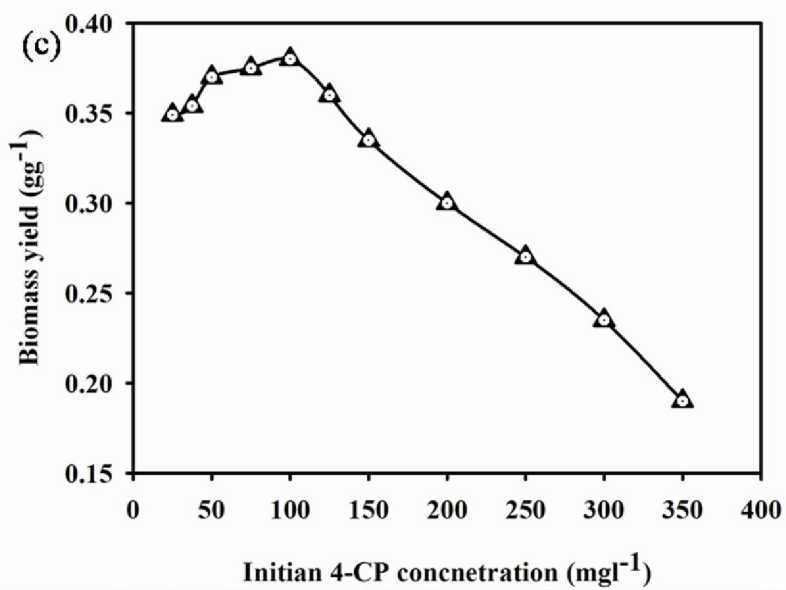
Furthermore, in the present study the smaller $\mu_{\max}$ value obtained by *A. chlorophenolicus* A6 may be attributed to the energy gained from substrate utilization being channelled towards maintenance energy rather than biomass formation. In addition, *A. chlorophenolicus* A6 biomass may be shunting very high percentage of the electrons for regeneration of NADPH which is generally used to activate the monooxygenase enzyme involved in substituted phenol biodegradation compared to other microorganism (Acuna-Arguelles et al., 2003; Kumaran and Paruchuri, 1997). This aspect however, requires further investigations to confirm. The value of the kinetic parameter K_i signify the degree of resistance of the microorganism to toxic effects of these substituted phenol and in general, a larger K_i value reveals that the biomass is highly resistant to inhibition by its substrate. In the present study the value of inhibitory constant (K_i) from the best fitted model was estimated to be 105 mg l⁻¹, 111 mg l⁻¹ and 125 mg l⁻¹ for 4-CP, 4-BP and 4-NP respectively. However, K_i values in the present study was found significantly low when compared with several literature reported values. For instance the K_i values of 4-CP, 4-NP, phenol and cresol on *C. tropicalis*, *Acinetobacter calcoaceticus*, *Pseudomonas species* was reported to be in the range of 145-516 mg l⁻¹ (Sahinkaya and Dilek, 2005; Acuna-Arguelles, et al., 2003; Livingston and Chase, 1989 ; Kumaran and Paruchuri, 1997). Thus it is surmised that the toxicity tolerance of *A. chlorophenolicus* A6 toward the substituted phenol is very poor.

5.3.1.3 Variation of Biomass Yield coefficient profile of *A. chlorophenolicus* A6

From the experimental result on biomass growth and substituted phenol degradation, yield coefficient (Y) was calculated using the following equation (Kumar et al., 2005):

$$Y = \frac{X - X_0}{S_0 - S} \quad (5.10)$$

where X_0 , X are the initial and final biomass concentration at the end of the culture with corresponding substrate concentrations S_0 and S. Figure 5. 11. (a), (b) and (c) is a plot of the cell mass yield coefficient versus initial substituted phenol concentration.



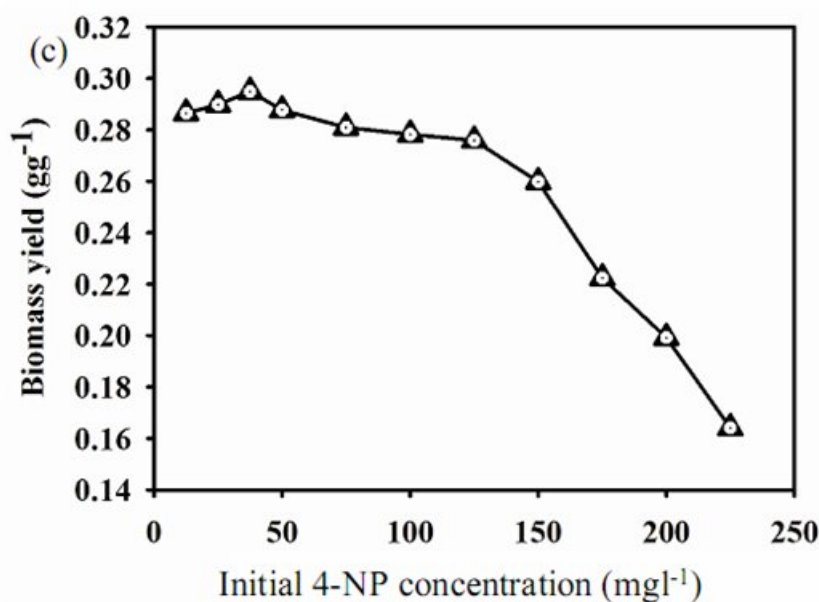


Figure 5.11 Effect of initial substituted phenol concentration on biomass yield (a) 4-CP (b) 4-BP and (c) 4-NP.

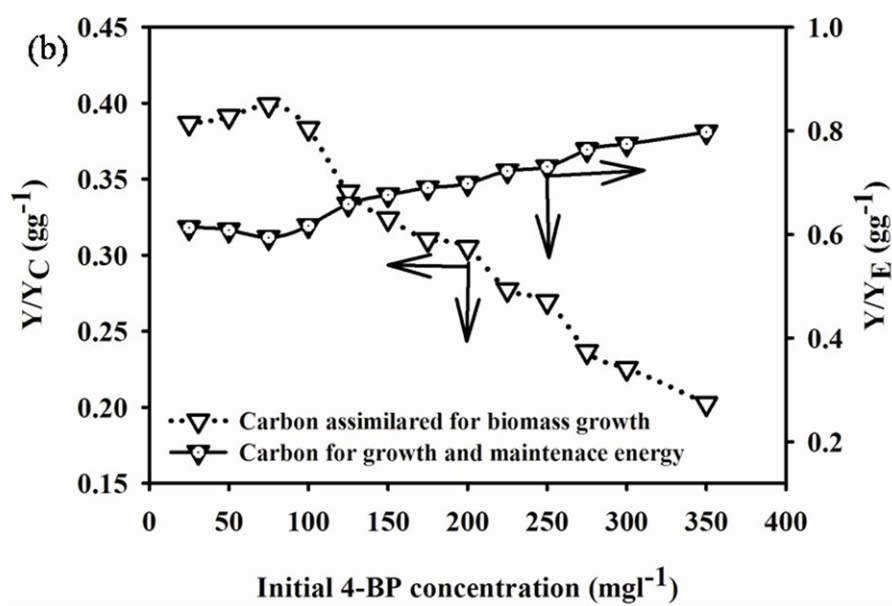
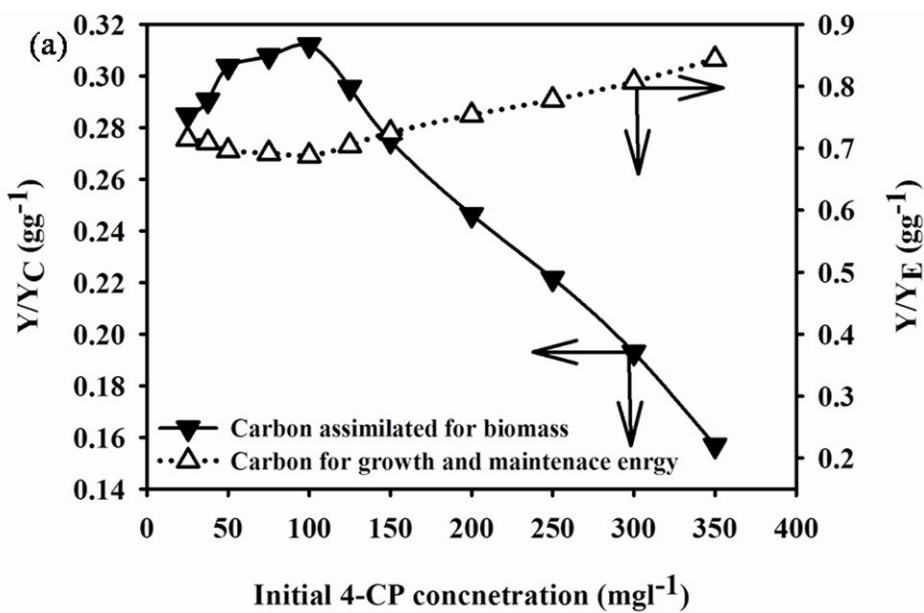
It can be seen, the yield coefficient varied from 0.21-0.39 and 0.183-0.359 g g⁻¹ with the initial 4-CP and 4-BP concentration from 25–350 mg l⁻¹ respectively. Where as, in case of 4-NP the yield value was found lower than that of 4-CP and 4-BP i.e. from 0.16-0.295 g g⁻¹. The lower biomass yield on 4-NP is correlated well with literature, as 4-NP is a metabolic uncoupler, and disassociate the energy coupling between catabolism and anabolism, thereby resulting in dissipation of certain portions of energy through futile cycles. This presents high potentialities for reducing excess sludge production (EPA, 1980). However, at lower initial concentration and below their respective inhibitory constant (K_i 105, 111 and 128 mg l⁻¹) of all the substituted phenols, the yield value remained almost constant, mainly due to absence of any inhibition to biomass growth of the culture at these lower concentrations. In the literature, several authors have also observed that biomass yield coefficient remains nearly constant for substrate concentrations below its inhibition level (Kumar et al., 2005; Chen and Johns, 1996; Onysko et al., 2000) probably due the requirement of lower maintenance energy at sub-inhibitory concentration than at higher concentration of the substrate (Chen and Johns, 1996).

At 4-CP, 4-BP and 4-NP concentrations above 105, 111 and 128 mg l⁻¹ respectively, considerable decrease in the value of yield coefficient was observed indicating the stronger inhibitory effect wherein the energy expended to maintain the cell activity is more. Therefore, the effect of maintenance energy requirements on substrate consumption and biomass production cannot be avoided.

The variation of cell mass yield with 4-CP, 4-BP and 4-NP concentration in their respective culture media can be further rationalized by a material balance on substrate consumption. Because these substituted phenols are consumed mainly for assimilation into biomass and for growth and maintenance energy, Eq. (5.11) can be used to described this aspect (Shuler and Kargi, 1992).

$$\frac{1}{Y} = \frac{1}{Y_C} + \frac{1}{Y_E} \quad (5.11)$$

where, Y_C represents the theoretical yield of cell mass on the substituted phenol, Y_E represents the yield of cell mass on substituted phenol consumed for energy, and Y is the observed cell yield on substituted phenol. Y_C is the calculated theoretical yield of 1.217, 0.9047 and 1.15 g g⁻¹ for 4-CP, 4-BP and 4-NP respectively based on the assumption that the carbon content of dry cells is about 45% (Rangaswami and Bagyaraj 2001). Y_E can, therefore, be determined according to Eq. (5.11). The percentage of the total substrate carbon converted to energy for cell growth and maintenance can be obtained as Y/Y_E , and the percentage of the total substrate carbon assimilated into biomass can be obtained as Y/Y_C . Figure 5. 12 (a), (b) and (c) plots Y/Y_C and Y/Y_E versus initial substituted phenol concentration. It can be clearly seen that Y/Y_E initially remained almost steady upto 4-CP, 4-BP and 4-NP concentration of 105, 111, and 128 mg l⁻¹, later, the value of Y/Y_E increased with a raise in the respective pollutants concentration. Also the relative proportion of the substrate consumed for energy (Y/Y_E) exceeded that for assimilation into cell mass (Y/Y_C) for these substituted phenol concentrations greater than their respective inhibitory concentration (K_i), which could be due to the requirement of high maintenance energy for overcoming the effect of substrate inhibition at high concentrations of these pollutants.



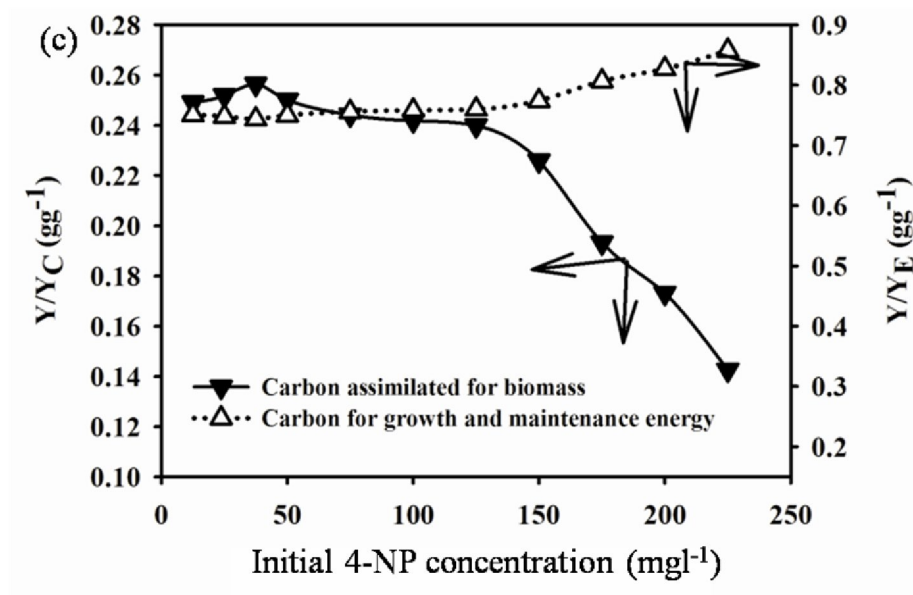


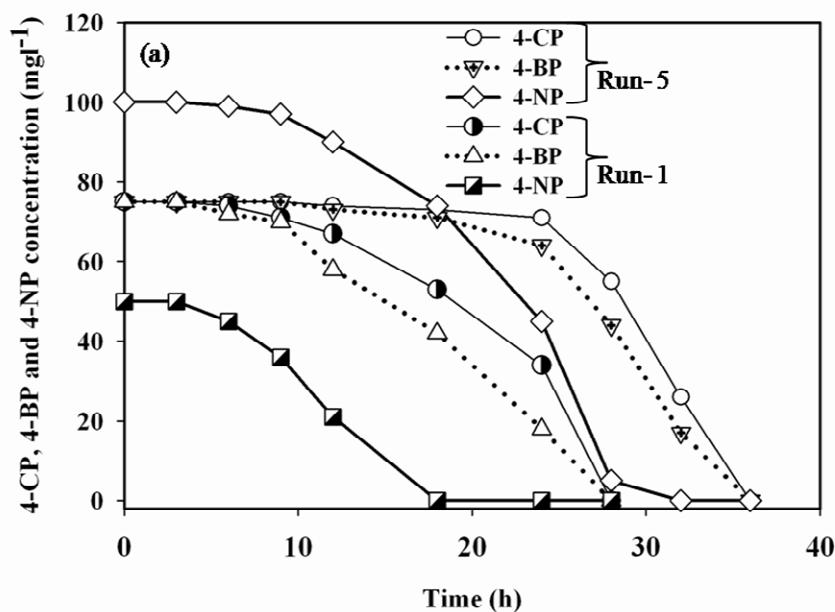
Figure 5. 12 Effect of initial substituted phenol concentration on proportion of substrate carbon diverted for biomass yield (Y/Y_C) and maintenance energy (Y/Y_E) (a) 4-CP (b) 4-BP and (c) 4-NP.

5.3.2 Culture Growth and Mixed Substrates Degradation Kinetics

5.3.2.1 Simultaneous Degradation Profiles of substituted phenols

Microbial biodegradation of pollutants is usually studied in the laboratory as single component system. However, most of the pollutants in nature commonly occur as mixtures, as a results degrdation rate of individual compounds gets affected (Kova'rova'-Kovar and Egli, 1998). Further, from the results of the previous sections on single substrate systems, it is evident that the culture took longer time to degrade 4-NP in comparison to 4-CP and 4-BP. The single substrate system also revealed competitive performance of the microbial culture to degrade a phenolic compound over others, where affinity of the culture towards degrading 4-NP was found high.

However, it is quite likely that in a mixed substrate system the culture may exhibit various other phenomena such as preferential utilization of a substrate and interaction among these substrates. Hence, in this study biodegradation experiments with media containing 4-CP, 4-BP and 4-NP at different concentrations combinations were performed. The biodegradation patterns of these substrates are presented in Figure 5.13 (a) and (b). It is observed that in all the cases of concentration combinations the microbial culture took longer duration to degrade 4-CP and 4-BP completely as compared to 4-NP. A prominent lag phase was observed during 4-CP and 4-BP degradation. Further the lag phase for 4-CP and 4-BP degradation was found to increase with increase in initial 4-NP concentration. Similar pattern of lag phase for 4-NP degradation was observed with increase in 4-CP concentration in the culture media. 4-NP degradation was slower when 4-CP was present as an additional substrate than when 4-NP was the sole substrate.



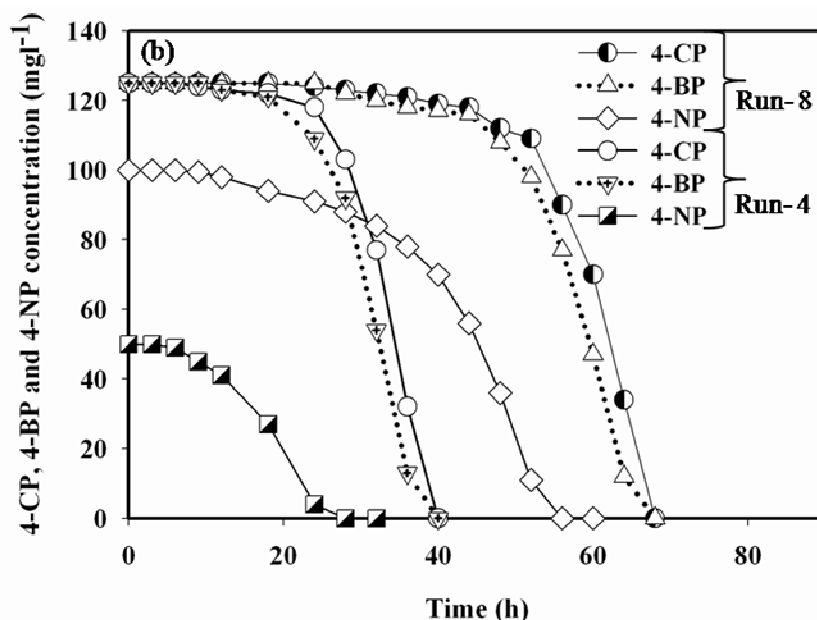


Figure 5. 13 Biodegradation of 4-CP, 4-BP and 4-NP by *A. chlorophenolicus* A6 in mixed substrate system for different initial concentration ranges. **(a)** At low concentration range (75+75+50 mg l⁻¹, run-1, and 75+75+100 mg l⁻¹, run-5) **(b)** at high concentration range (125+125+50 mg l⁻¹, run 4 and 125+125+100 mg l⁻¹, run- 8).

For instance in the single substrate system to degrade of 4-NP at an initial concentration of 100 mg l⁻¹ the culture took 8 h on compared with a minimum culture period of 18 h in mixed substrate system. The reason for slower degradation rates of pollutants in mixtures may be due to enhanced toxicity effect exerted by the phenolic in the mixture, for example, phenolics exert their toxicity by uncoupling oxidative phosphorylation (McLaughlin and Dilger 1980; Terada 1990; Escher et al. 1996), and formation of dimers between two differently substituted phenols can result in an uncoupling activity that is higher than the sum of the single compounds (Escher et al. 2001). In addition, formation of different intermediates in mixed substrate degradation system may inhibit enzymes vital for degradation, or its active binding site (Alexander, 1999). The degradation patterns of individual substrates in the mixed substrate system also differed considerably with those obtained in their respective single substrate systems (Pakshirajan et al., 2008).

Irrespective of the concentration levels of these three substrates, 4-NP was preferentially degraded over 4-CP and 4-BP. Between 4-CP and 4-BP, 4-BP degradation was found quicker than the other; however the difference was less significant. Unell et al. (2008) reported that although the same enzyme system is responsible for degradation of 4-CP, 4-BP and 4-NP by *A. chlorophenolicus* A6 one compound is preferentially degraded over others. For example, in the present case 4-NP is degraded preferentially over others.

A possible explanation may be due to the fact that the enzyme in the degradation pathway has a higher affinity towards 4-NP than those to 4-CP and 4-BP, alternatively, there could be transport-level interactions affecting degradation of the compound. For example, if *A. chlorophenolicus* A6 only takes up the phenolate ion form of these substituted phenols, the differing pKa values of the compounds would affect their order of degradation. 4-NP has a pKa of 7.1, whereas it is 9.3 for 4-CP and 9.17 for 4-BP. This means that at pH 7.2 – 7.5 of the culture media typically used in these experiments, about 55% of the 4-NP was present in its dissociated form, i.e. as a phenolate ion, whereas in the case of 4-CP and 4-BP it is less than 2%. This may be the possible reason for preferential degradation of 4-NP over these halo-substituted phenols. Further, simultaneous degradation of 4-CP and 4-BP in mixture could be caused by their similar availabilities due to their identical pKa values (9.41 and 9.37, respectively). In addition, this observation correlated well with results obtained in single substrate system where the values of half saturation constants (K_s) for 4-CP and 4-BP were nearly the same i.e. 30.83 and 30.77 mg l⁻¹ respectively; whereas, the values for 4-NP were lower (20.15 mg l⁻¹). Similar observation on preferential degradation of one aromatic compound over another in mixture found from the literature, even though the same degradation pathway is followed for these compounds by different microorganisms. For example, *P. putida* F1 uses the same degradation pathway for toluene and phenol, but when challenged with a mixture of these compounds, phenol degradation does not clearly start until almost all toluene has been removed from the medium (Reardon et al., 2000). Bai et al. (2007) studied biodegradation of phenol and *m*-cresol as mixed substrate using *Alcaligenes faecalis* and observed that between phenol and *m*-cresol, in the range of 50 - 150 mg l⁻¹ a rapid degradation of *m*-cresol took place towards the depletion of the phenol in the medium.

Yan et al. (2006) studied mixed substrate biodegradation of phenol and cresol with the yeast *C. tropicalis* and reported that the presence of phenol at low concentrations ($60 - 100 \text{ mg l}^{-1}$) enhanced the rate of *m*-cresol biodegradation. A similar effect was observed in the present study; where degradation time of 4-CP and 4-BP directly depend upon initial concentration of 4-NP i.e higher concentration of 4-NP resulted in quicker degradation of 4-CP and 4-BP towards the depletion of the 4-NP in the medium accompanied by an increase in lag phase. For an instant 250 mg l^{-1} of 4-CP or 4-BP takes about 16 h for completely degradation in single substrate system whereas in mixed substrate system (125 mg l^{-1} 4-CP and 125 mg l^{-1} 4-BP) it takes less time of 12 h containing with 50 mg l^{-1} 4-NP.

5.3.2.2 Biomass Growth of the Culture in the Mixed Substrate System

Biomass profiles obtained at all combinations of initial concentrations of the phenolics in the mixture are presented in Figure 5.14 in the form of OD₆₀₀ of the culture.

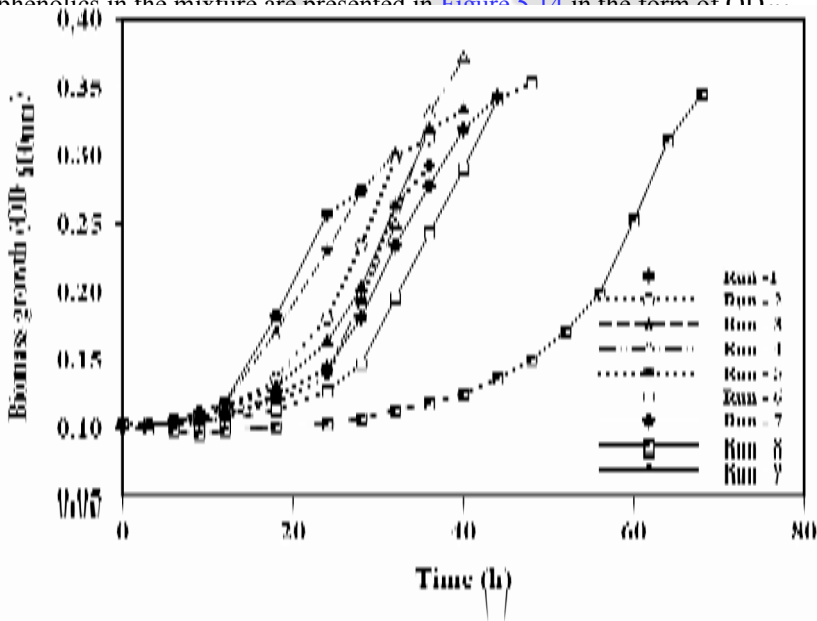


Figure 5.14 Biomass profile of *A. chlorophenolicus* A6 when grown in medium containing 4-CP, 4-BP and 4-NP.

It is clear from the figure that the culture took more time to grow when higher concentration 4-NP ($> 100 \text{ mg l}^{-1}$) was present in the media (together with 4-CP and 4-BP); nevertheless, the cell mass output was not comparatively high in such cases. The observation on the biomass profile of the culture in the mixed substrate system differed considerably with the patterns observed in the single substrate system. This phenomenon is, however, quite obvious due to more availability of carbon source in the medium along with increased toxicity. These observations were in close agreement with those of Bai et al. (2007), who studied similar mixed substrate effect on the growth of *Alcaligenes faecalis*. Moreover a lag phase was observed beyond certain concentrations combination of 100 mg l^{-1} for 4-CP, 4-BP each and 75 mg l^{-1} of 4-NP probably due to their combined toxicity effect of the phenolics. On the other hand, as discussed earlier with references to both Figure 5.13 (a) and (b) 4-CP and 4-BP were degraded simultaneously. When degradation patterns of these pollutants are thoroughly analyzed, the question immediately arises whether the observed behavior is due to diauxic growth or anything else. Two approaches may address this question first, if diauxic growth occurred, there would likely be a plateau in OD in the transition between growths on the different substituted phenols. No such plateau was detected by a visual inspection of the data depicted in Figure 5.14, clearly revealing no diauxic growth. Unell et al. (2008), employed a mutant strain of *A. chlorophenolicus* A6 (T99) carrying a transposon in a hydroxyquinol 1,2-dioxygenase gene which leads to severely impaired growth on 4-CP and observed that the T99 mutant behaved in exactly the same way when 4-NP or 4-BP was the sole source of carbon and energy, demonstrating that the same enzyme system was used as for 4-CP degradation and no diauxic growth.

5.3.2.3 Statistical Analysis of degradation results of the substituted phenols in mixed substrate system

Experimental data on the culture growth and degradation at various initial concentration combinations of these substituted phenols were used for calculating degradation rates of these substrates in mixture.

Figure 5.15 shows the degradation rates of these substituted phenols at different concentration combinations (run order number) as per the 2^2 level full factorial design of experiment (Table 4.4).

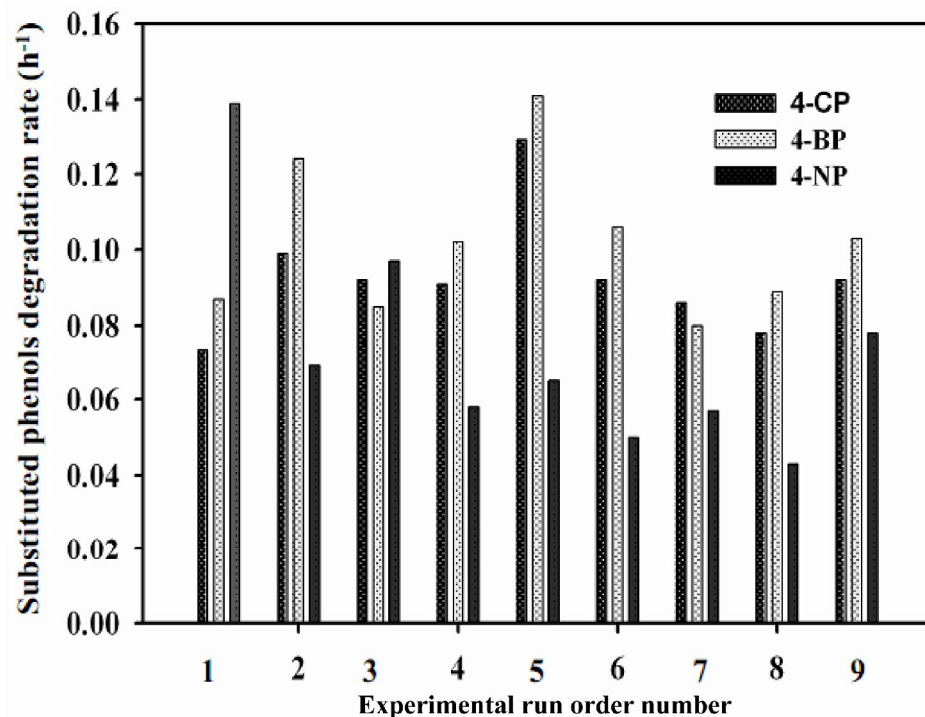


Figure 5.15 Degradation rates of 4-CP, 4-BP and 4-NP mixture obtained at different initial concentration ranges of these substrates.

The figure reveals that the degradation rates of 4-CP and 4-BP were higher than that of 4-NP except at their lower (experimental run number-1) concentration combination (75+75+50 mg l⁻¹ of 4-CP, 4-BP and 4-NP respectively). In general 4-BP degradation rates were found to be higher than that of 4-CP followed by 4-NP. Moreover, higher degradation rate of 4-CP and 4-BP were obtained in the higher concentration range of 4-NP which may be due high initial biomass produced during the 4-CP and 4-BP degradation process.

Further, these values were very low when the concentration ranges of the substrates were high. For instant degradation of 4-NP at lower concentration range (75+75+50 mg l⁻¹ of 4-CP, 4-BP and 4-NP respectively) was found to be 0.139 h⁻¹; on the contrary, the value was 0.043 h⁻¹ at higher concentration range (125+125+100 mg l⁻¹ of 4-CP, 4-BP and 4-NP respectively). This finding confirms the fact that substrate inhibition on the culture growth and degradation occurs only in the high concentration ranges of the substrates.

Statistical analyses in the form of analysis of variance (ANOVA) and Student's 't' test were performed based on the above obtained results. This analysis was done mainly to interpret the individual roles played by these substrates, and to model the variation in substituted phenols degradation rate due to these factors. This was felt necessary because although literature is replete with mechanistic models that deduce the interaction effects among these substrates on growth rates of microorganisms, there exists hardly any study, which deals with such type of statistical analysis that is proving useful for clear understanding of the main and interaction effects between the substrates in such type of mixed substrate degradation systems.

5.3.3.2 Analysis of Variance (ANOVA)

From this statistical analysis of the result, it is clear that both the individual (main) and two-way interaction terms for all of these phenolics were highly significant on the degradation activity at more than 97% confidence level ($P < 0.027$). On the other hand, except 4-NP degradation rate, the ANOVA results shown in Table 5.9 (a) and (b) revealed that the three-way interaction term was insignificant ($P > 0.05$). The ANOVA table for each pollutants degradation rate also displays an error term, which indicates that the error in the experiments was found to be significantly less. Moreover, determination coefficient ($R^2 > 99$) values presented in these tables suggest that the models were highly accurate in predicting the experimentally observed degradation rate. Further, while the Pareto chart shown in Figure 5.16 (a), (b) and (c) clearly indicated strong negative (inhibitory) individual effect of 4-CP on 4-NP as well as on its own degradation performance.

Tables 5.9 (a), (b) and (c) present the results of ANOVA of 4-CP, 4-BP and 4-NP degradation rates in the study.

Table 5.9 (a) ANOVA of 4-CP biodegradation in the mixture experiments

Source	df	SS	Adj MS	F	P	R ²
Main effect	3	4923.3	1641.12	1641.12	0.001	99.96
2-way interaction	3	322.37	107.46	107.46	0.009	
3-way interaction	1	15.12	15.12	15.12	0.060	
Residual error	2	2	1			
Pure error	2	2	1			
Total	10	5262.8				

Table 5.9 (b) ANOVA of 4-BP biodegradation in the mixture experiments

Source	df	SS	Adj MS	F	P	R ²
Main effect	3	2245.50	748.500	118.18	0.008	99.57
2-way interaction	3	681.50	227.167	35.87	0.027	
3-way interaction	1	4.50	4.500	0.71	0.488	
Residual error	2	12.67	6.333			
Pure error	2	12.67	6.333			
Total	10	2944.17				

Table 5.9 (c) ANOVA of 4-NP biodegradation in the mixture experiments

Source	df	SS	Adj MS	F	P	R ²
Main effect	3	520.375	173.458	520.37	0.002	99.9
2-way interaction	3	83.375	27.792	83.37	0.012	
3-way interaction	1	28.125	28.125	84.38	0.012	
Residual error	2	0.667	0.333			
Pure error	2	0.667	0.333			
Total	10	632.54				

df, degrees of freedom; SS, sum of squares; MS, mean sum of squares. F Fisher's F value (calculated by dividing the MS owing to the model by that due to error), P probability of incorrectly rejecting the null hypothesis when it is actually true.

Figure 5.16 (c) showed that the negative main effect of 4-NP on its degradation was relatively lower than that of 4-CP.

Moreover, interaction effect between 4-CP and 4-NP on 4-NP degradation performance and that between 4-CP and 4-BP on 4-BP degradation were significantly negative as compared with that between 4-CP and 4-BP on 4-CP degradation (Figure 5.16 a, b, c).

To further understand which of the individual substituted phenols (4-CP, 4-BP, and 4-NP) and their interactions played a crucial role on the degradation performance shown by the *A. chlorophenolicus* A6, Student's t test was performed. While the estimated coefficients of individual and interaction terms presented in Table 5.10 and the associated t and P values shown in the table were used to indicate significance of these coefficient terms. In general, a large t value associated with a low P value of a variable indicates a high significance of the corresponding model term. The 't' value coefficient for 4-NP degradation reveals strongly inhibition mainly due to 4-CP ($P = 0.001$) followed by 4-BP ($P = 0.015$).

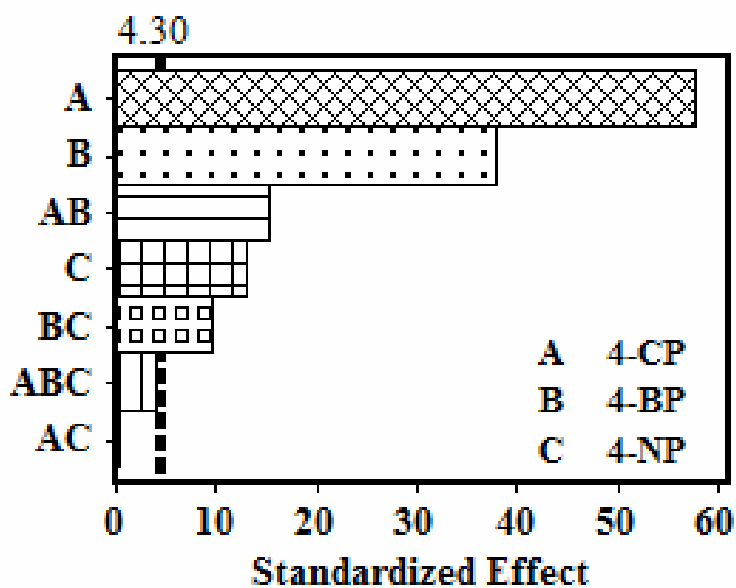


Figure 5.16 (a) Pareto chart of the standardized effects of the factors on 4-CP biodegradation rate in the mixture study ($\alpha=0.05$).

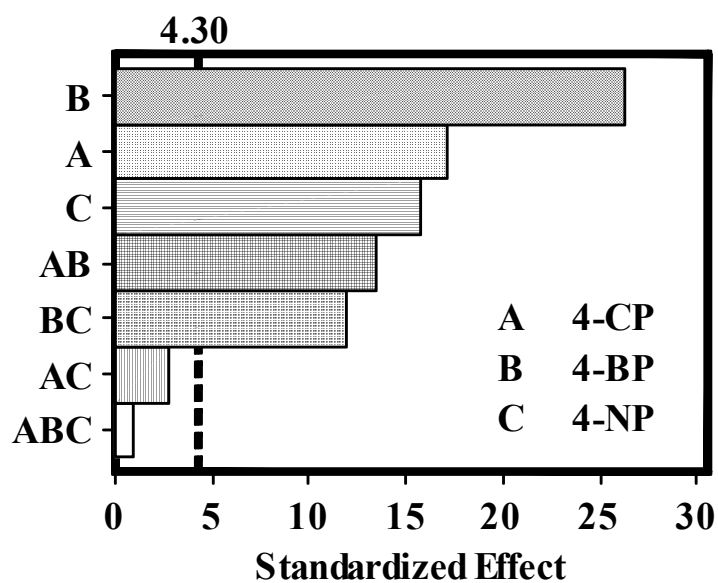


Figure 5.16 (b) Pareto chart of the standardized effects of the factors on 4-BP biodegradation rate in the mixture study ($\alpha=0.05$).

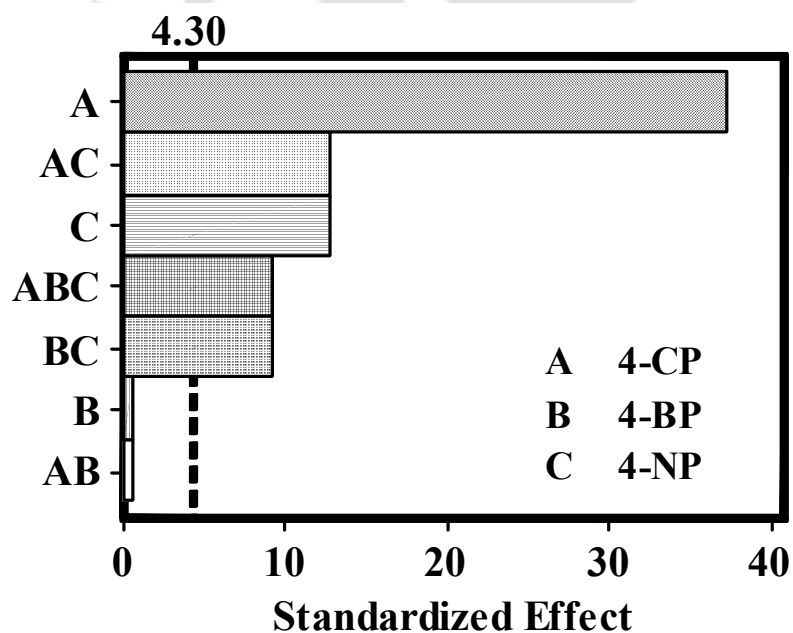


Figure 5.16 (c) Pareto chart of the standardized effects of the factors on 4-NP biodegradation rate in the mixture study ($\alpha=0.05$).

Table 5.10 Student's t test for estimating the coefficients of individual and interaction effects of the substituted phenols and their significance on degradation performance in the mixture study.

Terms	4-CP		4-BP		4-NP	
	degradation		degradation		degradation	
	t	p	t	p	t	p
constant	172.18	0.000	96.37	0.000	452.54	0.000
4-CP (X_1)	-57.63	0.000	-8.15	0.015	-37.35	0.001
4-BP (X_2)	-37.83	0.001	-15.4	0.004	0.61	0.603
4-NP (X_3)	-13.08	0.006	-7.02	0.020	-12.86	0.006
$X_1 \times X_2$	-15.20	0.004	-7.59	0.017	0.61	0.613
$X_1 \times X_3$	0.35	0.757	0.84	0.488	-2.86	0.063
$X_2 \times X_3$	-2.55	0.071	-7.02	0.020	4.2	0.0700
$X_1 \times X_2 \times X_3$	3.89	0.060	0.84	0.488	-9.19	0.012

Hence, it was concluded that 4-NP degradation performance was significantly inhibited in comparison with 4-BP due to presence of 4-CP. On the other hand, interaction between the other two factors viz. 4-CP and 4-BP ($X_1 \times X_2$) was found insignificant ($P = 0.613$) on 4-NP degradation but was negatively significant on their own degradation performance ($P = 0.004$ and 0.017). This analysis clearly indicated that 4-NP was significantly inhibited by 4-CP concentration in the mixture where as the vice-versa was not correct ($P = 0.757$). Other interaction effects except three way interaction effects were found to be significant at 95% confident level. In literature, similar statistical analysis and interpretation of results were applied for understanding the enhanced production of L-asparaginase by *Pectobacterium carotovorum* MTCC 1428 and *Escherichia coli* (Kumar et al. 2009).

5.3.2.4 Biomass Yield and Specific Degradation Rate of the Substrates

Biomass yield in the study was calculated as in previous section (5.3.1.3). The calculated biomass yield values for different concentrations ranges in the mixed substrate system are presented in Table 5.11

Table 5.11 Estimated biomass yield of the *A. chlorophenolicus* A6 in the mixture study

Run Order	Initial Conc. (mg l ⁻¹)			Yield (gg ⁻¹)
	4-CP	4-BP	4-NP	
1	75	75	50	0.2076
2	125	75	50	0.2016
3	75	125	50	0.1929
4	125	125	50	0.2152
5	75	75	100	0.1824
6	125	75	100	0.2000
7	75	125	100	0.1936
8	125	125	100	0.1673
9	100	100	75	0.2024
10	100	100	75	0.2029
11	100	100	75	0.2011

Although 4-NP was degraded prior to 4-CP and 4-BP, the kinetic data suggest that 4-NP had a stronger negative impact on the cells than the other two pollutants. For example, the net energy gain decreased with increasing concentrations of 4-NP, as seen from the high negative correlation between yield and 4-NP concentration. Although the cells consumed 4-NP faster, the yield was lower. This indicates that the cells are more stressed when grown on 4-NP compared to 4-CP and 4-BP. This observation is consistent with that previously obtained single substrate system ([section 5.3.11](#) and [5.3.13](#)). 4-NP is probably a more potent uncoupler than 4-CP and 4-BP, and thus affects cells more negatively; Escher et al. (1996) also found that the type of substituent group on a phenol and the position of substitution had an impact on the uncoupling activity. Further the lower biomass yield obtained with increased on 4-NP concentration keeping the other two pollutant at constant level is also correlated well with literature due to the fact that, 4-NP is a metabolic uncoupler, which is to disassociate the energy coupling between catabolism and anabolism, thereby resulting in dissipation of certain portions of energy through futile cycles. This presents high potentialities for reducing excess sludge production (EPA, 1980). However, irrespective of the combinations of initial 4CP, 4-BP and 4-NP concentrations, the biomass yield increased with an increase in the initial 4-CP and 4-BP concentration at a fixed 4-NP concentration in the mixture except in case of the experimental run number-8.

The lower biomass yield due to 4-CP, 4-BP and 4-NP at 125, 125 and 100 mg l⁻¹ may attributed to high initial concentration combination exerted elevated toxicity level on the microbial cell as consequence of which most of the carbon sources is diverted to maintenance energy rather than biomass growth, as discussed earlier. Abuhamed et al. (2004) observed a maximum biomass yield value of 0.75 gg⁻¹ due to benzene at an initial concentration of 700 mg l⁻¹ on the growth of a *Pseudomonas putida* strain. In the present study, a highest yield value of 0.2152 gg⁻¹ was obtained at higher concentration combination of 4-CP and 4-BP with low level of 4-NP (125+125+50 mg l⁻¹ of 4-CP, 4-BP and 4-NP respectively), and a very low value of 0.1673 at the same range of 4-CP and 4-BP but higher 4-NP concentration of 100 mg l⁻¹. These values clearly indicated that 4-CP and 4-BP concentrations particularly influence the biomass yield in the experiments. The yield values obtained in the present study was found inferior when compared with several literatures reported values ranging from 0.44-0.88 gg⁻¹ on phenol degradation using *Pseudomonas* species in mixed substrates system containing phenol, benzene and toluene (Kotturi et al., 1991; Reardon et al., 2000; Abuhamed et al., 2004). Such lower biomass yield, obtained in the present study, may be attributed two main reasons: (1) Toxicity of substituted phenol on microbial cell, as the aromatic ring with its shared resonance electrons is very stable and thus resistant to enzymatic attack. Halogen substituent on the aromatic ring stabilizes the structure further, as the halogens create a steric hindrance to enzymes and also have an electron withdrawing effect (Crossby, 1981; van den Berg, 1990; Copley, 1997; Uberoi and Bhattacharya, 1997). Therefore requirement of high maintenance energy for overcoming the toxic effect of these pollutants cannot be ignored. (2) The ratio of carbon content is lower than that of phenol compared to volatile hydrocarbon substrate benzene.

5.4 Biodegradation of substituted phenols as single substrate by *A. chlorophenolicus* A6 in the newly designed PBR

Although substituted phenol degradation by *A. chlorophenolicus* A6 is a growth associated process however, specific growth rate ($\mu_{\max}$) of the culture and substrate inhibition

constant (K_i) was found very low with compare to many literature, in addition, existence of strong negative main effect and interaction effect among the mixture of substrate strongly recommended the suitability of a packed bed reactor to enhance the toxic tolerance and higher biomass growth. Therefore in the present study a newly designed PBR was fabricated and detailed specification of the reactor is given in [Table 5. 12](#).

5.4.1 Hydrodynamic study in the newly designed PBR

Mixing is a physical operation which reduces non uniformity in fluid by eliminating gradient concentration of the substrate, gases, temperature and other properties ([Bailey and Ollis, 1986](#)). Mixing time (t_{m95}) is time to reach 95% homogeneity after a tracer input in a mixture. Therefore, smaller the mixing time results a better and quicker distribution as well as dispersion of oxygen, essential nutrients and temperature etc. to the microorganism for enhanced growth and degradation of the compounds.

Table 5.12 Detailed specifications of the reactor

Total reactor volume	6 L
Liquid working volume	4 L
Height of aeration port	at each 10 cm
Circular porous perplex disc	5 mm pore size
Nylon membrane	0.25 mm pore size
Fixed bed height	40 cm
Intra-particle porosity, ϵ_p	0.716
Inter-particle porosity, ϵ_e	0.2096
Total bed porosity, $\epsilon_{tot} = \epsilon_e + \epsilon_p$	0.9255
Dry material bulk density, $\rho_{tz}(\text{gcm}^{-3})$	0.033
Packed-bed volume, $V_{PB}(\text{cm}^3)$	4000
Packed-bed void volume, $V_{void} = V_{PB}(\epsilon_e + \epsilon_p)(\text{cm}^3)$	3702
Packed-bed circulating liquid volume, $V_{PB}(\epsilon_e)(\text{cm}^3)$	838
Packed-bed stagnant liquid volume, $V_{PB}(\epsilon_p)(\text{cm}^3)$	2864

* ϵ_e : inter particle porosity, ϵ_p : intra particle porosity, ϵ_{tot} : $\epsilon_e + \epsilon_p$ total bed porosity, ϵ_G : gas hold-up (%), V_{PB} : V_D packed-bed volume (L), t_{m95} : time to reach 95% homogeneity after a tracer input(s), UGR : superficial air velocity in riser (cm s^{-1}), V_{void} : $V_{PB}(\epsilon_e + \epsilon_p)$ void volume of packed-bed (L), kLa mass transfer coefficient in (h^{-1}) .

When the supply of oxygen is limited, both cell growth and degradation performance of packed bed reactor can be severely affected ([Gomez-DeJesus et al., 2009](#)).

For example, it Most nutrients required for cellular growth and metabolism are highly soluble in water, however, oxygen transfer often becomes a limiting step to the optimal performance of biological systems and also for scale-up because oxygen is only sparingly soluble in aqueous solutions. was reported that ceasing aeration in the medium during penicillin fermentation for just a few minutes seriously impacted the ability of the cells to produce the antibiotic (Nielsen and Villadsen, 1994). Similar result are also reported in many literature that the oxygen utilization by cell include synthesis of enzymes responsible for utilization of substrate, cell maintenance energy, as well electron acceptor for growth and metabolism of the microorganism (Gomez-DeJesus et al., 2009; Costura and Alvarez, 2000). However, Increase in hydrodynamic condition not necessarily mean to increase in DO concentration in the reactor rather it deals with how quickly and how uniformly DO in spite of its concentration get uniformly distributed and available for uptake by all the microoraginism present at different location for their metabolism. Thus, hydrodynamics conditions are important for growth of the microorganism and degradation performance of a PBR and evaluated in the present study.

The hydrodynamic experiment was performed in PBR under two different conditions viz., aeration only through the bottom aeration port (simple PBR); and aeration through all the 4 ports with the arrangement to get cross flow aeration along with perforated discs wrapped by nylon wire mess at the top with ceramic gas diffusers (newly designed PBR). Figure 5.17 compares the results of hydrodynamic study performed in these two conditions of the reactor. Figure 5.17 (a) shows the mixing profile in the newly designed PBR which clearly reveals a better t_{m95} value (time to reach 95% homogeneity after a tracer input) of 110 s when compared with 185 s in the simple PBR. This could be attributed due to its reduction of bubble formation, gas-liquid channeling effect and improved uniform dispersion of liquid and gas throughout the bed of the newly designed PBR. The mixing time (t_{m95}) obtained in both the reactors were plotted against the corresponding superficial gas flow velocity and are depicted in Figure 5.17. (b). The newly designed PBR had lower mixing time (t_{m95}) than the simple PBR for all the four gas flow rates investigated.

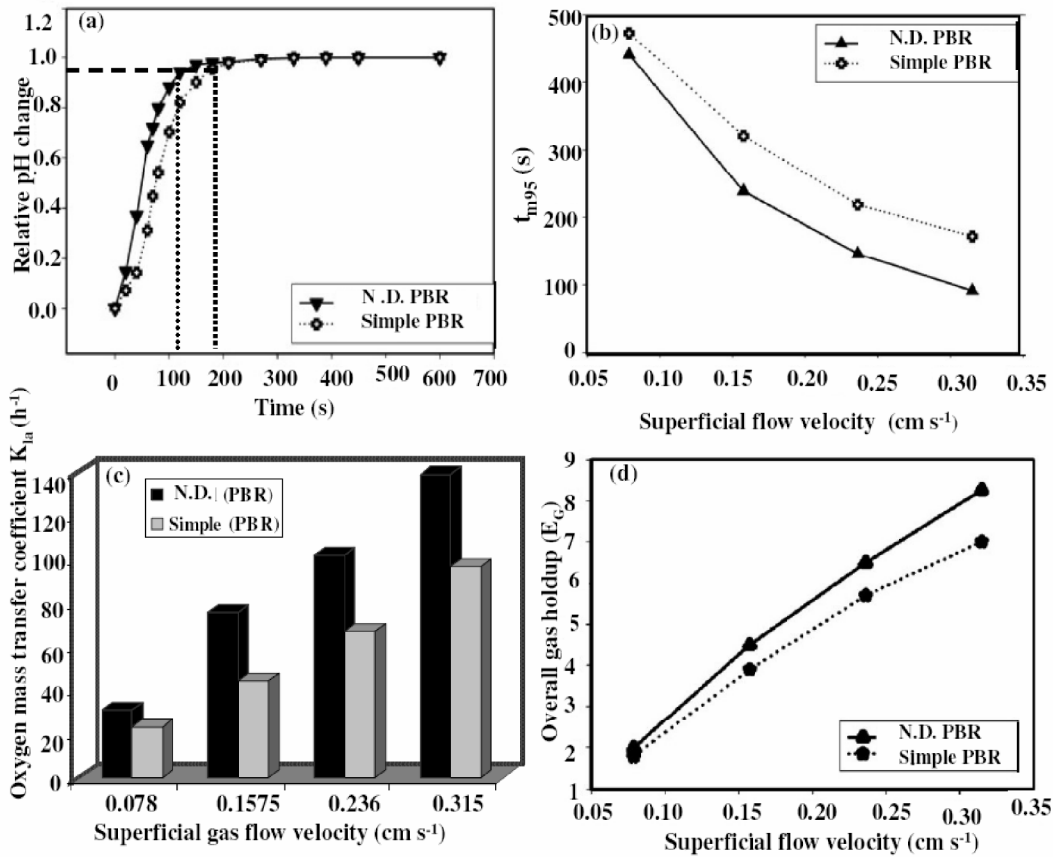


Figure 5.17 Comparison of hydrodynamic profiles obtained using the newly designed and simple PBRs in the study (a) mixing time profile at superficial flow velocities (U_{GR}) = 0.315 cm s⁻¹. (b) mixing time behavior at varying superficial air flow velocities (c) oxygen mass transfer coefficients (k_{La}) at U_{GR} values of 0.1575 cm s⁻¹ and 0.315 cm s⁻¹, (d) overall gas hold-up at different air flow velocities. (N.D.* PBR indicates newly designed PBR)

This indicated a better and uniform liquid circulation rate in the newly designed PBR than the simple PBR. The maximum difference in t_{m95} was observed when the superficial gas flow velocity was 0.315 cm s⁻¹. At this condition the mixing time (t_{m95}) in the newly designed PBR and simple PBR were 110 s and 185 s, respectively. Figure 5.17. (c) compares the values of oxygen mass transfer coefficient (k_{La}) between the newly designed PBR and simple PBR.

There was an enhancement of around 40% in $k_L a$ values in the newly designed PBR; from 97 h^{-1} to 139 h^{-1} , and 54 h^{-1} to 76 h^{-1} at the two different superficial gas flow rates of 0.315 cm s^{-1} and 0.157 cm s^{-1} respectively. This could be attributed to the reduction in pressure drop leading to enhancement in superficial gas liquid flow velocity in the newly designed PBR. This is well supported by literature that cross flow design providing by multiple points along the height in packed beds reduces pressure drop enabling the use of practical flow rates and even flow distribution across wide packed bed (Ajmera et al., 2002). In addition, due to the separation of the aeration zone (fragmented approach) at four levels along the height in the newly designed PBR presented a low shear stress compared to simple PBR at higher aeration rate using a single port (Fassnacht and Portner, 1999). Thus in the newly designed PBR with low shear stress minimizes erosion of the immobilized biomass on the PUF and enhanced biomass density for better biodegradation efficiency. Figure 5.17. (d) shows the overall gas hold up profile in both the reactors at 4 different superficial gas flow velocities, which reveals a higher value of gas hold up in the newly designed PBR than the simple PBR. A maximum of 17% enhancement in overall gas hold up (from 6.9 to 8.35) was observed in the newly designed PBR for a superficial gas flow velocity of 0.315 cm sec^{-1} . The increase in overall gas hold up capacity (ϵ_G) in the novel PBR may be due to the reduction in pressure drop due to intermittent uses of perforated discs wrapped by nylon wire mesh at the top with ceramic gas diffusers along the height, as a result of more volume expansion (ΔV) and greater gas hold up. An increase in overall gas holdup could also have affected the gas-liquid interfacial area and thus the oxygen mass transfer coefficient.

5.4.2 Abiotic removal of substituted phenols by adsorption and volatilization in the newly designed PBR

Substituted phenols viz., 4-CP, 4-BP and 4-NP removal in PBR by adsorption onto PUF was estimated from the breakthrough curve shown in Figure 5.18. The bed was saturated at the end of 60 h, 70 h and 80 h of operation for 4-CP, 4-BP and 4-NP respectively with an influent pollutant concentration of 200 mg l^{-1} .

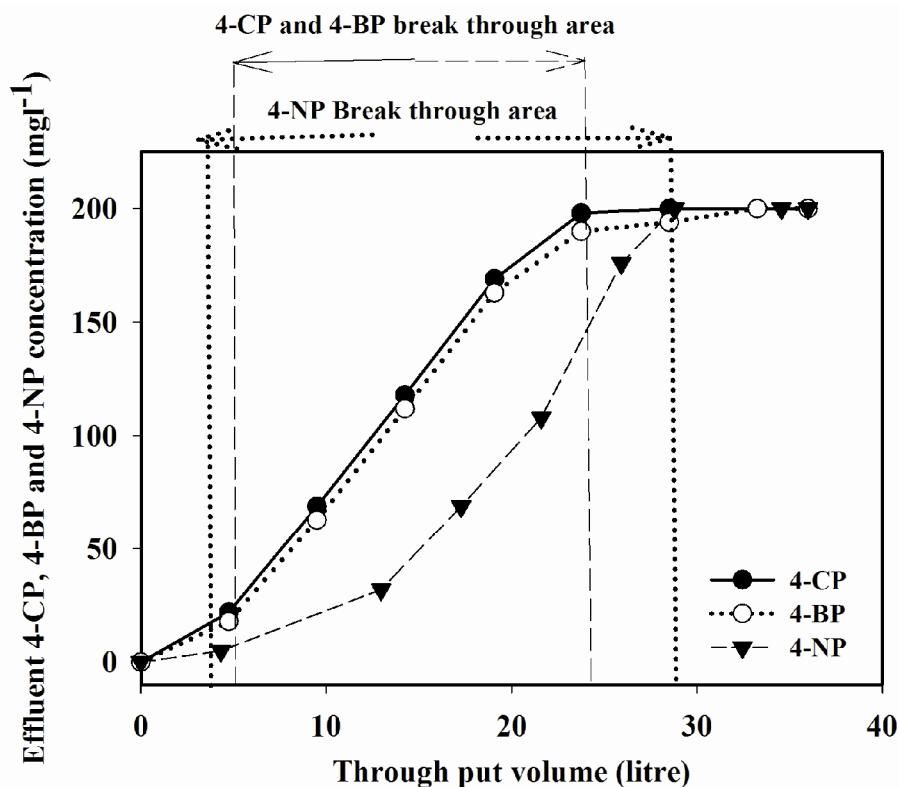


Figure 5.18 Breakthrough curve obtained for determining the adsorption capacity of the biosupport material used in the newly designed PBR on 4-CP, 4-BP and 4-NP (influent concentration = 200 mg l^{-1} , HRT = 10 h).

Details of abiotic loss of substituted phenol in newly designed PBR is given in [table 5.13](#). Thus, adsorption capacity of PUF for 4-CP, 4-BP and 4-NP, calculated to be 19, 21.90 and 37.84 mg of respective pollutant per gram of dry weight of PUF.

Table 5.13 Abiotic removal of substituted phenols by adsorption and volatilization in the newly designed PBR at initial concentration of 200 mg l^{-1} and HRT 10h.

Pollutants	Adsorption (mg gm^{-1} PUF)	Volatilization (mg l^{-1})
4-CP	19.04	0.49
4-BP	21.9	0.4
4-NP	37.84	3

Adsorption capacity of PUF on nitrophenol and nitrobenzene, removal were 1.5-2.67, 20 (mg l^{-1}) respectively (Shahawi and Nassif, 2003; Zheng et al., 2009). Details about the abiotic removal (adsorption and volatilization) of various phenolic pollutants on PUF and activated carbon by various reactor and batch shake flask study are given in Table 5.14. However, the adsorption capacity of activated carbon for chlorophenol, with an initial concentration 100 mg l^{-1} were 78.1 mg gm^{-1} of activated carbon (Hamdaoui and Naffrechoux, 2009; which is significantly higher than that of PUF in the present study (19 mg for 4-CP, 21.90 mg for 4-BP and 37.84 mg for 4-NP per gram of PUF). On the other hand, the adsorption capacity of PUF for 4-NP is comparatively little higher value of 37.84 mg of 4-NP gm^{-1} dry weight of PUF may be due to the different chemical nature of the compound. Volatilization loss of each of 4-CP and 4-BP was estimated to be 0.4 mg l^{-1} (0.2%) whereas, in case of 4-NP removal was 3 mg l^{-1} (1.5%). However, abiotics removal (due to adsorption and volatilization) was insignificant when compared with total removal due to biodegradation by *A.chlorophenolicus* A6. Several researchers reported insignificant loss of substituted phenol via volatilization (Jiang et al., 2010; Sahinkaya and Dilek, 2006; Lo et al., 2004).

5.4.3 Optimization of DO concentration

Figure 5.19 (a), (b) and (c) present the effect of DO on 4-CP, 4-BP and 4-NP removal in the newly designed PBR at their respective loading rate of 910, 910 and $1264 \text{ mg l}^{-1} \text{ day}^{-1}$. The 4-CP removal efficiency was found to be the maximum when DO level in the reactor was 3.5-4.0 mg l^{-1} . Similar optimum DO concentration of 3-4 and 3.4 mg l^{-1} were reported by several author on substituted phenol and phenol biodegradation using sequencing batch reactor and rotating biological contractor (RBC) (Tomei et al., 2004; Melo et al., 2005). The optimum DO levels for 4-BP and 4-NP were found to be 4.0 - 4.5 mg l^{-1} and 4.9-5.5 mg l^{-1} respectively, which is also correlated well with the literature reports of higher than 4.5 mg l^{-1} on dinitrotoluene degradation in fluidised bed reactor (Lendenmann et al., 1998).

Table 5.14 Abiotic removal (adsorption and volatilization) of various phenolic pollutants on PUF and activated carbon by various reactor and batch shake flask study.

Pollutants treated	Initial Conc. (mg l^{-1})	Removal efficiency (%) / Adsorption capacity (mg g^{-1})	Bioreactor type	Materials/ Microorganisms used	HRT (h)	References
4-CP	100	78.1 mg g^{-1}	Stirred tank reactor (400 rpm)	Granular Activated carbon	9h	Hamdaoui and Naffrechoux, 2009
4-NP, Benzoic acid	3.4 mol m^{-3} 4-NP, 3.98 mol m^{-3} benzoic acid	99.3% 4-NP	Column reactor	Granular Activated carbon		Chern and Chien, 2003
2-NP, 3-NP and 4-NP	10	1.5–2.67 mg g^{-1}	Column reactor (100 ml) packed with 3 gm of PUF	PUF		Shahawi and Nassif, 2003
Nitrobenzene	200	20 mg g^{-1}	Batch shake flask 180 rpm	PUF		Zheng et al., 2009
4-CP	200	No volatilization	Sequencing batch reactors	Mixed culture	28h	Sahinkaya and Dilek, 2006
4-NP	100	No significant loss of via volatilization or adsorption	Electrolytic cell. Electrochemical degradation	Glass electrode	2h	Zhou et al. 2010

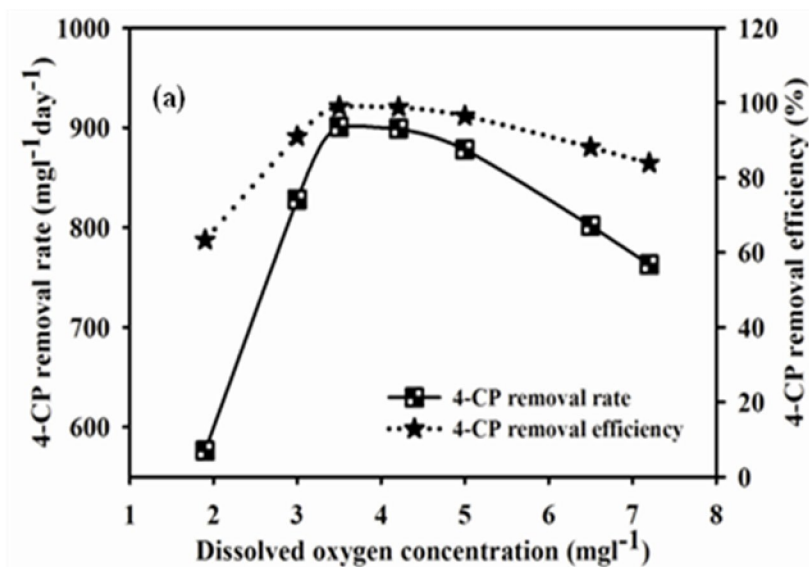


Figure 5. 19 Dissolved oxygen profile with respect to substituted phenol removal rate and removal efficiency at a constant loading rate of $910 \text{ mg l}^{-1} \text{day}^{-1}$ for **(a)** 4-CP.

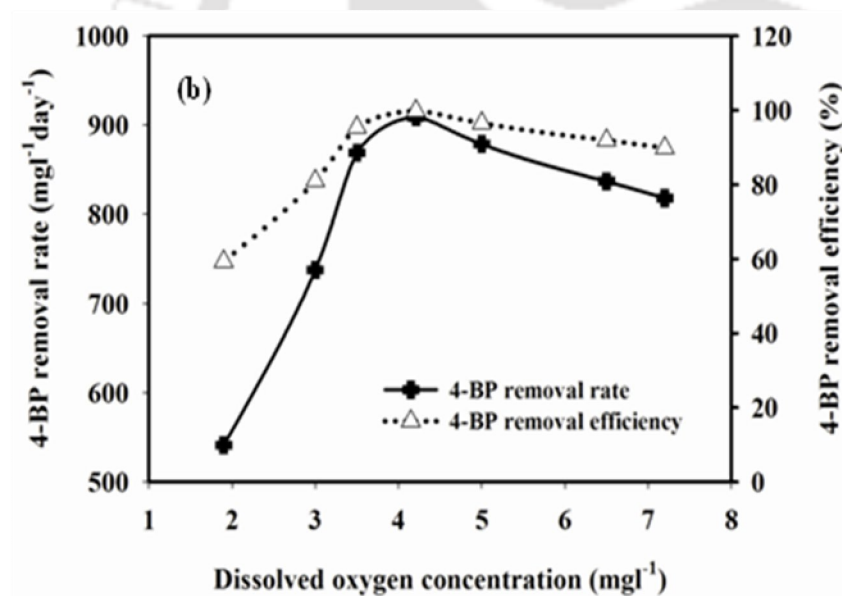


Figure 5. 19 Dissolved oxygen profile with respect to substituted phenol removal rate and removal efficiency at a constant loading rate of $910 \text{ mg l}^{-1} \text{day}^{-1}$ for **(b)** 4-BP.

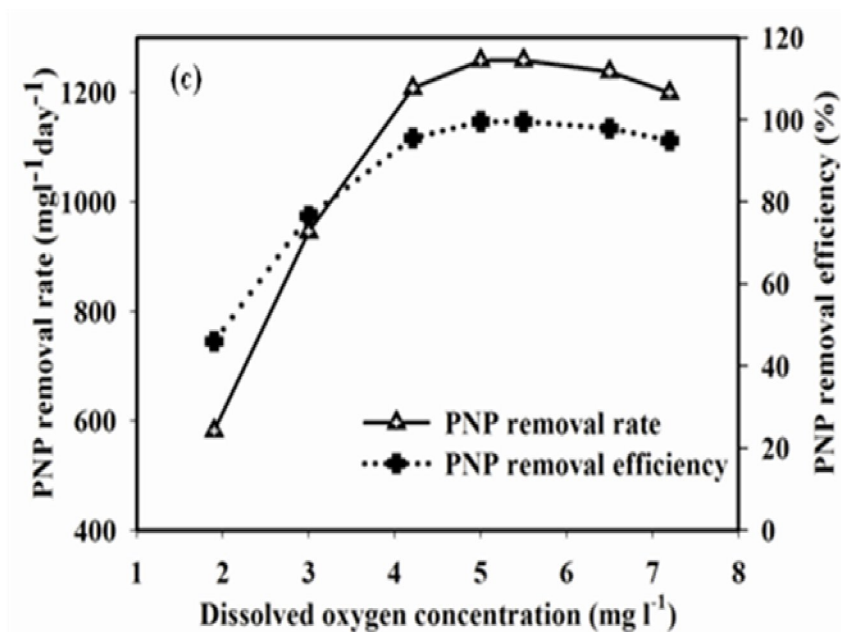


Figure 5.19 Dissolved oxygen profile with respect to substituted phenol removal rate and removal efficiency at a constant loading rate of $1264 \text{ mg l}^{-1} \text{ day}^{-1}$ for (c) 4-NP.

The lower value of optimum DO level for 4-CP degradation supported by several literatures that toxicity of substituted phenol on microbial cell. Firstly because of the aromatic ring with its shared resonance electrons is a very stable structure and thus resistant to enzymatic attack and halogen substituent on the aromatic ring stabilizes the structure further, as the halogens create a steric hindrance to enzymes and also have an electron withdrawing effect (van den Berg, 1990; Copley, 1997; Uberoi and Bhattacharya, 1997). Furthermore, among various halosubstituents, chloro group believed to be having strong electron withdrawing capacity (higher electro negativity) and makes the enzyme attack on aromatic ring more difficult (Pitter, 1985). Therefore, due to the higher toxicity nature the microbial metabolic activity slowed down as a result less DO requirement. Whereas, 4-BP and 4-NP might be exerted less toxic effect on the microbial cell as well as its metabolic activity as a result higher consumption of DO. This might be the possible reason for the variation in optimum DO level in the present study. However, this needs to be further investigated.

The reduction in the reactor performance at lower DO level might be due to the inadequate transfer of DO to the microorganisms present deep inside the biofilms formed in the pores of the PUF, causing anaerobic conditions (Costerton et al., 1995). However, any further increase in the DO concentration beyond the optimum values did not improve these substituted phenols removal efficiency in the PBR system. This could be attributed to the fact that *A. chlorophenolicus* A6 follows both aerobic and reductive dechlorination (inhibited by oxygen) metabolic pathway during 4-CP biodegradation (Nordin et al., 2005). Therefore, higher aeration rate might have hindered the anaerobic reductive dechlorination step of 4-CP biodegradation. Similar result was also reported by Shelley et al. (1996) on 4-NP degradation that at higher DO level. They reported that the oxygen acted as a strong electron acceptor for nitro-aromatics and thus reduced electron density of the aromatic ring resulting in inhibition of oxidative attack by the electrophilic oxygenases. The first 15 days of operation shown in Figure 5.20 (a, b, and c) represented the experiment related to optimization of DO concentration.

5.4.4 Biodegradation of 4-CP, 4-BP, and 4-NP by *A. Chlorophenolicus* A6 in the PBR

The PBR was fed with one among 4-CP, 4-BP and 4-NP, to evaluate its performance on single mono substituted phenol biodegradation under various operating conditions. The experiment was started by feeding the reactor with 4-CP. Performance of the reactor was then evaluated on 4-BP biodegradation followed by 4-NP. No external carbon source other than the specific mono substituted phenol was added into the reactor. COD equivalent of 4-CP, 4-BP, and 4-NP measured in the laboratory was 1.66, 1.43 and 1.53 respectively. The reactor was started with an influent pollutant concentration 400 mg l^{-1} for 4-CP and 4-BP whereas, in case of 4-NP it was 600 mg l^{-1} . Once the substituted phenol concentration in the effluent at any particular pollutant loading rate (i.e. influent pollutant concentration and HRT) remained stable for 3-4 cycles of run, it was assumed that the reactor had reached to a steady state condition. Then the reactor was then subjected to either the next higher pollutant concentration and/or next lower HRT.

4-CP biodegradation in PBR

As mentioned earlier the reactor was started with an influent 4-CP concentration of 400 mg l⁻¹ and after achieving steady state performance at 24h HRT, the HRT and/or the influent 4-CP concentration was increased/changed stepwise. The operational schedule of the PBR is given in Table 5.15 (a) and the performance of the reactor on 4-CP biodegradation is shown in Figure 5.20 (a).

Table 5.15 (a) Operational schedule of the PBR for 4-CP biodegradation

Operation period (day)	HRT (h)	Inf. conc. (mg l ⁻¹)	PLR (mg l ⁻¹ day ⁻¹)	Operation period (day)	HRT (h)	Inf. conc. (mg l ⁻¹)	PLR (mg l ⁻¹ day ⁻¹)
0-14 ^{a*}	12.5	400	910.70	67-72	24	1000	1183.03
14-17 ^{b*}	24	400	473.21	72-76	18	1000	1610.50
17-21	18	400	644.20	76-80	12.5	1000	2276.77
21-24	12.5	400	910.70	80-83	10	1000	2844.85
24-27	10	400	1137.94	83-86	7.5	1000	3759.12
27-30	7.5	400	1503.84	86-90	24	400	473.21
30-34	24	600	709.81	90-96 ^{c*}	24	400	473.21
34-38	18	600	966.30	96-98	24	1000	1183.03
38-41	12.5	600	1366.06	98-101	24	1000	1183.03
41-44	10	600	1706.91	101-103	24	1000	1183.03
44-47	7.5	600	2255.47	103-108	10	400	1138
47-52	24	800	946.42	108-110	10	1000	2845
52-56	18	800	1288.40	110-112	10	1000	2845
56-60	12.5	800	1821.41	112-114	10	1000	2845
60-63	10	800	2275.88	114-117	10	1000	2845
63-67	7.5	800	3007.39	117-119	10	1000	2845

PLR : pollutant loading rate, Inf. conc.: Influent concentration, *a, b* c*: PBR operational schedule during DO optimization, reactor

performance evaluation and shock loading conditions

Complete removal (below detection level) of 4-CP was achieved from influent concentrations of 400 mg l⁻¹, 600 mg l⁻¹, and 800 mg l⁻¹, when the PBR was operated at /or higher HRTs than 7.5h, 10h, and 18h, respectively. Thus, complete removal of 4-CP in the reactor was achieved at the maximum 4-CP loading rate of 1707 mg l⁻¹ d⁻¹ viz., when the reactor was operated at 10h HRT with 600 mg l⁻¹ influent 4-CP concentration.

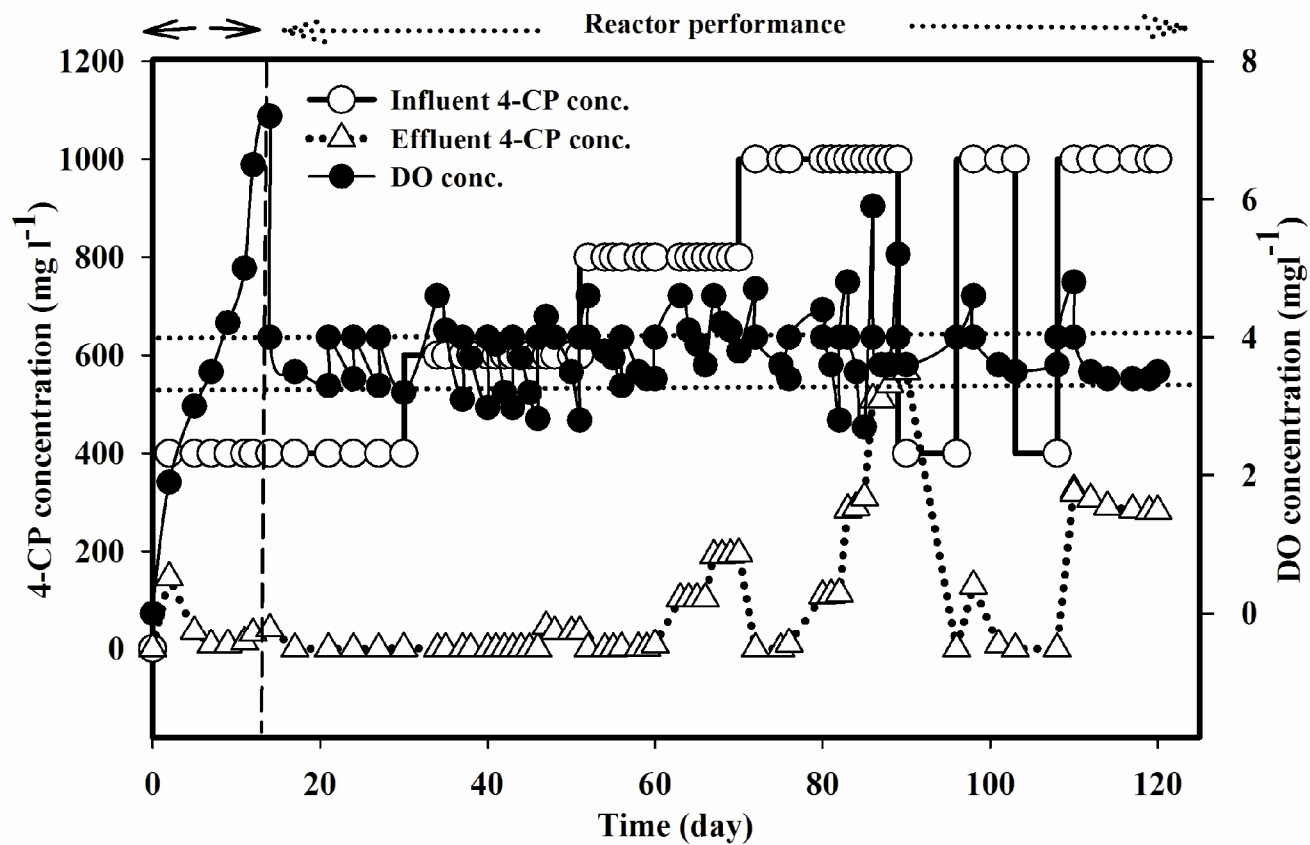


Figure 5. 20 (a) Biodegradation of 4-CP and DO profile in the newly designed PBR

This is further confirmed by $\approx 99\%$ COD removal at this optimum operating condition. Though, complete removal was not achieved, more than 99% removal occurred from influent 4-CP of 1000 mg l^{-1} in the reactor operated at HRT 18h and higher. The reactor performance was observed to be inferior when operated at any other influent concentrations and/or HRTs listed in Table 5.15 (a). For example, from initial 600 mg l^{-1} and 800 mg l^{-1} 36 mg l^{-1} and 192 mg l^{-1} of 4-CP was observed in the respective effluents, when the reactor was operated at 7.5 h HRT. In addition, the reactor became unstable at the influent 4-CP of 1000 mg l^{-1} and 10h HRT.

Table 5.15 (b) Operational schedule of the PBR for 4-BP biodegradation

PBR operation period (day)	HRT (h)	Inf. conc. (mg l^{-1})	PLR ($\text{mg l}^{-1} \text{ day}^{-1}$)	PBR operation period (day)	HRT (h)	Inf. conc. (mg l^{-1})	PLR ($\text{mg l}^{-1} \text{ day}^{-1}$)
0-15 ^a	12.5	400	910.70	76-80	12.5	1000	2276.77
15-18 ^b	24	400	473.21	80-83	10	1000	2844.85
18-21	18	400	644.20	83-86	7.5	1000	3759.12
21-24	12.5	400	910.70	86-90	24	1200	1419.63
24-27	10	400	1137.94	90-93	18	1200	1932.60
27-31	7.5	400	1503.84	93-97	12.5	1200	2732.12
31-35	24	600	709.81	97-101	10	1200	3413.82
35-39	18	600	966.30	101-104	7.5	1200	4510.94
39-42	12.5	600	1366.06	104-107	24	400	473.21
42-45	10	600	1706.91	107-111 ^c	24	400	473.21
45-48	7.5	600	2255.47	111-113	24	1000	1183.03
48-52	24	800	946.42	113-116	24	1000	1183.03
52-56	18	800	1288.40	116-118	24	1000	1183.03
56-60	12.5	800	1821.41	118-123	7.5	400	1138
60-64	10	800	2275.88	123-125	7.5	1000	3759.12
64-68	7.5	800	3007.39	125-128	7.5	1000	3759.12
68-72	24	1000	1183.03	128-131	7.5	1000	3759.12
72-76	18	1000	1610.50	131-133	7.5	1000	3759.12

PLR : pollutant loading rate , Inf. conc.: Influent concentration, ^a, ^b, ^c : PBR operational schedule during DO optimization, reactor performance evaluation and shock loading conditions

4-BP biodegradation in PBR

The reactor was operated in a very similar sequence to that of 4-CP biodegradation study.

The operational schedule of the PBR for 4-BP degradation study is given in Table 5.15 (b), and the performance is shown in Figure 5.20 (b). As given in the table 5.15 (b), the reactor was started with an influent 4-BP concentration of 400 mg l^{-1} at an HRT of 24h. Complete removal (below detection level) of 4-BP was achieved from influent concentrations of 400 mg l^{-1} , 600 mg l^{-1} , 800 mg l^{-1} , and 1000 mg l^{-1} when the PBR was operated at or higher HRTs than 7.5h, 10h, 12.5h and 18h respectively. Thus, complete removal of 4-BP in the reactor was achieved at the maximum 4-BP loading rate of $2277 \text{ mg l}^{-1} \text{ d}^{-1}$ viz., when the reactor was operated at 12.5h HRT with 1000 mg l^{-1} influent 4-BP concentration. COD removal at this operating condition was about 99%. Though, complete removal was not achieved, more than 99% removal occurred from influent 4-BP of 1200 mg l^{-1} in the reactor operated at HRT 24 h. The reactor performance was observed to be inferior when operated at any other influent concentrations and/or HRTs listed in Table 5.15 (b). For example, from initial 800 mg l^{-1} , 1000 mg l^{-1} and 1200 mg l^{-1} of 4-BP, 96 mg l^{-1} , 276 mg l^{-1} and 828 mg l^{-1} of 4-BP was observed in the respective effluents, when the reactor was operated at 7.5 h HRT. In addition, the reactor become unstable at the influent 4-BP of 1200 mg l^{-1} and 10 h HRT (97th -104th day).

4-NP biodegradation in PBR

The reactor was operated in a very similar sequence to that of 4-CP biodegradation study. However, the starting influent 4-NP concentration was kept little higher value of 600 mg l^{-1} at an HRT of 18h. The operational schedule of the PBR for 4-NP degradation study is given in Table 5. 15 (c), and the performance is shown in Figure 5.20 (c). Complete removal (below detection level) of 4-NP was achieved from influent concentrations of 800 mg l^{-1} , and 1000 mg l^{-1} when the PBR was operated at or higher HRTs than 10h or more.

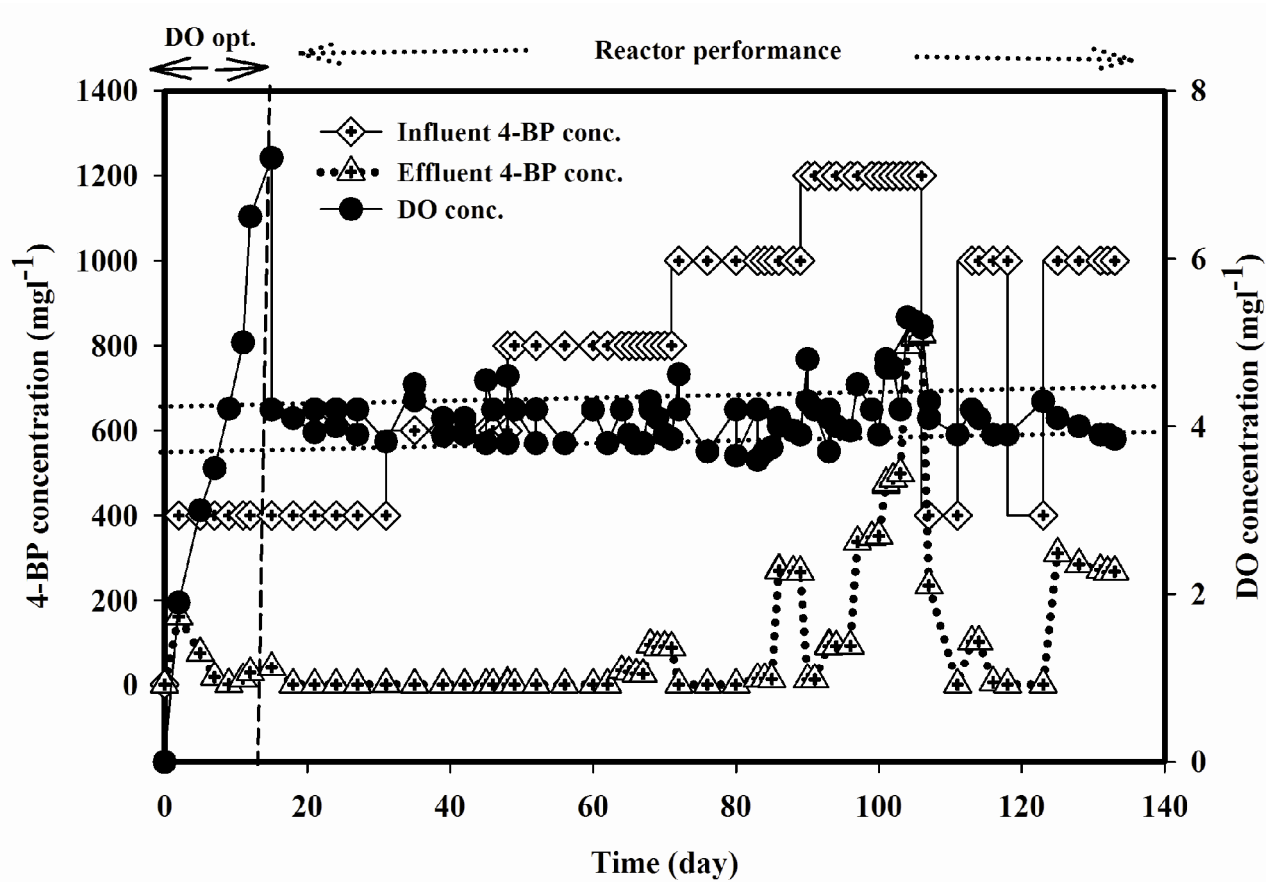


Figure 5. 20 (b) Biodegradation of 4-BP and DO profile in the newly designed PBR.

Thus, complete removal of 4-NP in the reactor was achieved at the maximum 4-NP loading rate of $2787 \text{ mg l}^{-1} \text{ d}^{-1}$ viz., when the reactor was operated at 10h HRT with 1000 mg l^{-1} influent 4-NP concentration. COD removal at this operating condition was about 99%. Though, complete removal was not achieved, more than 99% removal occurred from influent 4-NP of 1400 mg l^{-1} in the reactor operated at HRTs 18h. The reactor performance was observed to be inferior when operated at any other influent concentrations and/or HRTs listed in Table 5.15 (c). For example, from initial 800 mg l^{-1} , 1000 mg l^{-1} , 1200 mg l^{-1} and 1400 mg l^{-1} of 4-NP, 20 mg l^{-1} , 100 mg l^{-1} , 375 mg l^{-1} and 725 mg l^{-1} was observed in the respective effluents, when the reactor was operated at 7.5 h HRT. In addition, the reactor become unstable at the influent 4-NP of 1400 mg l^{-1} and 10 h HRT (75th -80th day).

Table 5.15 (c) Operational schedule of the PBR for 4-NP biodegradation

PBR operation period (day)	HRT (h)	Inf conc. (mg l^{-1})	PLR ($\text{mg l}^{-1} \text{ day}^{-1}$)	PBR operation period (day)	HRT (h)	Inf conc. (mg l^{-1})	PLR ($\text{mg l}^{-1} \text{ day}^{-1}$)
0-13 ^{*a}	12.5	600		63-66	7.5	1200	4492.48
13-16 ^{*b}	18	600	842.92	66-71	18	1400	1966.46
16-19	12.5	600	1264.39	71-75	12.5	1400	2949.69
19-21	10	600	1685.85	75-78	10	1400	4046.72
21-24	7.5	600	2247.80	78-80	7.5	1400	5243.9
24-28	18	800	1123.90	80-88 ^{*c}	18	600	842.92
28-31	12.5	800	1685.85	88-90	18	600	842.92
31-34	10	800	2247.37	90-92	18	1400	1966.46
34-37	7.5	800	2996.35	92-94	18	1400	1966.46
37-41	18	1000	1404.87	94-96	18	1400	1966.46
41-44	12.5	1000	2107.31	96-102	7.5	600	2247.8
44-47	10	1000	2786.47	102-104	7.5	1200	4492.48
47-51	7.5	1000	3745.63	104-106	7.5	1200	4492.48
51-55	18	1200	1685.85	106-108	7.5	1200	4492.48
55-59	12.5	1200	2409.94	108-111	7.5	1200	4492.48
59-63	10	1200	3371.07	111-114	7.5	1200	4492.48

PLR : pollutant loading rate , Inf. conc.: Influent concentration, *a, b* c* : PBR operational schedule during DO optimization, reactor performance evaluation and shock loading condition

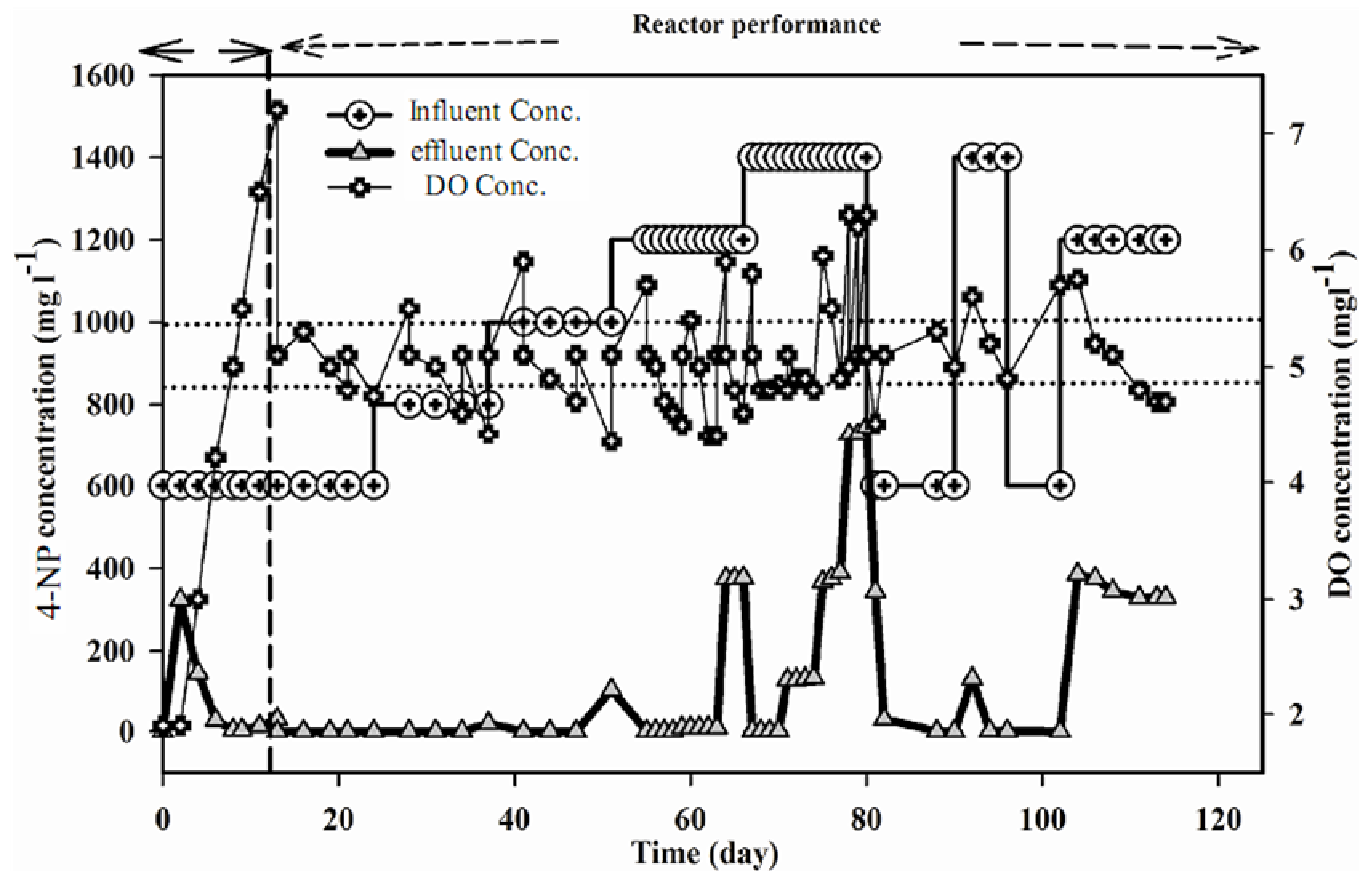


Figure 5. 20 (c) Biodegradation of 4-NP and DO profile in the newly designed PBR.

5.4.5 Effects of 4-CP, 4-BP and 4-NP loading rates on performance of PBR

In fact, the reactor performance was greatly dependent on the pollutant loading rate that in turn dependent upon the pollutant concentration and HRT. Experimental data were re-plotted in Figure 5.21, 5.22 and 5.23 showing the reactor performances in terms of pollutant removal efficiencies (%) and removal rates ($\text{mg l}^{-1}\text{day}^{-1}$) of 4-CP, 4-BP and 4-NP respectively, at different pollutant loading rates. Salient results on the performance of the PBR on these three mono- substituted phenol removal are given in Table 5.16. As given in Table 5.16, complete removal of 4-CP, 4-BP and 4-NP in the reactor were achieved at the maximum loading rate of 1707, 2277 and 2787 $\text{mg l}^{-1}\text{day}^{-1}$ of respective pollutants. The influent pollutant concentrations (mg l^{-1}), and HRTs (hour) maintained to get the above loading rate were (600, 10), (1000, 12.5) and (1000, 10) respectively.

Table 5.16 Performance of *A. chlorophenolicus* A6 in treating single substrate substituted phenol using the newly designed PBR.

Pollutant	Conc. (mg l^{-1})	HRT (h)	P L R ($\text{mg l}^{-1}\text{day}^{-1}$)	P R R ($\text{mg l}^{-1}\text{day}^{-1}$)	P R E (%)	COD R E (%)	Toxicity R E (%)	Remarks
4-CP	1000	24	1183	1183	100	98		a*
	600	10	1707	1707	100	99	93.5	b*
	800	12.5	1821	1803	99	97		c*
	800	7.5	3007	2285.85	76	73		d*
4-BP	1200	24	1419	1405	99	98		a*
	1000	12.5	2277	2277	100	99	95	b*
	1000	10	2815.02	2844.85	99	98		c*
	800	10	3007.39	2646.42	88	86		d*
4-NP	1400	18	1966.23	1966.23	100	98		a*
	1000	10	2786.47	2786.47	100	99	93	b*
	800	7.5	2996.35	2903.81	97	95		c*
	1000	7.5	3745.63	3371.07	90	87		d*

HRT: hydraulic retention time, P L R: pollutant loading rate, P L R: pollutant removal rate, P R E: pollutant removal efficiency a = Highest conc. 100% R E, b* = Optimum Condition, c* = Highest LR 99% R E and d* = Highest L R .

These loading rates were higher than those reported earlier on phenol and chlorophenol biodegradation by packed bed bioreactor, stirred reactor tank reactor, and fluidised bed reactor (Zilouei et al., 2006; Prieto et al., 2002; Tziotzios et al., 2005; Prieto et al., 2002; Gal'indez-Mayer et al., 2008), except the one where complete 4-CP degradation was achieved at a higher loading rate of $2305 \text{ mg l}^{-1}\text{day}^{-1}$, in a rotating biological contractor (Sahinkaya and Dilek, 2006). However, biogenic substrate like peptone was added in to the influent in that case (Sahinkaya and Dilek, 2006). The maximum 4-CP concentration investigated in any other bioreactor so far was 1050 mg l^{-1} and introduction of 4-CP higher than this, disturbed the reactor system and took about one month to recover (Buitro'n et al., 2005). Although, there was no report available so far on bromophenol biodegradation in any bioreactor system operated in continuous mode, this is one of the highest loading rate ($2277 \text{ mg l}^{-1}\text{day}^{-1}$) among many other substituted phenolic compounds, where complete removal was achieved. The maximum bromophenol degradation reported so far was only 60% and 85%, from initial 4-BP concentrations of 190 mg l^{-1} (Uhnáková et al., 2009) and 304 mg l^{-1} (Entezari and Petrier, 2003) respectively. In addition, to the best of our knowledge, this is the maximum 4-NP loading rate ($2787 \text{ mg l}^{-1}\text{day}^{-1}$) at which complete removal was achieved in any bioreactor reported so far. The maximum 4-NP that was completely removed from any continuous mode of bioreactor was only 320 mg l^{-1} (Tomei et al., 2003), whereas, in any batch shake flask it was 500 mg l^{-1} (Zheng et al., 2009). Furthermore, the maximum 4-NP concentration investigated in any bioreactor so far, with 99% degradation efficiency was 528.73 mg l^{-1} (Rezouga et al., 2009); beyond which inhibition of the microbial degradation performance was noted. In the present system, at 4-CP, 4-BP and 4-NP loading rates higher than 1707, 2277 and $2787 \text{ mg l}^{-1}\text{day}^{-1}$ respectively, removal efficiency was gradually reduced irrespective of the influent concentration. However, maximum removal rate of 2286, 2805 and $3371 \text{ mg l}^{-1}\text{day}^{-1}$ were obtained at the loading rate of 3007, 2845 and $3746 \text{ mg l}^{-1}\text{day}^{-1}$ of 4-CP, 4-BP and 4-NP respectively [Figure 5.21(b), 5.22 (b), 5.23 (b); Table 5.16]. At this condition the reactor was fed with influent 4-CP, 4-BP and 4-NP of 800, 1000 and 1000 mg l^{-1} and was operated at 7.5, 10 and 7.5 h of HRT, respectively (Table 5.16).

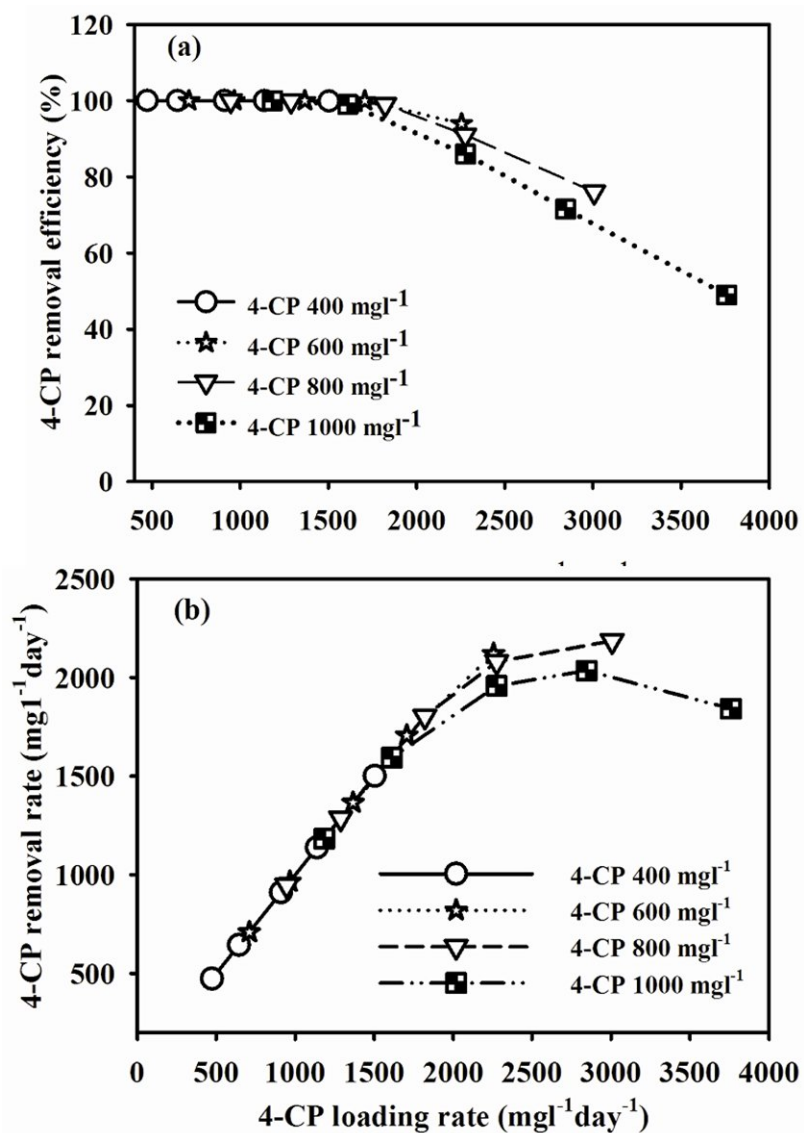


Figure 5. 21 (a) 4-CP removal efficiency, and **(b)** 4-CP biodegradation rate in the newly designed PBR at different 4-CP loading rates.

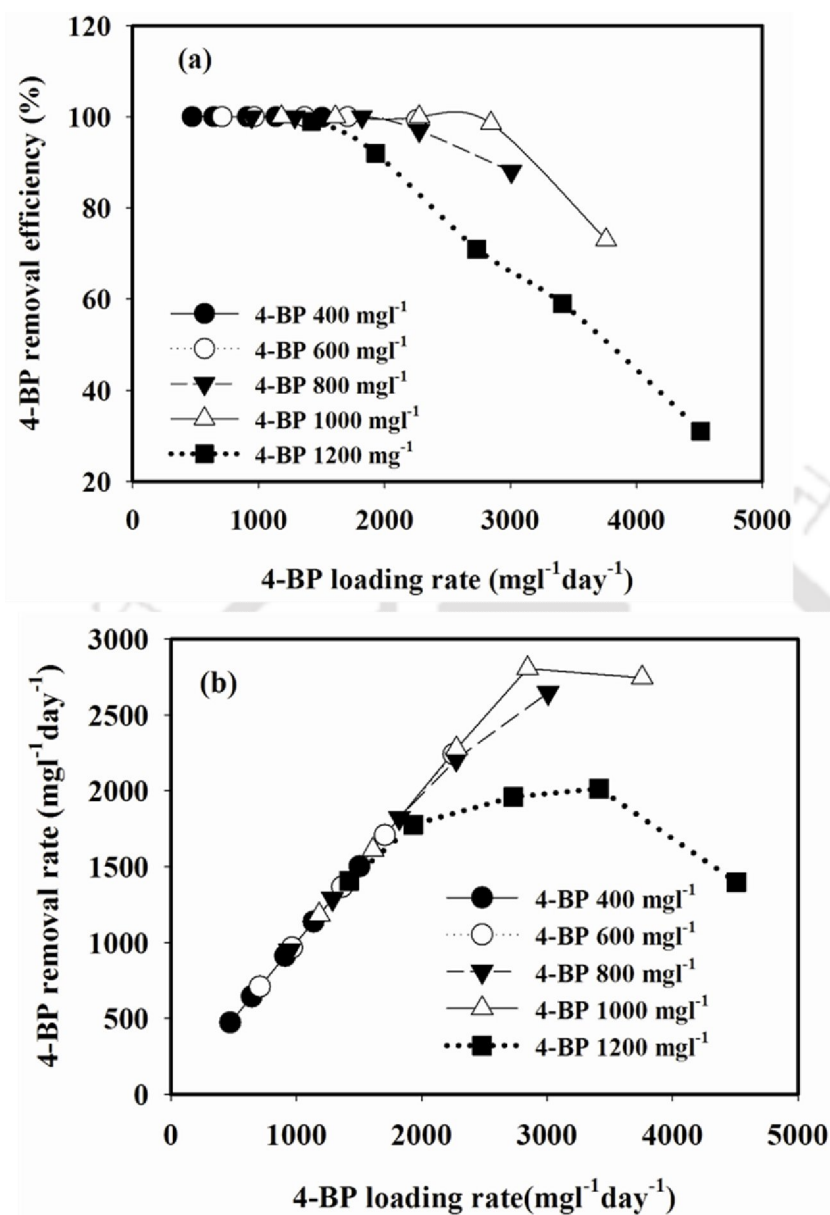


Figure 5. 22. (a) 4-BP removal efficiency, and (b) 4-BP biodegradation rate in the newly designed PBR at different 4-BP loading rates.

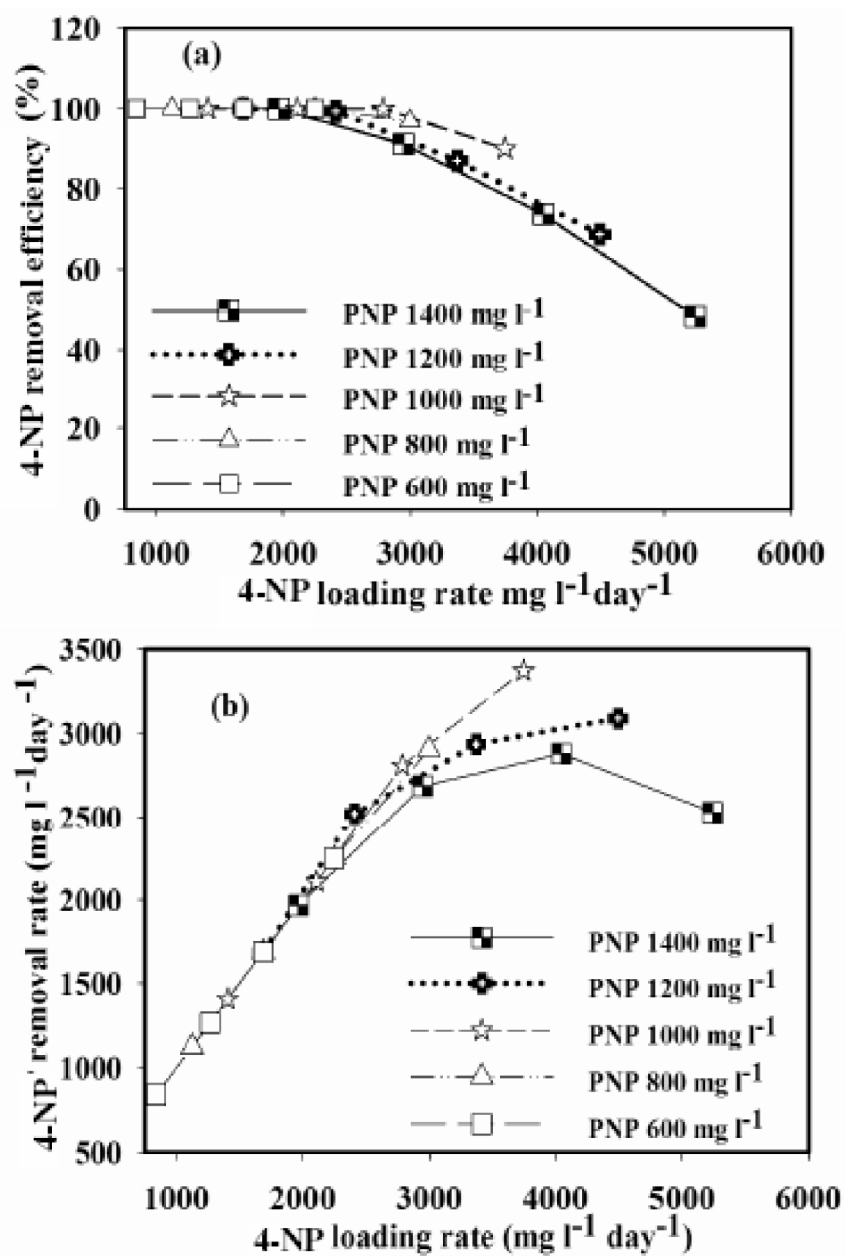


Figure 5. 23. (a) 4-NP removal efficiency, and **(b)** 4-NP biodegradation rate in the newly designed PBR at different 4-NP loading rates.

However, the reactor performance (% removal) decreased with increase in pollutant loading rates leaving high pollutant concentrations in the treated effluents [Figure 5.21 (a), 5. 22 (a), and 5. 23 (a)]. Thus, the optimum conditions of PBR operation for 4-CP, 4-BP and 4-NP removal were observed at the loading rates of 1707, 2277 and 2787 $\text{mg l}^{-1}\text{day}^{-1}$ for influent concentrations of 600, 1000 and 1000 mg l^{-1} respectively. Though, sponge-like structure of poly-urethane foam (PUF) has poor gas permeability (Matthew et al., 2008), the higher pollutant degradation efficiency observed in this study might be due to the enhancement of hydrodynamic conditions and gas liquid superficial flow velocity. Higher gas liquid superficial flow velocity might have facilitated better penetration of nutrient (4-CP/4-BP/4-NP) and oxygen (electron acceptor) deep inside the biosupport material (PUF) leading to high biomass growth and thus better pollutant biodegradation performance. As, abiotic loss due to adsorption and volatilization were insignificant (Table 5.13), 4-CP, 4-BP and 4-NP removal in the PBR were mainly due to biodegradation by *A. chlorophenolicus* A6.

Deleted: Figures 5.21 (b), 5.22 (b) and 5. 23 (b) also indicated that it could be possible to achieve better 4-CP, 4-BP and 4-NP removal rates had the reactor been operated at HRT lower than 7.5 h or lesser, with influent concentration of 800 mg l^{-1} , 800 mg l^{-1} and 1200 mg l^{-1}

5.4.6 Biomass growth in PBR

Biomass concentration for 4-CP, 4-BP and 4-NP were estimated to be 366, 414.5 and 380.5 mg of MLSS per gram of PUF which was more than 136 mg of MLSS g^{-1} of PUF reported by Fava et al. (1996) while treating nitrobenzene in a fixed bed bioreactor. The higher biomass growth might be due to production of higher amount of substituted phenol degrading enzymes such as hydroxyquinol 1,2-dioxygenase and monooxygenases etc (Nordin et al., 2005). Furthermore, it could well correlated with our earlier work that 4-chlorophenol biodegradation by *A. Chlorophenoloicus* A6 was a growth associated process (section 4.1).

5.4.7 Dissolved oxygen (DO) concentration profile in PBR

Dissolved oxygen (DO) profile of 4-CP, 4-BP and 4-NP during the operational period of the reactor is shown in Figure 5.20 (a), 5.20 (b), and 5.20 (c). Two dotted lines parallel to X axis in Figure 5.20 (a), 5.20 (b), and 5.20 (c) represents the optimum DO concentration range for 4-CP, 4-BP and 4-NP respectively. Whenever DO level in the reactor content was beyond this range it was brought back to this range by controlling rate of supply of air into the reactor. Sudden decrease or increase in DO concentration in the reactor was noticed because of change in organic loading rate either due to change in HRT or influent pollutant concentration, or due to change in both. Sudden decrease in DO concentration in the reactor were noticed whenever there was an increase in pollutant loading rate due to lowering of HRT of the reactor. This might be due to sudden increase in DO demand by the microorganism to degrade the extra amount of substituted phenol introduced to maintain a lower HRT. Similarly, sudden increases in DO concentration were noticed on 30th, 47th and 67th days for 4-CP introduction [Figure 5.20 (a)]; and 31th, 48th, 68th, and 86th days for 4-BP introduction [Figure 5.20 (b)]. That happened because of decrease in pollutant loading rate due to increase in HRT from 7.5 h to 24 h, even if the concentration of substituted phenols in feed were enhanced stepwise from 400 mgL⁻¹ to 600 mgL⁻¹, 800 mgL⁻¹ and 1000 mgL⁻¹ respectively for 4-CP and upto 1200 mgL⁻¹ in case of 4-BP. [Table 5.15. (a), (b) and (c)]. Similar increase in DO concentration was noticed on 24th, 37th, 51st, 66th days of the reactor run [Figure 5.20 (c)], as 4-NP loading rate was decreased due to increase in HRT from 7.5 h to 18 h, even if the 4-NP concentration in influent was enhanced stepwise from 600 mgL⁻¹ to 800, 1000, 1200 and 1400 mgL⁻¹, respectively. Similar changes in DO profile have been observed by other researchers (Salehi et al., 2010). However, whenever DO concentration in the reactor was beyond this range it was brought back to the above mentioned concentration range by controlling the air supply rate into the reactor. Though, the DO level was brought back to a value near to their respective optimum values as described above, a prominent ascends in DO concentration was observed on 80th – 86th day [Figure 5.20. (a)], 97th -104th day [Figure 5.20. (b)] and 75th -80th day [Figure 5.20 (c)] of run of the reactor while treating wastewater containing 1000 mgL⁻¹ of 4-CP, 1200 mgL⁻¹ of 4-BP and 1400 mgL⁻¹ 4-NP,

respectively, at an HRT of 10 h or shorter. This might be due to the toxicity effects of these substituted phenols at their respective concentration that inhibited the microorganisms from

normal metabolic activities, leaving a significant amount of DO and these pollutants unutilized. At this condition only 72%, 56% and 74% removal of 4-CP, 4-BP and 4-NP were achieved. Performance of the reactor was even worse when the HRT was further lowered to 7.5 h, where 4-CP, 4-BP and 4-NP removal was only 49%, 27% and 48% respectively.

5.4.8 Metabolic intermediate analysis

Furthermore, it is well understood that, *A. chlorophenolicus* A6 mineralized the substituted phenol like 4-CP by a metabolic pathway with 4-chlorocatechol as a major intermediate product (Nordin et al. 2005), and the formation of chlorinated catechols leads to process failure (Klec̃ka et al. 1981; Bartels et al. 1984). Similar result were also reported in many literature that 4-nitrocatechol and hydroxy-quinol, are the common intermediate products of 4-NP biodegradation by *Bacillus sphaericus* JS905 (Spain 1995) and *Arthrobacter* species (Jain et al. 1994). Therefore, in the present study possible accumulation of intermediate like 4-chlorocatechol, 4-bromocatechol and 4-nitrocatechol was investigated by LCMS (WATERS LC-MS/MS system, Q-ToF Premier). In the present study HPLC-MS analysis of the reactor effluent confirmed the formation of intermediates such as 4-chlorocatechol, hydroxyl-quinol from 4-CP and 4-nitrocatechol along with hydroxyl-quinol from 4-NP were the major intermediate as shown in (Figure 5.24 (a) and (b)), on contrary from 4-BP no such intermediate was noticed in the reactor effluent. Therefore, instability of the newly designed PBR at 1000 mg l⁻¹ of influent 4-CP could be due to combined toxic effects of 4-chlorocatechol, chloride and 4-CP, as the presence of even 100 mg l⁻¹ of chloride could inhibit *Sphingomonas* as well as *Arthrobacter* species on chlorophenol biodegradation (Stanlake and Finn, 1982; Cassidy et al. 1999). On the other hand the higher degradation performance for 4-BP at above 1000 mg l⁻¹ in the newly designed PBR may be due the absence of toxic intermediate accumulation such as 4-bromocatechol and chloride ion.

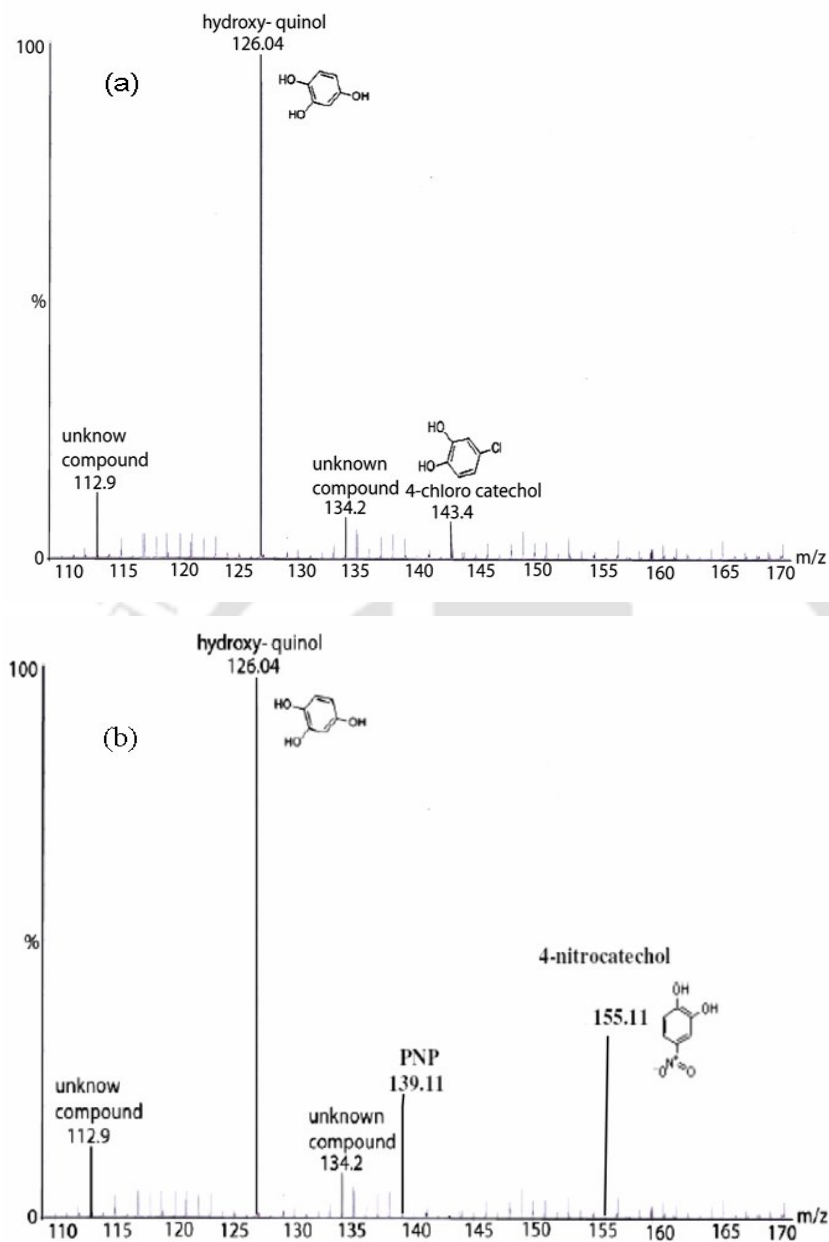


Figure 5.24 Mass spectra of substituted phenol metabolites cleavage pathway obtained by HPLC-MS, Hydroxy quinol (126.04), 4-chloro catechol (143.4) **(a)** 4-CP and **(b)** 4-NP.

Similarly higher degradation performance at 1400 mg l^{-1} was achieved in the present study may be due the fact that 4- nitrocatechol exerted less toxic effect on the *A. chlorophenolicus* A6 growth in compared to 4-chlorocatechol. Similar result also reported in literature that although 4-nitrocatechol is the major intermediate product of 4-NP degradation by several microorganisms however, its toxicity on the microbial process is not yet reported (Spain 1995; Jain et al. 1994).

5.4.9 Stability studies on the performance of the newly designed PBR under shock loading conditions

Any industrial wastewater treatment plant undergoes different intermittent organic and pollutant loading conditions, when concentrations of its pollutants changes abruptly. Performance of the newly designed PBR under shock loading conditions was evaluated by monitoring the reactor performance on these substituted phenols removal at two different shocks loadings. The reactor was brought back to a steady state condition at relatively lower organic loading before being subjected to a high organic loading as shock load. As given in Table 5.15 (a), 4-CP loading was reduced from 3759 to 473 on 86th-96th day and was operated until completely removal of 4-CP and steady state performance of the reactor was achieved for 5-7 cycles of run. The reactor was then subjected to 4-CP loading of $1183 \text{ mg l}^{-1} \text{ day}^{-1}$ on [day 96-103, Table 5.15 (a)] as shock load to the PBR. The reactor was then again subjected to a normal loading rate of $1138 \text{ mg l}^{-1} \text{ day}^{-1}$ [103th-108th day, Table 5.15 (a)] in order to achieve completely removal and stable condition of the reactor again before being subjected to another shock load, of $2845 \text{ mg l}^{-1} \text{ day}^{-1}$ to it, which was higher than the first shock load. Schedule of the experiments on stability of the reactor under shock loadings of 4-BP and 4-NP are indicated in Table 5.15 (b), and 5.15 (c), respectively. Performance of the reactor under shock loadings of 4-CP, 4-BP and 4-NP are plotted in figures Figure 5.25 (a), (b), and (c), respectively. Though the reactor was upset temporarily due to first shock load introduction as 4-CP, 4-BP and 4-NP removal efficiency was only 88%, 89.7% and 91 % respectively, more than 99% removal was observed within next 2 - 3 days of run of the reactor [Figure 5.25 (a), (b) and (c)], which remained stable for next 2 days.

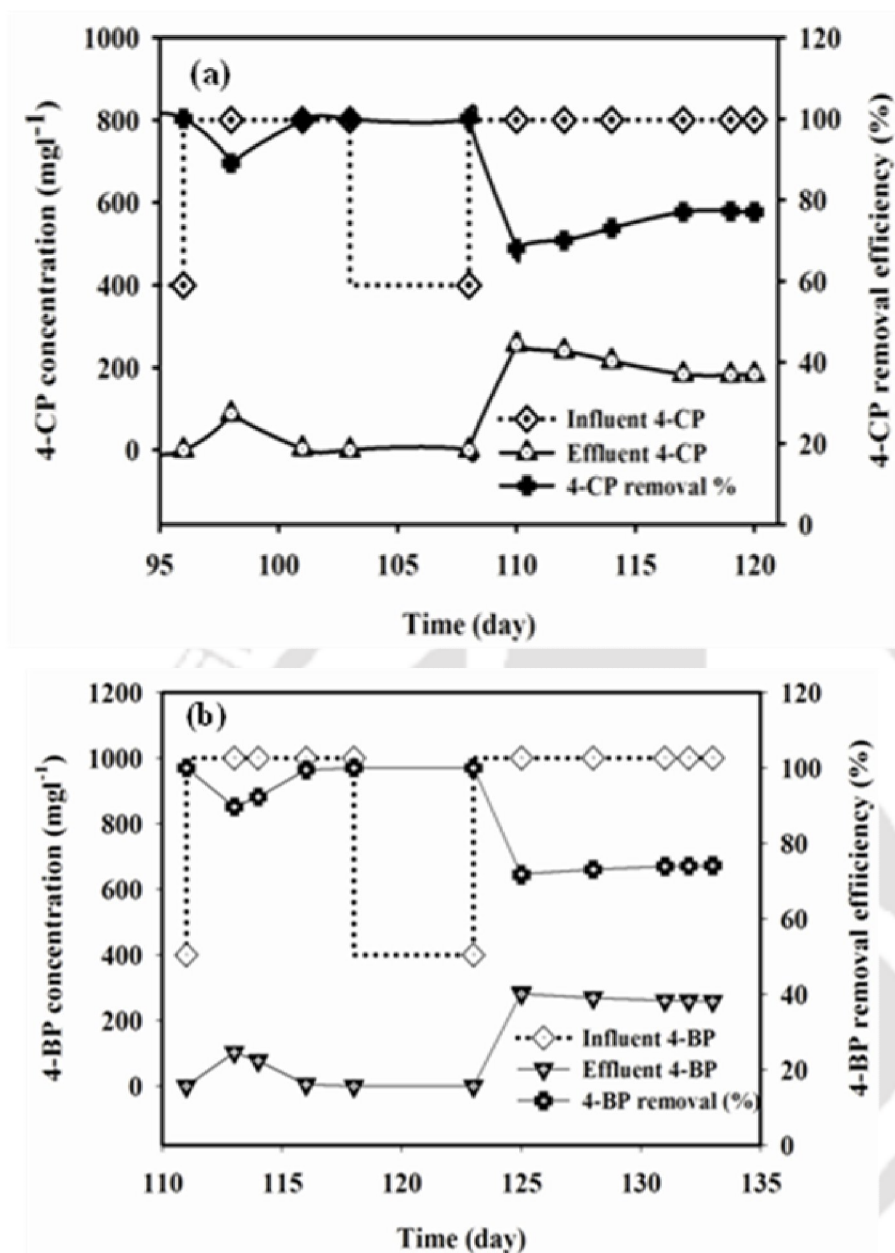


Figure 5. 25 Substituted phenol removal profile of the newly designed PBR under shock loading conditions operated at varying hydraulic loading rate **(a)** For 4-CP from 473- 1183 mgL⁻¹day⁻¹ and 1138-2845 mgL⁻¹day⁻¹ **(b)** For 4-BP from 473-1183 mgL⁻¹day⁻¹ and 1504-3759 mgL⁻¹day⁻¹.

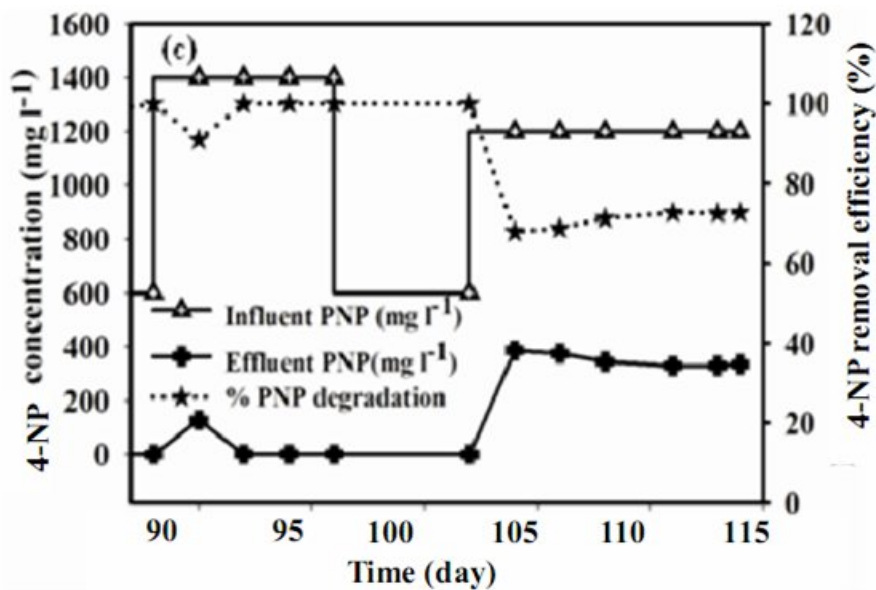


Figure 5. 25 Substituted phenol removal profile of the newly designed PBR under shock loading conditions operated at varying hydraulic loading rate **(c)** For 4-NP from 842-1966 $\text{mg l}^{-1}\text{day}^{-1}$ and 2248-4492 $\text{mg l}^{-1}\text{day}^{-1}$.

Under second shock loads condition as 4-CP, 4-BP and 4-NP removal efficiency was only 68%, 70 % and 67% respectively. Although the reactor performance was slightly disturbed due to this sudden change in operating condition, increases in loading rate, more than 77%, 72% and 74% removal was observed within next 4 - 6 days of run of the reactor [Figure 5.25. (a), (b) and (c)], which remained stable for next 2 days.

In general a toxic shock load produces an increase in the reaction time, diminishes the efficiency, and may inhibit the biomass (Buitrón et al., 2003). Buitrón et al. (2003) evaluated the degradation of 4-chlorophenol in a SBR operated with suspended biomass and reported that the treatment process was affected when the biomass was exposed to a shock load of 1050 mg l^{-1} , since the degradation time increased to 230%. The original degradation time and the activity of microorganisms after the peak was not recovered in the subsequent cycles operated with a lower 4-CP concentration.

Karim and Gupta (2001) reported reduction in efficiency of 4-NP degradation from 96% to 89.7% when HRT was reduced from 30 to 12 h to increase the loading rate) in an upflow anaerobic sludge blanket (UASB) reactor. In contrary, the present study suggested that the novel PBR was able to completely regain its original performance within a short period of operation, under the two shock loads applied. The performance of the PBR under shock loads was better than many other continuous bioreactor systems studied on substituted phenols biodegradation (Xing-yu et al., 2007; Tomei et al., 2008; Cho et al., 2000). This clearly revealed the potential of *A. chlorophenolicus* A6 on removal of mono substituted phenols from synthetic wastewater in newly designed bioreactor (PBR) system.

5.4.10 SEM characterization of PUF

Figure 5.26 shows the SEM image of the *A. chlorophenolicus* A6 cultured in batch shake flask using MSM before inculcating into the reactor.

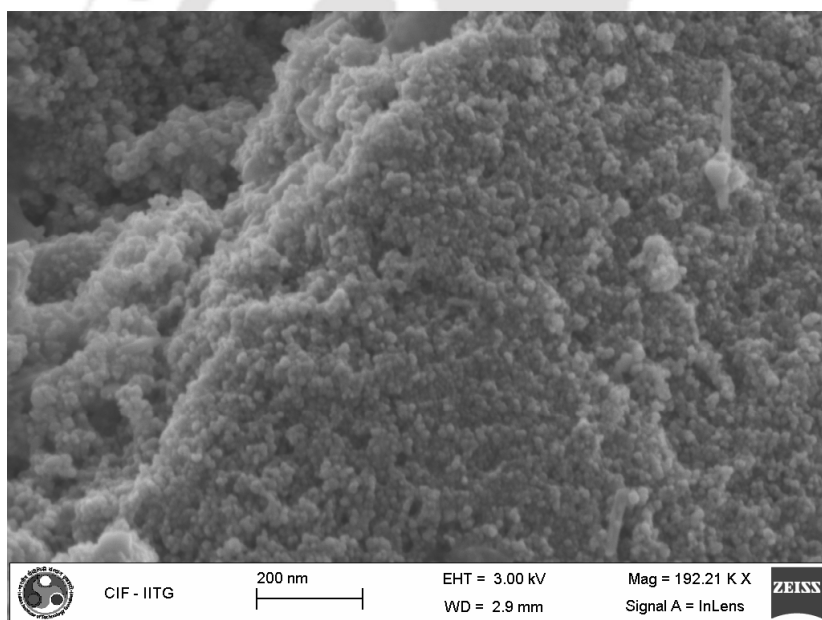


Figure 5.26 SEM images *A. chlorophenolicus* A6 growth on MSM in batch shake flask before inoculated in the PUF cube packed in the PBR.

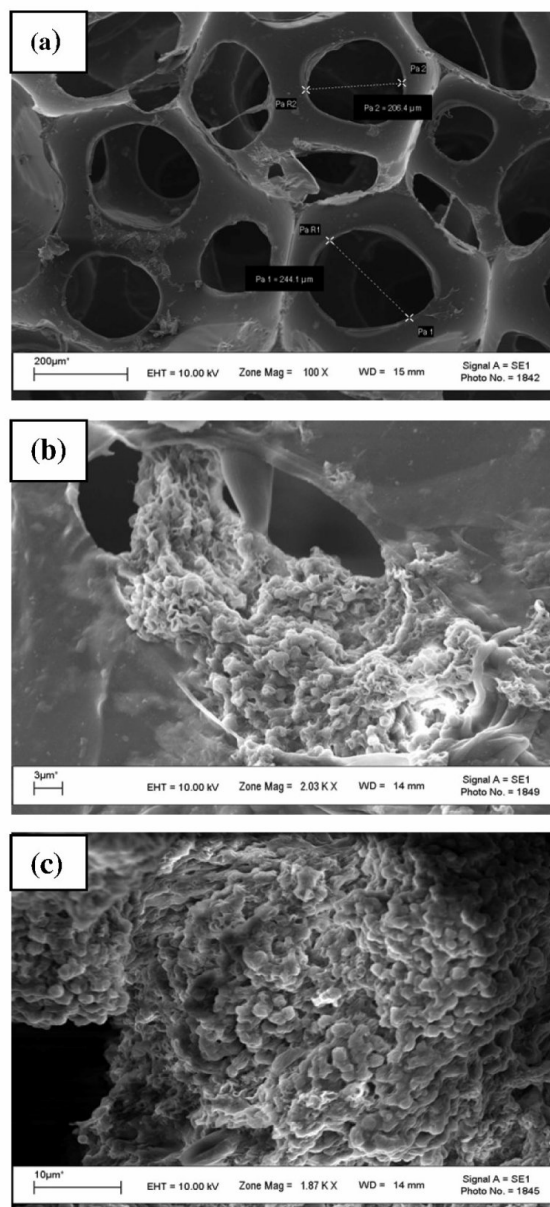
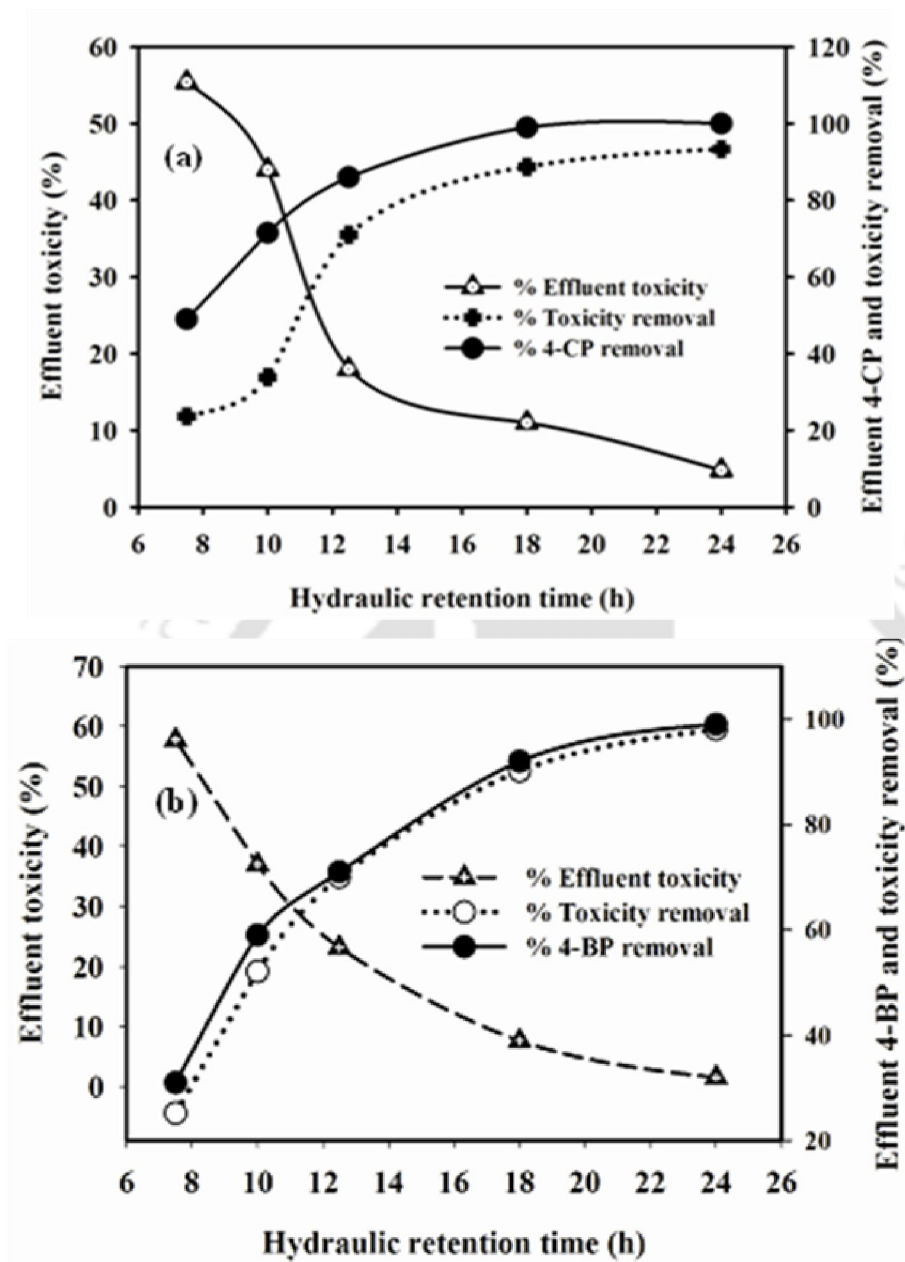


Figure 5.27 SEM images of PUF: (a) vesicular structure of PUF before inoculums, (b) bacterial growth on PUF, and (c) magnified picture of (b) showing more clear microbial morphology.

Figure 5.27 shows the scanning electron microscopic (SEM) images of PUF before and following the growth of *Arthrobacter chlorophenolicus* A6. The SEM picture confirmed microbial colonization on PUF. Observation of the biosupport material using SEM showed a succession of densely colonized areas shown in Figure 5.27 (b). Closer examination of the surface revealed the presence of dense biofilms embedded within the PUF matrix (Figure 5.27 (c)) and most colonies were observed to be large sized and cocci shaped. From these Figure 5.26 and 5.27 it is observed that the morphology of the microorganism remained similar (cocci shaped) which further confirmed the biodegradation of the substituted phenols occurs by *A. chlorophenolicus* A6

5.4.11 Toxicity removal in PBR

The percentage (%) toxicity of the influent at 1000, 1200 and 1400 mg l⁻¹ of 4-CP, 4-BP and 4-NP respectively, were tested on mixed microbial consortia from municipality sludge (say, MC) and was estimated to be 72 %, 77.23%, and 78%, respectively. Percentage (%) effluent toxicity and % toxicity removal tested on the above mentioned test species with respect to the influent to the newly designed PBR treating 1000, 1200, and 1400 mg l⁻¹ of 4-CP, 4-BP and 4-NP respectively, at different HRTs are shown in Figure 5.28. Percentage (%) toxicity removal was observed to be lesser with shorter HRTs. The minimum % of effluent toxicity was observed at the highest HRT of 24h, 24h, and 18h for 4-CP, 4-BP and 4-NP respectively, which were 4.8%, 1.5% and 1.17% respectively. On the other hand, the newly designed PBR could removed 93.5% 98% and 93% toxicity tested on mixed microbial consortia, for 4-CP, 4-BP and 4-NP respectively when operated at HRT of 24h for 4-CP and 4-BP whereas, 18h for 4-NP. Figure 5.28 (a), (b) and (c) also depicted the variations in effluent toxicity removal with residual substituted phenols removal in the effluent of the PBR. From the Fig. 5.28. (a) it was clearly revealed that the percentage effluent toxicity removal and with its respective residual 4-CP removal divergent each other particularly at lower HRTs indicating appearance of toxic metabolic intermediate formation such as 4-chlorocatechol.



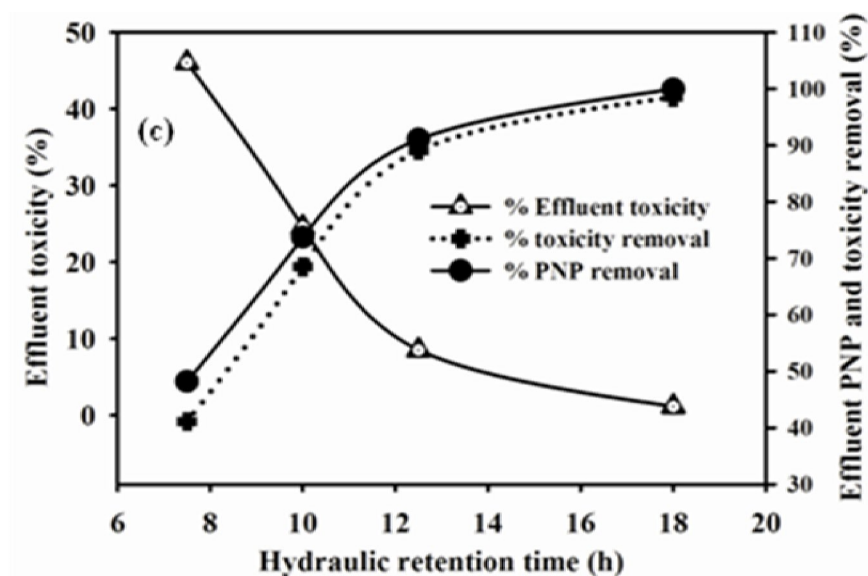


Figure 5. 28 Percentage toxicity in the effluent and percentage of substituted phenol removal with percentage of toxicity removal tested on mixed microbial consortia from municipal sludge in the newly designed PBR (a) 4-CP (b) 4-BP (c) 4-NP.

On contrary, for 4-BP and 4-NP [Figure 5.28. (b) and (c)] the variations in effluent toxicity removal with their respective pollutant removal followed somewhat similar trend indicating non toxic nature of the metabolites and/or non-appearance of toxic metabolites.

5.5.1 Simultaneously removal of 4-CP, 4-BP and 4-NP in PBR

Microbial degradation of pollutants is usually studied in laboratory using single target compound. However, most of the cases, more than one pollutant in nature are commonly occur as mixtures, which can affect the degradation of an individual compound (Kova'rova'-Kovar and Egli 1998). Therefore, in the present study degradation performance of *A.chlorophenolicus A6* in mixed substrate system was investigated in the PBR system. As the PBR system could completely remove from 600 to 1000 mgL⁻¹ of either of 4-CP, 4-BP or 4-NP when operated at 10-12.5h HRT, thus PBR was operated at an HRT of 12.5h for simultaneous removal of the above mentioned three pollutants.

Table 5.17 Operational schedule and performance of the PBR on biodegradation of mixtures of mono substituted phenols at 12.5 h HRT.

stages of run	Day of run	Influent phenolic conc. (mg l ⁻¹)			Effluent phenolic conc. (mg l ⁻¹)			Removal efficiency (%)		
		4-CP	4-BP	4-NP	4-CP	4-BP	4-NP	4-CP	4-BP	4NP
1	0	250	250	250	0	0	0	99.5	100	100
	4	250	250	250	1.25	0	0	99.5	100	100
	8	300	300	300	18	3	1.15	94	99	99.5
3	13	250	450	250	0.75	4.5	0.5	99.7	99	99.8
4	17	250	250	450	8.75	0.5	4.5	96.5	99.8	99
5	22	450	250	250	38.35	1.25	1.75	91.5	99.5	99.3
6	27	350	350	350	101.5	63	7	71	82	98
	28	250	250	250	31	0	0	87.6	100	100
	29	250	250	250	0	0	0	100	100	100
7	30	250	250	250	0	0	0	100	100	100
	32	250	450	450	82.5	193.5	13.5	67	57	97
	34	250	450	450	84	195	14.3	66	58	96.5
	35	250	450	450	81	194	13.8	67.7	57.4	96.8
	36	250	250	250	31	78	0	87.6	68.8	100
	37	250	250	250	0	0	0	100	100	100
	38	250	250	250	0	0	0	100	100	100
8	40	450	450	250	256.5	162	6.25	43	64	97.5
	42	450	450	250	258	164	6.9	42	65	97
	43	450	450	250	254	162	7.2	42.5	64	97.6
	45	250	250	250	95	42	0	62	83	100
	46	250	250	250	0	0	0	100	100	100
9	47	250	250	250	0	0	0	100	100	100
	48	450	250	450	283.5	47.5	27	37	81	94
	50	450	250	450	285	48	28	36.5	82	93
	52	450	250	450	281	49	29	38	83	94
	54	250	250	250	111	23	0	55.6	90.8	100
	55	250	250	250	0	0	0	100	100	100
	56	250	250	250	0	0	0	100	100	100
10	57	250	250	250	0	0	0	100	100	100
	58	450	450	450	302	285	100	32.28	36.66	77.7
	60	450	450	450	330	306	123	26.6	32	72.6
	63	450	450	450	357	333	156	20.66	26	65

Concentrations of individual mono- substituted phenols were varied between 250 - 450 mg l⁻¹

Table 5.17 shows details of the design matrix employed along with the performance of the reactor in the study at different stages.

Based on the results of the single substrate system the DO concentration in the reactor was maintained at 4-4.5 mg l⁻¹. The reactor was started with 250 mg l⁻¹ of each of 4-CP, 4-BP and 4-NP (stage 1) and was continued to run until it gave steady state performance. Once the effluent concentration of reactor at any stage, in terms of substituted phenols concentration were constant for 4-5 cycles of run, it was assumed that the reactor had reached to a steady state condition. After achieving steady state performance the concentration of pollutants mixture in influent was increased/changed stepwise from stage-1 to stage 10. Figure 5.29 illustrated the degradation performance of *A. chlorophenolicus* A6 using the PBR in treating synthetic wastewater containing mixture of these substituted phenols. From the figure it is clearly revealed that at lower concentration ranges of the pollutants (stage 1-5), degradation efficiencies of all the pollutants in the wastewater were considerably high.

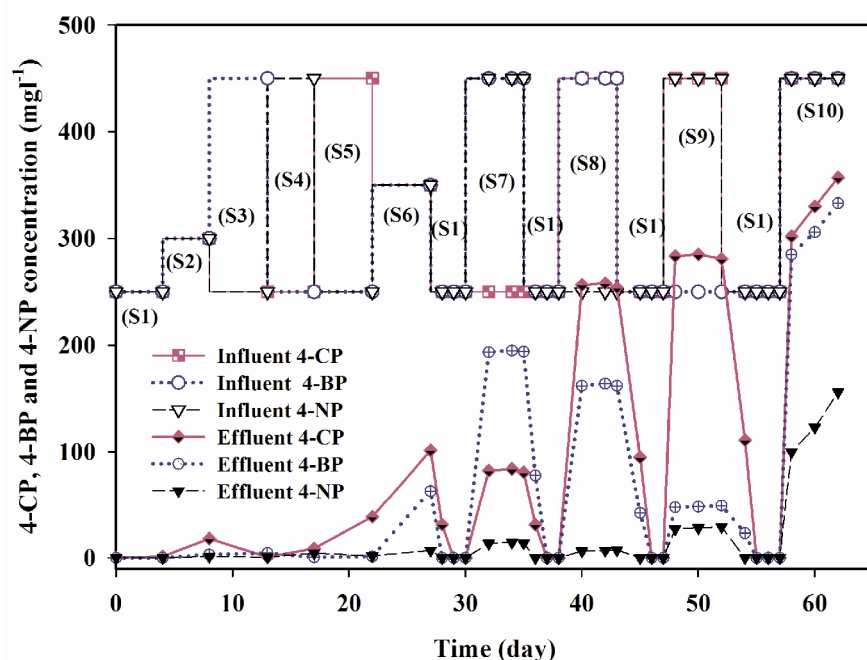


Figure 5.29 Degradation performance of *A. chlorophenolicus* A6 using the newly designed PBR treating synthetic wastewater containing mixture of 4-CP, 4-BP and 4-NP at 12.5h HRT. (*S indicates stages of operation).

Complete simultaneous removal of all the substituted phenols was achieved when the reactor was operated at stage 1 (of each of 4-CP, 4-BP and 4-NP was 250 mg l⁻¹). Though, complete removal was not achieved, more than 99% removal occurred at stages 2-5, except for 4-CP, which was removed by 94%. Chlorophenol removal efficiency of the reactor was further reduced to 91.5% at stage 5. Poor degradation of the 4-CP in the mixed substrate system with respect to single substrate system in the reactor might be due to the increased toxicity caused due to presence of other substituted phenols. For example, phenolics exert their toxicity by uncoupling oxidative phosphorylation (McLaughlin and Dilger 1980; Terada 1990; Escher et al. 1996), and formation of dimers between two differently substituted phenols can result in an uncoupling activity that is higher than the sum of the single compounds (Escher et al. 2001). In addition the formation of different intermediates and their combined toxic effects might have inhibited secretion of enzymes vital for their degradation, and/or competition for uptake into the cells (Alexander, 1999). The reactor performance was even worse at stages 6 onwards, especially on 4-CP and 4-BP removal. Because of poor performance, before going to the next higher stage from stage 6 to stage 10, the reactor was brought back to steady state conditions at stage 1 (Table 5.17). At stage 6 and stage 7, 101 and 83 mg l⁻¹ of 4-CP; 63 and 194 mg l⁻¹ of 4-BP; and 7 and 13.5 mg l⁻¹ of 4-NP were observed in the respective effluents. This might be due to the combined toxicity effects of these three pollutant mixture at this concentration that inhibited the microorganisms from normal metabolic activities, leaving a significant amount of substituted phenols unutilized.

The reactor became completely unstable and inefficient at stage 10 (450 mg l⁻¹ of each of the three substituted phenols). Poor performance and instability of the PBR at higher influent concentration combinations (higher stages) could be due to combined toxic effects of the pollutants, their intermediates and accumulation of chloride ions (Klec̃ka et al., 1981; Stanlake and Finn, 1982; Bartels et al., 1984; Jain et al., 1994; Spain 1995; Cassidy et al., 1999; Nordin et al., 2005). Similar toxic effects were reported on simultaneous removal of phenol and *m*-cresol (Bai et al., 2007). Figure 5.29 clearly demonstrated that irrespective of the concentration levels of these three substituted phenols; 4-NP was preferentially degraded over 4-CP and 4-BP.

This degradation performance of the PBR on 4-NP removal was found better than that of 4-CP and 4-BP. Further, this observation was also found to be consistent with the previous experiments on the wastewater containing a mixed substrate system in batch shake flask (section 5.3.21.). From the Table 5.17 it is clearly revealed that for most of the concentration combinations of these pollutants when total concentration fall below or equal to 950 mg l^{-1} (i.e. stage 1-5), the reactor could performed well. However, when the total concentration exceeded 950 mg l^{-1} , the efficiency was reduced from 91 to 37% for 4-CP and 99.5 to 57% in case of 4-BP respectively. On the other hand reduction in 4-NP removal was from 99.3 to 93%. Which clearly indicated the preferential reduction of 4-NP over the other two pollutants. Quan et al. (2004) studied on simultaneous biodegradation of 2,4-dichlorophenol (DCP) and phenol by *Achromobacter* sp in an internal loop airlift bioreactor at 8h HRT. In presence of 200 mg l^{-1} phenol, they observed a decrease in 2,4-DCP degradation efficiency from 100% to 87.9% from an initial 10 mg l^{-1} of 2,4-DCP. However, phenol degradation efficiency was not affected but remained at about 99.6%. The present study the PBR was conducted at much higher concentration of substituted phenol. Hence it is quite clear that our reactor system showed better potential on removal of phenolic compounds. Table 5.18 showed the efficiency of various bioreactor used on phenolic substances removal. It is clear from the table that newly designed PBR could be a better alternative to the other reactors on phenolic pollutants removal.

As mentioned earlier, the performance of a reactor dependent greatly upon the pollutant loading rates, experimental data were re-plotted in Figure 5.30 showing the reactor performance in terms of percentage removal, and pollutant removal rate ($\text{mg l}^{-1} \text{ day}^{-1}$), at different loading rates of the mixture. Complete removal of all these three pollutants in the reactor was achieved at the maximum individual pollutant loading rate of $569 \text{ mg l}^{-1} \text{ day}^{-1}$ or $1707 \text{ mg l}^{-1} \text{ day}^{-1}$ in terms of combined loading rate of the three pollutants. The performance of the PBR was found better than many others literature reports as given in Table 5.18. However, Sahinkaya et al. (2006) reported complete simultaneous removal of 4-CP and phenol in a rotating biological contractor (RBC) at a loading rate as high as $2305 \text{ mg l}^{-1} \text{ day}^{-1}$. However, they used biogenic substrate like peptone as a feed component for growth of the microorganism

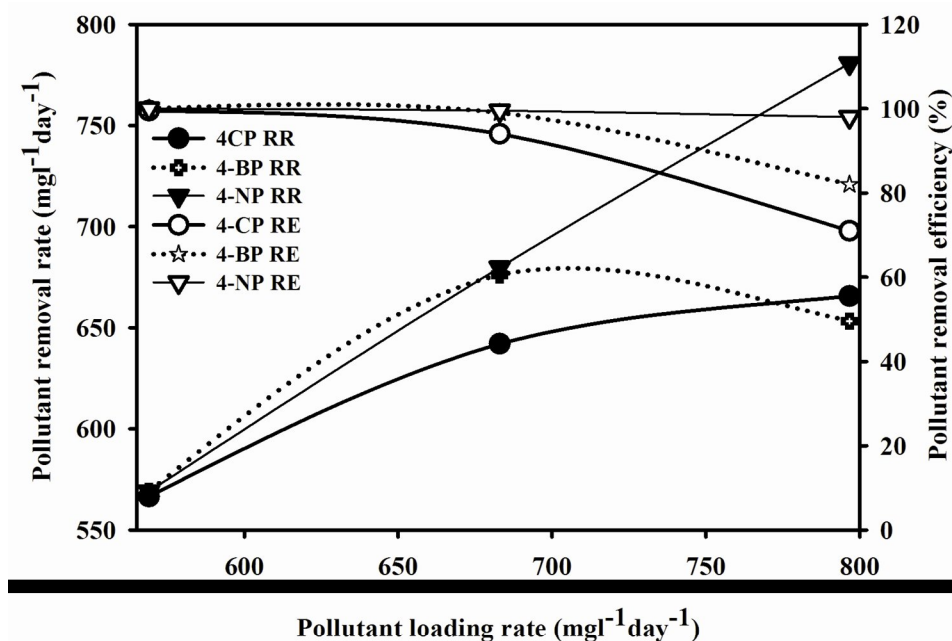


Figure 5.30 Substituted phenols removal efficiency and removal rate of *A. chlorophenolicus* A6 in the PBR at different loading rates, particularly stage 1, 2 and 6. (*RR indicated removal rate and RE indicated removal efficiency).

But in our system, individual loading rates greater than $569 \text{ mg l}^{-1} \text{ day}^{-1}$, (combined loading rate $1707 \text{ mg l}^{-1} \text{ day}^{-1}$) substituted phenols removal efficiency was gradually reduced in all the concentration matrices tested (Figure 5.30). On the other hand, the pollutant removal rate was found to increase with increases in loading rate of individual pollutants up to $683 \text{ mg l}^{-1} \text{ day}^{-1}$, but beyond that degradation rate of 4-CP and 4-BP fell down. On the contrary, there is an increase in pollutant removal rate of 4-NP in all the loading rates investigated. That further revealed the preferential uptake of 4-NP over the other two pollutants. Figure 5.30 also indicated that it could be possible to achieve better 4-NP removal rates in the mixture had the reactor been operated at HRTs lower than 12.5 h with influent pollutants concentration defined at stage 1.

Table 5.18 Performance of various bioreactors used on substituted phenols degradation in mixed substrate system

Pollutants treated	Initial Conc. (mg l ⁻¹)	Maximum Removal efficiency	Bioreactor type	Microorganisms used	HRT (h)	References
4-NP, 2,4-DNP, Aniline	50 to 180	99%	Sequencing batch reactor	<i>Bacterioidetes</i> , Candidate division TM7, <i>Proteobacteria</i>	12h	Xing-yu et al., 2007
4-CP, 2,4DCP, Peptone	220 & 110	>98% 2305 and 1202 mg l ⁻¹ day ⁻¹ respectively	2 Stage RBC	<i>Pseudomonas stutzeri</i>	16.8h	Sahinkaya et al., 2006
4-CP and Phenol	200 4-CP & 1600 phenol	-	(EFBAB) GAC fluidized bed	<i>Pseudomonas putida</i> ATCC 49451	10–15 h	Loh and Ranganath, 2006
Mixture 2CP, 4CP, 2,4 DCP, 2,4,6-TCP	107	99.7%	Packed-bed bioreactor	Mixed culture	2.5	Zilouei et al., 2006
2,4-DCP & Phenol	21, 2,4-DCP 400 phenol	94–99% 14.55 mg l ⁻¹ day ⁻¹ for Phenol 2.59 mg l ⁻¹ day ⁻¹ for 2,4-DCP	Honeycomb-like ceramic column packed air-lift bioreactor.	<i>Achromobacter</i> sp.	36 h	Xiangchun et al., 2003
2,4,6-TCP, 2,3,4,6-Te CP & PCP	16.8 TCP, 28.8 TeCP, 9.1 PCP Total CP 54.7	99.9% Degradation rate 31.0, 43.6 and 5.6 mg l ⁻¹ day ⁻¹	Batch-fed Air-lift percolator	Polychlorophenol-degrading bacterium K48	64 days	Langwaldt et al., 1998

Formatted: Portuguese (Brazil)

Phenol and 4-CP	98 and 1320 mg l ⁻¹ day ⁻¹ 4-CP and Phenol	>98%	Fluidized bed reactor	Yeast <i>Candida tropicalis</i>	-	Gal'indez-Mayer et al., 2008)
Phenol, 4-CP, 2,4-DCP	300 phenol, 50 each 4-CP & 2,4-DCP	100% of both phenol and 4-CP & 98% of 2,4-CP	Chemostat reactor	<i>Burkholderia</i> sp., <i>Microbacterium phyllosphaerae</i> , and <i>Candida tropicalis</i>	-	Salmer'on-Alcocer et al., 2007
ETBE, MTBE, TAME	140±5, 132±2 and 135±2 mg l ⁻¹ day ⁻¹ each	>99%	Uflow fixed bed reactor	Mixed culture		Kharoune et al., 2001
Phenol, 4CP	20 mg l ⁻¹ Each.	100%	Packed bed reactor	<i>P. testosteroni</i> CPW301	10 h	Kim et al., 2001
4-CP, 4-BP and 4-NP	250 mg l ⁻¹ each	100%	PBR	<i>A. chlorophenolicus</i> A6	12.5 h	Present study

The maximum 4-CP and 4-BP removal rate was estimated to be 642 and 676 $\text{mg l}^{-1}\text{day}^{-1}$, respectively, at the loading rate of 683 $\text{mg l}^{-1}\text{day}^{-1}$ of individual components. In case of 4-NP, the maximum removal rate was 780 $\text{mg l}^{-1}\text{day}^{-1}$ at the loading rate of 796 $\text{mg l}^{-1}\text{day}^{-1}$.

5.5.2 Toxicity removal efficiency in mixed substrate system in the PBR

The percentage (%) toxicity of the influent at stage 1 (250 mg l^{-1} each of 4-CP, 4-BP and 4-NP) was tested on mixed microbial consortia and found to be 81.23%. Percentage effluent toxicity and % toxicity removal tested on the above mentioned test species with respect to above mentioned influent concentration was estimated to be 1.8% and 97% respectively. Revealing the potential of the *A. chlorophenolicus* A6 using the PBR system in treating synthetic wastewater with mixed substrates.

5.6 Treatment of refinery industrial wastewater

After establishing the potential of *A. chlorophenolicus* A6 in treating synthetic wastewater spiked with mono- substituted phenols, both with single and mixed substrate systems, its performance in raw petroleum refinery wastewater treatment was evaluated. The characteristic of raw wastewater collected from Guwahati, India is given in [Table 5.19](#).

5.6.1 Preliminary batch study results

As the pH of the raw wastewater was 8.2, it was adjusted to 7.5 by using dilute HCl and NaOH solutions. This was done because the optimum pH for the growth of *A. chlorophenolicus* A6 was found to be 7.5 ([section 5.4.2](#)). Results of preliminary batch study revealed that the actinomycetes failed to remove COD from raw refinery wastewater as well as refinery wastewater diluted with distilled water. This might be due to deficiency of one or more components of MSM in raw wastewater essential for growth of *A. chlorophenolicus* A6. Toxicity exerted by other pollutants and heavy metals present in the real refinery wastewater might be the other cause for the poor performance.

Table 5.19 Characteristics of the petroleum refinery industry wastewater used in the study

Parameter	Concentration
Turbidity	327 NTU
Total dissolved solids	559 mg l ⁻¹
Conductivity	0.825 mS/cm
Dissolved oxygen concentration	0.95 mg l ⁻¹
pH	8.20
Oxidation reduction potential	201.8 mv
NH ₄ ⁺ -N	9.5 mg l ⁻¹
NH ₃ -N	0.753 mg l ⁻¹
Cl ⁻	740 mg l ⁻¹
NO ₃ ⁻	162 mg l ⁻¹
Ni	2 mg l ⁻¹
Pb	8 mg l ⁻¹
Sr	15 mg l ⁻¹
Phenol	20 mg l ⁻¹
COD	190 mg l ⁻¹

NTU* = Nephelo Turbidity Unit.

Figure 5.31 demonstrated the effect of mineral salt media MSM on COD removal efficiency (%). From the figure it was observed that, the COD removal efficiency was gradually increased when the refinery wastewater was diluted with MSM. More than 98% COD removal was observed from the refinery wastewater containing an initial COD of 447 mg l⁻¹ within 20h of culture, when mixed with equal volume of MSM. Although the refinery wastewater was spiked with mixture of substituted phenols (75 mg l⁻¹ of each of 4-CP, 4-BP and 4-NP), their concentrations were not measured any time during the shake flasks experiment. However the degradation of the substituted phenols was expressed in terms of equivalent COD removal performance. As 1 gm of 4-CP, 4-BP and 4-NP was found equivalent to 1.70, 1.46 and 1.53 gm of COD respectively. Hence, the refinery wastewater was mixed with equal volume of MSM before being treated in continuous mode of operation using the PBR system. Though, several reports suggested the presence of 30 - 200 mg l⁻¹ of phenolic compounds in petroleum refinery wastewater (Jou and Huang, 2003; Coelho et al., 2006; Al Zarooni and Elshorbagy, 2006; Toril, 2001; Cormack et al., 1998), in fact effluent concentration of phenolics reached even up to 2123 mg l⁻¹ (Almendariz, et al., 2005).

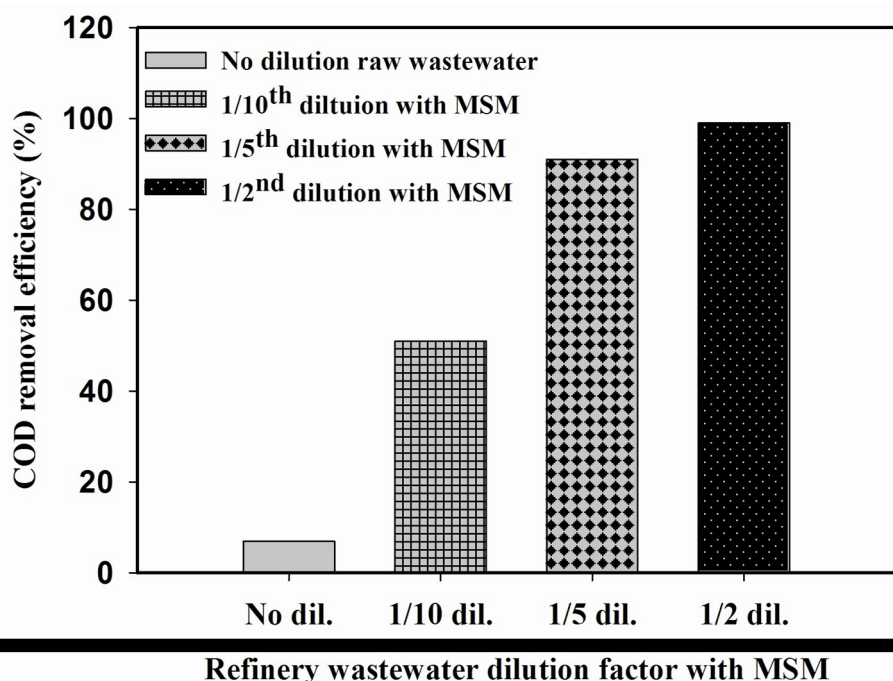


Figure 5.31 Effect of mineral salt media (MSM) on COD removal efficiency (%).

As 4-CP, 4-BP and 4-NP are the target pollutants of the present project, but neither of these pollutants were present in the refinery wastewater collected from Guwahati, India; therefore, the refinery wastewater was spiked with a mixture of these substituted phenols before being fed into the PBR.

5.6.2 Refinery wastewater treatment in PBR

Table 5.20 shows the operational schedule and performance of the newly designed PBR treating phenolic refinery wastewater spiked with substituted phenols. Supplemented with 250 mg l^{-1} of each of these three pollutants (stage 1). Once the effluent concentration of the reactor at any stage, in terms of substituted phenols concentration, was constant for 3–4 cycle of run, the influent pollutants mixture concentration was increased stepwise from stage-1 to stage 3.

Table 5.20 Operational schedule and performance of the newly designed PBR treating petroleum refinery wastewater spiked with substituted phenol at HRT 12.5h.

Stages of run	Day of run	Influent concentration (mgL ⁻¹)			Effluent concentration (mgL ⁻¹)			% removal efficiency		
		4-CP	4-BP	4-NP	4-CP	4-BP	4-NP	4-CP	4-BP	4-NP
1	1	250	250	250	0.800	0.00	0.00	99.68	100	100
	2	250	250	250	0.900	0.00	0.00	99.64	100	100
	3	250	250	250	0.820	0.00	0.00	99.67	100	100
	4	250	250	250	0.840	0.00	0.00	99.66	100	100
2	5	300	300	300	28.00	5.50	1.15	90.6	98.16	99.5
	6	300	300	300	26	4.5	1.2	91.33	98.5	99.6
	7	300	300	300	25.4	4.2	1.2	91.53	98.6	99.6
	8	300	300	300	25.5	4.25	1.4	91.5	98.6	99.4
	9	300	300	300	25.7	4.3	1.2	91.43	98.56	99.5
3	10	350	350	350	115.5	73.0	7.0	67.64	79.14	98.0
	11	350	350	350	113	71	7.5	67.71	79.7	97.8
	12	350	350	350	111	71.5	7.2	68.28	79.57	97.95
	13	350	350	350	111.5	71.9	7.25	68.14	79.45	97.92
	14	350	350	350	111.8	72	7.15	68.06	79.42	97.95
	15	350	350	350	111.7	71.7	7.25	68.05	79.51	97.92

HRT of the reactor was kept at 12.5h. The DO level of the reactor was maintained between 4-4.5 mgL⁻¹ throughout the operations. The reactor was started feeding refinery wastewater. The performance of the reactor on treating the refinery wastewater containing mixture of these three pollutants is shown in Fig. 5.32. The results revealed that at lower concentrations at stages 1 and 2, degradation efficiencies of all the pollutants in the wastewater were considerably high. Complete removal of all the three pollutants from the refinery wastewater was achieved at maximum individual pollutant loading rate of 569 mgL⁻¹day⁻¹ (stage 1). Figure 5.33 shows COD removal profile that suggests 97% removal at stages 1 and 2. This confirmed the use of substituted phenols as sole source of carbon by *A. chlorophenolicus* A6. At higher stages, substituted phenols removal efficiency was reduced especially for 4-CP and 4-BP. The efficiency of PBR on degradation of these three substituted phenols were once again found to be in the following order: 4-CP<4-BP<4-NP (Table 5.20).

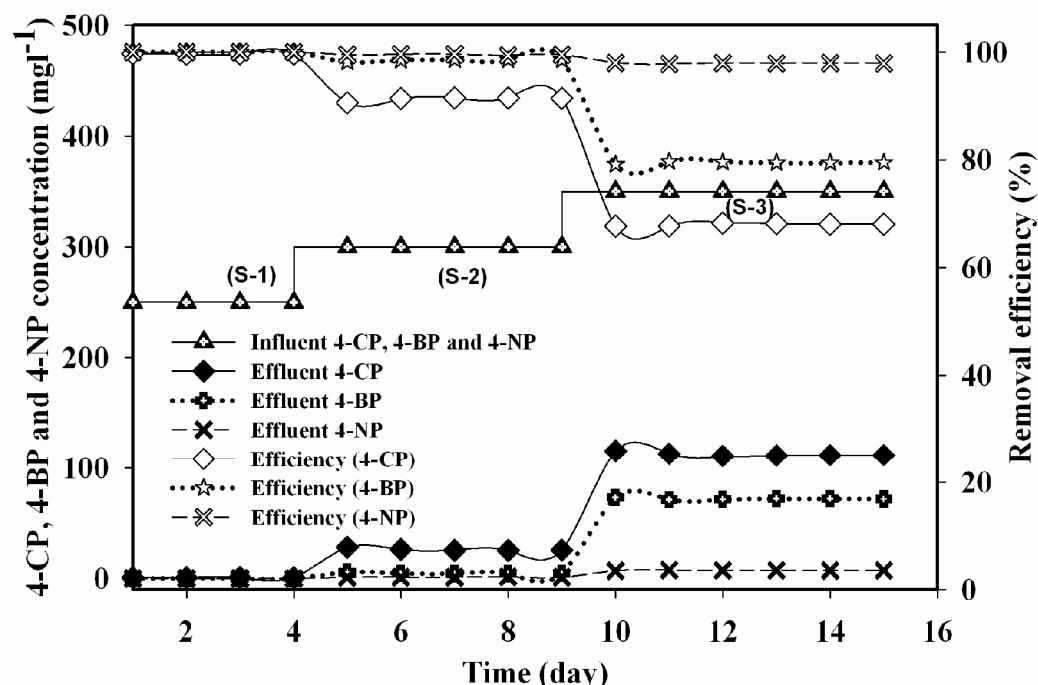


Fig. 5.32 Degradation performance of PBR in treating petroleum refinery wastewater containing mixture of 4-CP, 4-BP and 4-NP at 12.5h HRT by *A. chlorophenolicus* A6. (*S indicates stage of operation).

The reason for inferior degradation of 4-CP could be due to toxicity caused by the presence of high concentration chloride in raw refinery wastewater (Table 5.19). It was reported that the presence of chloride ion inhibited microbial activity (Cassidy et al., 1999; Stanlake and Fin 1982). A performance of the PBR on simultaneous removal of substituted phenols from synthetic wastewater (Table 5.17) was better than that of refinery wastewater (Table 5.20). This might be due to the presence of other foreign materials including heavy metals in refinery wastewater (Alexander, 1999), that might have affected the performance of *A. chlorophenolicus* A6. However, the toxic effect due to toxic metals, chloride and other foreign materials was not very significant especially on 4-BP and 4-NP biodegradation (Figure 5.32).

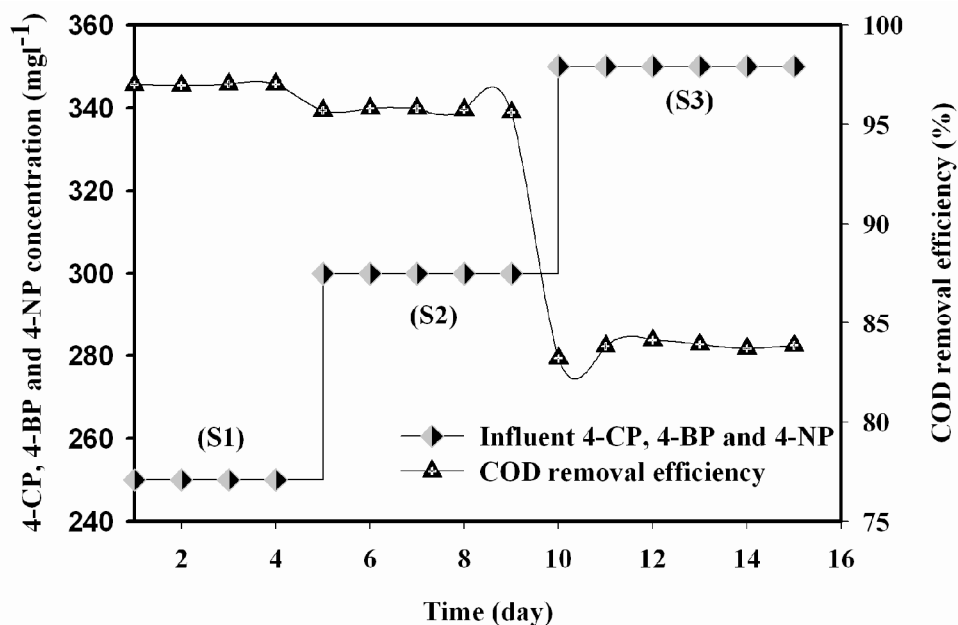


Fig. 5.33 COD removal performance of PBR in treating petroleum refinery wastewater containing mixture of 4-CP, 4-BP and 4-NP at 12.5h HRT by *A. chlorophenolicus* A6. (*S indicates stage of operation)

Figure 5.34 shows the reactor performance in terms of percentage removal efficiency and pollutant removal rate ($\text{mg l}^{-1} \text{day}^{-1}$) at different loading rates of these substituted phenols mixture spiked in the refinery wastewater. The degradation profile was found to be similar to that of mixed substrate system in synthetic wastewater (Figure 5.30, section 5.5.1). Complete removal of all these three pollutants was achieved at the maximum individual loading rate of $569 \text{ mg l}^{-1} \text{day}^{-1}$. However, when individual loading rates of the substituted phenols exceeded $569 \text{ mg l}^{-1} \text{day}^{-1}$, removal efficiency was gradually reduced in all the concentration combination tested. On other hand, the degradation rate was increased with the increase in loading rate of the individual pollutant, upto $683 \text{ mg l}^{-1} \text{day}^{-1}$. At loading rate more than $683 \text{ mg l}^{-1} \text{day}^{-1}$ the degradation rates of 4-CP and 4-BP were reduced. On contrary, there was a prominent increase in degradation rate of 4-NP at its entire loading rates investigated in the study. This further demonstrated the preferential uptake up 4-NP over the other two pollutants.

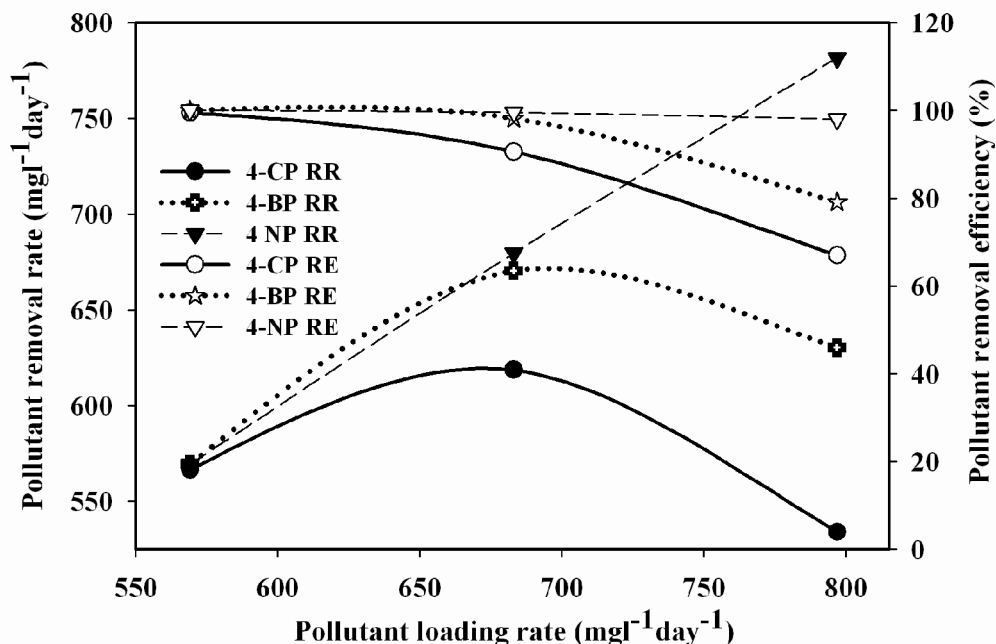


Figure 5.34 Substituted phenols removal efficiency and biodegradation rate in the PBR at different loading rates. (*RR = Removal rate; RE = Removal efficiency).

Figure 5.34 also indicates that it could be possible to achieve better 4-NP removal rate in the mixture had the reactor been operated at HRTs lower than 12.5 h at stage 1. As discussed earlier (section 5.5.1), the maximum 4-CP and 4-BP removal rate from synthetic wastewater was 618 and 670 mg l⁻¹ day⁻¹ at respective pollutant loading rate of 683 mg l⁻¹ day⁻¹; whereas, 4-NP removal was 781 mg l⁻¹ day⁻¹ at the loading rate of 796 mg l⁻¹ day⁻¹. Thus, the optimum conditions of PBR operation was observed at individual pollutant loading rate of 569 mg l⁻¹ day⁻¹ (stage 1) when concentration of individual pollutant was 250 mg l⁻¹ in the mixture.

Various reactors used on treatment of refinery wastewater and their performances are listed in Table 5.21. Most of these reactors were operated at low concentrations of phenol and/or substituted phenols. However, the PBR used in the present study was found to be more efficient than that of others, even if it was fed with higher substituted phenol loadings.

Thus, in the present study the degradation performance of the PBR treating the refinery wastewater spiked with these pollutant mixture was found better than activate sludge process, fixed film bioreactor system, expanded anaerobic sludge bed reactor, membrane bioreactor and airlift loop bioreactor systems (Table 5.21).

5.6.3 Toxicity removal efficiency in mixed substrate system in the PBR

The percentage (%) toxicity of influent refinery wastewater containing substituted phenols mixture at stage 1 (at 250 mg l⁻¹ each of 4-CP, 4-BP and 4-NP) was tested on mixed microbial consortia and found to be 84.23%. Percentage effluent toxicity and % toxicity removal with respect to the above mentioned influent was estimated to be 4.5% and 94.65%, respectively. Further, the substituted phenols toxicity removal from the refinery wastewater spiked with these pollutants using the PBR was found better than many other batch and continuous types of reactors used for treatment of various pollutants (Table 5.22). Through Gallego et al. (2003) reported 100% toxicity removal efficiency, concentration of pollutants mixture in the feed were less as shown in table 5.22.

5.7 Biodegradation of Substituted phenol using *A.chlorophenolicus* A6 cells immobilized with calcium alginate beads

In order to further extend the scope of this actinomycete in treating these phenolics, studies were aimed to compare the efficiency of the culture to degrade the substituted phenols with that of an emerging technique such as cell immobilization using a calcium alginate beads were also investigated in a continuous mode of operation using a simple PBR in the present study. Before starting the reactor study in continuous mode of operation using the cell immobilization calcium alginate bead system some preliminary batch experiment was carried out.

Table 5.21 Performance of various bioreactor systems for treatment of oil refinery wastewater

Waste Details	Influent Phenolics Conc. (mg l ⁻¹)	Phenolics Removal efficiency (%)	Influent COD Conc. (mg l ⁻¹)	COD Removal efficiency (%)	Bioreactors	Microorganisms	HRT (h)	References
Oil refinery wastewater	31	99	420	70	Activated sludge	Mixed culture	25	Jou and Huang, 2003
Oil refinery wastewater	30	100	510	85-90	pilot scale fixed-film bioreactor system	Mixed culture	8	Jou and Huang, 2003
Oil refinery wastewater Mixture of 2123, 2730 and 1379 mg l ⁻¹ of phenol, p-cresol and o-cresol	Supplemented with Nutrients		1.5 g COD l ⁻¹ day ⁻¹	90	Expanded granular sludge bed reactor Anaerobically	Mixed culture	-	Almendariz et al., 2005
Oil refinery wastewater	750	100	1268	97	PBR	<i>A. chlorophenolicus A6</i>	12.5	Present study

Table 5.22 Toxicity removal profile of in treating various pollutants with different biological system.

Waste Details	Influent Phenolics Conc. (mg l ⁻¹)	Toxicity Removal efficiency (%)	Bioreactors	Microorganisms	HRT (h)	References
2-4, DNP	10	72	Anaerobic systems	<i>Mixed culture</i>	-	Haghighi-Poden et al., 1995
2-4, DNP	40	94	Anaerobic Chemostat	<i>Mixed culture</i>	-	Uberoi and Bhattacharya, 1997
2,4,6-TCP	390	70	Batch shake flask	<i>Rhodococcus species</i>	240	Kargi and Pamukoglu 2008
2-CP, phenol and m-cresol	100, 50, 50	100	Fluidised bed reactor	Mixed culture	36	Gallego et al., 2003
Oil refinery wastewater 4-CP, 4-BP and 4-NP	250 each	94.39	Packed bed reactor	<i>A. chlorophenolicus A6</i>	12.5	Present study

* TCP = trichlorophenol, DNP = dinitrophenol

5. 7.1 Optimization of pH

Effect of pH on removal of 4-CP by cell immobilized alginate beads is shown in Figure 5.35.

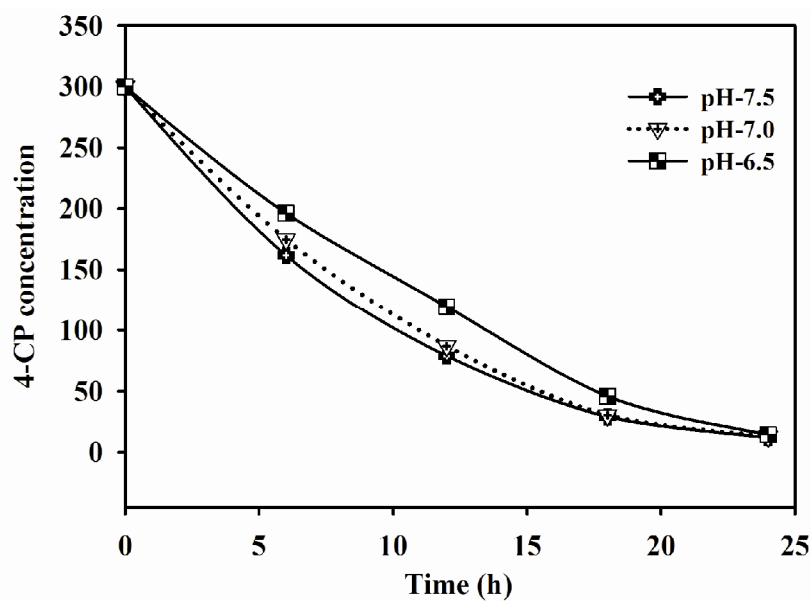


Figure 5.35 Effect of pH on removal of 4-CP by *A. chlorophenolicus* A6 cells immobilized with calcium alginate bead.

From figure it is clearly illustrated that the 4-CP degradation performance was found superior at higher pH (7.5 and 7) throughout the culture period. For instant percentage of 4-CP removal at pH 7.5, 7 was found to be 90.38 %, 89.89% respectively where as in case of pH 6.5 a lower value of 84.78% after 18 h of culture (Figure 5.35). However, the stability of the cell immobilized beads was found poor at higher pH. For example the alginate beads were found dissolved found dissolved after 15 h of culture. Based on these criteria, further studies were carried out at an optimized pH 6.5.

5.7.2 Biodegradation of Substituted phenol using *A. chlorophenolicus* A6 cells immobilized with calcium alginate beads in a simple PBR system.

The reactor was started with an influent 4-CP concentration of 100 mg l^{-1} . Once 4-CP concentration in the effluent of the reactor was constant for about 4-6 cycles of run, at a particular 4-CP loading rate the EBCT and/or the influent 4-CP concentration was increased/changed stepwise from 100 mg l^{-1} to 250 mg l^{-1} . The performance of the reactor on 4-CP biodegradation is shown in Figure 5.36. Complete removal (below detection level) of 4-CP was achieved from influent concentrations of 100 mg l^{-1} , 150 mg l^{-1} , and 200 mg l^{-1} , when the reactor was operated at HRTs 2 h, 3 h, and 8 h respectively, or higher.

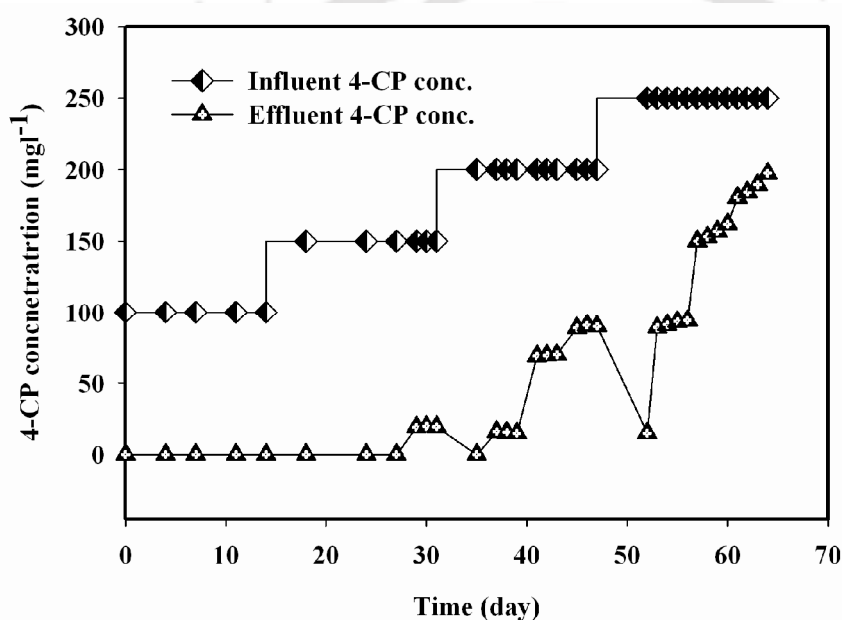


Figure 5.36 4-CP biodegradation profile of cell immobilized calcium alginate beads in the PBR

Thus, complete removal of 4-CP in the reactor was achieved at the maximum 4-CP loading rate of $1275.5 \text{ mg l}^{-1} \text{ day}^{-1}$ viz., when the reactor was operated at 3 h HRT with 150 mg l^{-1} influent 4-CP concentration.

Though, complete removal was not achieved, more than 94% removal occurred from influent 4-CP of 250 mg l^{-1} in the reactor operated at HRT of 8h. The reactor performance was observed to be inferior when operated at any other influent concentrations and/or HRTs listed in Table 5.23. The reactor performance became unstable at the influent 4-CP of 250 mg l^{-1} and 3h HRT. A prominent ascends in 4-CP concentration in the reactor effluent was observed on 57th day of run (Figure 5.36), when the reactor was introduced with wastewater containing 250 mg l^{-1} of 4-CP and operated with an HRT of 3 h (Table 5.23). This might be due to the toxicity effects of 4-CP at this concentration that inhibited the microorganisms from normal metabolic activities, leaving a significant amount of 4-CP unutilized. Removal of 4-CP was only 38% at this condition. Performance of the reactor was even worse when the HRT was lowered to 2 h, where 4-CP removal was only 24.5%.

Table 5.23 Operational schedule of the column reactor packed with *A. chlorophenolicus* A6 immobilized with calcium alginate beads.

Operation stages	Operation period (day)	EBCT (h)	Influent conc. 4-CP (mg l^{-1})
1	0-4	8	100
	5-7	4	100
	8-11	3	100
	12-15	2	100
	16-19	8	150
2	20-23	4	150
	24-27	3	150
	28-31	2	150
	32-35	8	200
3	36-38	4	200
	39-43	3	200
	44-47	2	200
	48-52	8	250
4	53-56	4	250
	57-60	3	250
	61-65	2	250

As described earlier, instability of the PBR in the present study at higher pollutant loading rate of $2125 \text{ mg l}^{-1} \text{ day}^{-1}$ (4-CP at 250 mg l^{-1} and HRT of 2h) could be due to combined toxic effects of chloride, 4-chlorocatechol, and 4-CP, as the presence of even 100 mg l^{-1} of chloride could inhibit *Sphingomonas* as well as *Arthrobacter* species on chlorophenol biodegradation (Klečka et al., 1981; Stanlake and Finn, 1982; Bartels et al., 1984; Cassidy et al., 1999). Further, instability of the reactor might be due to the diffusion limitation of the alginate beads with respect to the high loading rate of the 4-CP. Similar diffusion limitation was also reported by several authors using cell immobilized with calcium alginate beads (Tanaka et al., 1984; Shuler and Kargi, 1992; Chen et al., 1993; Aksu and Bulbul, 1999). Experimental data are re-plotted in Fig. 5.37 (a) and (b), showing the reactor performance in terms of 4-CP removal efficiency (%) and removal rate ($\text{mg l}^{-1} \text{ day}^{-1}$), respectively, at different 4-CP loading rates. Complete removal of 4-CP in the reactor was achieved at the maximum 4-CP loading rate of $1275.5 \text{ mg l}^{-1} \text{ day}^{-1}$ when the reactor was operated at 3 h HRT and 150 mg l^{-1} influent 4-CP concentration [Fig. 5.37 (a)]. In the present system, at loading rates greater than $1275.5 \text{ mg l}^{-1} \text{ day}^{-1}$, 4-CP removal efficiency was gradually reduced irrespective of the influent concentration. Though, complete 4-CP removal was observed at the 4-CP loading rate of $1275.5 \text{ mg l}^{-1} \text{ day}^{-1}$, maximum 4-CP removal rate of $1474 \text{ mg l}^{-1} \text{ day}^{-1}$ was obtained at the loading rate of $1700.5 \text{ mg l}^{-1} \text{ day}^{-1}$ (4-CP = 150 mg l^{-1} , HRT = 2 h) [Figure 5.37 (b)]. Fig. 5.37 (b) also indicates that it could be possible to achieve better 4-CP removal rate had the reactor been operated at HRTs lower than 2h with 4-CP influent concentration of 100 mg l^{-1} or lesser. However, the reactor performance (% removal) decreased with increase in 4-CP loading rate leaving high 4-CP concentration in the treated effluent [Fig. 5.37 (a)]. Thus, the optimum conditions of PBR operation was observed at the 4-CP loading rate of $1275.5 \text{ mg l}^{-1} \text{ day}^{-1}$ for an inlet concentration of 150 mg l^{-1} .

Although there are several reports available on phenol biodegradation in bioreactor system using cell immobilized alginate beads, reports on substituted phenol removal specifically on 4-CP, 4-BP and 4-NP removal is scanty.

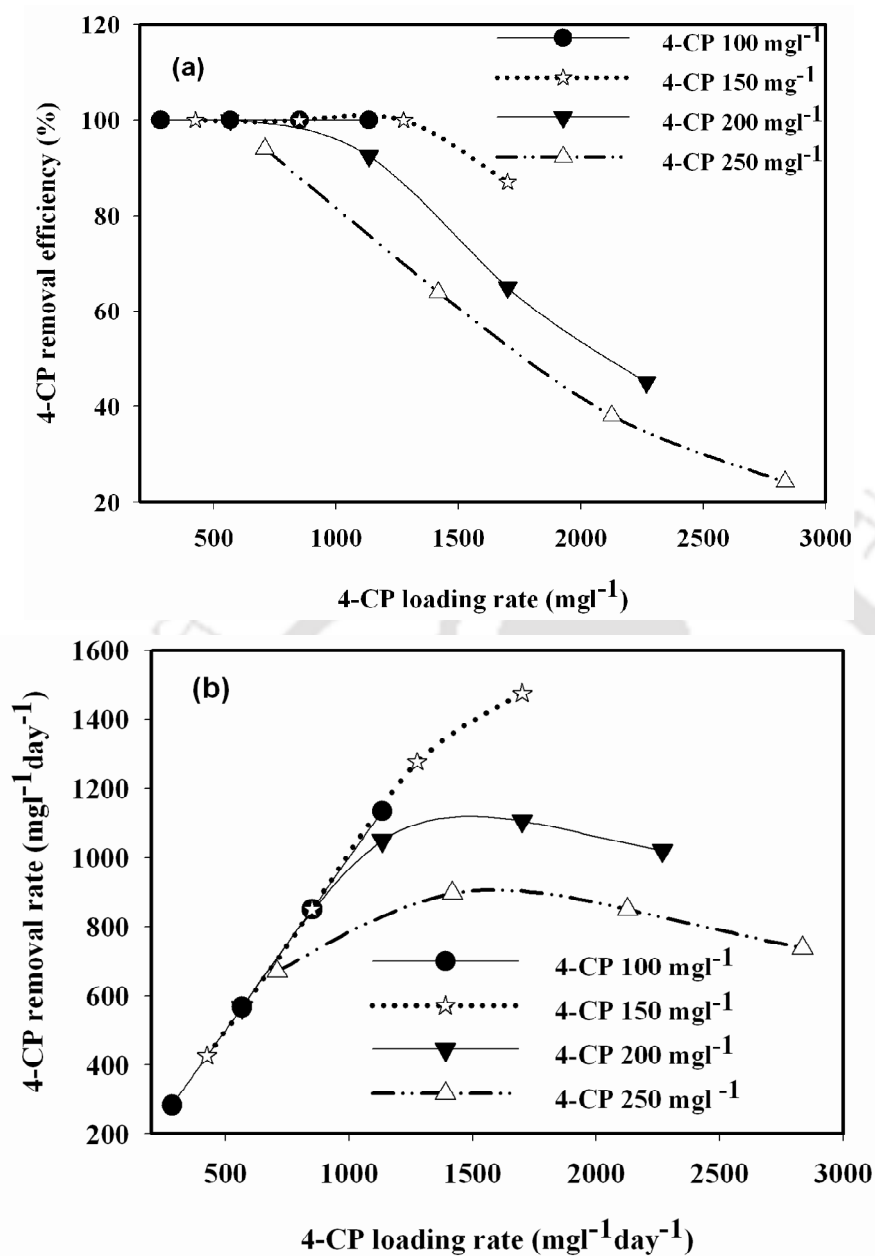


Figure 5.37 (a) Variation of 4-CP removal efficiency and (b) 4-CP biodegradation rate with respect to different 4-CP loading rates in the PBR using cell immobilized calcium alginate beads.

Further, the optimum pollutant loading rate ($1275 \text{ mg l}^{-1} \text{ day}^{-1}$) and the concentration level (150 mg l^{-1}) of 4-CP at which the calcium alginate immobilized cells degraded completely was however; lower than that in newly designed PBR where completely removal was achieved at pollutant loading rate $1707 \text{ mg l}^{-1} \text{ day}^{-1}$ and at an influent 4-CP concentration of 600 mg l^{-1} (section 5.4.5).

The higher 4-CP degradation efficiency observed in the newly designed PBR might be due to high biomass growth (366 mg g^{-1} of PUF with respect to 6.5 mg g^{-1} of alginate bead) and thus better 4-CP biodegradation performance. On the other hand, lower degradation performance in cell immobilized with calcium alginate bead system may be due to the fact such as; (i) lower operational pH of 6.5 to that of newly designed PBR (pH of 7.5). (ii) Alginate beads provide lower surface area as well as porosity for biomass growth and substrate diffusion with compared to PUF and this might have causes pollutant diffusion limitation (Tanaka et al., 1984; Shuler and Kargi, 1992; Chen et al., 1993; Aksu and Bulbul, 1999). However, these aspects need to investigate further to confirm. Overall the substituted phenol degradation performance of the newly designed PBR with cell immobilized PUF is found superior to that of the simple PBR reactor using cell immobilized with calcium alginate beads.

SUMMARY AND CONCLUSION

- Substituted phenols such as 4-CP, 4-BP and 4-NP are highly toxic and are identified as major phenolic pollutants that emanate from various industrial effluents. An efficient treatment method for treatment these pollutants is sought. Biological treatment using microorganism as compared to physico-chemical methods, are showing more promise for treating such wastewater. Among the aerobic substituted phenols degrading microorganisms *A. chlorophenolicus* A6 is found to be one of the most potential candidate and degrade the compound by a novel route via hydroxyquinol pathway with reductive dechlorination being one of the key intermediate steps in the process.
- For enhancing its biodegradation efficiency, optimization of the media components and culture conditions were first carried out in batch shake flasks employing statistical design of experiments including 2-level factorial design, central composite design and response surface methodology (RSM). At the optimized settings of the parameters, not only very good enhancement in the 4-CP biodegradation efficiency was achieved but also could degrade the highest concentration of 4-CP reported so far in literature in batch shake flask mode.
- Batch degradation kinetics of the individual phenolic compounds were established for initial concentrations in the range of 25 – 350 mg l⁻¹ for 4-CP and 4BP, and 25-225 mg l⁻¹ for 4-NP in batch shake flasks. Various bio-kinetic parameters such as maximum specific growth rate (μ_m), half saturation constant (k_s) and inhibition coefficient (K_i) were estimated by fitting suitable substrate inhibition models to the experimental data. All these parameters values established very good performance of the microorganism in degrading sufficient high concentration of the phenolics within a reasonable short period of time. While 4-CP and 4-BP resulted in good biomass yield of the culture with increase in concentration of the substrate, any increase in 4-NP concentration did not improve the yield value much. However, at high concentration of the substrates the microorganism utilized the carbon sources mainly for its maintenance purpose.

Summary and Conclusion

- Degradation of a mixture of phenolics at various initial concentrations combinations was studied in batch shake flasks for finding the ability of the microorganism to grow and degrade these compounds simultaneously. Experiments in this particular study were based on statistically planned full factorial design of experiments. The obtained results were also analysed statistically in the form of ANOVA and student 't' test, which rendered useful interpretations of the main and interaction effects of the two substrates on each other degradations as well as the culture growth. In general, 4-NP was preferentially degraded by the microorganism followed by 4-BP and 4-CP in the mixture.
- Proportion of 4-CP, 4-BP and 4-NP in the mixture was observed to be an important factor affecting their degradation performance by the microorganism, high concentration of 4-CP strongly inhibited the degradation of 4-NP. Whereas, very high concentration of 4-CP and 4-NP were self inhibitory towards their own degradation and degradation of 4-BP was not affected much due to an increase in its concentration in the mixture.
- In order to investigate the performance of the *A. chlorophenolicus* A6 in continuous treatment of phenolics wastewater a newly designed packed bed reactor (PBR) was fabricated. The hydrodynamic condition of the newly designed PBR was found improved by employing cross flow design and perforated disc wrapped by nylon mesh together with ceramic gas diffuser. The microorganism was immobilized by growing on to PUF that was used as packing material for the reactor.
- The performance of the newly designed PBR in continuous mode of operation was found to be competent in treating the phenolics with 100% efficiency even at low value of HRTs. However, a slight fluctuation and instability in the reactor performance at high concentrations particularly for 4-CP was noticed. Further, continuous mode of operation proved highly efficient than simple batch shake flask mode in degrading phenolics at much high concentration thus overcoming the problem of substrate inhibition.
- Bioreactor stability in the study was evaluated by subjecting it to operations leading to intermittent and shock loading conditions. Where continuous experiments were performed at two different conditions by sudden increase in loading rate of the pollutants.

Summary and Conclusion

The reactor regained its original stable performance in handling the shock condition even at high pollutant loading rates.

- Industrial wastewater sample collected from a petroleum refinery and spiked with the substituted phenols was tested for evaluating the potential of *A.chlorophenolicus A6* in treating real industrial effluents. Results revealed nearly same degradation efficiency of refinery wastewater as that of synthetic wastewater but only up to a concentration of 300 mgL⁻¹ each of the substituted phenols. Completely substituted phenol removal, more than 97% COD removal and 94.6 % toxicity removal were achieved in the study. The toxic effects of heavy metals and other pollutants present in the refinery wastewater were attributed to the reduced performance of the microorganism in treating the phenolic at concentration higher than 300 mgL⁻¹ each.
- Performance of *A. chlorophenolicus A6* to degrade 4-CP was also investigated in a column reactor packed with the microorganism immobilized inside calcium alginate beads. Completely removal was achieved at an influent concentration of 250 mgL⁻¹ for an empty bed contact time of 3 h. However, the degradation performance was not good when compared with that of the newly designed PBR mainly due to its improved hydrodynamic conditions.
- High degradation efficiency in continuous treatment of both industrial wastewater (from petroleum refinery) as well as synthetic wastewater containing the substituted phenol was achieved using the *A. chlorophenolicus A6* in the newly designed PBR. Thus establishing its potential to treat phenolics containing wastewaters and its industrial application.

SUGESTION FOR FUTURE WORK

.....

Based on the findings of the present investigation, the following suggestions are made for future studies:

- In order to confirm the microbial colonization in PUF in the PBR system clone library analysis or quantitative real-time PCR using specific primer set of strain A6 is need to investigate further.
- Estimation of kinetic parameters in multiple substrate system.
- Scale up studies with the newly designed PBR for field applications.
- Mathematical modeling of the phenolic degradation and iimobilized biomass growth in the continuously operated PBR for better understanding and predictability of the system performance.
- Feasibility of using sequential aerobic and anerobic degradation for phenolics containing wastewater.
- Microbial community analysis during long continous operation of the bioreactor system treating the phenolic contaminated wastewater.
- Mechanism details of preferential uptake of 4-NP over 4-CP and 4-BP, analysis of cellular fatty acid content in response to 4-NP, 4-CP and 4-BP uptake by the microorganism.

BIBLIOGRAPHY

- [1] **Abdel-Fattah, Y.R., Saeed, H.M., Gohar, Y.M., El-Baz, M.A.** (2005) Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. *Process Biochemistry* 40: 1707-1714
- [2] **Abuhamed, T., Bayraktar, E., Mehmetolu, T., and Mehmetolu, U.** (2004) Kinetics model for the growth of *Pseudomonas putida* F1 during benzene, toluene and phenol biodegradation. *Process Biochemistry* 39: 983-988.
- [3] **Acuna-Askar, K., Englande Jr, A.J., Ramirez-Medrano, A., Coronado-Guardiola, J.E., Chavez-Gomez, B.** (2003) Evaluation of biomass production in unleaded gasoline and BTEX-fed batch reactors. *Water Science and Technology* 48:127–133.
- [4] **Ahn, Y., Rhee, S., Fennell, D., Kerkhof, L.J., Hentschel, U., Haeggblom, M.** (2003) Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge *Aplysina aerophoba*. *Applied Environmental Microbiology* 69: 4159–4166.
- [5] **Aiba, S., Shoda, M., Nagalani, M.** (1968) Kinetics of product inhibition in alcohol Fermentation. *Biotechnology and Bioengineering* 10: 845–864.
- [6] **Aiken, B.S., and Logan, B.E.** (1996) Degradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium* grown in ammonium lignosulphonate media. *Biodegradation* 7:175–182.
- [7] **Ajmera, S.K., Delattre, C., Schmidt, M.A., Jensen, K.F.** (2002) Micro fabricated cross-flow chemical reactor for catalyst testing. *Sensors and Actuators, B: Chemical* 82: 297–306.
- [8] **Akdogan, H.A., and Pazarlioglu, N.K.** (2011) Fluorene biodegradation by *P. osteratus* – Part II: Biodegradation by immobilized cells in a recycled packed bed reactor. *Process Biochemistry*. 46:840-846.

- [9] **Aksu, Z., and Bu"lbu"l, G.** (1999) Determination of the effective diffusion coefficient of phenol in Ca-alginate-immobilized *P. putida* beads. *Enzyme and Microbial Technology* 25: 344–348.
- [10] **Alexandar, M., and Lustigman, B.K.** (1966) Effect of chemical structure on microbial degradation of substituted benzenes. *Journals of Agriculture and Food Chemistry* 14: 410-413.
- [11] **Alexander, M.** (1999) Biodegradation and Bioremediation, 2nd ed. Academic Press, San Diego, CA. UK.
- [12] **Alleman, B.C., Logan, B.E., Gilbertson, R.L.** (1992) Toxicity of pentachlorophenol to six species of white rot fungi as a function of chemical dose. *Applied Environmental Microbiology* 58: 4048-4050.
- [13] **Alleman, B.C., Logan, B.E., Gilbertson, R.L.** (1995) Degradation of pentachlorophenol by fixed films of white-rot fungi in rotating tube bioreactors. *Water Research* 29:61–67.
- [14] **Almendariz, F.J., Meraz, M., Olmos, A.D., Monroy, O.** (2005) Phenolic refinery wastewater biodegradation by an expanded granular sludge bed reactor. *Water Science and Technology* 52:391-396.
- [15] **Alnaizy, R. and Akgerman, A.** (2000) Advanced oxidation of phenolic compounds. *Advances in Environmental Research* 4(3): 233-244.
- [16] **Al-Zarooni, M., and Elshorbagy, W.** (2006) Characterization and assessment of Al Ruwais refinery wastewater. *Journal of Hazardous Materials* 136: 398–405.
- [17] **Andrews, J.F.** (1968) A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. *Biotechnology and Bioengineering* 10:707-723.
- [18] **Annachhatre, A.P., and Gheewala, S.H.** (1996) Biodegradation of chlorinated phenolic compounds. *Biotechnology Advanced* 14: 35–56.
- [19] **Apajalahti, J.H.A., and Salkinoja-Salonen, M.S.** (1986) Degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. *Applied Microbiology and Biotechnology* 25:62–67.
- [20] **APHA.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, DC.

- [21] **Backman, A., and Jansson, J.K.** (2004) Degradation of 4-chlorophenol at low temperature and during extreme temperature fluctuations by *Arthrobacter chlorophenolicus* A6. *Microbial Ecology* 48:246–253.
- [22] **Backman, A., Maraha, N., Jansson, J. K.** (2004) Impact of temperature on the physiological status of a potential bioremediation inoculant, *Arthrobacter chlorophenolicus* A6. *Applied and Environmental Microbiology* 70: 2952-2958.
- [23] **Bae, H.S., Lee, J.M. Lee, S.T.** (1996a) Biodegradation of 4-chlorophenol via a hydroquinone pathway by *Arthrobacter ureafaciens* CPR706. *FEMS Microbiology Letters* 145:125-129.
- [24] **Bae, H.S., Lee, J.M., Kim, Y.B., Lee, S.T.** (1996b) Biodegradation of the mixtures of 4- chlorophenol and phenol by *Comamonas testosteroni* CPW301. *Biodegradation* 7: 463-469.
- [25] **Bae, H.S., Yamagishi, T., Suwa, Y.** (2002) Evidence for degradation of 2-chlorophenol by enrichment cultures under denitrifying conditions. *Microbiology* 148: 221–227.
- [26] **Bae, S.C.** (1996) Biodegradation of 4-chlorophenol via a hydroquinone pathway by *Arthrobacter ureafaciens* CPR706. *FEMS Microbiology Letters* 145: 125-129.
- [27] **Bahnmann, D.W.** (2004) Photocatalytic water treatment: solar energy applications. *Solar Energy* 77: 445-459.
- [28] **Bai, J., Wen, J.P., Li, H.M. Jiang, Y.** (2007) Kinetic modeling of growth and biodegradation of phenol and *m*-cresol using *Alcaligenes faecalis*. *Process Biochemistry* 42: 510-517.
- [29] **Bailey, J.E. and Ollis, D.F.** (1986) *Biochemical Engineering Fundamentals* (second edition), McGraw-Hill, New York, NY.
- [30] **Bandyopadhyay, K., Das, D., Maiti, B.R.** (1998) Kinetics of phenol degradation using *Pseudomonas putida* MTCC 1194. *Bioprocess Engineering* 18: 373 – 377.
- [31] **Banerjee, I., Modak, J.M., Bandyopadhyay, K., Das, D., Maiti, B.R.** (2001) Mathematical model for evaluation of mass transfer limitations in phenol biodegradation by immobilized *Pseudomonas putida*. *Journal of Biotechnology* 87: 211-223.
- [32] **Banerji, S.K., and Bajpai, R.K.** (1994) Cometabolism of pentachlorophenol by

- microbial species. *Journal of Hazardous Materials* 39: 19–31.
- [33] **Barros, L.M., Macedo, G.R., Duarte, M.M.L., Silva, E.P., Lobato, A.K.C.L.** (2003) Biosorption of cadmium using the fungus *Aspergillus niger*. *Brazilian Journal of Chemical Engineering* 20: 229-239.
- [34] **Bartels, I., Knackmuss, H.J., Reineke, W.** (1984) Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols, *Applied Environmental Microbiology* 47: 500–505.
- [35] **Battersby, N.S. and Wilson, V.** (1989) Survey of anaerobic biodegradation potential of organic chemicals in digesting sludge. *Applied Environmental Microbiology* 55: 433–9.
- [36] **Becker, J.G., Stahl, D.A., Rittmann, B.E.** (1999) Reductive dehalogenation and conversion of 2-chlorophenol to 3- chlorobenzoate in a methanogenic sediment community: Implications for predicting the environmental fate of chlorinated pollutants. *Applied Environmental Microbiology* 65: 5169–5172.
- [37] **Beltrame, P., Beltrame, P.L., Carniti, P., Pitea, D.** (1982) Kinetics of biodegradation of mixtures containing 2,4-di chloro phenol in a continuous stirred reactor. *Water Research* 16: 429–434.
- [38] **Bergman, A.** (1990) Brominated flame retardants in a global environmental perspective. In: Freij L, ed. *Proceedings of the workshop on brominated aromatic flame retardants, Skokloster, 24–26 October 1989*. Solna, National Chemicals Inspectorate (KEMI).
- [39] **Bhatti, Z.I., Toda, H., Furukawa, K.** (2002) p-Nitrophenol degradation by activated sludge attached on nonwovens. *Water Research* 36: 1135–1142.
- [40] **Bhushan, B., Chauhan, A., Samanta, S.K., Jain, R.K.** (2000). Kinetics of biodegradation of p-nitrophenol by different bacteria. *Biochemical and Biophysical Research Communication* 274: 626–630.
- [41] **Bisaillon, J.G., Lepine, F., Beaudet, R., Sylvestre, M.** (1993) Potential for carboxylation-dehydroxylation of phenolic compounds by a methanogenic consortium. *Canadian Journal of Microbiology* 39: 642–648.
- [42] **Blanch, H.W. and Clark, D.S.** (1996) Biochemical engineering. Marcel dekker Inc, New York.

- [43] **Booth, R.A. and Lester, J.N.** (1995) The potential formation of halogenated by-products during peracetic acid treatment of final sewage effluent. *Water Research* 29:1793–1801.
- [44] **Borras, C., Laredo, T., Scharifker, B.R.** (2003) Competitive electrochemical oxidation of p-chlorophenol and p-nitrophenol on Bi-doped PbO₂. *Electrochimica Acta* 48: 2775- 2780.
- [45] **Boudenne, J., Cercleir, O., Galéa, J., Van Der Vlist, E.** (1996) Electrochemical oxidation of aqueous phenol at a carbon black slurry electrode. *Applied Catalysis A: General* 143: 185-202.
- [46] **Boyle, J.L., Lindsay, R.C., Stuiber, D.A.** (1992) Bromophenol distribution in salmon and selected seafoods of fresh water and saltwater origin. *Journal of Food Science* 57: 918–922.
- [47] **Bradford, M.M.** (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
- [48] **Briglia, M., Nurmiäho-Lassila, E.L, Vallini, G., Salkinoja-Salonen, M.S .**(1990) The survival of the pentachlorophenol-degrading *Rhodococcus chlorophenolicus* PCPol and *Flavobacterium* sp. in natural soil. *Biodegradation* 1: 273-281
- [49] **Brillas, E., Sauleda, R., Casado, J.** (1998) Degradation of 4-chlorophenol by anodic oxidation, electro-Fenton, photo electro-Fenton and peroxicoagulation processes. *Journal of the Electrochemical Society* 145: 2253-2262.
- [50] **Bruning, T., Chronz, C., Their, R., Havelka, J., Ko, Y., Bolt, H.M.** (1999) Occurrence of urinary tract tumors in miners highly exposed to dinitrotoluene. *Occupational and Environmental Medicine* 41: 144–149.
- [51] **Buitro'n, G., Schoeb, M.E., Moreno-Andrade, I., Moreno, J.A.** (2005) Evaluation of two control strategies for a sequencing batch reactor degrading high concentration peaks of 4-chlorophenol. *Water Research* 39: 1015–1024.
- [52] **Bumpus, I. and Tatarko, M.** (1994) Biodegradation of 2,4,6-trinitrotoluene by Phanerochaete chrysosporium: identification of initial degradation products and the discovery of a TNT metabo-Me that inhibits lignin peroxidase. *Current Microbiology* 28:185-190.

- [53] **Burns, L.A., Bradley, S.G., White J.K.L.** (1994) Immunotoxicity of mono-nitrotoluenes in female B6C3F1 mice: I. Para-nitrotoluene, *Drug and Chemical Toxicology* 17: 317–358.
- [54] **Calleja, G., Serna, J. and Rodríguez, J.** (1993) Kinetics of adsorption of phenolic compounds from wastewater onto activated carbon. *Carbon* 31: 691-697.
- [55] **Carmona, M.R., da Silva, M.A.P., Leite, S.G.F.** (2005) Biosorption of chromium using factorial experimental design. *Process Biochemistry* 40: 779-788.
- [56] **Carvalho, M.F., Vasconcelos, I., Bull, A.T., Castro, P.M.L.** (2001) A GAC biofilm reactor for the continuous degradation of 4-chlorophenol: treatment efficiency and microbial analysis, *Applied Microbiology and Biotechnology* 57: 419–426.
- [57] **Cassidy, M.B., Lee, H., Trevors, J.T., Zablotowicz, R.B.** (1999) Chlorophenol and nitrophenol metabolism by *Sphingomonas* sp UG30. *Journal of Industrial Microbiology and Biotechnology* 23: 232-241.
- [58] **Cejkova, A., Masak, J., Jirku, V., Vesely, M., Patek, M., Nesvera, J.** (2005) Potential of *Rhodococcus erythropolis* as a bioremediation organism. *World Journal of Microbiology and Biotechnology* 21: 317-321.
- [59] **Chalmers, J.J. and Bavarian, F.** (1991) *Biotechnology Progress* 7: 151.
- [60] **Chatonnet, P., Bonnet, S., Boutou, S., Labadie, M.D.** (2004) Identification and responsibility of 2,4,6-tribromoanisole in musty, corked odors in wine. *Journal of Agricultural and Food Chemistry* 52:1255–1262.
- [61] **Chen, D. and Ray, A.K.** (1998) Photodegradation kinetics of 4-nitrophenol in TiO₂, suspension. *Water Research* 32: 3223–3234.
- [62] **Chen, D., Lewandowski, Z., Roe, F., Surapaneni, P.** (1993) Diffusivity of Cu²⁺ in Ca-alginate gel beads. *Biotechnology and Bioengineering* 41: 755– 60.
- [63] **Chen, F. and Johns, M.R.** (1996) Relationship between substrate inhibition and maintenance energy of *Chlamydomonas reinhardtii* in heterotrophic culture. *Journal of Applied Phycology* 8: 15–19.
- [64] **Chern, M. and Chien, Y.** (2003) Competitive adsorption of benzoic acid and p-nitrophenol onto activated carbon: isotherm and breakthrough curves. *Water research* 37, 2347–2356.

- [65] **Cherry, R.S. and Hull, C.T.** (1992) *Biotechnology Progress* 8: 11.
- [66] **Chih-Jen, L., Chi-Mei, L., Chiou-Zong, H.** (1996) Biodegradation of chlorophenols by immobilized pure-culture microorganisms. *Water Science and Technology* 34: 67-72.
- [67] **Chisti, Y.** (2001) *Critical Review of Biotechnology* 21: 67.
- [68] **Cho, Y.G., Rhee, S.K., Lee, S.T.** (2000) Influence of phenol on biodegradation of *p*-nitrophenol by freely suspended and immobilized *Nocardioides* sp. NSP41. *Biodegradation* 11: 21–28.
- [69] **Cho, Y.G., Yoon, J.H., Park, Y.H., Lee, S.T.** (1998) Simultaneous degradation of *p*nitrophenol and phenol by a newly isolated *Nocardioides* sp. *Journal of General and Applied Microbiology* 44: 303-309.
- [70] **Chudoba, J., Albokova, J., Lentge, B., Kummel, R.** (1989) Biodegradation of 2,4-dichlorophenol by activated sludge microorganisms. *Water Research* 23: 1439–1442
- [71] **Chung, H.Y., Ma, W.C.J., Kim, J.S.** (2003b) Seasonal distribution of bromophenols in selected Hong Kong seafood. *Journal of Agricultural and Food Chemistry* 51:6752–6760.
- [72] **Chung, N., Kang, G.Y., Kim, G.H., Lee, I.S., Bang, W.G.** (2001) Effect of nutrient nitrogen on the degradation of pentachlorophenol by white rot fungus, *Phanerochaete chrysosporium*. *Journal of Microbiology and Biotechnology* 11: 704–708.
- [73] **Clayton, G.D., Clayton, F.E., eds.** (1993) *Patty's industrial hygiene and toxicology. Vol. 2. Toxicology.* New York, NY, John Wiley and Sons.
- [74] **Cobos-Vasconcelos, D.D.L., Santoyo-Tepole, F., Juarez-Ramirez, C., Ruiz-Ordaz, N., Galindez-Mayer, C.J.J.** (2006) Cometabolic degradation of chlorophenols by a strain of *Burkholderia* in fed-batch culture. *Enzyme and Microbiol Technology* 40: 57–60.
- [75] **Coelho, A., Castro, A.V., Dezotti, M., Sant'Anna Jr., G.L.** (2006) Treatment of petroleum refinery sourwater by advanced oxidation processes. *Journal of Hazardous Materials* 137: 178–184.

- [76] **Copley, S.** (1997) Diverse mechanistic approaches to difficult chemical transformations: microbial dehalogenation of chlorinated aromatic compounds. *Chemistry & Biology* 4:169-174.
- [77] **Copley, S.D.** (2000) Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. *Trends in Biochemical Sciences* 25: 261-265.
- [78] **Cormack, P.M., Jones, P., Hetheridge, M.J., Rowland, S.J.** (1998) Analysis of oilfield produced waters and production chemicals by electrospray ionization multi-stage mass spectrometry. *Water Research* 35: 3567–78.
- [79] **Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M.** (1995) Microbial biofilms. *Annual Review of Microbiology* 49: 711–745.
- [80] **Costura, R.K. and Alvarez, P.J.** (2000) Expression and longevity of toluene dioxygenase in *pseudomonas putida* fl induced at different dissolved oxygen concentrations *Water Research* 34, 3014-3018.
- [81] **Coulter, C., Kennedy, J.T., McRoberts, W.C., Harper, D.B.** (1993) Purification and properties of an s-adenosylmethionine 2,4- disubstituted phenol o-methyltransferase from *Phanerochaete chrysosporium*. *Applied Environmental Microbiology* 59: 706–711.
- [82] **Crosby, D.G.** (1981) Environmental chemistry of pentachlorophenol. *Pure and Applied Chemistry* 53: 1051-1080.
- [83] **Danuta, W., Urszula, G., Izabela, G., Magdalena, P., Katarzyna, H.K.** (2011) Induction of aromatic ring: cleavage dioxygenases in *Stenotrophomonas maltophilia* strain KB2 in cometabolic systems. *World Journal of Microbiology and Biotechnology* 27: 805–811.
- [84] **Dargaville, T.R., Guerzoni, F.N., Looney, M.G., Solomon D.H.** (1996) The adsorption of multinuclear phenolic compounds on activated carbon. *Journal of Colloid and Interface Science* 182: 17-25.
- [85] **Dash, S.S. and Sathyanarayana, N.** (2007) Enhanced biodegradation of caffeine by *Pseudomonas* sp. using response surface methodology. *Biochemical Engineering Journal* 36: 288–293.
- [86] **de Wit, C.A.** (2000) Brominated Flame Retardants. Swedish Environmental Protection Agency, Report 5065, Stockholm, Sweden.

- [87] **Delia, U. and Aysen, M.** (2005) Treatment of 2,4-dichlorophenol (DCP) in a sequential anaerobic (upflow anaerobic sludge blanket) aerobic (completely stirred tank) reactor process. *Process Biochemistry* 40: 3419–3428.
- [88] **Derringer, G. and Suich, R.** (1980) Simultaneous optimization of several response variables. *Journal of Quality Technology* 12: 214-219.
- [89] **Deschler, C., Duran, R., Junqua, M., Landou, G., Salvado, J.C., Goulas, P.** (1998) Involvement of 3,4-dichlorophenol hydroxylase in degradation of 3,4-dichlorophenol by the white rot fungus *Phanerochaete chrysosporium*. *Journal of Molecular Catalysis B Enzyme* 5: 423–428.
- [90] **Dolon, B.A., Razo-Flares, E., Lettiga, G., Field, J.A.** (1996) Continuous detoxification, transformation and degradation of nitrophenol in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnology and Bioengineering* 51: 439-449.
- [91] **Donlon, B.A., Razo-Flores, E., Field, J.A., Lettinga, G.** (1995) Toxicity of N substituted aromatics to acetoclastic methanogenic activity in granular sludge. *Applied Environmental Microbiology* 61: 3889–93.
- [92] **Donoso, C., Becerra, J., Martínez, M., Garrido, N., Silva, M.** (2008) Degradative ability of 2,4,6-tribromophenol by saprophytic fungi *Trametes versicolor* and *Agaricus augustus* isolated from Chilean forestry. *World Journal of Microbiology and Biotechnology* 24: 961–968.
- [93] **Dunnick, J.K., Elwell, M.R., Bucher, J.R.** (1994) Comparative toxicities of o-, m-, and p-nitrotoluene in 13-week feed studies in F344 rats and B6C3F1 mice. *Fundamental and Applied Toxicology* 22: 411–421.
- [94] **Eckenfelder, W., Argaman, Y., Miller, E.** (1989) Process selection criteria for the biological treatment of industrial wastewaters. *Environmental Progress* 8: 40- 45.
- [95] **Edgehill, R.U., Finn, R.K.** (1983a) Activated sludge treatment of synthetic waste water containing pentachlorophenol. *Biotechnology and Bioengineering* 25: 2165–2176.
- [96] **Edgehill, R.U.** (1994) Pentachlorophenol removal from slightly acidic mineral salts, commercial sand, and clay soil by recovered *Arthrobacter* strain ATCC 33790. *Applied Microbiology Biotechnology* 41: 142-148.

- [97] **Edwards, V.H.** (1970) The influence of high substrate concentrations on microbial kinetics. *Biotechnology and Bioengineering* 12: 679–712.
- [98] **Egli, T.** (1995) The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. *Advances in Microbial Ecology* 14: 305-386.
- [99] **Eker, S. and Kargi, F.** (2007) Performance of a hybrid-loop bioreactor system in biological treatment of 2,4,6-tri-chlorophenol containing synthetic wastewater: Effects of hydraulic residence time. *Journal of Hazardous Materials* 144: 86–92.
- [100] **Elibol, M.** (2004) Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3 (2) with response surface methodology. *Process Biochemistry* 39: 1057–1062.
- [101] **Ellis, F.** (2002) Paracetamol. a curriculum resource. Cambridge: Royal Society of Chemistry. ISBN 0-85404-375-6.
- [102] **Ellis, L.B.M. and Wackett L.P.** (1997) The University of Minnesota Biocatalysis/Biodegradation Database. <http://umbbd.ahc.umn.edu>.
- [103] **Ellis, T.G. and Anselm, C.V.** (1999) Effect of batch discharges on extant biodegradation kinetics in activated-sludge systems. *Water Environmental Research*. 71: 290–298.
- [104] **Ellis, T.G., Barbeau, D.S., Smets, B.F., Grady, J.C.P.L.** (1996b) Respirometric technique for determination of extant kinetic parameters describing biodegradation. *Water Environmental Research* 68:917–926
- [105] **Ellis, T.G., Smets, B.F., Magbanua, J.B.S., Grady, J.C.P.L.** (1996a) Changes in measured biodegradation kinetics during the long-term operation of completely mixed activated sludge (CMAS) bioreactors. *Water Science and Technology* 34:35–42.
- [106] **Entezari, M.H. and Petrier, C.** (2003) A combination of ultrasound and oxidative enzyme: sono-biodegradation of substituted phenols. *Ultrasonics Sonochemistry* 10: 241–246.
- [107] **Environmental Protection Agency.** (1976) Water quality criteria. Washington DC.
- [108] **Environmental Protection Agency.** (1984) Federal Register, EPA Method 604, Phenols, Part VIII, 40 CFR part 136, Washington, DC, p. 58.

- [109] **Environmental Protection Agency.** (1988) Effluent guidelines and standards, Organic chemicals, plastics, and synthetic fibers. 40 CFR part 414, Washington, DC.
- [110] **EPA** (1980) Environmental Protection Agency, Ambient water quality for nitrophenols, EPA/440/5 80-063, Washington, DC.
- [111] **EPA.** (1985) Health and environmental effects profile for nitrophenols. Final draft. Cincinnati, OH: U.S. Environmental Protection Agency, Environmental Criteria and Assessment Office.
- [112] **EPA.** (1998) Handbook Advanced Photochemical Oxidation Processes United States Environmental Protection Agency. Office of Research and Development Washington, USA.
- [113] **Erba, A.D., Falsanisi, D., Liberti, L., Notarnicola, M., Santoro, D.** (2007) Disinfection by-products formation during wastewater disinfection with peracetic acid. *Desalination* 215, 177–186.
- [114] **Escher, B.I., Hunziker, R.W., Schwarzenbach, R.P.** (2001) Interaction of phenolic uncouplers in binary mixtures: concentration-additive and synergistic effects. *Environmental Science and Technology* 35: 3905-3914.
- [115] **Escher, B.I., Snozzi, M., Schwarzenbach, R.P.** (1996) Uptake, speciation, and uncoupling activity of substituted phenols in energy transducing membranes. *Environmental Science and Technology* 30: 3071-3079.
- [116] **Fahr, K., Wetzstein, H.G., Grey, R., Schlosser, D.** (1999) Degradation of 2,4-dichlorophenol and pentachlorophenol by two *brown rot fungi*. *FEMS Microbiology Letters* 175: 127–132.
- [117] **Farhadian, M., Larroche, C., Borghei, M., Troquet, J., Vachelard, C.** (2006) Bioremediation of BTEX-contaminated groundwater through bioreactors. 4e^{me} Colloque Franco-Roumain de chimie applique^e, Universite^e Blaise Pascal, Clermont-Ferrand, France, 28 Juin–2 Juillet, p. 438.
- [118] **Farrell, A. and B. Quilty.** (1999) Degradation of mono-chlorophenols by a mixed microbial community via a *meta*-cleavage pathway. *Biodegradation* 10: 353-362.

- [119] **Farrell, A. and Quilty, B.** (2002) Substrate-dependent autoaggregation of *Pseudomonas putida* CP1 during the degradation of mono-chlorophenols and phenol. *Journal of Industrial Microbiology & Biotechnology* 28: 316-324.
- [120] **Fassnacht, D. and Portner, R.** (1999) Experimental and theoretical considerations on oxygen supply for animal cell growth in fixed-bed reactors. *Journal of Biotechnology* 72: 169–184.
- [121] **Fava, F., Armenante, P.M., Kafkewitz, E.** (1995) Aerobic degradation and dechlorination of 2- chlorophenol, 3-chlorophenol and 4-chlorophenol by a *Pseudomonas pickettii* strain. *Letters in Applied Microbiology* 21: 307-312.
- [122] **Fava, F., Di Gioia, D., Marehetti, L., Quattroni, G.** (1996) Aerobic dechlorination of low-chlorinated biphenyls by bacterial biofilms in packed-bed batch bioreactors. *Applied Microbiology and Biotechnology* 45: 562-568.
- [123] **Federal Register.** (1984) EPA Method 604, Phenols, Part VIII, 40 CFR Part 136. Environmental Protection Agency, Washington DC p. 58.
- [124] **Fernando, J., Beltrán, F., Rivas, J., Gimeno, O.** (2005) Comparison between photocatalytic ozonation and other oxidation processes for the removal of phenols from water. *Journal of Chemical Technology and Biotechnology* 80: 973–984.
- [125] **Ferraroni, M., Kolomytseva, M.P., Solyanikova, I.P., Scozzafava, A., Golovleva, L.A., Briganti, F.** (2006) Crystal Structure of 3-Chlorocatechol 1,2-dioxygenase Key Enzyme of a New Modified Ortho-pathway from the Gram-positive *Rhodococcus opacus* ICP Grown on 2-chlorophenol. *Journal of Molecular Biology* 360:788–799.
- [126] **Flodin, C. and Whitfield, F.B.** (2000) Brominated anisoles and cresols in the red alga *Polysiphonia sphaerocarpa*. *Phytochemistry* 53: 77–80.
- [127] **Flodin, C., Helidoniotis, F., Whitfield, F.B.** (1999) Seasonal variation in bromophenol content and bromoperoxidase activity in *Ulva lactuca*. *Phytochemistry* 51: 135–138.
- [128] **Fox, M.A. and. Dulay, M.T.** (1993) Heterogeneous photocatalysis. *Chemical Reviews* 93: 341 357.
- [129] **Fujishima, A. and Fujiwara, M.** (1999) Single dose oral toxicity test of 2,4,6-tribromophenol in rats. Shizuoka, Japan Ministry of Health & Welfare, Biosafety

- Research Centre (Toxicity Testing Reports of Environmental Chemicals No. 7; (<http://wwwdb.mhlw.go.jp/ginc/dbfile1/paper/paper118-79-6a.html>) (in Japanese).
- [130] **Galíndez-Mayer, J., Ramón-Gallegos, J., Ruiz-Ordaz, N., Juárez-Ramírez, C., Salmerón-Alcocer, A., Poggi-Varaldo, H.M.** (2008) Phenol and 4-chlorophenol biodegradation by yeast *Candida tropicalis* in a fluidized bed reactor. *Biochemical Engineering Journal* 38: 147–157.
- [131] **Gallego, A., Fortunato, M.S., Foglia, J., Rossi, S., Gemini, V., Gomez, L., Gomez, C.E., Higa, L.E., Korol, S.E.** (2003) Biodegradation and detoxification of phenolic compounds by pure and mixed indigenous cultures in aerobic reactors. *International Biodeterioration & Biodegradation* 52: 261-267.
- [132] **Genthner, B.R.S., Price, W.A., Pritchard, P.H.** (1989) Characterization of anaerobic dechlorinating consortia derived from aquatic sediments. *Applied Environmental Microbiology* 55: 1472–1476.
- [133] **Givens, S., Brown, E., Gelman, S., Grady, C. and Skedsvold, D.** (1991) Biological process design and pilot testing for carbon oxidation, nitrification and denitrification system. *Environmental Progress* 10(1): 133-146.
- [134] **Glaze, W., Lay, Y., Kang, J.** (1995) Advanced oxidation processes. A kinetic model for the oxidation of 1,2-dibromo-3-chloropropane in water by the combination of hydrogen peroxide and UV radiation. *Industrial and Engineering Chemistry Research* 34: 2314-2323.
- [135] **Goerke, H. and Weber, K.** (1991) Bromophenols in *Lanice conchilega* (Polychaeta, Terebellidae) the influence of sex, weight and season. *Bulletin of Marine Science*, 48: 517–523.
- [136] **Golovleva, L.A. Zaborina, O., Pertsova, R., Baskunov, B., Schurukhin, Y., Kuzmin, S.** (1992) Degradation of polychlorinated phenols by *Streptomyces rochei* 303. *Biodegradation* 2: 201–208.
- [137] **Gómez-De Jesús, A., Romano-Baez, F.J., Leyva-Amezcu, L., Juárez-Ramírez, C., Ruiz-Ordaz, N., Galíndez-Mayer, J.** (2009) Biodegradation of 2,4,6-trichlorophenol in a packed-bed biofilm reactor equipped with an internal net draft tube riser for aeration and liquid circulation. *Journal of Hazardous Materials* 161: 1140-1149.

- [138] **Gorontzy, T., Kuver, J., Blotevogel, K.H.** (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *Journal of General Microbiology* 139: 1331–6.
- [139] **Goswami, M., Shivaraman, N., Singh, R.P.** (2002) Kinetics of chlorophenol degradation by benzoate-induced culture of *Rhodococcus erythropolis* M1. *World Journal of Microbiology and Biotechnology* 18: 779–783.
- [140] **Goudar, C. T., Ganji, S. H., Pujar, B. G., Strevett, K. A.** (2000) Substrate inhibition kinetics of phenol biodegradation. *Water Environment Research* 7: 50- 55.
- [141] **Grady, J.C.P.L., Smets, B.F., Barbeau, D.S.** (1996) Variability in kinetic parameter estimates: a review of possible causes and a proposed terminology. *Water Research* 30:742–748.
- [142] **Grimwood, M. and Mascarenhas, R.** (1997) Proposed Environmental Quality Standards for 2-, 3- and 4-chlorophenol and 2,4-Dichlorophenol in Water. Environment Agency Technical Report P46/i688. WRC Report No EA4215.
- [143] **Grove, R.S., Faeder, E.J., Ospital, J., Bean, M.** (1985) Halogenated compounds discharged from a coastal power plant. In: Jolley RL, Bull RJ, Davies WP, Katz S, Roberts MH, Jacobs VA, eds. *Water chlorination: Chemistry, environmental impact and health effects. Vol. 5.* Chelsea, MI, Lewis Publishers, pp. 1371–1379.
- [144] **Guerin, T.F.** (2002) A pilot study for the selection of a bioreactor for remediation of groundwater from a coal tar contaminated site. *Journal of Hazardous Materials* 89: 241–252.
- [145] **Guieysse, B. and Mattiasson, B.** (1999) Fast remediation of coal-tar related compounds in biofilm bioreactors. *Applied Microbiology and Biotechnology* 52:600–607.
- [146] **Guieysse, B., Bernhoft, I., Andersson, B.E., Henrysson, T., Olsson, S., Mattiasson, B.** (2000) Degradation of acenaphthene, phenanthrene and pyrene in a packed-bed biofilm reactor. *Applied Microbiology and Biotechnology* 54: 826–831.
- [147] **Guittonneau, S., Glaze, W., Duguet, J., Wable, O.** (1991) Characterization of natural waters for potential to oxidize organic pollutants with ozone *Proceedings of 10th Ozone World Congress, Zürich, Switzerland.*

- [148] **Gunasekara, A.S., Tenbrook, P.L., Palumbo, A.J., Johnson, C.S. Tjeerdema, R.S.** (2005) Influence of phosphate and copper on reductive dechlorination of thiobencarb in California rice field soils. *Journal of Agriculture and Food Chemistry* 53: 10113-9.
- [149] **Häggblom, M.M. and Valo, R.J.** (1995) Bioremediation of Chlorophenol Wastes. In *Microbial Transformation and Degradation of Toxic Organic Chemicals*, ed by Lily Y. Young, Carle. Cerniglia, pp. 389-434. Wiley-Liss Inc.
- [150] **Haggbloom, M.M., Apajalahti, J.H.A., Salkinoja-Salonen, M.S.** (1988a) O-methylation of chlorinated p hydroquinones by *Rhodococcus chlorophenolicus*. *Applied Environmental Microbiology* 54: 1818–1824.
- [151] **Haggbloom, M.M., Nohynek, L.J., Salkinojasalonen, M.S.** (1988b) Degradation and o-methylation of chlorinated phenolic compounds by *Rhodococcus* and *Mycobacterium* strains. *Applied Environmental Microbiology* 54: 3043–3052.
- [152] **Haggbloom, M.M., Rivera, M.D., Young, L.Y.** (1993) Influence of alternative electron-acceptors on the anaerobic biodegradability of chlorinated phenols and benzoic acids. *Applied Environmental Microbiology* 59: 1162–1167.
- [153] **Haghighi-Poden, M.R. and Bhattacharya, S.K.** (1996) Fate and toxic effects of nitrophenols on anaerobic treatment systems. *Water Science and Technology* 34: 345–50.
- [154] **Haghighi-Poden, M.R., Bhattacharya, S.K., Qu, M.** (1995) Effects of nitrophenols on acetate utilizing methanogenic systems. *Water Research* 29: 391–399.
- [155] **Haldane, J.B.S.** (1965) *Enzymes*. MIT Press, Cambridge, MA, pp. 84.
- [156] **Hamdaoui, Q. and Naffrechoux, E.** (2008) Sonochemical and photosonochemical degradation of 4-chlorophenol in aqueous media *Ultrasonics Sonochemistry* 15: 981–987.
- [157] **Hamdaoui, Q. and Naffrechoux, E.** (2009) Adsorption kinetics of 4-chlorophenol onto granular activated carbon in the presence of high frequency ultrasound. *Ultrasonics Sonochemistry* 16: 15–22.

- [158] **Hammel, K.E. and Tardone, P.J.** (1988) The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidases. *Biochemistry* 27: 6563–6568.
- [159] **Hannaford, A.M. and Kuek, C.** (1999) Aerobic batch degradation of phenol using immobilized *Pseudomonas putida*. *Journal of Industrial Microbiology & Biotechnology* 22: 121-126.
- [160] **Hao, O.J., Kim, M.H., Seagren, E.A., Kim, H.** (2002) Kinetics of phenol and chlorophenol utilization by *Acinetobacter* species. *Chemosphere* 46:797-807
- [161] **Harrington, E.C.** (1965) The desirability function. *Industrial Quality Control* 21: 494- 498.
- [162] **Hart, S.** (1996) In situ bioremediation: defining the limits. *Environmental Science and Technology* 30: 398-401.
- [163] **Hassenklover, T., Predehl, S., Pilli, J., Ledwolorz, J., Assmann, M., Bickmeyer, U.** (2006) Bromophenols, both present in marine organisms and industrial flame retardants, disturb cellular Ca²⁺ signaling in neuroendocrine cells (PC12). *Aquatic Toxicology* 76: 37–45.
- [164] **Heimann, C.A., Friis, K.A., Jakobsen, R.** (2005) Effects of sulfate on anaerobic chloroethene degradation by an enriched culture under transient and steady-state hydrogen supply. *Water Research* 39: 3579–3586.
- [165] **Heinaru, E., Viggor, S., Vedler, E., Truu, J., Merimaa, M., Heinaru, A.** (2001) Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads. *FEMS Microbiology Ecology* 37: 79-89.
- [166] **Henze, M., Harremoes, P., Jansen, J., Arvin, E.** (2002) Wastewater treatment. *Biological and Chemical Processes*. Springer, Berlin, Heidelberg.
- [167] **Herrmann, J.M., Duchamp, C., Karkmaz, M., Hoai, B.T., Lachheb, H., Puzenat, E. and Guillard, C.** (2007) Environmental green chemistry as defined by photocatalysis. *Journal of Hazardous Materials* 146(3):624-629.
- [168] **Higson, F.K.** (1992) Microbial degradation of nitroaromatic compounds. *Advanced Applied Microbiology* 37: 1–9.

- [169] **Hincapié, M., Maldonado, M.I., Oller, I., Gernjak, W., Sánchez-Pérez, J.A. Ballesteros, M.M., Malato, S.** (2005) Solar photocatalytic degradation and detoxification of EU priority substances. *Catalysis Today* 101: 230-210.
- [170] **Hoffmann, M.R, Martin,S.T., Choi,W. and Bahnemann, D.W.** (1995.) Environmental Applications of semiconductor catalysis. *Chemical Reviews* 95(1): 69- 96.
- [171] **Hofrichter, M., Bublitz, F., Fritsche, W.** (1994) Unspecific degradation of halogenated phenols by the soil fungus *Penicillium frequentans*-Bi-7/2. *Journal of Basic Microbiology* 34: 163–172.
- [172] **Hofrichter, M., Gunther, T., Fritsche, W.** (1993) Metabolism of phenol, chloro- and nitrophenols by the *Penicillium strain Bi 7/2* isolated from a contaminated soil *Biodegradation* 3: 415–421.
- [173] **Hollender, J., Dott, W., Hopp, J.** (1994) Regulation of chloro- and methylphenol degradation in *Comamonas testosteroni* JH5. *Applied and Environmental Microbiology* 60: 2330-2338.
- [174] **Hollender, J., Hopp, J., Dott, W.** (2000) Cooxidation of chloro- and methylphenols by *Alcaligenes xylosoxidans* JH1. *World Journal of Microbiology and Biotechnology* 16: 445–450.
- [175] **Hoq. M.M., Solomon, B.O., Hempel, C., Rinas, U., Deckwer, W.D.** (1995) The kinetics of cellulase-free xylanase excretion by *Thermomyces lanuginosus* RT 9. *Journal of Chemical Technology & Biotechnology* 63: 229-236.
- [176] **Hovander, L., Malmberg, T., Athanasiadou, M., Athanassiadis, I., Rahm, S., Bergman, Å., Wehler, E.K.** (2002) Identification of hydroxylated PCB metabolites and other phenolic 65 halogenated pollutants in human blood plasma. *Archives of Environmental Contamination and Toxicology* 42: 105-117.
- [177] **Howe, P.D., Dobson, S., Malcolm, H.M.** (2005) 2,4,6-Tribromophenol and Other Simple Brominated Phenols. World Health Organization, Geneva.
- [178] **HSDB.** (1989) Hazardous Substances Data Bank. National Library of Medicine. National Toxicology Information Program, Bethesda, MD. September 11.
- [179] **HSDB.** (2003) Hazardous substances data bank. Bethesda, MD, National Institutes of Health, National Library of Medicine. Industrial Bio-Test (1976a) Report to

- Michigan Chemical Corporation 48 hour dynamic aquatic toxicity study with 2,4,6-tribromophenol in *Daphnia*. Northbrook, IL, Industrial Bio-Test IRDC (1974c) Acute oral toxicity LD50 study in albino rats.
- [180] **Hu, Z.C, Korus, R.A, Levinson, W.E., Crawford, R.L.** (1994) Adsorption and biodegradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium*. *Environmental Science and Technology* 28: 491–496.
- [181] **Hussain, S., Arshad, M., Saleem, M., Zahir, Z.A.** (2007) Screening of soil fungi for in vitro degradation of endosulfan. *World Journal of Microbiology and Biotechnology* 23: 939-945.
- [182] **Iandolo, D., Amore, A., Biolo, L., Leo, G., Olivieri, G., Faraco, V.** (2011) Fungal solid state fermentation on agro-industrial wastes for acid wastewater decolourization in a continuous flow packed-bed bioreactor. *Bioresource Technology* DOI: 10.1016/j.biortech.2011.05.029
- [183] **Imandi, S.B., Bandaru, V.V.R., Somalanka, S.R., Bandaru, S.R., Garapati, H.R.** (2008) Application of statistical experimental designs for the optimization of medium constituents for the production of citric acid from pineapple waste. *Bioresource Technology* 99: 4445-4450.
- [184] IRDC (1974c) Acute oral toxicity LD50 study in albino rats. Mattawan, MI, IRDC (Report No. EPA/OTS 86-900000307; NTIS/OTS 0523299; submitted to US EPA by Great Lakes Chemical Corporation, West Lafayette, IN, March 1990)”
- [185] **Israni, S.H., Koli, S.S., Patwardhan, A.W., Melo, J.S., Dsouza, S.F.** (2002) Phenol degradation in rotating biological contactors, *Journal of Chemical Technology and Biotechnology* 77: 1050–1057.
- [186] **IUCLID**, (2003) Data set for 2,4,6-tribromophenol. Ispra, European Chemicals Bureau, International Uniform Chemical Information Database.
- [187] **Jahani, M., Alizadeh, M., Pirozifard, M., Qudsevali, A.** (2008) Optimization of enzymatic degumming process for rice bran oil using response surface methodology. *LWT - Food Science and Technology* 41: 1892-1898.
- [188] **Jain, R.K., Dreisbach, J.H., Spain, J.C.** (1994) Biodegradation of *p*-nitrophenol via 1,2,4- benzenetriol by an *Arthrobacter* sp. *Applied and Environmental Microbiology* 60: 3030-3032.

- [189] **Jajuee, B., Margaritas, A., Karamanev, D., Bergougnou, M.A.** (2007) Kinetics of biodegradation of *p*-xylene and naphthalene and oxygen transfer in a novel airlift immobilized bioreactor. *Biotechnology and Bioengineering* 96: 232 – 243.
- [190] **Jarvinen, K.T. and Puhakka, J.A.** (1994) Bioremediation of chlorophenol contaminated groundwater, *Environmental Technology* 15: 823–832.
- [191] **Jarvinen, K.T., Melin, E.S., Puhakka, J.A.** (1994) High-rate bioremediation of chlorophenol-contaminated groundwater at low-temperatures. *Environmental Science and Technology* 28: 2387–2392.
- [192] **Jensen, P., Emrich, R., Weber, K.** (1992) Brominated metabolites and reduced numbers of meiofauna organisms in the burrow wall lining of the deep-sea enteropneust *Stereobalanus canadensis*. *Deep-Sea Research Part A – Oceanographic Research Papers*, 39:1247–1253.
- [193] **Jernberg, C. and Jansson, J.K.** (2002) Impact of 4-chlorophenol contamination and/or inoculation with the 4-chlorophenol-degrading strain, *Arthrobacter chlorophenolicus* A6L, on soil bacterial community structure. *FEMS Microbiology Ecology* 42: 387-397.
- [194] **Johnson, G.R. and Olsen, R.H.** (1997) Multiple pathways for toluene degradation in *Burkholderia cepacia* sp. strain JS150. *Applied and Environmental Microbiology* 63: 4047- 4052.
- [195] **Jordan, M., Sucker, H., Einsele, A., Widmer, F., Eppenberger, H.M.** (1994) *Biotechnology and Bioengineering* 43: 446.
- [196] **Joshi, D.K. and Gold, M.H.** (1993) Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Applied Environmental Microbiology* 59: 1779–1785.
- [197] **Jou, C.J.G. and Huang, GC.** (2003) A pilot study for oil refinery wastewater treatment using a fixedfilm bioreactor. *Advanced in Environmental engineering Research* 7: 463-469.
- [198] **Kappeler, J. and Gujer, W.** (1992) Estimation of kinetic parameters of heterotrophic biomass under aerobic conditions and characterization of wastewater for activated sludge modelling. *Water Science and Technology* 25: 125–139.

- [199] **Karamanev, D.G. and Samson, R.** (1998) High-rate biodegradation of pentachlorophenol by biofilm developed in the immobilized soil bioreactor. *Environmental Science and Technology* 32: 994–999.
- [200] **Kargi, F. and Eker, S.** (2004) Toxicity and batch biodegradation kinetics of 2,4-dichlorophenol by pure *Pseudomonas putida* culture. *Enzyme Microbiol Technology* 35: 424–428.
- [201] **Kargi, F. and Eker, S.** (2005) Removal of 2,4-dichlorophenol and toxicity from synthetic wastewater in a rotating perforated tube biofilm reactor. *Process Biochemistry* 40: 2105–2111.
- [202] **Karim, K and Gupta, S.K.** (2003) Continuous biotransformation and removal of nitrophenols under denitrifying conditions. *Water Research* 37: 2953–9.
- [203] **Karim, K. and Gupta, S.K.** (2001) Biotransformation of nitrophenols in upflow anaerobic sludge blanket reactors. *Bioresource Technology* 80: 179–186.
- [204] **Kermanshahipour, A., Karamanev, D., Margaritis, A.** (2005) Biodegradation of petroleum hydrocarbons in an immobilized cell airlift bioreactor. *Water Research* 39: 3704–3714.
- [205] **Kharoune, M., Pauss, A., Lebeault, J.M.** (2001) Aerobic biodegradation of an oxygenates mixture: ETBE, MTBE and TAME in an upflow fixed-bed reactor. *Water Research* 35: 1665–1674.
- [206] **Khleifat, K.M.** (2006) Biodegradation of phenol by *Ewingella americana*: Effect of carbon starvation and some growth conditions. *Process Biochemistry* 41: 2010–2016.
- [207] **Kidak, R. and Ince, N.H.** (2006) Ultrasonic destruction of phenol and substituted phenols: A review of current research. *Ultrasonics Sonochemistry* 13(3): 195–199.
- [208] **Kim, J.H., Oh, K.K., Lee, S.T., Kim, S.W., Hong, S.I.** (2002) Biodegradation of phenol and chlorophenols with defined mixed culture in shake-flasks and a packed bed reactor. *Process Biochemistry* 37: 1367–1373.
- [209] **Kiyohara, H., Hatta, T., Ogawa, Y., Kakuda, T., Yokoyama, H., Takizawa, N.** (1992) Isolation of *Pseudomonas pickettii* strains that degrade 2,4,6-trichlorophenol and their dechlorination of chlorophenols. *Applied Environmental Microbiology* 58: 1276–1283.

- [210] **Klečka, G.M. and Gibson, D.T.** (1981) Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. *Applied Environmental Microbiology* 41: 1159–1165.
- [211] **Klecka, G.M. and Maier, W.J.** (1985) Kinetics of microbial growth on pentachlorophenol. *Applied Environmental Microbiology* 49: 46–53.
- [212] **Kohring, G.W., Rogers, J.E., Wiegel, J.** (1989) Anaerobic biodegradation of 2,4-dichlorophenol in fresh-water lakesediments at different temperatures. *Applied Environmental Microbiology* 55: 348–353.
- [213] **Kotturi, G., Robinson, C.W., Inniss, W.E.** (1991) Phenol degradation by a psychrotrophic strain of *Pseudomonas putida*. *Applied Microbiology & Biotechnology* 34: 539-543.
- [214] **Kovárová-Kovar, K. and Egli, T.** (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiology and Molecular Biology Reviews* 62:646-666.
- [215] **Kreft, J.U. and Wimpenny, J.W.T.** (2001) Effect of EPS on biofilm structure and function as revealed by an individual-based model of biofilm growth, *Water Science and Technology* 43: 135–141.
- [216] **Kretzmer, G.** (2002) Industrial processes with animal cells. *Applied Microbiology and Biotechnology* 59: 135–142.
- [217] **Kuiper, J. and Arnbjörn, O.** (1984) Hanstveit. Fate and effects of 4-chlorophenol and 2,4-dichlorophenol in marine plankton communities in experimental enclosures. *Ecotoxicology and Environmental Safety*. 8: 15-33.
- [218] **Kuipers, B., Cullen, W.R., Mohn, W.W.** (1999) Reductive dechlorination of nonachlorobiphenyls and selected octachlorobiphenyls by microbial enrichments cultures. *Environmental Science and Technology* 33: 3577-3583.
- [219] **Kumar, A., Kumar, S., Kumar, S.** (2005) Biodegradation kinetics of phenol and catechol using *Pseudomonas putida* MTCC 1194. *Biochemical Engineering Journal* 22: 151-159.
- [220] **Kumar, S., Pakshirajan, K., Dasu, V.V.** (2009) Development of medium for enhanced production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Applied Microbiology and Biotechnology* 84:477–486.

- [221] **Kumaran, P. and Paruchuri, Y.L.** (1996) Kinetics of phenol biotransformation. *Water Research* 31: 11-22.
- [222] **Kumaran, P. and Paruchuri, Y.L.** (1997) Kinetics of phenol biotransformation. *Water Research* 31: 11-22.
- [223] **Kwon, S.H., Kim, J.H., Cho, D.** (2009). An analysis method for degradation kinetics of lowly concentrated PAH solutions under UV light and ultrasonication. *Journal of Industrial and Engineering Chemistry* 15: 157-162.
- [224] **Lachheb, H., Houas, A., Herrmann, J.-M.** (2008) Photocatalytic degradation of polynitrophenols on various commercial suspended or deposited titanium catalyst using artificial and solar light. *International Journal of Photoenergy* Article ID 497895. doi:1155/2008/497895.
- [225] **Lallai, A. and Mura, G.** (1989) pH variation during phenol biodegradation in mixed cultures of microorganisms. *Water Research* 23: 1335-1338.
- [226] **Langwaldt, J.H. and Puhakka, J.A.** (2000) On-site biological remediation of contaminated groundwater: a review. *Environmental Pollution* 107: 187-197.
- [227] **Langwaldt, J.H., Mannisto, M.K., Wichmann, R., Puhakka, J.A.** (1998) Simulation of in situ subsurface biodegradation of polychlorophenols in air-lift percolators. *Applied Microbiology and Biotechnology* 49: 663-668.
- [228] **Lee, S.Y. and Pang-Tsui, Y.** (1999) Succeed at gas/liquid contacting. *Chemical Engineering Progress* 95: 23-49.
- [229] **Leeuwen, C.J.V. and Vermeire, T.** (2007) Risk assessment of chemicals: An introduction. second edition, Springer, Dordrecht, The Netherlands -p329.
- [230] **Legrini, O., Oliveros, E., Braun, A.M.** (1993) Photochemical processes for water treatment. *Chemical Reviews* 93: 671- 698.
- [231] **Lendenmann, U., Spain, J.C., Smets, B.F.** (1998) Simultaneous degradation of 2,4-dinitrotoluene and 2,6-dinitrotoluene in an aerobic fluidized-bed biofilm reactor. *Environmental Science and Technology* 32: 82-87.
- [232] **Letourneau, L., Bisaillon, J.G., Lepine, F., Beaudet, R.** (1995) Sporeforming bacteria that carboxylate phenol to benzoic-acid under anaerobic conditions. *Canadian Journal of Microbiology* 41: 66-272.

- [233] **Leung, K., Errampalli, D., Cassidy, M., Lee, H., Trevors, J.T., Okamura, H., Bach, H.J., Hall, B.** (1997) A case study of bioremediation of polluted soil: Biodegradation and toxicity of chlorophenols in soil. *In* J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.), *Modern soil microbiology*. Marcel Dekker, New York, N.Y.
- [234] **Leung, K.T., Cambell, S., Gan, Y., White, D.C., Lee, H., Trevors, J.T.** (1999) The role of the *Sphingomonas* sp UG30 pentachlorophenol-4- monooxygenase in *p*-nitrophenol degradation. *FEMS Microbiology Letter* 173: 247–253.
- [235] **Leung, K.T., Cassidy, M.B., Shaw, K.W., Lee, H., Trevors, J.T., Lohmeier, Vogel. E.M., Vogel, H.J.** (1997) Pentachlorophenol biodegradation by *Pseudomonas* spp. UG25 and UG30. *World Journal of Microbiology and Biotechnology* 13: 305–313.
- [236] **Leyva, E., Moctezuma, E, Ruíz, M. G., Torres-Martínez, L.** (1998) Photo degradation of phenol and 4-chlorophenol by BaO–LiO₂–TiO₂ catalysts. *Catalysis Today* 40: 367–376
- [237] **Li, D.Y., Eberspacher, J., Wagner, B., Kuntzer, J., Lingens, F.** (1991) Degradation of 2,4,6-trichlorophenol by *Azotobacter* sp strain GP1. *Applied Environmental Microbiology* 57: 1920–1928.
- [238] **Li, K., Chang, S., Yu, C.** (2000) Treatment of chlorohydrocarbon contaminated groundwater by air stripping. *International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, 2nd, Monterey, CA, United States, May 22-25:293-300.
- [239] **Lin, J.E., Wang, H.Y., Hickey, R.F.** (1990) Degradation kinetics of pentachlorophenol by *Phanerochaete chrysosporium*. *Biotechnology and Bioengineering* 35: 1125–1134.
- [240] **Liu, X.Y., Wang, B.J., Jiang, C.Y., Zhao, K.X., Harold, L.D., Liu, S.J.** (2007) Simultaneous biodegradation of nitrogen-containing aromatic compounds in a sequencing batch bioreactor. *Journal of Environmental science* 19: 530-535.
- [241] **Livingston A.G. and Chase H.A.** (1989) Modeling phenol degradation in a fluidized bed bioreactor. *AIChE* 35 (12): 1980-1992.

- [242] **Loa, S.C., Lin, C.F., Wu, C.H., Hsieh, P.H.** (2004) Capability of coupled CdSe/TiO₂ for photocatalytic degradation of 4-chlorophenol. *Journal of Hazardous Materials* 114: 183–190.
- [243] **Loh, K. C. and Ranganath, S.** (2005) External-loop fluidized bed airlift bioreactor (EFBAB) for the co metabolic biotransformation of 4-chlorophenol (4-cp) in the presence of phenol. *Chemical Engineering Science* 60: 6313 - 6319.
- [244] **Loh, K.C., and Wu, T.T.** (2006) Cometabolic transformation of 2- chlorophenol and 4-chlorophenol in the presence of phenol by *Pseudomonas putida*. *Canadian Journal of Chemical engineering* 84:356–367.
- [245] **Londry, K.L. and Fedorak, P.M.** (1992) Benzoic-acid intermediates in the anaerobic biodegradation of phenols. *Canadian Journal of Microbiology* 38: 1–11.
- [246] **Londry, K.L. and Fedorak, P.M.** (1993) Fluorophenols and 3-fluorobenzoate in phenol-degrading methanogenic cultures. *Arch of Microbiology* 160: 137–143.
- [247] **Lora, P.O., Sjolund, M., Traco, I C., Morvan, J.** (2000) Adaptation of an inoculum to 2,4,6-trichlorophenol biodegradation in an activated-sludge bioreactor. *Water Science and Technology* 42: 179–183.
- [248] **Lovanh, N., Hunt, C.S., Alvarez, P.J.J.** (2002) Effect of ethanol on BTEX biodegradation kinetics: aerobic continuous culture experiments. *Water Research* 36:3739-3746.
- [249] **Luong, J.H.T.** (1987). Generalization of Monod kinetics for analysis of growth data with substrate inhibition. *Biotechnology Bioengineering* 29: 242-248.
- [250] **Luthy, R.G., Stamoudis, V.C., Campbell, J.R. et al.,** (1983) Removal of organic contaminants from coal conversion process condensates. *Water Pollution Control Fed* 55: 196–207.
- [251] **Lynch, J.M. and Moffat, A.J.** (2005) Bioremediation—prospects for the future application of innovative applied biological research. *Annals of Applied Biology* 146: 217–221.
- [252] **Madsen, T. and Aamand, J.** (1991) Effects of sulfuroxy anions on degradation of pentachlorophenol by a methanogenic enrichment culture. *Applied Environmental Microbiology* 57: 2453–2458.

- [253] **Maellaro, E., DelBello, B., Casini, A.F., Comporti, M., Ceccarelli, D., Muscatello, U., Masini, A.** (1990) Early mitochondrial disfunction in bromobenzene treated mice: a possible factor of liver injury. *Biochemical Pharmacology* 40: 1491–1497.
- [254] **Magar, V.S., Stensel, H.D., Puhakka, J.A., Ferguson, J.F.** (1999) Sequential anaerobic dechlorination of pentachlorophenol: competitive inhibition effect and a kinetic model. *Environmental Science and Technology* 33: 1604–1611.
- [255] **Mahanty, B., Pakshirajan, K., Dasu, V.V.** (2008) Biodegradation of pyrene by *Mycobacterium frederiksbergense* in a two-phase partitioning bioreactor system. *Bioresource Technology* 99: 2694–2698.
- [256] **Mannisto, M.K., Tirola, M.A., Puhakka, J.A.** (2001) Degradation of 2,3,4,6-tetrachlorophenol at low temperature and low dioxygen concentrations by phylogenetically different groundwater and bioreactor bacteria. *Biodegradation* 12: 291–301.
- [257] **Margesin, R. and Schinner, F.** (1997) Effect of temperature on oil degradation by a psychrotrophic yeast in liquid culture and in soil. *FEMS Microbiology Ecology* 24: 243–249.
- [258] **Marr, J., Kremer, S., Sterner, O., Anke, H.** (1996) Transformation and mineralization of halophenols by *Penicillium simplicissimum* SK9117. *Biodegradation* 7: 165–171.
- [259] **Mattawan, M.I.** (1990) International Research Development Corporation (Report No. EPA/OTS 86-900000307; NTIS/OTS 0523299; submitted to US EPA with test data and cover letter by Great Lakes Chemical Corporation, West Lafayette, IN).
- [260] **Matthew, J.L., McMahon, A., Daugulis, J.** (2008) Enhancement of biogenic sulfide production in a packed-bed bioreactor via critical inoculum design and carrier material selection. *Biotechnology and Bioengineering* 100: 855–863.
- [261] **Matthews, R.** (1990) Purification of water with near-UV illuminated suspensions of titanium dioxide. *Water Research* 24: 653–660.
- [262] **Matus, V., Sanchez, M.A., Martinez, M., Gonzalez, B.** (2003) Efficient degradation of 2,4,6-trichlorophenol requires a set of catabolic genes related to tcp genes from

- Ralstonia eutropha* JMP134(pJP4). *Applied Environmental Microbiology* 69: 7108–7115.
- [263] **May, H.D., Boyle, A.W., Price, W.A., Blake, C.K.** (1992) Subculturing of a Polychlorinated biphenyl-dechlorinating anaerobic enrichment on solid media. *Applied and Environmental Microbiology* 58: 4051-4054.
- [264] **McAllister, K.A., Lee, H., Trevors, J.T.** (1996) Microbial degradation of pentachlorophenol. *Biodegradation* 7: 1–40.
- [265] **McBain, A., Cui, E., Herbert, L., Ruddick, J.N.R** (1995) The microbial degradation of chlorophenolic preservatives in spent, pressure treated timber. *Biodegradation* 6: 47-55.
- [266] **McCarthy, D.L., Claude, A.A., Copley, S.D.** (1997) In vivo levels of chlorinated hydroquinones in a pentachlorophenol-degrading bacterium. *Applied Environmental Microbiology* 63:1883–1888.
- [267] **McLaughlin, S.G. and Dilger, J.P.** (1980) Transport of protons across membranes by weak acids. *Physiological Reviews* 60: 825-863.
- [268] **Meerts, I.A.T.M., Letcher, R.J., Hoving, S., Marsh, G., Bergman, Å., Lemmen, J.G., van der Burg, B., Brouwer, A.** (2001) *In vitro* estrogenicity of polybrominated diphenyl ethers (PBDEs), hydroxylated PBDEs. *Environmental Health Perspectives* 109:399-407.
- [269] **Melin, E.S., Ferguson, J.F., Puhakka, J.A.** (1997) Pentachlorophenol biodegradation kinetics of an oligotrophic fluidizedbed enrichment culture. *Applied Microbiology and Biotechnology* 47: 675–682.
- [270] **Melin, E.S., Jarvinen, K.T., Puhakka, J.A.** (1998a) Effects of temperature on chlorophenol biodegradation kinetics in fluidized-bed reactors with different biomass carriers. *Water Research* 32:81–90.
- [271] **Melin, E.S., Puhakka, J.A., Ferguson, J.F.** (1998b) Enrichment and operation strategies for polychlorophenol degrading microbial cultures in an aerobic fluidized-bed reactor. *Water Environmnetal Research* 70: 171–180.
- [272] **Melo, J.S., Kholi, S., Patwardhan, A.W., D’Souza, S.F.** (2005) Effect of oxygen transfer limitations in phenol biodegradation. *Process Biochemistry*. 40: 625–628.

- [273] **Menke, B. and Rehm, H.J.** (1992) Degradation of mixtures of monochlorophenols and phenol as substrates for free and immobilized cells of *Alcaligenes sp A7-2*. *Applied Microbiology and Biotechnology* 37: 655–661.
- [274] **Metcalf and Eddy. Inc** (2003) Wastewater engineering: Treatment, disposal and reuse 4 th eds. Tata-McGraw-Hill, New Delhi, India.
- [275] **Meyer, B.A. and Smith, D.W.** (1985) Flow through porous media: comparison of consolidated and unconsolidated materials. *Industrial Engineering Chemistry Fundamental* 24: 360–368.
- [276] **Michels, J. and Gottschalk, G.** (1994) Inhibition of the lignin peroxidase of *Phanerochaete chrysosporium* by the hydroxylamino-dinitrotoluene intermediate in the degradation of 2,4,6 trinitrotoluene. *Applied Environmental Microbiology* 60: 18-194.
- [277] **Mileski, G.J., Bumpus, J.A., Jurek, M.A., Aust, S.D.** (1988) Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. *Applied Environmental Microbiology* 54: 2885–2889.
- [278] **Millette, D., Barker, J.F., Comeau, Y., Butler, B.J., Frind, E.O., Clément, B., Samson, R.** (1995) Substrate interaction during aerobic biodegradation of creosote-related compounds: a factorial batch experiment. *Environmental Science and Technology* 29: 1944-1952.
- [279] **Minero, C., Pelizzetti, C., Piccini, P., Vinceti, M.** (1994) Photocatalyzed transformation of nitrobenzene on TiO₂ and ZnO *Chemosphere* 28: 1229-1244.
- [280] **Minhalma, M., Domínguez, J.R., dePinho, M.N.** (2006) Cork processing wastewaters treatment by an ozonization/ ultrafiltration integrated process. *Desalination* 191: 148–152.
- [281] **Mohapatra, D.P., Brar, S.K., Tyagi, R.D., Surampalli, R.Y.** (2011) Concomitant degradation of bisphenol A during ultrasonication and Fenton oxidation and production of biofertilizer from wastewater sludge. *Ultrasonics Sonochemistry* doi:10.1016/j.ultsonch.2011.03.013.
- [282] **Mohn, H., Puhakka, J.A., Ferguson, J.F.** (1999) Effects of electron donors on degradation of pentachlorophenol in a methanogenic fluidized, bed, reactor. *Environmental Technology* 20: 909–920.

- [283] **Mohn, W.W. and Tiedje, J.M.** (1992) Microbial reductive dehalogenation. *Microbiol Review* 56: 482–507.
- [284] **Monod, J.** (1949) The growth of bacterial cultures. *Annual Reviews of Microbiology* 3: 371-394.
- [285] **Monteiro, A.A.M.G., Boaventura, R.A.R., Rodrigues, A.E.** (2000) Phenol biodegradation by *Pseudomonas putida* DSM 548 in a batch reactor. *Biochemical Engineering Journal* 6: 45-49.
- [286] **Montgomery D. C.** (2004) Design and analysis of experiments. 6th edition. John Wiley & Sons.
- [287] **Mori, M., Sayama, M., Futagami, M., Naito, T.M., Kozuka, H.** (1986) Mutagenicity of some hydroxylaminotoluene derivatives towards *Salmonella typhimurium* esterification systems, *Journal of Pharmacobiodynamics* 9 : 1036–1039.
- [288] **Morris, P.J., Mohn, W.W., Quensen III, J.F., Tiedje, J.M., Boyd, S.A.** (1992) Establishment of a polychlorinated biphenyl-degrading enrichment culture with predominantly meta dechlorination. *Applied and Environmental Microbiology* 58: 3088-3094.
- [289] **Müller, C., Petruschka, L., Cuypers, H., Burchhardt, G., Herrmann, H.** (1996) Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein PhlR. *Journal of Bacteriology* 178: 2030-2036.
- [290] **Mulchandani, A. and Luong, J.H.T.** (1989) Microbial inhibition kinetics revisited *Enzyme Microbial Technology* 11: 66-73.
- [291] **Muller, M.D. and Buser, H.R.** (1986) *Environmental Science and Technology* 20: 1151-7.
- [292] **Murthy, M.S.R.C., Swaminathan, T., Rakshit, K.Y.** (2000) Statistical optimization of lipase catalyzed hydrolysis of methyloleate by response surface methodology. *Bioprocess Engineering* 22:35–39.
- [293] **Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J.** (2000) Wastewater treatment with particulate biofilm reactors. *Journal of Biotechnology* 80: 1-33.

- [294] **Niccolai, A., Fontani, S., Kapat, A., Olivier, R.** (2003) Maximization of recombinant *Helicobacter pylori* neutrophil activating protein production in *Escherichia coli*: improvement of a chemically defined medium using response surface methodology. *FEMS microbiology Letters* 22: 257-262.
- [295] **Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J.** (2000) Wastewater treatment with particulate biofilm reactors. *Journal of Biotechnology* 80: 1-33
- [296] **Nielsen, J. and Villadsen, J.** (1994) *Bioreaction Engineering Principles*. Plenum Press, New York.
- [297] **Ning, Z., Kennedy, K.J., Fernandes, L.** (1997) Anaerobic degradation kinetics of 2,4-dichlorophenol (2,4-DCP) with linear sorption. *Water Science and Technology* 35: 67-75.
- [298] **Nomani, A.A., Ajmal, M., Ahmad, S.** (1996) Gas chromatography mass spectrometric analysis of four polluted river waters for phenolic and organic compounds. *Environmental Monitoring and Assessment* 40: 1-9.
- [299] **Nordin, K., Unell, M., Jansson, J.K.** (2005) Novel 4-chlorophenol degradation gene cluster and degradation route via hydroxyquinol in *Arthrobacter chlorophenolicus* A6. *Applied Environmental Microbiology* 71: 6538-6544.
- [300] **Nuhoglu, N. and Yalcin, B.** (2005). Modeling of phenol removal in a batch reactor. *Process Biochemistry*, 40: 1233-1239.
- [301] **O'Brien, P.J. and Herschlag, D.** (1999) Catalytic promiscuity and the evolution of new enzymatic activities. *Chemistry & Biology* 6: 91-105.
- [302] **O'Connor, O.A. and Young, L.Y.** (1993) Effect of nitrogen limitation on the biodegradability and toxicity of nitro- and aminophenol isomers to methanogenesis. *Archives of Environmental Contamination and Toxicology* 25: 285-91.
- [303] **O'Reilly, K.T. and Crawford, R.L.** (1989) Degradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium* cells. *Applied Environmental Microbiology* 55: 2113-2118.
- [304] **Öberg, T., Warman, K., Bergström, J.** (1987) Brominated aromatics from combustion. *Chemosphere* 16: 2451-2465.

- [305] **Ohlen, K., Chang, Y.K., Hegemann, W., Yin, C.R., Lee, S.T.** (2005) Enhanced degradation of chlorinated ethylenes in groundwater from a paint contaminated site by two-stage fluidizedbed reactor. *Chemosphere* 58: 373–377.
- [306] **Ohtsubo, Y., Miyauchi, K., Kanda, K., Hatta, T., Kiyohara, H., Senda, T., Nagata, Y., Mitsui, Y., Takagi, M.** (1999) PcpA, which is involved in the degradation of pentachlorophenol in *Sphingomonas chlorophenolica* ATCC39723, is a novel type of ring-cleavage dioxygenase. *FEBS Letters* 459: 395-398.
- [307] **Okeke, B.C., Paterson, A., Smith, J.E., Watson, C.I.A.** (1997) Comparative biotransformation of pentachlorophenol in soils by solid substrate cultures of *Lentinula edodes*. *Applied Microbiology and Biotechnology* 48: 563–569.
- [308] **Okpokwasili, G.C. and Nweke, C.O.** (2005) Microbial growth and substrate utilization kinetics. *African Journal of Biotechnology* 5: 305-317.
- [309] **O'Neil, M.J.** (2006) The Merck Index. 14th ed., Whitehouse Station, NJ: Merck and Co., Inc., p. 233.
- [310] **Onysko, K.A., Budman, H.M., Robinson, C.W** (2000) Effect of temperature on the inhibition kinetics of phenol biodegradation by *Pseudomonas putida* Q5. *Biotechnology and Bioengineering* 70: 291–299.
- [311] **Oren, A., Gurevich, P., Henis, Y.** (1991) Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui*. *Applied Environmental Microbiology* 57: 3367–70.
- [312] **Orser, C.S. and Lange, C.C.** (1994) Molecular analysis of pentachlorophenol degradation. *Biodegradation* 5: 277–288.
- [313] **Orser, C.S., Dutton, J., Lange, C., Jablonski, P., Xun, L., Hargis, M.** (1993) Characterization of a *Flavobacterium* glutathione S-transferase gene involved in reductive dechlorination. *Journal of Bacteriology* 175: 2640–2644.
- [314] **Paca, J., Halecky, M., Barta, J., Bajpai, R.** (2009) Aerobic biodegradation of 2,4-DNT and 2,6-DNT: Performance characteristics and biofilm composition changes in continuous packed-bedbioreactors. *Journal of Hazardous Materials* 163: 848-854.
- [315] **Padilla, L., Matus, V., Zenteno P., González, B.** (2000) Degradation of 2,4,6-trichlorophenol via chlorohydroxyquinol in *Ralstonia eutropha* JMP134 and JMP222. *Journal of Basic Microbiology* 4: 243-249.

- [316] **Pakshirajan, K., Chugh, D. and Saravanan, P.** (2008) Feasibility of *m* –cresol degradation using an indigenous mixed microbial culture with glucose as co-substrate. *Clean Technology Environmental policy* 3:303:308.
- [317] **Pamukoglu, M.Y., Kargi, F.** (2008) Biodegradation kinetics of 2,4,6-trichlorophenol by *Rhodococcus rhodochrous* in batch culture. *Enzyme Microbiol Technology* 43: 43-47.
- [318] **Papoutsakis, E.T.** (1991) Media additives for protecting freely suspended animal cells against agitation and aeration damage. *Trends Biotechnology* 9: 316-324.
- [319] **Park, H.-J. and Kim, E.-S.** (2003) An inducible *Streptomyces* gene cluster involved in aromatic compound metabolism. *FEMS Microbiology Letters* 226: 151-157.
- [320] **Pedersen, A.R. and Arvin, E.** (1995) Removal of toluene in waste gases using a biological trickling filter. *Biodegradation* 6: 109–118.
- [321] **Penttinen, O.P.** (1995) Chlorophenols in aquatic environments: structure activity Correlations. *Annales zoologici fennici* 32: 287-294.
- [322] **Perez, M., Rodri'guez-Cano, R., Romero, L.I., Sales, D.** (2006) Anaerobic thermophilic digestion of cutting oil wastewater: effect of co-substrate. *Biochemical Engineering Journal* 29: 250–257.
- [323] **Peternele, W.S., Winkler-Hechenleitner, A.A., Pineda, E.A.G.** (1999) Adsorption of Cd (II) and Pb (II) onto functionalized formic lignin from sugarcane bagasse. *Bioresource Technology*, 68: 95–100.
- [324] **Peyton, G. and Glaze, W.** (1988) Destruction of pollutants in water with ozone in combination with ultraviolet radiation. 3. Photolysis of aqueous ozone. *Environmental Science and Technology* 229: 761-767.
- [325] **Pitter, P.** (1975) Determination of biological degradability of organic substances. *Water Research* 10: 231–235. **Pitter, P.** (1985) Correlation of microbial degradation rates with the chemical structure. *Acta Hydrochim Hydrobiol* 13: 453–460.
- [326] **Piyushkumar, M., Kiran, D., Lele, S.S.** (2007) Application of response surface methodology to cell immobilization for the production of palatinose. *Bioresource Technology* 98: 2892–2896.

- [327] **Polnisch, E., Kneifel, H., Frankze, H., Hofman, K.H.** (1992) Degradation and dehalogenation of monochlorophenols by the phenol-assimilating yeast *Candida maltosa*. *Biodegradation* 2: 193–199.
- [328] **Prieto, M.B., Hidalgo, A., Rodriguez-Fernández, C., Serra, J.L., Llama, M.J.** (2002) Biodegradation of phenol in synthetic and industrial wastewater by *Rhodococcus erythropolis* UPV-1 immobilized in an air-stirred reactor with clarifier, *Applied Microbiology and Biotechnology* 58: 853–859.
- [329] **Pruden, A., Sedran, M., Cuidan, M., Venosa, A.** (2003) Biodegradation of MTBE and BTEX in an aerobic fluidized bed reactor. *Water Science and Technology* 47: 123–128.
- [330] **Przepiórski, J.** (2006) Enhanced adsorption of phenol from water by ammoniatreated activated carbon. *Journal of Hazardous materials* 135: 453–456.
- [331] **Puhakka, J.A., Melin, E.S., Jarvinen, K.T., Koro. P.M., Rintala, J.A., Hartikainen. P., Shieh, W.K., Ferguson, J.F** (1995b) Fluidizedbed biofilms for chlorophenol mineralization. *Water Science and Technology* 31: 227–235.
- [332] **Qader, A., Aman, S.U., Bano, A., Syed, S., Azhar, N.A.** (2008) Role of calcium ions and temperature on dextransucrase production. *Indian Journal of Biotechnology* 7: 404–406.
- [333] **Qiu, X., Zhonga, Q., Li, M., Baia, W., Baotong, L.B.** (2007) Biodegradation of p-nitrophenol by methyl parathion-degrading *Ochrobactrum* sp. B2. *International Biodeterioration & Biodegradation* 59: 297–301.
- [334] **Quan, X., Shi, H., Zang, Y., Wang, J., Qian, Y.** (2004) Biodegradation of 2,4 dichlorophenol and phenol in an airlift inner loop bioreactor immobilized with *Achromobacter* sp. *Separation and Purification Technology* 34: 97–103.
- [335] **Quiroz, M.A., Reyna, S., Martí'nez-Huitle, C.A., Ferr, S., Battisti, A.D.** (2005) Electrocatalytic oxidation of p-nitrophenol from aqueous solutions at Pb/PbO₂ anodes *Applied Catalysis B: Environmental* 59: 259–266.
- [336] **Ra, J.S., Oh, S.Y., Lee, B.C., Kim, S.D.** (2008).The effect of suspended particles coated by humic acid on the toxicity of pharmaceuticals, estrogens, and phenolic compounds. *Environmental international* 34: 184–192.

- [337] **Radehaus, P.M., Schmidt, S.K.** (1992) Characterization of a novel *Pseudomonas* sp. that mineralizes high concentrations of pentachlorophenol. *Applied and Environmental Microbiology* 58: 2879-2885.
- [338] **Radilla, G., Fourar, M., Larachi, F.** (2005) Correlating gas-liquid co-current flow hydrodynamics in packed beds using the F-function concept. *Journal of Chemical Technology and Biotechnology* 80: 107–112.
- [339] **Rangaswami, G. and Bagyaraj, D.J.** (2001) Agricultural Microbiology. Second edition. Prentice-Hall of India, Private Ltd, New Delhi, pp. 112.
- [340] **Ravikumar, K., Pakshirajan, K., Swaminathan, T., Balu, K.** (2005) Optimization of batch process parameters using response surface methodology for dye removal by a novel adsorbent. *Chemical Engineering Journal* 105: 131–138.
- [341] **Ray, P., Oubelli, A.M., Loser, C.** (1999) Aerobic 4-nitrophenol degradation by microorganisms fixed in a continuously working aerated solid-bed reactor. *Applied Microbiology and Biotechnology* 51: 284–290.
- [342] **Razo-Flores, E., Donlon, B., Lettinga, G., Field, J.** (1997) Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge. *FEMS Microbiology Review* 20: 525–538.
- [343] **Reardon, K.F., Mosteller, D.C., Rogers, J.D.B.** (2000) Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for *Pseudomonas putida* F1. *Biotechnology and Bioengineering* 69: 385-400.
- [344] **Reddy, G.V.B. and Gold, M.H.** (2000) Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved. *Microbiology* 146: 405–413.
- [345] **Reddy, G.V.B., Gelpke, M.D.S., Gold, M.H.** (1998) Degradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*: involvement of reductive dechlorination. *Journal of Bacteriology* 180: 5159–5164.
- [346] **Reddy, L.V.A., Wee, Y.J., Yun, J.S., Ryu, H.W.** (2008) Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett–Burman and response surface methodological approaches. *Bioresource Technology* 99: 2242-2249.

- [347] **Reid, W.D.** (1973) Mechanism of renal necrosis induced by bromobenzene or chlorobenzene. *Experimental. Molecular Pathology* 19: 197-214.
- [348] **Rezouga, F., Hamdi, M., Sperandio, M.** (2009) Variability of kinetic parameters due to biomass acclimation: Case of para-nitrophenol biodegradation by stirred tank bioreactor. *Bioresource Technology* 100: 5021–5029.
- [349] **Rhee, G.Y., Bush, B., Bethoney, C.M., Denucci, A., Oh, H.M., Sokol, R.C.** (1993) Anaerobic dechlorination of Aroclor 1242 as effected by some environmental conditions. *Environmental Toxicology and Chemistry* 12: 1033-1039.
- [350] **Rigot, J. and Matsumura, F.** (2002) Assessment of the rhizosphere competency and pentachlorophenol-metabolizing activity of a pesticide-degrading strain of *Trichoderma harzianum* introduced into the root zone of corn seedlings. *Journal of Environmental Science and Health B* 37: 201–210.
- [351] **Robert, D. and Malato.S.** (2002) Solar photocatalysis: a clean process for water detoxification *The Science of The Total Environment* 291: 85-97.
- [352] **Rodriguez, G.V., Youssef, C.B., Vilanova, J.W.** (2006). Two-step modeling of the biodegradation of phenol by an acclimated activated sludge. *Chemical Engineering Journal* 117: 245-252.
- [353] **Ronen, Z., Visnovsky, S., Nejdat, A.** (2005) Soil extracts and co-culture assist biodegradation of 2,4,6-tribromophenol in culture and soil by an auxotrophic *Achromobacter piechaudii* strain TBPZ. *Soil Biology and Biochemistry* 37: 1640–1647.
- [354] **Ruiz-Ordaz, N., Ruiz-Lagunez, J.C., Castañón-González, J.H., Hernández-Manzano, E., Cristiani-Urbina, E., Galíndez-Mayer, J.** (2001) Phenol biodegradation using a repeated batch culture of *Candida tropicalis* in a multistage bubble column. *Revista Latinoamericana de Microbiología* 43: 19-25.
- [355] **Rush, G.F., Newton, J.R., Maita, K., Kuo, C.H., Hook, J.B.** (1984) Nephrotoxicity of phenolic bromobenzene metabolites in the mouse. *Toxicology* 30: 259–272.
- [356] **Rutgers, M., Breure, A.M., vanAndel, J.G., Duetz, W.A.** (1997) Growth yield coefficients of *Sphingomonas sp. strain P5* on various chlorophenols in chemostat culture. *Applied Microbiology and Biotechnology* 48: 656–661.

- [357] **Rutgers, M., Gooch, D.D., Breure, A.M., VanAndel, J.G.** (1996) Assessment of inhibition kinetics of the growth of strain P5 on pentachlorophenol under steady-state conditions in a nutristat. *Arch of Microbiology* 165:194–200.
- [358] **Ryan, D.R., Leukes, W.D., Burton, S.G.** (2005) Fungal bioremediation of phenolics wastewaters in an airlift reactor. *Biotechnology Progress* 21: 1066-1074.
- [359] **Sahinkaya, E. and Dilek, F. B.** (2007) Biodegradation kinetics of 2,4-dichlorophenol by acclimated mixed cultures. *Journal of Biotechnology* 127: 716–726.
- [360] **Sahinkaya, E. and Dilek, F.B.** (2005) Biodegradation of 4-chlorophenol by acclimated and unacclimated activated sludge—Evaluation of biokinetic coefficients. *Environmental Research* 99: 243–252.
- [361] **Sahinkaya, E. and Dilek, F.B.** (2006) Biodegradation of 4-CP and 2,4-DCP mixture in a rotating biological contactor (RBC). *Biochemical Engineering Journal* 31: 141–147.
- [362] **Sahinkaya, E. and Dilek, F.B.** (2006) Effect of biogenic substrate concentration on 4-chlorophenol degradation kinetics in sequencing batch reactors with instantaneous feed. *Journal of Hazardous Material* 137: 282–287.
- [363] **Saia, F.T.** Damianovic, M.H.R.Z., Cattony, E.B.M., Brucha, G., Foresti, E., Vazoller, R.F. (2007) Anaerobic biodegradation of pentachlorophenol in a fixed-film reactor inoculated with polluted sediment from Santos-São Vicente Estuary, Brazil. *Environmental Biotechnology*. doi:[10.1007/s00253-007-0841-z](https://doi.org/10.1007/s00253-007-0841-z).
- [364] **Salkinoja-Salonen, M.** (1996) Description of four pentachlorophenoldegrading bacterial strains as *Sphingomonas chlorophenolica* sp nov. *System Applied Microbiology* 18: 527–538.
- [365] **Salkinoja-Salonen, M.S.** (1990) Biochemistry and ecology of the clean-up of pentachlorophenol from contaminated soils. Fifth Colloquium on Pulp and Paper Mill Effluents. Sato K (1983) Effect of a pesticide, pentachlorophenol (PCP) on soil microflora. *Plant & Soil* 75:417-426.
- [366] **Salehi, Z., Sohrabi, M., Vahabzadeh, F., Fatemi, S., Kawase, Y.** (2010) Modeling of p-nitrophenol biodegradation by *Ralstonia eutropha* via application of the substrate inhibition concept. *Journal of Hazardous Materials* 177: 582–585.

- [367] **Salehi, Z., Yoshikawa, H., Mineta, R., Kawase, Y.** (2010) Aerobic biodegradation of nitrophenol by acclimated waste activated sludge in a slurry bubble column. *Process Biochemistry* doi:10.1016/j.procbio.2010.08.024
- [368] **Salokannel, A., Heikkinen, J., Kumpulainen, M., Sillanpää, M., Turunen, J.** (2007) Tertiary treatment of pulp and paper mill wastewaters by ozonation and O₃/H₂O₂. *Paper Timber* 89:348–351.
- [369] **Schmidt, S.K., Simkins, S., Alexander, M.** (1985) Models for the kinetics of biodegradation of organic compounds not supporting growth. *Applied and Environmental Microbiology* 50: 323–331.
- [370] **Seiglemurandi, F., Steiman, R., Benoitguyod, J.L.** (1991) Biodegradation potential of some micromycetes for pentachlorophenol. *Ecotoxicology and Environmental Safety* 21: 290–300.
- [371] **Selvam, K., Muruganandham, M., Muthuvel, I., Swaminathan, M.** (2007) The influence of inorganic oxidants and metal ions on semiconductor sensitized photodegradation of 4-fluorophenol. *Chemical Engineering Journal* 128: 51–57.
- [372] **Sen, R. and Swaminathan, T.** (2004) Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production. *Biochemical Engineering Journal* 21: 141–148.
- [373] **Shahawi, M.S. E.I. and Nassif, H.** (2003) *Anal. Chim. Acta* 481–29.
- [374] **Sharma, S.L. and Pant, A.** (2001) Crude oil degradation by marine actinomycetes *Rhodococcus* species. *Indian Journal of Marine Science* 30: 146–150.
- [375] **Sheeja, R.Y. and Murugesan, T.** (2002) Mass transfer studies on the biodegradation of phenols in up-flow packed bed reactors. *Journal of Hazardous Materials*. 89: 287–301.
- [376] **Sheikh, Y.M. and Djerassi, C.** (1975) 2,6-Dibromophenol and 2,4,6-tribromophenols antiseptic secondary metabolites of *Phoronopsis viridis*. *Experientia* 31: 265–266.
- [377] **Shelley, M.D., Autenrieth, R.L., Wild, J.R., Dale, B.E.** (1996) Thermodynamics analysis of trinitrotoluene biodegradation and mineralization pathways. *Biotechnology and Bioengineering* 50: 198–205.

- [378] **Shelton, D.R. and Tiedje J.M.** (1984) General method for determining anaerobic biodegradation potential. *Applied Environmental Microbiology* 47: 850–7.
- [379] **Shim, H., Shin, E.B., Yang, S.T.** (2002) A continuous fibrous-bed bioreactor for BTEX biodegradation by a co-culture of *Pseudomonas putida* and *Pseudomonas fluorescens*. *Advanced Environmental Research* 7: 203–216.
- [380] **Shuler, M.L. and Kargi, F.** (1992) Bioprocess engineering. Upper Saddle River, NJ: Prentice Hall, 1992. pp. 254–61.
- [381] **Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Causack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs, V.C., Wallenstein, M.D., Zak, D.R., Zeglin, L.H.** (2008) Stoichiometry of soil enzyme Activity at global scale. *Ecology letter* 11:1252-1264.
- [382] **Snyder, C.J.P., Asghar, M., Scharer, J.M., Legge, R.L.** (2006) Biodegradation kinetics of 2,4,6-trichlorophenol by an acclimated mixed microbial culture under aerobic conditions. *Biodegradation* 17: 535–544.
- [383] **Solyanikova, I.P. and Golovleva, L.A.** (2004) Bacterial degradation of chlorophenols: pathways, biochemica, and genetic aspects. *Journal of Environmental Science and Health B* 39: 333–351
- [384] **Song, H.Y., Liu, J.Z., Xiong, Y.H., Weng, L.P., Ji, L.N.** (2003) Treatment of aqueous chlorophenol by phthalic anhydride-modified horseradish peroxidase. *Journal of Molecular Catalysis B: Enzymatic* 22: 37–44.
- [385] **Spain, J.C.** (1995) Biodegradation of nitroaromatic compounds. *Annual Reviews of Microbiology* 49: 523-555.
- [386] **Spain, J.D.** (1991) Gibson: Pathway for biodegradation of p-nitrophenol in a *Morexeffa* sp. *Applied Environmental Microbiology* 57: 634-641.
- [387] **Sprenger, C., Lorenzen, G., Pekdeger, A.** (2008) Occurrence and fate of microbial pathogens and organic trace compounds at riverbank filtration sites in Delhi, India., TECHNIAU.
- [388] **SRI.** (1989) Directory of Chemical Producers, United States of America. Stanford Research Institute International, Menlo Park, CA, 808.

- [389] **Stanlake, G.J. and Finn, R.K.** (1982) Isolation and characterization of a penta chloro phenol degrading bacterium. *Applied Environmental Microbiology* 44: 1421–1427.
- [390] **Stringfellow, W.T. and Aitken, M.D.** (1995) Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading *pseudomonads*. *Applied and Environmental Microbiology* 61: 357-362.
- [391] **Strotmann, U.J., Butz, B., Bias, W.R.** (1993) The dehydrogenase assay with resazurin: practical performance as a monitoring system and pH. Dependent toxicity of phenolic compounds. *Ecotoxicology and Environmental Safety* 25: 79–89.
- [392] **Stuart, S.L. and Woods, S.L.** (1998) Kinetic evidence for pentachlorophenol-dependent growth of a dehalogenating population in a pentachlorophenol- and acetate-fed methanogenic culture. *Biotechnology and Bioengineering* 57: 420–429.
- [393] **Suidan, M.T., Strubler, C.E., Kao, S.W., Pfeffer, J.T.** (1983) Treatment of coal gasification wastewater with anaerobic filter technology. *Journal of Water Pollution Control Federation* 55: 1263–1270.
- [394] **Sweetman, J.A. and Simmons, M.S.** (1980) The production of bromophenols resulting from chlorination of waters containing bromide ion and phenol. *Water Research* 14: 287–290.
- [395] **Swoboda-Colberg, N.G.** (1995) Chemical contamination of the environment: Sources, types, and fate of synthetic organic compounds, p. 27-74. In L. Y. Young and C. E. Cerniglia (ed.), *Microbial Transformation and Degradation of Toxic Organic Chemicals*. Wiley-Liss, New York, N.Y.
- [396] **Tabatabai, M.A.** (1994) Soil enzymes. In: Weaver RW, Angle S, Bottomley P (eds) *Methods of soil analysis. Part 2: Microbiological and biochemical properties*. Soil Science Society of America, Madison, pp 775–833.
- [397] **Taguchi, H. and Humphrey, A.E.** (1996) Dynamic measurement of the volumetric oxygen transfer coefficients in fermentation systems. *Journal of Fermentation Technology* 44: 881–889.
- [398] **Takenaka, S., Okugawa, S., Kadowaki, M., Murakami, S., Aoki, K.** (2003) The metabolic pathway of 4-aminophenol in *Burkholderia sp. strain AK-5* differs from

- that of aniline and aniline with C-4 substituents. *Applied and Environmental Microbiology* 69: 5410-5413.
- [399] **Tan, H.M.** (1999) Bacterial catabolic plasmids. *Applied Microbiology and Biotechnology* 51: 1- 12.
- [400] **Tanaka, H., Matsumura, M., Veliky, I.A.** (1984) Diffusion characteristics of substrates in Ca-alginate gel beads. *Biotechnology and Bioengineering* 26: 53– 8.
- [401] **Tani, K., Muneta, M., Nakamura, K., Shibuya, K., Nasu, M.** (2002) Monitoring of *Ralstonia eutropha* KT1 in groundwater in an experimental bioaugmentation field by in situ PCR. *Applied and Environmental Microbiology* 68: 412-416.
- [402] **Tanyildizim, M.S., Ozer, D., Elibol, M.** (2005) Optimization of α -amylase production by *Bacillus* sp. using response surface methodology. *Process Biochemistry* 40: 2291–2296.
- [403] **Tartakovsky, B., Manuel, M.F., Beaumier, D., Greer, C.W., Guiot, S.R.** (2001) Enhanced selection of an anaerobic pentachlorophenol- degrading consortium. *Biotechnology and Bioengineering* 73: 476–483.
- [404] **Tchobanoglous, G.** (1998) Wastewater Engineering, Treatment and Reuse, third ed. McGraw-Hill, New York (Metcalf& Eddy).
- [405] **TenBrook, P.L., Kendall, S.M., Viant, M.R., Tjeerdema, R.S.** (2003) Toxicokinetics and biotransformation of p-nitrophenol in red abalone (*Haliotis rufescens*) *Aquatic Toxicology* 62: 329-336.
- [406] **Tepe, O. And Dursun, A. Y.** (2008) Combined effects of external mass transfer and biodegradation rates on removal of phenol by immobilized *Ralstonia eutropha* in a packed bed reactor. *Journal of Hazardous Materials* 151: 9-16.
- [407] **Terada, H.** (1990) Uncouplers of oxidative phosphorylation. *Environmental Health Perspectives* 87: 213-218.
- [408] **Terada, Ii.** (1993) The interaction of highly active uncouplers with mitochondria. *Biochemical and Biophysic Acta* 639: 225-242.
- [409] **Thakur, I.S., Verma, P., Upadhyaya, K.** (2002) Molecular cloning and characterization of pentachlorophenol-degrading monooxygenase genes of *Pseudomonas* sp from the chemostat. *Biochemical and Biophysic Resesearch Communication* 290: 770–774.

- [410] **Tiedje, J.M., Duxbury, J.M., Alexander, M., Dawson, J.E.** (1969) 2,4- D Metabolism – pathway of degradation of chlorocatechols by *Arthrobacter sp.* *Journal of Agriculture and Food Chemistry* 17: 1021–1026.
- [411] **Tirola, M.A., Busse, H.J., Kampfer, P., Mannisto, M.K.** (2005) *Novosphingobium lentum sp nov.*, a psychrotolerant bacterium from a polychlorophenol bioremediation process. *Int Journal of Systematic Evolution and Microbiology* 55: 583–588.
- [412] **Tirola, M.A., Wang, H., Paulin, L., Kulomaa, M.S.** (2002) Evidence for natural horizontal transfer of the pcpB gene in the evolution of polychlorophenol-degrading *sphingomonads*. *Applied Environmental Microbiology* 68: 4495–4501.
- [413] **Timmis, K.N. and Pieper, D.H.** (1999) Bacteria designed for bioremediation. *Trends in Biotechnology* 17: 201-204.
- [414] **Timmis, K.N., Steffan, R.J., Unterman, R.** (1994) Designing microorganisms for the treatment of toxic wastes. *Annual Review of Microbiology* 48: 525–557.
- [415] **Tolosa, I., Bayona, J.M., Albaiges, J.** (1991) Identification and occurrence of brominated and nitrated phenols in estuarine sediments. *Marine Pollution Bulletin* 22: 603–607.
- [416] **Tomei, M.C. and Annesini, M.C.** (2008) Biodegradation of phenolic mixtures in a sequencing batch reactor a kinetic study. *Envnmental Scince and Pollution Research* 15: 188 – 195.
- [417] **Tomei, M.C. Annesini, M.C. Lubertia, R., Cento, G., Senia, A.** (2003) Kinetics of 4-nitrophenol biodegradation in a sequencing batch reactor. *Water Reserach* 37: 3803–3814.
- [418] **Tomei, M.C., Annesini, M.C., Bussolletti, S.** (2004) 4-nitrophenol biodegradation in a sequencing batch reactor: kinetice study asnd effect of filling time. *Water research* 38: 375-384.
- [419] **Toril, I.R.U.** (2001) Chemical characterization of produced water from four offshore oil production platforms in the North Sea. *Chemosphere* 39: 2593–606.
- [420] **Trapido, M. and Kallas, J.** (2000) Advanced oxidation processes for the degradation and the detoxification of 4-nitrophenol. *Environmental Technology* 21: 799–808.
- [421] **TRI.** (1989) The Toxic Release Inventory. Office of Toxic Substances, Washington, DC. November 8.

- [422] **TRI.** (1998) Toxics release inventory. Washington, DC, US Environmental Protection Agency, Office of Toxic Substances (17 December).
- [423] **Tsai, K.C. and Folsom, C.M.** (1982) Treatment of coal liquefaction waste with PAC-enhanced activated sludge process. In: Proceedings of the 39th Industrial Waste Conference, Purdue University, Purdue, May, pp 497–505.
- [424] **Tsai, S.Y. and Juang, R.S.** (2006) Biodegradation of phenol and sodium salicylate mixtures by suspended *Pseudomonas Putida* CCRC 14365. *Journal of Hazardous Materials*, 138: 125-132.
- [425] **Tuomela, M., Lyytikainen, M., Oivanen, P., Hatakka, A.** (1999) Mineralization and conversion of pentachlorophenol (PCP) in soil inoculated with the *white-rot fungus Trametes versicolor*. *Soil Biology and Biochemistry* 31: 65–74.
- [426] **Turhan, K., and Zuhail, T.** (2009) Decolorization of direct dye in textile wastewater by ozonization in a semi-batch bubble column reactor. *Desalination* 242: 256–263.
- [427] **Tyler, J.E. and Finn, R.K.** (1974) Growth rates of a *Pseudomonad* on 2,4- D and 2,4 dichlorophenol. *Applied Microbiology* 28: 181–184.
- [428] **Tziotziou, G., Teliou, M., Kaltsouni, V., Lyberatos, G., Vayenas, D.V.** (2005) Biological phenol removal using suspended growth and packed bed reactors. *Biochemical Engineering Journal* 26: 65–71.
- [429] **U.S. Public Health Service.** (1992) Toxicological profile for nitrophenols: 2-nitrophenol, 4-nitrophenol. *Agency for Toxic Substances and Disease Registry*. page - 3
- [430] **Uberoi, V. and Bhattacharya, S.K.** (1997) Toxicity and degradability of nitrophenols in anaerobic systems. *Water Environment Research* 69: 146–54.
- [431] **Uhnáková, B., Petrůčková, A., Biedermann, D., Homolka, L., Vejvoda, V., Bednár, P., Papoušková, B., Šulc, M., Martínková, L.** (2009) Biodegradation of brominated aromatics by cultures and laccase of *Trametes versicolor*. *Chemosphere* 76: 826–832.
- [432] **Unell, A., Nordin, K., Jernberg, C., Stenstrom, J., Jansson, J.K.** (2008) Degradation of mixtures of phenolic compounds by *Arthrobacter chlorophenolicus* A6. *Biodegradation* 19: 495–505.

- [433] **Uotila, J.S., Kitunen, V.H., Coote, T., Saastamoinen, T., Salkinoja-Salonen, M., Apajalahti, J.H.A.** (1995) Metabolism of halohydroquinones in *Rhodococcus chlorophenolicus* PCP-1. *Biodegradation* 6: 119-126.
- [434] **Uotila, J.S., Kitunen, V.H., Saastamoinen, T., Coote, T., Haggblom, M.M., Salkinoja-Salonen, M.S.** (1992) Characterization of aromatic dehalogenases of *Mycobacterium fortuitum* CG- 2. *Journal of Bacteriology* 174: 5669–5675.
- [435] **Uotila, J.S., Salkinoja-Salonen, M.S., Apajalahti, J.H.A.** (1991) Dechlorination of pentachlorophenol by membrane bound enzymes of *Rhodococcus chlorophenolicus* PCP-I. *Biodegradation* 2: 25–31.
- [436] **Valli, K, Gold, M.H.** (1991) Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium*. *Journal of Bacteriology* 173: 345–352.
- [437] **Valo, R. and Salkinoja-Salonen, M.** (1984) Clean up of chemically contaminated soil using microbes. *Kemia- Kemi* 11: 948-949.
- [438] **Valo, R.J., Haggblom, M.M., Salkinoja-Salonen, M.S.** (1990) Bioremediation of chlorophenol containing simulated ground water by immobilized bacteria. *Water Reserach* 24: 253–258.
- [439] **Van, H.J.D. and Ward, O.P.** (2001) Volatile hydrocarbon biodegradation by a mixed-bacterial culture during growth on crude oil. *Journal of Industrial Microbiology and Biotechnology* 26: 356–362.
- [440] **VanBriesen, J.M., Blough, M.S., Brown, W.E., Minkley, E.G.** (2004) Critical oxygen concentrations for biodegradation of PCBs. Symposia Papers Presented Before the Division of Environmental Chemistry, American Chemical Society, Philadelphia, PA August 22-26.
- [441] **vanden Berg, K.J.** (1990) Interaction of chlorinated phenols with thyroxine binding sites o human transthyretin, albumin and thyroid binding globulin. *Chemico-Biological Interactions* 76: 63- 75.
- [442] **Vanderberg, L.A., Krieger-Grumbine, R., Taylor, M.N.** (2000) Evidence for divers oxidations in the catabolism of toluene by *Rhodococcus rhodocrous* strain OFS. *Applied Microbiology and Biotechnology* 53: 447-452.

- [443] **Vasconcelos, J.M.T., Rodrigues, J.M.L., Orvalho, S.C.P., Alves, S.S., Mendes, R.L., Reis, A.** (2003) Effect of contaminants on mass transfer coefficients in bubble column and airlift contactors. *Chemical Engineering and Science* 58: 1431–1440.
- [444] **Vázquez, I., Rodríguez-Iglesias, J., Marañón, E., Castrillón L., Álvarez, M.** (2007). Removal of residual phenols from coke wastewater by adsorption. *Journal of Hazardous materials* 147: 395-400.
- [445] **Venkatachalam, N., Palanichamy, M., Murugesan, V.** (2007a) Sol–gel preparation and characterization of alkaline earth metal doped nano TiO₂: Efficient photocatalytic degradation of 4-chlorophenol. *Journal of Molecular Catalysis A: Chemical* 273: 177–185.
- [446] **Vetter, W. and Janussen, D.** (2005) Halogenated natural products in five species of antarctic sponges: compounds with POP-like properties? *Environmental Science and Technology* 39: 3889–3895.
- [447] **Vidali, M.** (2001) Bioremediation an overview. *Pure Applied Chemistry* 73: 1163–1172.
- [448] **Vinod, V.A. and Reddy, G.V.** (2005) Simulation of biodegradation process of phenolic wastewater at higher concentrations in a fluidized-bed bioreactor. *Biochemical Engineering Journal* 24: 1-10.
- [449] **Vione, D., Minero, C., Maurino, V., Carlotti, M.E., Picatotto, T., Pelizzetti, E.** (2005) Degradation of phenol and benzoic acid in the presence of a TiO₂-based heterogeneous photocatalyst. *Applied Catalysis B: Environmental* 58: 79-88.
- [450] **Viraraghavan, T. and Alfaro, F.de.M.** (1998) Adsorption of phenol from wastewater by peat, fly ash and bentonite. *Journal of Hazardous materials* 57: 59-70.
- [451] **Visvanathan, C., Thu, L.N., Jegatheesan, V., Anotai, J.** (2005) Biodegradation of pentachlorophenol in a membrane bioreactor. *Desalination* 183: 455–464.
- [452] **Wan, N., Gu, J.D., Yan, Y.** (2007) Degradation of p-nitrophenol by *Achromobacter xylosoxidans* Ns isolated from wetland sediment. *International Biodeterioration and Biodegradation* 59: 90–96.
- [453] **Wang S.J. and Zhong, J.J.** (2006) In: S. T. Yang, Eds., *Bioprocessing for value-added products from renewable resources*. Elsevier, Amsterdam, 131.

- [454] **Wang, Q., Hou, Y., Xu, Z., Miao, J., Li, G.** (2008a) Optimization of cold-active protease production by the psychrophilic *bacterium Colwellia* sp. NJ341 with surface response methodology. *Bioresource Technology* 99: 1926-1931.
- [455] **Wang, S. J. and Zhong, J. J.** (2006) In: S. T. Yang, Eds., *Bioprocessing for value-added products from renewable resources*, Elsevier, Amsterdam, 131.
- [456] **Wang, S.J. and Loh K.C.** (1999) Modeling the role of metabolic intermediates in kinetics of phenol biodegradation. *Enzyme and Microbial Technology* 25: 177-184.
- [457] **Wang, S.J. and Loh, K.C.** (1999) Facilitation of cometabolic degradation of 4-chlorophenol using glucose as an added growth substrate. *Biodegradation* 10: 261–269.
- [458] **Wang, Y.** (1992) Effect of chemical oxidation on anaerobic biodegradation of model phenolic compounds. *Water Environment Research* 64: 268-273.
- [459] **Wang, Y.H., Chan, K.Y., Li, X.Y., So, S.K.** (2006). Electrochemical degradation of 4-chlorophenol at nickel–antimony doped tin oxide electrode. *Chemosphere* 65: 1087–1093.
- [460] **Wang, Y.H., Li, Y.P., Zhang, O., Zhang, X.** (2008b) Enhanced antibiotic activity of *Xenorhabdus nematophila* by medium optimization. *Bioresource Technology* 99: 1708-1715.
- [461] **Watanabe, .I, Takahashi, K., Tatsukawa, R.** (1985) Brominated phenols and anisoles in river and marine sediments in Japan. *Bulletin of Environmental Contamination and Toxicology* 35: 272–278.
- [462] **Watanabe, I., Kashimoto, T., Tatsukawa, R.** (1984) Brominated phenol production from the chlorination of wastewater containing bromide ions. *Bulletin of Environmental Contamination and Toxicology* 33: 395–399.
- [463] **Waul, C., Arvin, E., Schmidt, J.E.** (2008) Model description and kinetic parameter analysis of MTBE biodegradation in a packed bed reactor. *Water Research, Volume* 42: 3122-3134.
- [464] **Webb, J.L.** (1963) *Enzyme and Metabolic Inhibitors*. Academic Press, Boston, USA.
- [465] **Wegman, R.C.C. and Hofstee, A.W.M.** (1979) Chlorophenols in surface waters of the Netherlands (1976-1977). *Water Research* 13: 651-657.

- [466] **Wegman, R.C.C. and vanden Broek, H.H.** (1983) Chlorophenols in river sediment in the Netherlands. *Water Research* 17: 227-230.
- [467] **Wei, Q., Liu, H., Zhang, J.J., Wang, S.H., Xiao, Y., Zhou, N.Y.** (2010) Characterization of a para-nitrophenol catabolic cluster in *Pseudomonas sp.* strain NyZ402 and construction of an engineered strain capable of simultaneously mineralizing both para- and ortho-nitrophenols. *Biodegradation* doi:10.1007/s10532-009-9325-4.
- [468] **Weihua, S., Zheng, Z., Rami, A.S., Tao, Z., Desheng, H.** (2002) Degradation and detoxification of aqueous nitrophenol solutions by electron beam irradiation, *Radiation Physics and Chemistry* 65: 559–563.
- [469] **Wester, R.C., Maibach, H.I., Sedik, L., Melendres, J., Wade, M., Dizio, S.** (1993) Percutaneous absorption of pentachlorophenol from soil. *Fundamental of Applied Toxicology* 20: 68-71.
- [470] **Westerberg, K., Elvang, A.M., Stackebrandt, E., Jansson, J.K.** (2000) *Arthrobacter chlorophenolicus* sp nov., a new species capable of degrading high concentrations of 4-chlorophenol. *International Journal of Systematic Evolution Microbiology* 50: 2083–2092.
- [471] **Whitfield, F.B., Helidoniotis, F., Shaw, K.J., Svoronos, D.** (1999) Distribution of bromophenols in species of marine algae from eastern Australia. *Journal of Agricultural and Food Chemistry* 47: 2367–2373.
- [472] **Whitfield, F.B., Last, J.H., Shaw, K.J., Tindale, C.R.** (1988) 2,6- Dibromophenols: the cause of an iodoform-like flavour in some Australian crustacea. *Journal of the Science of Food and Agriculture* 46: 29–42.
- [473] **Whitfield, F.B., Shaw, K.J., Walker, D.I.** (1992) The source of 2,6- dibromophenol: cause of an iodoform taint in Australian prawns. *Water Science and Technology* 25: 131–138.
- [474] **WHO.** (1989) Chlorophenols other than pentachlorophenol. World Health Organization, Geneva
- [475] **WHO.** 2005 2,4,6-tribromophenol and other simple brominated phenols. P.D. Howe, Dr S. Dobson, and Mr H.M. Malcolm, Centre for Ecology & Hydrology, Monks Wood, United Kingdom.

- [476] **Wiesmann, U. and Putnaerglis, A.** (1986) Influence of oxygen concentration on substrate degradation in activated sludge reactors. *German Chemical Engineering* 9: 284-291.
- [477] **Wild, S.R., Harrad, S.J., Jones, K.C.** (1993) Chlorophenols in digested U.K. sewage sludges. *Water Reserach* 27: 1527-1534.
- [478] **Wu, J., Bewtra, J., Biswas, N., Taylor, K.** (1994) Effect of H₂O₂ addition mode on enzymatic removal of phenol from wastewater in the presence of polyethylene glycol. *Canadian Journal of Chemical Engineering* 72: 881-886.
- [479] **Wu, W.M., Bhatnagar, L., Zeikus, J.G.** (1993) Performance of anaerobic granules for degradation of pentachlorophenol. *Applied Environmental Microbiology* 59: 389–397.
- [480] **Xiangchun, Q., Hanchang, S., Zhang, J.W., Yi, Q.** (2003) Biodegradation of 2,4 dichlorophenol in an airlift honeycomb like ceramic reactor. *Process Biochemistry* 38:1545-1551.
- [481] **Xing-yu, L., Bao-jun, W., Cheng-ying, J., Ke-xin, Z., Drake, H.L., Shuang-Jiang, L.** (2007) Simultaneous biodegradation of nitrogen-containing aromatic compounds in a sequencing batch bioreactor. *Journal of Environmental Science* 19: 530–535.
- [482] **Xu, L., Resing, K., Lawson, S.L., Babbitt, P.C., Copley, S.D.** (1999) Evidence that pcpA encodes 2,6-dichlorohydroquinone dioxygenase, the ring cleavage enzyme required for pentachlorophenol degradation in *Sphingomonas chlorophenolica* strain ATCC 39723. *Biochemistry* 38: 7659–7669.
- [483] **Xun, L., Bohuslavek, J., Cai, M.** (1999) Characterization of 2,6-dichloro-p-hydroquinone 1,2- dioxygenase (PcpA) of *Sphingomonas chlorophenolica* ATCC 39723. *Biochemical and Biophysichal Research Communications* 266: 322-325.
- [484] **Xun, L., Topp, E., Orser, C.S.** (1992) Diverse substrate range of a *Flavobacterium* pentachlorophenol hydroxylase and reaction stoichiometries. *Journal of Bacteriology* 174: 2898–2902.
- [485] **Xun, L., Topp, E., Orser, C.S.** (1992) Purification and characterization of a tetrachloro-phydroquinone reductive dehalogenase from a *Flavobacterium* sp. *Journal of Bacteriology* 174: 8003-8007.

- [486] **Xun, L.Y and Webster, C.M.** (2004) A monooxygenase catalyzes sequential dechlorinations of 2,4,6-trichlorophenol by oxidative and hydrolytic reactions. *Journal of Biological Chemistry* 279: 6696–6700.
- [487] **Xun, L.Y., Topp, E., Orser, C.S.** (1992a) Confirmation of oxidative dehalogenation of pentachlorophenol by a *Flavobacterium* pentachlorophenol hydroxylase. *Journal of Bacteriology* 174: 5745–5747.
- [488] **Xun, L.Y., Topp, E., Orser, C.S.** (1992b) Purification and characterization of a tetrachloro-p-hydroquinone reductive dehalogenase from a *Flavobacterium* sp. *Journal of Bacteriology* 174: 8003–8007.
- [489] **Yamada, T., Takahama, Y., Yamada, Y.** (2008) Biodegradation of 2,4,6-tribromophenol by *Ochrobactrum* sp. strain TB01. *Bioscience Biotechnology and Biochemistry* 72: 1264–1271.
- [490] **Yan, J., Jianping, W., Bai, J., Daoquan, W. Zongding, H.** (2006) Phenol biodegradation by the yeast *Candida tropicalis* in the presence of *m*-cresol. *Biochemical Engineering Journal* 29: 227-23.
- [491] **Yang, C.F., Lee, C.M., Wang, C.C.** (2005) Degradation of chlorophenols using pentachlorophenol-degrading bacteria *Sphingomonas chlorophenolica* in a batch reactor. *Current Microbiology* 51:156–160.
- [492] **Yang, R. D. and Humphrey, A. E.** (1975) Dynamic and steady state studies of phenol biodegradation in pure and mixed cultures. *Biotechnology and Bioengineering* 17: 1211-1235.
- [493] **Yano, T. and Koga, S.** (1969) Dynamic behaviour of the chemostat subject to substrate inhibition. *Biotechnology and Bioengineering* 11: 139-153.
- [494] **Yano, T., Nakahara, T., Kamiyama, S., Yamada, K.** (1996) Kinetic studies on microbial activities in concentrated solutions and Effect of excess sugars on oxygen uptake rate of a cell-free respiratory system. *Agricultural and Biological Chemistry* 30: 42–48.
- [495] **Ye, F.X., Shen, D.S., Feng, X.S.** (2004) Anaerobic granule development for removal of pentachlorophenol in an upflow anaerobic sludge blanket (UASB) reactor. *Process Biochemistry* 39: 1249–1256.

- [496] **Zaborina, O., Daubaras, D.L., Zago, A., Xun, L., Saido, K., Klem, T., Nikolic, D., Chakrabarty, A.M.** (1998) Novel pathway for conversion of chlorohydroxyquinol to maleylacetate in *Burkholderia cepacia* AC1100. *Journal of Bacteriology* 180: 4667-4675.
- [497] **Zechendorf, B.** (1999) Sustainable development: how can biotechnology contribute? *Trends in Biotechnology* 17: 219-225.
- [498] **Zhang, X and Wiegel, J.** (1990) Sequential anaerobic degradation of 2,4-dichlorophenol in freshwater sediments. *Applied Environmental Microbiology* 56: 1119-1127.
- [499] **Zhao, X., Hickey, R.F., Voice, T.C.** (1999) Long-term evaluation of adsorption capacity in a biological activated carbon fluidized bed reactor system. *Water Research* 33: 2983-2991.
- [500] **Zheng, Y., Liu, D., Liu, Shiwang., Xu, Shangying., Yuan, Y., Xiong, Li.** (2009) Kinetics and mechanisms of *p*-nitrophenol biodegradation by *Pseudomonas aeruginosa* HS-D38. *Journal of Environmental Sciences* 21: 1194-1199.
- [501] **Zheng, Y., Liu, D., Xu, H., Zhong, Y., Yuan, Y., Xiong, L., Li, W.** (2009) Biodegradation of *p*-nitrophenol by *Pseudomonas aeruginosa* HS-D38 and analysis of metabolites with HPLC-ESI/MS. *International Biodeterioration and Biodegradation* 63: 1125-1129.
- [502] **Zhong, J.J.** (2010). Recent advances in bioreactor engineering *Korean Journal of Chemical Engineering* 27: 1035-1041.
- [503] **Zhou, T.C., Zhou, W.W., Hu, W.W., Zhong, J.J.** (2010) Encyclopedia of Industrial Biotechnology, Wiley, 2, 913.
- [504] **Zilouei, H., Guieysse, B., Mattiasson, B.** (2006) Biological degradation of chlorophenols in packed-bed bioreactors using mixed bacterial consortia. *Process Biochemistry* 41:1083-108.
- [505] **Zilouei, H., Soares, A., Murto, M.,** (2006) Influence of temperature on process efficiency and microbial community response during the biological removal of chlorophenols in a packed-bed bioreactor. *Applied Microbiology and Biotechnology* 72: 591-599.

- [501] **IRDC** (1974c) Acute oral toxicity LD50 study in albino rats. Mattawan, MI, International Research Deveopcorporationn (Report No. EPA/OTS 86-900000307; NTIS/OTS 0523299; submitted to US EPA by Great Lakes Chemical Corporation, West Lafayette, IN, March 1990)”
- [502] **Nicolella, C., van Loosdrecht, M.C.M., Heijnen, J.J.** (2000) Wastewater treatment with particulate biofilm reactors. *Journal of Biotechnology* 80: 1-33.
- [503] **Salkinoja-Salonen, M.S.** (1990) Biochemistry and ecology of the clean-up of pentachlorophenol from contaminated soils. Fifth Colloquium on Pulp and Paper Mill Effluents. Sato K (1983) Effect of a pesticide, pentachlorophenol (PCP) on soil microflora. *Plant & Soil* 75:417-426.
- [504] **Shahawi, M.S. E.I. and Nassif, H.** (2003) *Anal. Chim. Acta* 481-29.

LIST OF PUBLICATIONS

Published/Accepted in Refereed International Journals

1. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K.** (2010) Medium Development for Enhancing the Biodegradation of 4-Chlorophenol by *Arthrobacter chlorophenolicus* A6. (2010). *International Biodeterioration and Biodegradation* 64: 474-480.
2. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K., Ghosh, A.** (2011) Biodegradation of 4-Chlorophenol by *Arthrobacter chlorophenolicus* A6: Effect of Culture Conditions and Degradation Kinetics. *Biodegradation*. 22: 275-286.
3. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K.** (2010) Biodegradation of para-nitrophenol using *Arthrobacter chlorophenolicus* A6 in a novel upflow packed bed reactor . *Journal of Hazardous materials* 190:729-737.
4. **Ghosh, A., Pakshirajan, K., Ghosh, P.K., Sahoo, N. K.** (2011) Perchlorate degradation by an indigenous microbial consortium predominantly *Burkholderia* sp. *Journal of Hazardous Materials*.187:133-139.
5. **Pakshirajan, K., Sivasankar, A., Sahoo, N.K.** (2011) Decolourization of synthetic wastewater containing azo dyes by immobilized *Phanerochaete chrysosporium* in a continuously operated RBC reactor. *Applied Microbiology and Biotechnology*. 89: 1223-1232.
6. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K.** (2011). Biodegradation kinetics of paranitrophenol by *Arthrobacter chlorophenolicus* A6. *Applied Biochemistry and Biotechnology*. 165: 1587-1596.
7. **Sahoo, N.K., Ghosh, P.K., Pakshirajan, K.** (2011). Kinetics of 4-bromophenol degradation using calcium alginate immobilized *Arthrobacter chlorophenolicus* A6. *International journal of Earth Science and Engineering*. 4: 663-668.

Communicated/Under Review in Refereed International Journals

1. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K. (2011)** Biodegradation of para-bromo phenol using *Arthrobacter chlorophenolicus* A6 in a novel upflow packed bed reactor *journal of Bioscience and Bioengineering*.
2. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K. (2011)** Biodegradation of para-chloro phenol using *Arthrobacter chlorophenolicus* A6 in a novel upflow packed bed reactor. *Journal of Environmental Management*.
3. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K. (2011)** Biodegradation kinetics of para-bromophenol by *Arthrobacter chlorophenolicus* A6. *Journal of Chemical Technology and Biotechnology*.

In International Conferences

1. **Sahoo, N.K., Ghosh, P.K., Pakshirajan, K. (2011).** Kinetics of 4-bromophenol degradation using calcium alginate immobilized *Arthrobacter chlorophenolicus* A6. *International conference on advanced in civil engineering*. KL University, Guntur Andra pradesh India, during 21th-23rd October (2011).
2. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K. (2011)** Biodegradation of p-bromophenol using Ca-alginate immobilized *Arthrobacter chlorophenolicus* A6 gel beads (Poster). International conference on Climate change and Water. Organized by Department of humanities science of Indian institute of Technology Guwahati During 3rd -5th January 2011.
3. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K. (2010)** Batch biodegradation of para-Bromophenol by *Arthrobacter chlorophenolicus* A6. International conference on genomics sciences (Poster). VII Convention of BRSI organized by Department of Biotechnology of Madurai Kamraj University, Madurai During 12th- 14th November 2010.

4. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K.** (2010) Kinetics of growth and biodegradation of p-nitrophenol and p-chlorophenol by *Arthrobacter chlorophenolicus* A6 (Oral). Ninth International Conference on Hydro-Science and Engineering. Organized by Department of Ocean Engineering, Indian institute of Technology Madras, Chennai During 2nd-5th August 2010.
5. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K.** (2010) Growth and biodegradation kinetics of p-bromophenol and p-nitrophenol by *Arthrobacter chlorophenolicus* A6 (Poster). International conference Environmental Health and Toxicity. Organized by Department of Civil Engineering of Indian institute of Technology, Kanpur During 15th -17th March 2010.
6. **Sahoo, N.K., Pakshirajan, K., Ghosh, P.K.** (2009) Effect of Culture Conditions and Degradation Kinetics of *Arthrobacter chlorophenolicus* A6 Enhancing the Biodegradation of 4-Chlorophenol (Poster). International conference on Emerging Trend in Biotechnology. VI Convention of BRSI, Organized by Department of Biotechnology of Banaras Hindu University Varanasi During 4th - 6th December 2009.

In International Conferences

1. **Sahoo, N., Pakshirajan, K. and Ghosh, P. K.** (2008) Screening of media constituents for chlorophenol biodegradation by *Arthrubactor chlorophenolicus* A6 employing full factorial design of experiments (Oral). ChEmference'08, organized at Department of Chemical Engineering of Indian Institute of Technology Kanpur during 5th-6th July 2008.
2. **Sahoo, N.K., Pakshirajan, K., Ghosh, P. K.** (2007) Evaluation of actinomycetes and bacteria isolated from soil samples in biodegradation of chlorophenols (Poster). 48th Annual conference of AMI, Organized by Department of Biotechnology of Indian Institute of Technology Madras, Chennai During 18th-21st December 2007.