STUDIES ON MICROBIAL REDUCTION OF PERCHLORATE IN BATCH AND CONTINIOUS SYSTEMS

A Thesis Submitted to the Indian Institute of Technology Guwahati, India, for the Award of the Degree of

DOCTOR OF PHILOSOPHY

By

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I dedicate this thesis to my family and friends

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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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. Pranab Kumar Ghosh, Associate Professor of Department of Civil Engineering and Dr. Kannan Pakshirajan, Associate Professor of Department of Biotechnology.

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

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CERTIFICATE

This is to certify that the work presented in this Thesis entitled "Studies on microbial reduction of perchlorate in batch and continuous systems" by Atreyi Ghosh (Roll No. 06615203) 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. Atreyi Ghosh has worked in the "Centre for Environment" of IIT Guwahati and this Thesis, in our opinion is of the standard required for the award of the degree of Doctor of Philosophy. The results in this Thesis have not been submitted elsewhere for any degree or diploma.

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ABSTRACT

Perchlorate contamination of the ground water, soil and surface water is a public health concern of recent times due to their detrimental effect of human health and other living entities. Therefore, its removal from contaminated systems is necessary for the human well being and environment. Perchlorate salts (especially ammonium and sodium) are extensively used mostly in military ammunitions and fireworks industries all over the world.

Several removal techniques such as ion exchange, adsorption and precipitation, chemical and electro-chemical processes have been tested for perchlorate from water systems. Those techniques majorly suffer from one or more disadvantages like, high maintenance cost, regeneration of brines and inefficient removal of perchlorate etc. In this scenario, the eco-friendly bioreduction using microbes in pure or mixed cultures shows promising future in the field of perchlorate removal from water and wastewater. The main focus of the present investigation was to develop a complete anaerobic treatment system for perchlorate bearing wastewater in anaerobic process using a mixed microbial consortium. The mixed microbial consortium was collected from an activated sludge reactor and investigated for its potential in perchlorate degradation from synthetic wastewater in batch shake flasks and as well as continuous systems.

The capacity of the mixed microbial consortium to reduce perchlorate using phenol and other five different carbon sources and phenol was tested in batch system. Compared to other tested carbon-sources used in this study, succinate has proven to be better for perchlorate degradation by the mixed consortium predominantly *Burkholderia* sp. on the other hand the mixed microbial consortium predominantly *Pseudoxanthomonas* sp. was found to utilize phenol as sole C-source

for perchlorate bioreduction which is reported for the first time. The mixed microbial culture grown in batch mode with perchlorate along with succinate and phenol separately was isolated to identify the predominant strains. The 16S rDNA analysis of the predominant strain showed to be *Burkholderia*. sp. using succiante as sole C-source in the mixed culture. For the first time *Burkholderia*. sp., predominantly present in this mixed culture, has been reported to be involved in perchlorate degradation. Newly isolated bacterial species *Pseudoxanthomonas* sp. isolated from a sewage sludge consortium was found to reduce perchlorate while taking phenol as electron acceptor. The optimum conditions for perchlorate reduction by the enriched mixed culture predominantly *Burkholderia* sp. was found to be 30^{o}C and pH 7.0. Plackett–Burman screening and Taguchi design was employed for screening 5 parameters for perchlorate degradation from two isolated bacterial strains *Burkholderia* sp. and *Pseudoxanthomonas* sp., utilizing succinate and phenol as C-source. Four physical parameters: temperature, pH, inoculums age and inoculums volume were selected along with the ration of carbon source and perchlorate concentration. Coefficients and sum of squares ratio in percentage (%) of these variables were calculated by subjecting the experimental data to statistical analysis. Temperature, inoculum age and carbon to perchlorate ration showed significant importance in perchlorate degradation by mixed consortium predominantly with *Burkholderia* sp. using succinate as C-source. Temperature, inoculum age and pH showed significant effect in case of mixed consortium predominantly *Pseudoxanthomonas* sp. using phenol as C-source. Those factors were further analyzed for optimization by Taguchi method to get the optimum culture conditions. Use of this design is scarce in perchlorate degradation and has not been attempted previously.

The effect of co-pollutants (which are commonly present in the wastewaters along with perchlorate (e.g., nitrate, chlorate, phosphate and sulfate) on perchlorate removal using succinate

as well as phenol as electron donor by the mixed cultures predominant with respective strains was also investigated at initial perchlorate concentration of 500 mg /L. Results have shown that the degradation of perchlorate was affected to different extent in presence of an equal concentration (500 mg/L of each) of co-pollutants such as nitrate, chlorate and phosphate. The presence of nitrate showed considerable effect on perchlorate reduction.

A mixed microbial consortium predominantly *Burkholderia* sp. capable to degrade perchlorate under anaerobic condition was enriched from domestic sewage sludge. The biodegradation kinetics of the enriched culture to degrade perchlorate using succinate and phenol as sole C-source in a synthetic wastewater system was determined separately in batch mode. The initial concentration of the perchlorate was in the range from 100 to 600 mg/L with interval of 100 mg/L in both the cases. The results indicated probable inhibitory effect of initial ClO_4^- concentration on its reduction, which has not been reported till date and needs further investigation which emphases on metabolic aspect of PRB (perchlorate reducing bacteria).

On the other hand the zero order reaction showed to be followed for ClO_4^- reduction with succinate as sole C-source.

Perchlorate bioreduction by mixed bacterial consortia, predominantly *Pseudoxanthomonas* sp. (phenol as carbon source) and *Burkholderia* sp. (succinate as carbon source) in flow through reactors were also evaluated in two laboratory scale PBR (packed bed reactor) operated in different conditions. Up to 96 % perchlorate removal could be achieved in the PBR using succinate as electron acceptor by the mixed consortium predominantly *Burkholderia* sp. Simultaneous removal of phenol and perchlorate was also achieved by the mixed consortium predominantly *Pseudoxanthomonas* sp. in another continuous PBR system.

CHAPTER 1: INTRODUCTION

Perchlorate is a naturally occurring and man-made anion commonly associated with the solid salts of ammonium, potassium, and sodium perchlorate. These salts are highly soluble in water, and because perchlorate sorbs poorly to mineral surfaces and organic material, it can be very mobile in surface and subsurface aqueous systems. Perchlorate contamination may persist for extended periods of time in the environment, since it is relatively inert under typical groundwater and surface water conditions. Ammonium perchlorate and the other perchlorate salts are used in a wide range of applications, including pyrotechnics and fireworks, blasting agents, matches, lubricating oils, textile dye fixing, nuclear reactors, electronic tubes, tanning and finishing leather, rubber manufacturing, electroplating, aluminum refinishing, automobile air bag inflators, paint and enamel production, and pharmaceuticals. The most common use for ammonium perchlorate is in explosives and rocket propellant. Because it has a limited shelf life, the ammonium perchlorate used in the rocket and missile supply must occasionally be replaced. As a result, large amounts of the compound are periodically disposed of in a broad sense, Perchlorate is a highly oxidized chlorine oxy-anion manufactured for use as the oxidizer in solid propellants for rockets, missiles, explosives and pyrotechnics (Urbansky, 2000; Gullick et al., 2001; Logan, 2001). Approximately 90% of all perchlorate salts are manufactured as ammonium perchlorate for use in rocket and missile propellants. The periodic replacement and use of solid propellant has resulted in the discharge of more than 15.9 million kg of perchlorate salts into the environment since the 1950s. Perchlorate salts are highly soluble in water. Sodium perchlorate has a solubility of about 2 kg/L, allowing large amounts to be readily transported through surface and ground waters. The United States Environmental Protection Agency (U.S. EPA) has identified perchlorate users and manufacturers in 44 states, and perchlorate releases in at least 20

states (U.S. EPA, 2005). Such perchlorate releases are estimated to have affected the drinking water of 15 million people. Perchlorate is of concern for the following reasons: it has potential human health effects at low concentrations; it may be widespread in the environment; removing it from water and soil may be costly; and it may have deleterious effects on ecosystems.

Perchlorate can be detected by many methods including ion-selective electrodes, ion chromatography, capillary electrophoresis, HPLC, and spectrophotometry (Urbansky, 2000) and among these methods, ion chromatography is the most commonly used detection method for perchlorate. Besides the anthropogenic substrates perchlorate is known to occur in nature only in Chilean caliche, a material that is used in some fertilizers. The EPA developed a method for measuring perchlorate concentrations in fertilizers. It was concluded that most fertilizers did not contain perchlorate, which therefore, could not be attributed to the observed extensive perchlorate contamination in the environment (Urbansky et al., 2000).

Although there is currently no federal drinking water standard for perchlorate, perchlorate has been included on the federal Contaminant Candidate List (U.S. EPA, 1998). High concentrations of perchlorate are known to affect the function of the thyroid gland in humans by inhibiting the uptake of iodide. Recent studies have also indicated that low concentrations of perchlorate significantly inhibit iodide uptake in humans and animals (Losi et al., 2002; U.S. EPA, 2005). The Office of Environmental Health Hazard Assessment in California EPA has proposed a public health goal of 6 mg/L for perchlorate in drinking water. In a recent perchlorate risk assessment draft report, the U.S. EPA (2002) proposed a draft reference dose of 0.03 mg/kg of body weight per day, which could produce a drinking water equivalent level of 1 mg/L to protect human health. Based on this information, the California Department of Health Service in California decreased the action level for perchlorate in drinking water from 18 to 4 mg/L (US

EPA, 2002). In New Mexico, the action level was set at 1 mg/L. In the last 15 years, several reviews have been published on various perchlorate issues that include: bacterial degradation (Herman and Frankenberger, 1998) chemistry and analytical chemistry (Urbansky, 2000) toxicological studies and drinking water standards (Urbansky, 2000; U.S. EPA, 2005); and contamination sources and occurrence data (Wolff, 1998; Urbansky, 2000; Gullick et al., 2001; Logan, 2001). However, there have been important advances made in the treatment of perchlorate-contaminated water since the microbiology and existing treatment technologies were reviewed in 1998 (Herman and Frankenberger, 1998). One of the most important developments since these reviews have been the reports of microbial treatment processes capable of removing perchlorate down to levels expected to be suitable for drinking water (4 µg/L). These processes are the basis of several recent patents on biological treatment processes for perchlorate treatment (Logan, 2001).

Based on the current state of process development, it can be concluded that perchlorate decontamination can be realized only through integration of one or more of physico-chemical and biological processes. However, biological reduction processes needs further optimization in the terms of reactor detention time, loading rate, selection of appropriate electron donor and identification of minimum electron donor concentrations.

Chapter 1: Introduction

Presentation and Layout of the thesis

The proposed thesis contains the following chapters with appropriate sections and subsections and also contains references, and the list of publications.

Chapter 1: Introduction

Chapter 2: Literature review and aim of the study

Thorough and updated literature on the relevant subject has been presented in this chapter. Aim and scopes of the study are pointed out at the last of this chapter.

Chapter 3: Materials and Methods:

Various materials used, analytical and experimental procedures adopted are presented in details in this chapter.

Chapter 4: Result and Discussion:

Experimental results obtained through batch, fed batch and continuous mode of operation of different lab scale reactors are presented in this chapter. Thorough discussions have been made to each of those results.

Chapter 5: Conclusions and scope of future study

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 2: LITERATURE REVIEW

2.1. Perchlorate anion

Perchlorate ion consists of a tetrahedral array of oxygen atoms around a central chlorine atom. It is a strong oxidizing agent owing to the oxidation state of the chlorine as +7. In this respect, perchlorate is slightly weaker than dichromate and permanganate. Perchlorate is usually found as an anion component of a salt most often associated with either of these common cations: ammonium, sodium, or potassium. The ion has large ionic size and low charge density and is kinetically inert to reduction which makes it a non-complexion anion that poorly adsorbs to mineral or organic surfaces (Urbansky, 1998). Low charge density, larger ionic size and its tetrahedral structure makes it more stable to get reduced.Perchlorate anion forms odorless, colorless and highly soluble salts, with ammonium, magnesium, potassium, sodium and lithium. Understanding of nature and properties of perchlorate salts is of great importance when one is dealing with perchlorate pollution. Typical chlorinated aliphatic compounds, e.g. trichloroethylene (TCE), are relatively insoluble, strictly used for industrial purposes. In addition, those compounds are volatile in nature and capable of being absorbed and can be reduced by metals such as zero valent iron. In contrast, perchlorate is a highly soluble inorganic anion (2.09 kg/L for $NaClO_4$) that adsorbs poorly to mineral surfaces and activated carbon and is not removed during groundwater transport. The high solubility of perchlorate in aqueous medium and its kinetic stability makes perchlorate very difficult to remove from water or wastewater effectively. Once perchlorate is introduced into the environment, perchlorate is expected to be very recalcitrant and mobile in surface water and groundwater and it may exist in the environment for many decades (Wang et al., 2007).

2.1.1 Redox properties of chlorine compounds

Perchlorate reduction is an extremely slow process and can usually be observed only under strong acidic condition. The redox behavior of perchlorate is so rarely observed in chemical solutions that sodium perchlorate is often used to adjust the ionic strength of solutions prior to electrochemical or other laboratory studies (Urbansky, 1998). Oxidation state of the element chlorine is the highest (+7) in perchloric acid. As an oxidant, perchlorate ion is kinetically nonlabile, which means the reduction of the central chlorine atom from an oxidation state of +7 (perchlorate) to -1 (chloride ion) is an extremely slow process. A Latimer diagram for chlorine in acid solution Fig. 2.2 is a convenient way to summarize the redox potentials relating stable compounds of chlorine. The standard reduction potentials presented in here refer to 1 M acid solution at 25°C. The convention here in is that if a reaction combined from one or more half reactions of complementary electron count has a positive change in reduction potential then the reaction as written is thermodynamically favorable (Gu and Coates, 2006).

The Gibbs free energy change for a combination of half-cell reactions at 25°C can be computed from the equation

$G = -nFE$

Where,

G = Gibbs free energy change.

n = number of electrochemical equivalents in the half-cell reaction.

F= Faraday constant equal to 9.6485×10^4 J $V^{-1}mole^{-1}$.

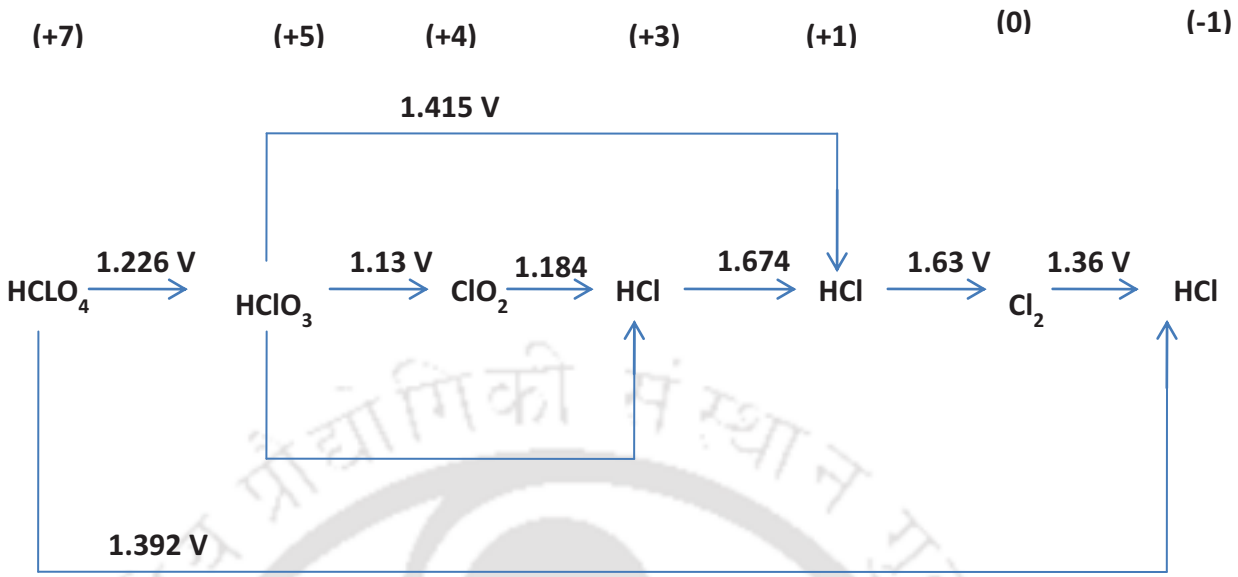


Figure 2.1: Latimer diagram giving the standard reduction potentials for stable species of chlorine in aqueous 1 M acid solution at 25°C. The formal oxidation state of chlorine is given by number above each species (Gu and Coates, 2006)

2.1.2. Sources of perchlorate in the environment

Perchlorate may appear to be just another addition to a growing list of halogenated chemicals that persist in the environment and the chemical nature of this inorganic anion make it quite unusual. Perchlorate salts shares both natural and anthropogenic sources. The natural sources of perchlorate are mostly confined to arid and semi arid environmental conditions. Natural perchlorate was first identified in Chilean nitrates over 100 years ago. Most of the naturally occurring perchlorate is assumed to be concentrated in Atacama Desert. Many studies have been conducted investigating the occurrence of natural non-chilean perchlorate, it is likely that perchlorate is much more widespread than previously thought, may in low levels. Perchlorate has also been detected in both drinking water and saliva samples collected in India. Concentrations

of perchlorate measured in drinking water in India are one to two orders of magnitude lower than the concentrations reported for more industrialized countries like USA, Japan and Korea. However, concentrations of perchlorate in water samples did not exceed the US EPA's interim health advisory level of 15 µg/L in drinkingwater. Studies have reported foodstuffs to be a source of perchlorate in the United States (Sanchez et al., 2005). Based on the mean concentration of 0.1 µg/L in drinking water from India, exposure of perchlorate for a 70-kg adult drinking 2 L/d of water would be 0.003 µg/kg b w/d, which is <1% of the reference dose established by the EPA. However, concentrations in saliva exceeded the concentrations in the water samples, with several saliva samples containing concentrations above 1 µg/L, suggesting the presence of other sources of perchlorate for the Indian population and further investigation is needed to examine the sources of exposure of the Indian population to perchlorate. Perchlorate is manufactured in large quantities as salt of ammonium ion, primarily for use as an oxidizer in solid rocket propellants. Its contamination is mostly associated with military activities or defense contracts (Gullick et al, 2001). It is also used in vehicles, electroplating operations, perchloric acid production, electro-polishing, production of matches, flash powder for photography, bleaching agent, leather tanning, oxygen generators, ejection seats, paints and enamels, etching of copper and brass, road flares and fireworks. Wastes from the manufacturing industries and improper disposal of perchlorate-containing chemicals are increasingly being discovered in soil and water. Ammonium perchlorate (NH_4ClO_4) has been used as an energetic booster in rocket fuels, and it appears that most perchlorate contamination is the result of discharge from rocket fuel manufacturing plants or from the demilitarization of weaponry (missiles). Potassium perchlorate ($KClO_4$) can be used as oxidant for rocket propulsion, and it was the majorsource for perchlorate contamination. However, most of the contamination appears to have come from the

industrial discharge decades ago of then unregulated waste effluents containing high levels of ammonium perchlorate. Although ammonium perchlorate was released initially, the salt is highly soluble and dissociates completely to ammonium and perchlorate ions upon dissolving in water. Perchlorate has been found in ground waters in the United States, at typical concentrations of 50-200 mg/L, primarily as a result of production and use in solid rocket propellant (Urbansky, 2000). Most of the affected regions have perchlorate concentrations below 0.5 g/L; however, concentrations as high as 3.7 g/L have also been encountered. Perchlorate in sewage sludge, rice, bottled drinking water and milk was detected in China (Shi et al, 2007).

2.1.3 Groundwater mobilization of perchlorate

Releasing mechanisms for perchlorate pollution from various anthropogenic sources may include spillage, residue, usage, aerial deposition and disposal in mediums like soil, surface water and groundwater. The perchlorate anion being highly stable in aqueous medium it remains in there without going under reduction for several years. Perchlorate can be removed in anaerobic conditions and also expected to be removed on some soils which has got anion exchange capacity, again removal is subjected to anion exchange capacity of soils. Whatever may be the introducing medium of perchlorate, it is bound to meet water may be surface water or groundwater on account of its high solubility. When perchlorate comes in contact with water, flows with water and contaminates water bodies.

The arid environment serves both to concentrate as well as prevent anaerobic conditions in which perchlorate can be microbiologically degraded. In very arid areas perchlorate may be stored in subsurface unsaturated zone much as nitrate. In other areas in which conditions allows for infiltration into groundwater, perchlorate may be present even if significant outflow exists or if

anaerobic condition exist the perchlorate would not be expected to accumulate significantly. Interestingly, an area which may be most impacted are those in which irrigated agriculture has been established in semi-arid or arid areas. The irrigation can serve to both mobilize stored perchlorate allowing its infiltration into groundwater as well as increase the effect of evaporative concentration.

2.1.4 Fate of perchlorate in the environment

Despite the strong oxidizing nature of perchlorate, it is known to be stable and nonreactive in aqueous systems. The high stability of in water is due to its large kinetic barrier to reduction as well as its poor coordinating ability to bind to surfaces (poor nucleophile). Perchlorate has reportedly been reduced by some transitional metal ions, but concentrations of these reduced transition metal ions in natural waters and geologic media are perhaps too low to impact the fate of perchlorate in the environment. Although the reduction of perchlorate by Fe (II) has been reported at elevated temperatures, it does not occur at ambient temperatures and near-neutral pH condition. The reaction was found to accelerate under acidic pH conditions but was inhibited by the presence of soluble chloride ion. Nevertheless, no measureable reduction of perchlorate occurred using bulk iron filings, micro scale iron powders, or Fe (II) ion alone. Therefore role of iron or ironcontaining minerals in the natural attenuation of perchlorate is also likely to be insignificant in the subsurface environment.

Perchlorate is poorly retained or sorbed by sediment minerals in the subsurface because of its negative charge and its non-complexion nature with metal ions (Urbansky, 1998). Its large ionic size and low charge density reduces its affinity for metal cations and make it highly soluble and

thus exceedingly mobile in natural aqueous systems. Perchlorate sorption is not expected to attenuate because it absorbs weakly to most of the minerals present in soil. Thus chemical reduction of perchlorate in the environment is not significant. These two factors account for perchlorate being both very mobile in aqueous systems and persistent for many decades under typical ground and surface water conditions. The activation energy to perchlorate reduction is so high that it cannot be an oxidant under human physiological conditions (i.e. at dilute solution, ambient temperatures and neutral pH). This is supported by absorption, distribution, metabolism, and elimination studies that show perchlorate is excreted almost unchanged in the urine after absorption. The comprehensive study of perchlorate retention and mobility in soils reported the adsorption and release of in a variety of soils and minerals at varying perchlorate concentrations (Urbansky, 2002). Their findings support the widely-accepted view that perchlorate does not appreciably sorbs to soils or minerals and that its mobility and fate are largely influenced by hydrological and biological factors. However, sorption of perchlorate appears to occur in soils with appreciable anion-exchange capacities (AEC). These soils act like anion exchangers, and perchlorate is sorbed by the replacement of other anions such as chloride, sulfate and nitrate. Therefore, unless appreciable anion exchange capacity is confirmed, sorption and retention of perchlorate in soil sediments will likely be negligible.

One of the most significant factors affecting perchlorate fate in the subsurface is likely to be dilution as it migrates from the source, biological uptake, and possibly degradation under anaerobic conditions. In the very similar way like many other contaminants perchlorate also tend to dilute from source, by which it originated.

A commonly studied mechanism for natural attenuation of perchlorate in the subsurface environment is anaerobic microbial degradation. It has been found quite effective for treatment high strength perchlorate wastewater. Perchlorate is used as an electron acceptor by some bacteria for cellular respiration and is degraded completely to chloride ion. Perchlorate uptake by plants, trees, forage and edible vegetation is known to occur and thus represents another natural attenuation and redistribution pathway. The studies have revealed that perchlorate is found in plants tissues which incorporate mechanism of attenuation (Yu et al., 2004). Table2.3 describes different mechanisms of attenuation.

2.2. Health effects of perchlorate

Perchlorate is known to interfere with the uptake of iodine in the thyroid at the sodium (Na^+)-iodide (I^-) symporter, or NIS of thyroid gland, thereby causing a reduction in the hormones thyroxine (T4) and tri-iodothyronine (T3) (U.S EPA, 2005). Hyperthyroidism due to iodine deficiency during pregnancy is a cause of permanent cognitive impairment of the developing fetus, known as cretinism (Utiger and Braveman, 2000). Perchlorate is thought to be responsible for abnormal fetal and child growth and as well as its development (Urbansky, 2000). In some cases, thyroid gland tumors can also be caused due to the disruption in the levels of thyroid hormones. Fig.1. Shows he schematic diagram of the effect of perchlorate anion on human health.

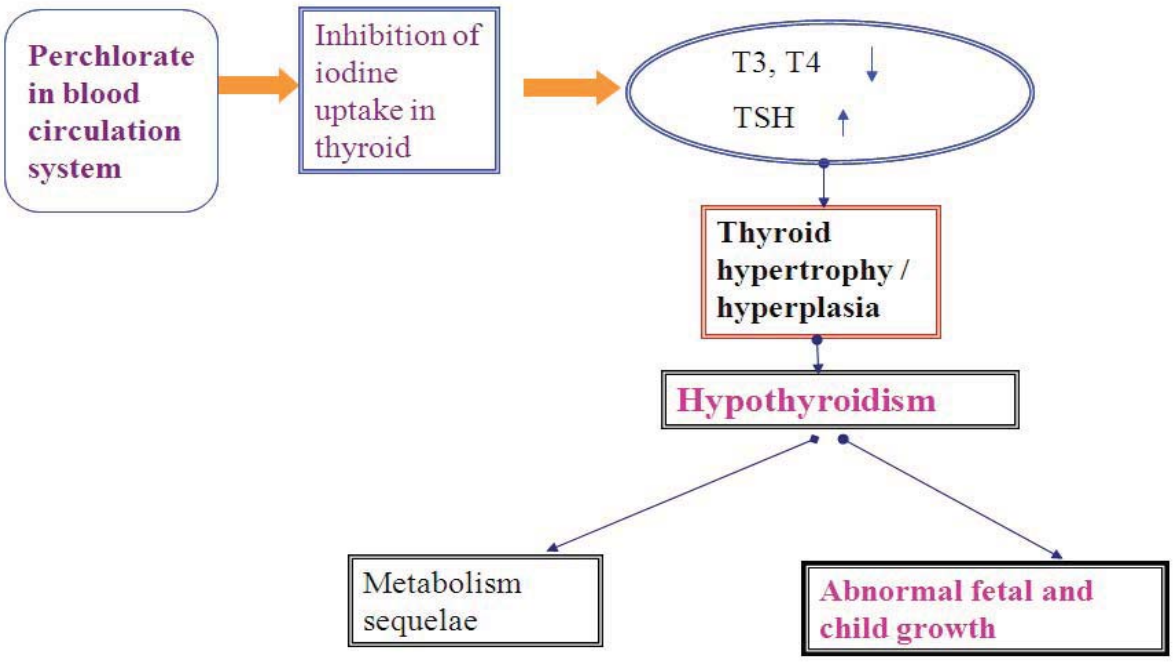


Fig 2.2: Flow chart showing interference of perchlorate with the uptake of iodine in the thyroid at the (Na^+)-iodide (I^-) symporter thereby causing a reduction in the hormones thyroxine (T4) and tri-iodothyronine (T3).

2.3. Permssible limits of perchlorate in drinking water

Despite of the fact that the appreciation of widespread perchlorate contamination emerged only in a few years ago, considerable progress has been made in the hazard identification and quantitative dose-response characterization for both the human health and ecotoxicological risks for potential perchlorate exposure. The thyroid gland has been confirmed as the target tissue in

humans, laboratory animals. The key event of the mode of action for perchlorate is iodide uptake inhibition at the NIS (Sodium iodine symporter) with the potential for both subsequent neurodevelopmental and neoplastic sequelae. A human health reference dose has been proposed to be protective for both sequelae based on a mode of action model. Additional research is needed to determine the contribution of sources of perchlorate other than drinking water. This requires more progress in the area of analytical methods to extend current approaches to other media of perchlorate contamination. The office of Environmental Health Hazard Assessment in California EPA has proposed a Public Health Goal of 6µg/L (Table.2.1) for perchlorate in drinking water. In the National Academy of Science's (NAS's) January 2005 report, maximum permissible dose for perchlorate was proposed to be 0.7 µg/kg/d (Gu et. al, 2007).

Table 2.1: Permissible limits of perchlorate ion in drinking water standardized by some states in USA according to US EPA, 2005.

State	Advisory level
Arizona	14 µg/L
California	6 µg/L
Massachusetts	1 µg/L
Maryland	1 µg/L
New Mexico	1 µg/L
New York	5 µg/L
Nevada	17 µg/L

2.4. Perchlorate treatment technologies

Perchlorate treatment technologies can be divided into two primary categories, incineration and removal. Removal technologies encompass broadly the physic-chemical and biological treatment. The physic-chemical methods include electrochemical reduction, ion-exchange, membrane filtration, electrodialysis, catalytic reduction, and biological treatment includes phytoremediation and microbiological treatment processes. A recent report by U.S EPA indicates that ion exchange and bioremediation are among the most commonly used technologies to remove or degrade perchlorate from contaminated water systems.

2.4.1 Membrane-based technologies

Membrane-based techniques are effective for the remediation of perchlorate from ground water systems, but they generally suffer from several drawbacks as well. It can be impractical for municipal treatment systems because of the fouling of membranes and the associated treatment cost (Urbansky, 2002). Moreover RO (reverse osmosis) treated water has to be remineralized with sodium chloride, sodium bicarbonate, and other salts to prevent degradation of the distribution system and to make the water palatable, because deionized water generally is considered to have an unpleasant taste. Therefore, as long as sufficient salts are taken from food and other sources, consumption of deionized water is not likely to cause a threat to the normal electrolyte balance. As with RO, electrodialysis also may be used for perchlorate removal. These two techniques are possibly best suited for point-of-use or small systems contaminated with perchlorate.

2.4.2 Anion exchange technology

It has been found that perchlorate ion is strongly retained by quaternary ammonium resins. Assuming that a chloride-form resin is used, the presence of phosphates, carbonates, and sulfate remains an issue. Although it may be possible to produce a resin salt that matches the proportions of the major anions in the incoming water, it may result in extremely inconvenient operation. In addition, the low concentration of perchlorate in the raw water (in ppb level) substantially reduces the driving force for its removal. In other words, to adequate removal of the perchlorate ion may require essentially demineralizing and remineralizing in the water system, depending on its anion contents. It is possible to modify resins to improve their selectivity for particular anions. Dowex 1X-8 is used to selectively preconcentrate perchlorate; the selectivity of the resins for perchlorate is about 100 times that of chloride and 10 times that of nitrate. In addition to selectivity in a thermodynamic context, there is the matter of rapid equilibrium and anion exchange which are. If the rate of exchange is too slow, a resin will not be usable no matter how high its selectivity. The U.S. Department of Energy developed a mixed triethylammonium-trihexylammonium resin that is capable of removing pertechnetate down to the parts-per-trillion level (Brown et. al, 2003). Tethered triphenylarsonium or phosphonium moieties or a tethered nitron (through a phenyl group) might work in an anion-exchange resin to selectively preconcentrate perchlorate as a step in before remediation. The disadvantage of the tethered nitron group is that normal degradation of the resin would lead to the release of arsenic into the treated water. Although the health effects of nitron are unknown, it would be expected to undergo biodegradation; furthermore, it would be destroyed readily by UV irradiation (185 nm), whereas arsenic would remain as an inorganic oxy anion in the water even if the organic portion of the species is destroyed.

2.4.3 Precipitation

In this technique the low solubility of the $HNitClO_4$ (ion pair of protonated nitroncation and the perchlorate anion) has been used. It may be possible to explore this pairing for purposes of perchlorate remediation. If the addition of nitron to perchlorate-containing waters results in formation of the soluble ion pair, it may be possible to subsequently induce an intramolecular reaction in which both the perchlorate and the nitron are destroyed. Photoactivation of the perchlorate by UV or laser irradiation may promote an intramolecular redox reaction (probably by oxygen atom transfer). The proximity of the $HNit^{+}$-ClO_4^{-} ion pair within a solvent (water) cage means that it is not necessary to form an encounter complex. In addition, the local concentration of the two species is very high within the solvent cage. This helps in reducing the effects of the perchlorate kinetic barrier (discussed below). Irradiation with UV light also promotes destruction of the nitron by hydroxyl radical formation. Ideally, the largest possible wavelength (lowest frequency and energy) light would be used to reduce side reactions that would destroy the nitron. Unfortunately, nitron has potential to remediate only those sites with very high perchlorate concentrations (in ppm level). At present, nitron is about 52 times more expensive than an equal mass of reagent-grade sodium chloride (Urbensky, 2002). At some of the sites where the perchorate concentration is 0.037 M, nitron could readily be used as a precipitant since the nitron-hydrogen perchlorate salt has a solubility of only 0.19 mM. In addition to cost, all physical separation processes have one major problem i.e., waste disposal. Most likely, the regenerant from ion exchange and the concentrate from RO or electrodialysis

would contain perchlorate at concentrations too high to be released into a sewage system. Although these techniques take out the perchlorate, they concentrate it somewhere else. This waste presents a problem in terms of cost and post-treatment needs.

2.4.4. Chemical and electrochemical reduction

Chemical reduction specifically by adding electrons in water system is simply too slow in case of perchlorate and therefore do not appear to play any role to the drinking water treatment unless safe catalysts are available. Commonly used reductants such as iron metal, thiosulfate, sulfite, iodide and ferrous ions do not react at any observable rate with perchlorate, and the more reactive species are found to be too toxic. In addition, any reducing agent will obviously have oxidized by-products and the toxicity of the by-products must be considered before its application. Thus there is more hope for electrochemical reduction over chemical reduction. A definite advantage of the technique is the large amount of control over kinetics that results from control of the operating potential. Electrode reduction kinetics is limited by three factors: (1) diffusion of the ions to the electrode surface, (2) association with the electrode surface, and (3) activation crossing the over-potential required to reach the transition state (Urbansky, 2002). Among these factors over-potential is a major limiting factor. Although some may be affected by electro-reduction, this probably does not result in a significant obstacle. To date, this option has not been explored for low-concentration treatment in a pilot scale at large extent. Although electrochemical technologies are well established for industries (e.g., electroplating of metals, electrolysis of brine) they are not applied in drinking water treatment. Most successful strategies for remediating perchlorate contamination by reduction method will utilize metal cation-catalyzed reduction either by varying chemical or by electrochemical means. Several metal chelates have found to be potential for this purpose, especially if embedded in an electrode used in the electrochemical reduction process.

2.4.5 Biological treatment method

All physicochemical techniques to treat perchlorate contaminates environment which includes high capital and maintenance cost, generation of large quantity of brines and spent resin having high perchlorate concentration. In addition, membrane fouling by alkaline earth and transitional metal compounds can present a problem depending on their concentration in the water. Bioreduction of perchlorate in engineered systems offers the greatest potential for inexpensive and complete perchlorate removal. Several reactor technologies have been shown to treat

perchlorate, for which three patents have been obtained (Attaway and Smith, 1993; Logan, 2001).There are total five full scale and fifteen pilot scale in situ bioreactors implemented at different states in the U.S where perchlorate contaminated ground water has been detected above permissible limit. Although bioreactor system is technically feasible for perchlorate removal, it can prove to be ineffective or costly for treatment at its low concentration (e.g., at 100 ppb) because a highly reducing environment is required for perchlorate removal using microorganism. Additionally, certain microbiota can irreversibility foul or damage the membrane material, necessitating complete replacement. Among the pure strains of PRB which has been used for perchlorate bio reduction from water systems are *Vibrio dechloritans* Cuznesove B-1168 Korenkov et al. (1976), *Wolilella succinogenes* HAP-1 (Wallace et al., 1996), *Dechloromonas agitata* CKB (Bruce et al., 1999), *Dechloromonas* sp. SIUL (Coates et al., 1999), *Dechlorosoma* sp. JM (Miller and Logan, 2000), *Citrobacter* sp. IsoCock 1 (Okeke et al., 2002) and *Dechloromonas* sp. JDS5 (Shrout and Parkin, 2002).

2.4.5.1 *Perchlorate bioreduction pathway*

Perchlorate respiring bacteria (PRB) have been found in many different environments making it possible to bioremediate perchlorate-contaminated environments (Attaway and Smith, 1993; Herman and Frankenberger, 1999; Hatzinger et al, 2000). Perchlorate is used as a terminal electron acceptor by pure and mixed cultures of microorganisms (Logan, 2000; Herman and Frankenberger, 1998) as showing is the following schematics in Fig.2.3. The reduction of perchlorate or chlorate to chloride by bacteria was also subsequently confirmed by other researchers (Korenkov et al., 1976; Rikken et al., 1996). None of the intermediates accumulate in solution (Attaway and Smith, 1993; Logan, 2000; Herman and Frankenberger, 1998). Chlorite disproportionation to chloride and oxygen is a non-energy yielding step catalyzed by chlorite dismutase enzyme (Rikken, 1996).

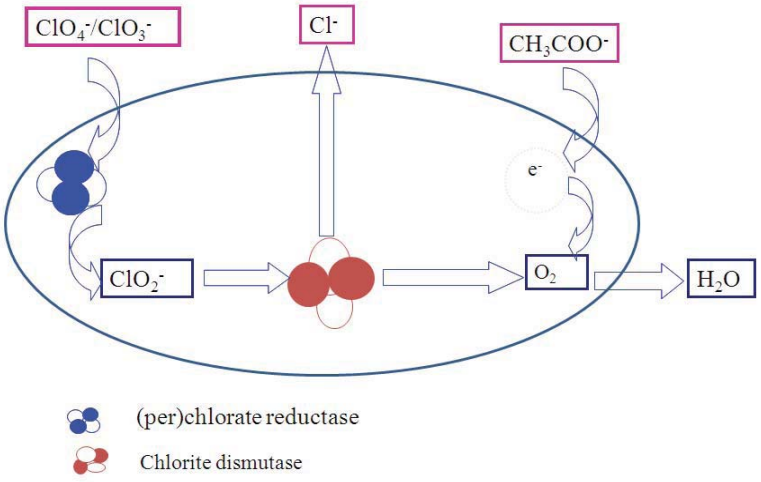


Fig. 2.3: Proposed model of pathway involved in respiratory reduction of perchlorate.

2.4.5.2. *Use of different carbon sources for microbial perchlorate bioreduction*

Phenol has been used as sole C-source for denitrification by some microbial cultures (Bak and Widdel, 1986; Tschech and Fuchs, 1987; Wang Y. T. et al., 1986; WojciechHolub, et al., 2000; Mette M. Broholm and Arvin, 2000; Pe´terKesseru et al., 2005). These nitrate reducers are capable of accepting phenol and also other known aromatic hydrocarbons for their metabolism. The perchlorate reducers share many similar metabolic features with the denitrifiers including their choice of electron donors (C-sources). Till date, there is no report of perchlorate reducers accepting phenol or any other aromatic hydrocarbons while degrading perchlorate.

In the past 20 years there have been many reports on the biodegradation of phenol under anaerobic conditions.

2.4.5.3 *Bioprocess for perchlorate remediation*

The conventional bioreactors for ex situ treatment of perchlorate employ either fixed-or fluidized-film bioreactors in plug flow or recirculation mode with acetate or H_2 as the electron donor. In the fixed film packed-bed bioreactors sand, plastic, glass bead, activated carbon or elemental sulfur is used as support media (Wallace et al., 1998; Min et al., 2004; Sahu et al., 2009; Chung et al., 2010). Fluidized-bed bioreactors use either sand or GAC for microbial colonization and high recycle rates to keep the support medium suspended and mixed (Xiao et al., 2010). Many of these bioreactors are efficient in reducing perchlorate to acceptable levels along with removal of several co-contaminants. Positive enrichment of highly specific perchlorate reducing bacteria had been demonstrated under different operational conditions (Nerenberg et al., 2008; Ahn et al., 2009; Xiao et al., 2010). Recently, McCarty and developed a numerical model based on the relative penetration of competing electron acceptors (O_2, nitrate and perchlorate) in the biofilm of a fluidized-bed reactor for the treatment of ground water. The ex situ treatment process is particularly suitable for the treatment of highly concentrated waste streams originating from the perchlorate manufacturing units or the munitions handling facilities. However, direct application of this process for the treatment of drinking water is questionable at the moment. The high operational cost and excess biomass build up and clogging resulting from the use of organic electron donor limits large scale propagation of this technology. Possibility of secondary contamination of treated water with microbial cells and their metabolic by-products

are also some other concerns. Carryover organic residuals can increase demand for chlorine for disinfection which can lead to formation of unacceptable disinfection by-products in the treated water. Moreover, presence of residual ethanol and methanol can be unacceptable in drinking water supplies. To circumvent some of these problems, in recent years a whole range of newer generation bioreactors have been developed. These include H_2-based hollow-fiber membrane biofilm reactor (-MBfR) (Chung et al., 2007; Nerenberg et al., 2008; van Ginkel et al., 2008; Sahu et al., 2009; van Ginkel et al., 2010), ion exchange membrane bioreactor (IEMB) (Matos et al., 2006), and bioelectrical reactor (BER) (Thrash et al., 2007) or a microbial fuel cell (MFC) (Butler et al. 2010). The (MBfR) systems offer benefits in terms of less biomass production and lower solubility of H_2 in water (1.62 mg/L at 25^{o}C and 1 atm H_2), thereby eliminating the need for post-treatment removal. Similarly, the insoluble elemental S°-based chemolithotrophic perchlorate reduction can be suitable for ensuring a long-term sustained supply of electron donor in low maintenance bioreactors (Ju et al., 2008; Sahu et al., 2009). The (IEMB) process simultaneously combines advantages of Donnan dialysis and biological perchlorate reduction and selectively removes ionic pollutants.

Many heterotrophic biological treatment systems have been tested to degrade perchlorate including suspended, fixed-bed, and fluidized-bed reactors (Attaway and Smith, 1993; Wallace et al. 1998; Green and Pitre, 2000; Hatzinger et al., 2000; Logan et al., 2001). Organic electron donors that have been used include simple compounds such as acetate and ethanol, as well as more complex organic substrates such as those found in compost piles. Perchlorate degradation has also been achieved in bioreactors using only inorganic. These reactors are sustained by hydrogen gas delivered by pressurization, gas transfer across liquid films or synthetic

membranes (Miller and Logan, 2000; Nerenberg et al., 2004). These hydrogen-based technologies are promising technologies for water treatment because less biomass is produced by autotrophic processes than heterotrophic processes. Large-scale tests are needed to evaluate process efficiency and the economics of these different hydrogen-based systems. Although at least one biological treatment process has been approved for use in the state of California for drinking water treatment (DHS, 2002), little has been done to study the removal of PRB from the treated water. Membrane bioreactors can be used to keep the bacteria separated from the contaminated water (Batista and Liu, 2001), but these systems are at a less advanced stage of development than other biological perchlorate treatment systems. It has been suggested that reactors based on enzymes to reduce perchlorate could avoid the potential health problems associated with biological treatment. Perchlorate reductase, which can reduce both perchlorate and chlorate, and chlorite dismutase (van Ginkel et al., 1996; Coates et al., 1999; Stenklo et al., 2001) have both been purified. However, no such enzyme-based systems have been reported in the literature for treatment of perchlorate contaminated water. The presence of alternate electron acceptors in perchlorate contaminated water will be an issue for all types of biological reactors. Oxygen is an important intermediate in the perchlorate degradation pathway (Rikken et al., 1996). It is well known that for PRB oxygen is a preferential electron acceptor to perchlorate, and that high concentrations of dissolved oxygen inhibit perchlorate reduction (Song and Logan, 2004). It is not clear what concentration of dissolved oxygen will completely inhibit perchlorate reduction, how long bacteria can withstand exposure to high concentrations of oxygen before losing the ability to reduce perchlorate, or how long it would take oxygen-exposed bacteria to regain the ability to reduce perchlorate. However, the presence of oxygen, nitrate, or sulfate in bioreactor feed streams does not appear to be a problem for the steady operation of such systems.

In a pilot-scale test for ex situ groundwater treatment, it was found that oxygen, nitrate and perchlorate were all completely reduced but that sulfate was not measurably degraded (Logan et al., 2001). Thus, it is likely that the main impact of oxygen and nitrate on a treatment system will be to increase the requirement of substrate (such as acetate or hydrogen) that is oxidized by the bacteria. Table.2 summaries the different laboratory scale reactors which has been studied to treat perchlorate contaminated waters.

Table 2.2: Lab scale bioreactor systems applied for perchlorate remediation.

Reactor Type	Culture type	Types of Water	Hydraulic retention time (HRT)	Influent ClO_4^- (mg/L)	Effluent ClO_4^- (mg/L)	Electron Donor (s)	References
Fluidized bed reactor with sand and activated carbon media	Mixed culture	Drinking water well	3.1hr	6.7	<0.400	Acetate, Methanol, Ethanol	Greene and Ptre, 1999
Fixed film reactor with celite R-635		Synthetic water	1 hr	0.74	<0.004	Hydrogen	Giblin et al., 2000
Up flow reactor Packed with sand	Mixed culture	Synthetic water	51min	100-1000	<0.005	Lactate	Logan, 2001
Autotrophic packed bed biofilm reactor	Pure culture	Synthetic water	1.1 to 1.3 min	0.740	0.460	Hydrogen	Miller Logan, 2001
Suspended growth reactor	*Wollinellasuccinogenes* HAP-1 in mixed culture	Synthetic water	3 hrs	0.13,0.738	0.01, 0.031	BYF-100	Attaway and Smith, 1993
Fixed bed (sand GAC)	Mixed culture	Synthetic water	10hrs	0.13, 0.738	0.01, 0.031	Acetate	Herman and Frankenberger ,1999; Giblin

Reactor Type	Culture type	Types of Water	Hydraulic retention time (HRT)	Influent ClO_4^- (mg/L)	Effluent ClO_4^- (mg/L)	Electron Donor (s)	References
							et al., 2000
Fixed bed (GAC)	Tapwater	Synthetic water	1hr	0.051	<0.004	A mixture of acetate, lactate and pyruvate	Brown et al., 2000
Fixed bed (cylindrical pall rings)	Primary sludge and and effluent from waste water treatment plant	Synthetic water		0.1,1	<0.004	Acetate	Burns et al, 2001
Fixed bed (Celite pellets)	Perclace	Synthetic water		0.8	<0.004	Acetate	Losi et al., 2002
Fixed bed (Glass beads)	Mixed culture	Synthetic waste water		0.073	<0.004	Hydrogen	Logan and LaPoint, 2002
Fluidized bed (sand or GAC)	Biological solids from an anaerobic digester	Synthetic waste water		25		Ethanol, methanol or mixture of the two alcohols	Hatzinger et al., 2000
Fluidized bed (GAC)	Mixed culture	Synthetic waste water	2hrs	11-23	<0.004	Acetic acid and ethenol	Tonga et al., 2001

Reactor Type	Culture type	Types of Water	Hydraulic retention time (HRT)	Influent ClO_4^- (mg/L)	Effluent ClO_4^- (mg/L)	Electron Donor (s)	References
Hollow-fiber membrane bioreactor.	*Ralstoniaeutropha*	Synthetic waste water	1 hr	0.1	<0.0003	Hydrogen	Nereberg et al., 2002
Fixed bed (sand)	*Dechlorosoma*KJ.	Synthetic wastewater	2.1 mins	20	<.004	Acetate	Kim and Logan, 2000
Fixed bed (GAC)	Mixed culture	Drinking water	18 mins	20	<0.004	Acetate	Kim and Logan, 2001
Fluidized bed ractor with GAC as packing material	Mixed culture	Synthetic wastewater	154 mins	300	<0.3	Acetate	Patel et al., 2008
Hollow fibre Membrane biofilm reactor (MBfRs)	Contaminated drinking water	Contaminated drinking water	90 mins	1	0.01	Hydrogen	Nerenberg and Rittman, 2004
Hollow fibre Membrane biofilm reactor (MBfRs)	Synthetic wastewater	Mixed consortium	4, 8, 20, 24, 29 hrs.	5, 30, 40, 100	<0.004	Acetate	Ginkel, 2010

Reactor Type	Culture type	Types of Water	Hydraulic retention time (HRT)	Influent ClO_4^- (mg/L)	Effluent ClO_4^- (mg/L)	Electron Donor (s)	References
Ion exchange membrane	Polluted water	Enriched mixed culture from municipal sludge	0.25, 1.4, 2.0, 4.0, 8.3 hrs.	1	<.004	Ethenol	Matos et al., 2005
Ion-exchange brine using the membrane biofilm reactor (MBfR)	Synthetic wastewater	Backwash sludge from perchlorate degrading packed bed reactor	1, 0.4 hrs.	20, 100, 8.2	2, 45,6	Yeast extract , citric acid	Ginkel et al., 2008

2.4.5.4 *Phenol as an alternative C-source for perchlorate biodegradation*

Phenol is difficult to be decomposed biologically. It is toxic to plants, microorganisms, animals and humans, causing serious environmental problems (Liu et al., 2009). In addition, it is water soluble and highly mobile, and so it is likely to reach drinking water sources downstream from discharges, where, even at low concentrations, it can cause severe odor and taste problems and pose risks to populations. Phenolic compounds are present in the liquid effluent of coal gasification plants, coking plants, petroleum refineries, pharmaceutical, fertilizer and dye manufacturing plants, as well as degreasing and paint stripping operations (Khan et al., 1981) and fiber-board manufacturing. As a result of growing awareness over pollution caused by phenol release, efforts are being made to minimize their adverse effect.

Currently, many treatment techniques such as activated carbon adsorption, solvent extraction, chemical oxidation, electrochemical oxidation and biodegradation have been developed to remove phenol from contaminated environment (Ra et al., 2008). Of these options, physicochemical methods have proven to be costly and have the inherent drawbacks of causing a secondary pollution. However, biodegradation technique, which is environmental friendly and cost effective, has turned out to be a favorable alternative (Ariana et al., 2004). In the past 20 years there have been many reports on the biodegradation of phenol under anaerobic conditions. It has been shown that phenol is utilized by sulphate-reducing bacteria, microorganisms participating in methanogenic fermentation and denitrifying bacteria (Tschech and Fuchs, 1987). Although not found to be bioaccumulative, humans exposed to phenol in well water at concentrations of 1300 mg/L exhibited a statistically significant increase in diarrhoea, mouth

sores, dark urine and burning of the mouth (US EPA, 1998). The process has been carried out for many years either aerobically or anaerobically. Between the two, the latter is preferred because it saves the energy needed for aeration and produces substantially lower amount of sludge.Under aerobic conditions, phenol and *o*-cresol have been observed to degrade in in situ microcosm experiments in a sand aquifer material. In aerobic laboratory microcosm and column experiments with aquifer sediments and groundwater degradation of phenol, *o*-cresol, and *p*-cresol was observed and degradation of phenol, *o*-cresol, and 2,4-xylenol was observed in laboratory microcosms with groundwater. Phenol, *o*-cresol, 2,6- and 3,5-xylenol were also found to degrade in a laboratory column experiment and a field infiltration experiment in fractured clayey till. All of the phenols _phenol, *o*-, *m*-, and *p*-cresol, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 2,3-, 2, 2,6-, 3,4- and 3,5-xylenol have been observed to be degradable under aerobic conditions in laboratory studies with other media containing surface soils, waste water sludge, etc. (Arvin et al., 1991). Under nitrate reducing conditions degradation of phenol was observed in some in situ microcosms but not in all whereas *o*-cresol was persistent in all in situ microcosms. Degradation of phenol, *m*- and *p*-cresol, and 2, 4- and 3,4-xylenol was observed in laboratory microcosm with groundwater but no sediment. Degradation of phenol and *o*-cresol under nitrate reducing conditions was observed in a column experiment with fractured clayey till (Broholm et al., 1999). Persistence of 2,3-, 2,5-, 2,6-, and 3,5-xylenol was observed under nitrate reducing conditions . Phenol have been observed to degrade under nitrate reducing conditions in laboratory studies with other media surface soils, waste water sludge, etc.. or with enrichment culture srspecific bacteria (Tschech and Fuchs, 1987; Flyvbjerg et al., 1993b;). Phenol has been used as sole C-source for denitrification by

some microbial cultures (Tschech and Fuchs, 1987; Wang. et al., 1986; Wojciech et al., 2000;). It has been shown that phenol is utilized by sulphate-reducing bacteria, microorganisms participating in methanogenic fermentation (Wang et al., 1986) and denitrifying bacteria (Tschech and Fuchs, 1987). These nitrate reducers are capable of accepting phenol and also other known aromatic hydrocarbons for their metabolism. The perchlorate reducers share many similar metabolic features with the denitrifiers including their choice of electron donors (C-sources).Two possible anaerobic degradation pathways of phenol under mesophilic condition have been reported. In one suggested pathway (Kobayashi et al, 1989), phenol is first converted through carboxylation to produce benzoate. The latter is then de-aromatized to form cyclohexanecarboxylate which is further cleaved to form heptanoate. Heptanoate is then degraded either through b-oxidization to form valerate, propionate and acetate (Keith et al, 1978), or directly to form propionate and butyrate, both of which can be further degraded into acetate (Fina et al, 1978). This pathway was supported by the presence of enzymes performing carboxylation, decarboxylation and dehydroxylation reactions during phenol anaerobic degradation (Gallert and Winter, 1992). In the other degradation pathway (Bakker, 1977), phenol is reduced in the presence of nitrate to cyclohexanone and then n-caproate, which is subsequently undergone β-oxidation to form lower volatile fatty acids (VFAs). Anaerobic treatment of phenol-containing wastewater in upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB)-based bioreactors has been well documented. Although, perchlorate is a good electron acceptor in biological process, till date, there is no report of perchlorate reducers accepting phenol or any other aromatic hydrocarbons while degrading perchlorate.

REMARKS:

It is very clear that above researchers have focused on treating wastewater contaminated with perchlorate using bioreactor systems and in batch scales by employing different microbial cultures; however, the reduction of perchlorate utilizing cheaper C-sources has not been explored elaborately in the past studies. Therefore it is feasible to adopt such systems for perchlorate remediation from water and wastewater systems. The previous research has also left scope for exploring further investigation encompasses the effect of co-contaminants which are normally present in the wastewater like, nitrate on the biological reduction of perchlorate ion by microbial cultures.

Therefore, all the unexplored research directions were extensively studied and reported in the present thesis. The present thesis focused to develop a microbial culture capable of perchlorate degradation in water systems using different organic and aromatic C-sources. Phenol was used by an enriched mixed microbial consortium for perchlorate degradation. Industrial waste water containing phenol can be used for perchlorate bioreduction by microbial consortium minimizing the removal cost in a large amount. The effects of co-pollutants on perchlorate reduction were also evaluated. In addition the thesis contains a performance of newly fabricated packed bed reactors which were continuously operated for perchlorate reduction from synthetic and refinery waste water.

Based on the extensive literature review the present study aims at development of a bioreactor system for effective removal of perchlorate by mixed bacterial culture using different electron donors as carbon sources.

To achieve the above objective, following investigations were performed.

- Collection of mixed microbial consortia from a sewage treatment plant and acclimation for perchlorate reduction using different carbon sources.
- Batch studies on perchlorate removal by acclimatized mixed bacterial consortia in presence and absence of various co-pollutants (such as nitrate, nitrite, chlorate, phosphate and sulfate) using different carbon sources (such as succinate, malate, formate, citrate and phenol)
- Isolation of predominant strains of perchlorate reducing microbe(s) from the acclimatized mixed consortium in selected batch reactors.
- Investigation of the kinetics of perchlorate reduction by mixed bacterial consortia predominantly the above isolated strains.
- Fabrication of a packed bed reactor (PBR) system and evaluation of its performance with the enriched mixed consortium on perchlorate reduction under different feeding and operating conditions.

CHAPTER 3: MATERIALS AND METHODS

3.1 Material

3.1.1 Chemicals and reagents

All the chemicals and reagents used in the present study were either of analytical reagent (AR) grade or laboratory reagent (LR) grades. Sodium perchlorate ($NaClO_4.H_2O$) was used to prepare perchlorate stock solution for analysis as well as preparation of wastewater. Double distilled water or ultrapure water was used for preparation of all the chemical reagents.

3.1.2 Glassware, apparatus and instruments

All glasswares used in this study were of borosilicate and were kept immersed overnight in chromic acid solution (2.25 gm $K_2Cr_2O_7$ and 20 ml concentrated H_2SO_4 in 1L of distilled water) followed by washing with tap water and then distilled water. For drying cleaned glassware were kept in oven at 110^oC for 2 - 4 hours before being used in the experiment. Various instruments used in the present study are listed in Table 3.1.

Table 3.1: Instruments and equipment used in the present investigation.

Instrument/Equipments	Parameters tested/measured	Model/Manufacturer Specification
Ion chromatography	Estimation of perchlorate, chlorate, nitrate, phosphate, sulphate, succinic acid, citric acid, acetic acid, malic acid, formic acid etc.	Metrohm792 AG, Harisau, Switzerland
Ion meter	Estimation of Perchlorate ion.	Orion 3 star-QY-14478 Thermo Scientific, Singapore
Scanning electron microscopy	Photograph of microorganisms, reactor packing media.	Make: LEO, Model: 1430 VP, U.S.A
UV-visible spectrophotometer	Biomass concentration, phenol estimation	Model lambda-45, Perkin Elmer, USA
Centrifuge	To separate biomass and other suspended solids from solution	Remi C-24-BL Mumbai, India
Autoclave	Sterilization	Equitron-7407PAD India
Digital pH meter	pH	Orion 3 star-QY-14478 Thermo Scientific, Singapore
Electronic balance	weight	Sartorius BT-224S
Hot air oven	MLSS, Drying other materials	Tonco-PLT-125, India
Peristaltic pump	Feeding into PBR (packed bed reactor)	Miclin India Ltd. PP10
Incubator shaker	For optimization of media components , culture condition and batch studies	Daihan Lab tech, LSI-100SR Korea

Laminar Air flow hood	To transfer pure culture in aseptic manner	Clean air system, CAH-1800, Chennai, India
BOD incubutor	To prepare slant culture in agar media	Delux model- IK-120, Delhi, India

3.1.3 Sludge biomass and growth media

Sludge from the wastewater treatment plant of Indian Institute of Technology Guwahati was collected, kept in a 2 L aspirator glass bottle containing about 1.5 L of culture media with acetate as carbon source. The culture media prepared in distilled water contains the followings: Sodium perchlorate, 1.00 g/L, Sodium acetate, 1.00 g/L Nitrogen gas was supplied to the media from a nitrogen gas cylinder to expel out any dissolved oxygen present. Supernatant was replaced with fresh media at an interval of 15 days.

3.1.4 Packed bed reactor (PBR)

Two nos. laboratory scale upflow PBR were fabricated to study the biodegradation of perchlorate by the enriched mixed culture consortium in flow through mode. Schematic diagram of PBR with its detailed specifications are shown in figure 3.1. The different components of the reactor are described below:

Main Reactor, sampling, effluent ports, biogas outlet arrangement and influent tank:

The main reactor was made up of Perspex tubes with an internal and external diameter of 7.0 cm and 5.0 cm respectively. Total length of the reactors was 60 cm. Thus the total volume of the reactor was 1275 ml. Working volume of the reactor was kept as 750 ml. For sampling one outlet port was provided at 7.5 cm length below the top of the reactor. Polyurethene foam (PUF) cut into small sizes of approximately 2.5 cm x 1.5cm x 1.5 cm were loosely packed into the reactors up to the height of 60 cm. Thus the volume of the packing media in the reactor was 1000 ml. The PUF was washed with de-ionized water, autoclaved (20 min, 120^{o}C), rewashed, and dried overnight at 70^{o}C in hot air oven before being used as bio-support material in the 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 of the reactor.

An inverted T-joint with a long silicon pipe at the top was connected to the effluent port of the reactor to facilitate escape of biogas formed in the effluent pipe. The inverted T-joint thus prevented accumulation of biogas in the pipe that could otherwise blocked the treated wastewater from free flow. The biogas formed in the reactor was allowed to pass through the cone shaped top of the reactor, a liquid trap and finally through a gas trap as shown in Fig 3.1. An actual photograph of the reactor set-up in the laboratory is shown in Fig 3.2.

A 10 L polypropylene carboy was used as influent tank for the PBR. Freshly prepared wastewater was supplied with nitrogen gas from a nitrogen cylinder to purge out dissolved oxygen (DO), if any. In fact, nitrogen gas was supplied daily for 10 – 20 minutes to keep the feed (influent) under anaerobic condition.

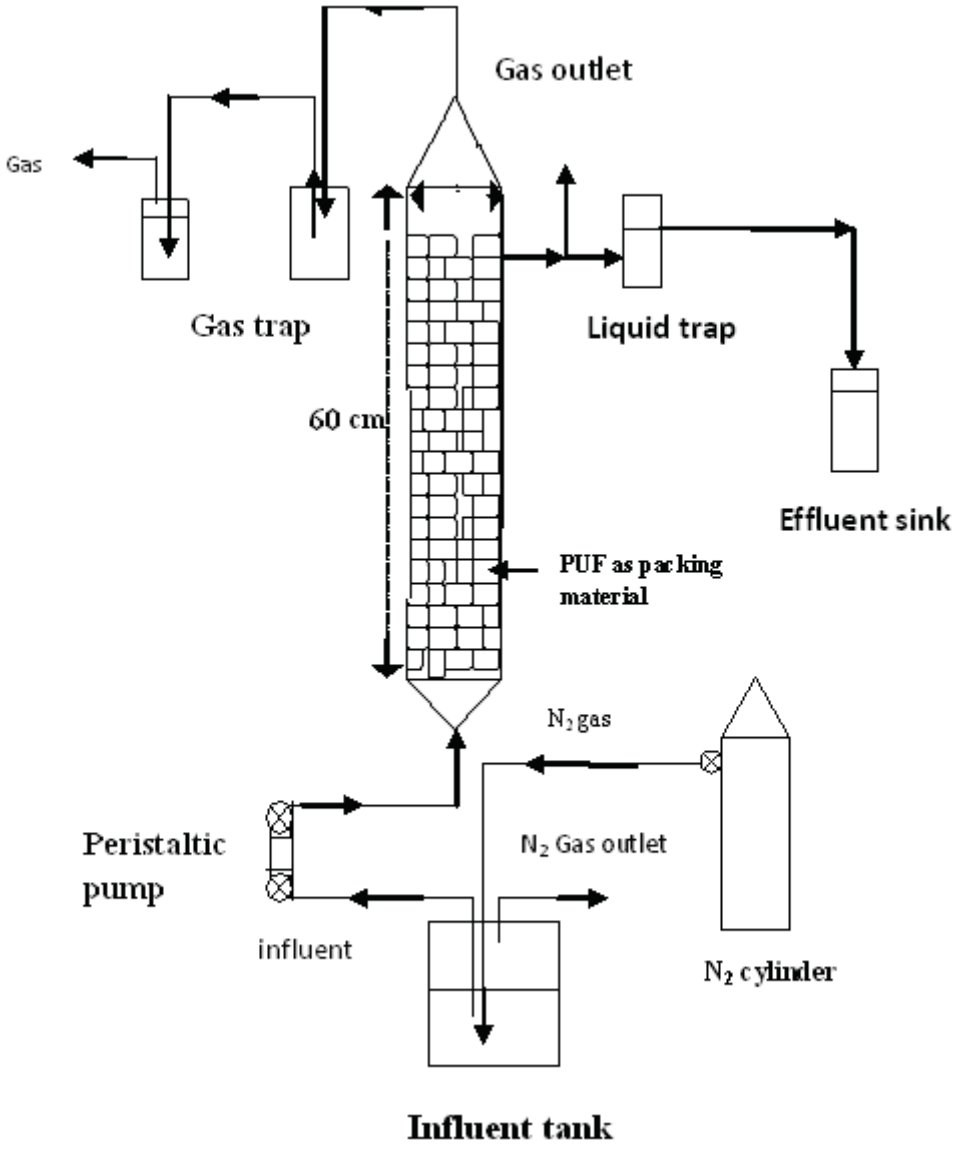


Fig 3.1: Schematic diagram of the PBR system used for studying perchlorate bioreduction under continuous flow through mode.

Fig 3.2: Photograph of the PBR system used for studying perchlorate bioreduction under continues flow through system.

Porosity and void volume of PUF was determined according to the method described by as follows: 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 determination with a graduated cylinder (1L). The sample was weighed with 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.

$$\% \text{ porosity} = \frac{\text{void space volume}}{\text{volume of the sample}}$$

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

3.2. Experimental Methodologies

3.2.1 Seed culture development

Media used for developing seed culture of the microorganism for use in biodegradation experiments contained specific media. Perchlorate 1.0 g/L acetate, 1.0 g/L or other C-sources as mentioned, 1 g/L and 1x phosphate buffer saline (10X PBS gm/L; 14.4 Na_2HPO_4, 2.4 K_2HPO_4, 8.0 NaCl, 2.0 KCl). The seed culture medium(100 ml) taken in a 250 ml Erlenmeyer flask was inocululated to fresh medium and cultured for 48 h at 28^{o}C.

3.2.2 Inoculum preparation

Seed culture cells obtained as before were harvested at late exponential phase (48h) by centrifugation (6000g, 20 min at 22^{o}C), washed in 1x phosphate buffer saline (10X PBS gm/L; 14.4 Na_2HPO_4, 2.4 K_2HPO_4, 8.0 NaCl, 2.0 KCl) pH 7.4, added to the 250 ml Erlenmeyer flasks with 100 ml of specific media containing 500 mg/L the sole source of carbon and energy. The flasks were incubated for overnight in an orbital incubator shaker and were re-grown at 28^{o}C, 180rpm. 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}

3.2.3 Culture condition

All the enrichments and growth of mixed cultures were performed in anaerobic medium which consists of the following chemicals in distilled water. Sodium perchlorate, 1.00 g/L; Sodium acetate, g/L or other C-sources as mentioned, 1 g/L, 1x phosphate buffer saline (10X PBS gm/L; 14.4 Na_2HPO_4, 2.4 K_2HPO_4, 8.0 NaCl, 2.0 KCl) and 10 ml/L of trace mineral supplement: 2.00 g/L ; EDTA, 0.5 g/L; $MgSO_4.7H_2O$, 3.0 g/L; $MnSO_4.H_2O$, 0.5 g/L; NaCl, 1.0 g/L; $FeSO_4.7H_2O$, 0.1 g/L; $Co(NO_3)_2.6H_2O$, 0.1 g/L; $CaCl_2$ (anhydrous), 0.1 g/L; $ZnSO_4.7H_2O$, 0.1 g/L; $CuSO_4.5H_2O$, 0.01 g/L; $AlK(SO_4)_2$ (anhydrous), 0.01 g/L; H_3BO_3, 0.01g/L; $Na_2MoO_4.2H_2O$, 0.01 g /L; Na_2SeO_3 (anhydrous), 0.001 g /L; $Na_2WO_4.2H_2O$, 0.01 g /L; $NiCl_2.6H_2O$, 0.02 g /L. Initial pH of media was adjusted by adding required amount of 1M H_2SO_4 or 1M NaOH

solution. The enrichment medium was then transferred to 2.0 l conical flask crimp-sealed with butyl-rubber stoppers and needles.

3.2.4 Acclimatization

Activated sludge collected from a wastewater treatment plant treating domestic wastewater was acclimated for perchlorate degradation. An amount of 0.5-L sludge and 1.0-L medium were added to a 2.0-L conical flask, incubated at 28^{o}C in an incubator so that the final concentration of the biomasss in the flask was 200 mg/L. A magnetic agitator was used to stir the solution continuously throughout the experiment, and 100 ml of fresh medium was replaced daily. To maintain anaerobic condition, these cultures were purged with oxygen-free nitrogen gas at regular intervals. The experiment was conducted about one and half months.

3.2.5 Batch studies to evaluate the effect of different C-sources on perchlorate bioreduction

The potential of the enriched mixed consortium to utilize different organic acids (such as succinic acid, acetic acid, oxalic acid, formic acid and citric acid) and phenol as sole source of carbon in degrading perchlorate was analyzed in a series of batch shake flasks. The sodium salts of each of these acids and phenol of 500 mg/L as COD were added to the respective flasks containing culture media spiked with 1000 mg/Lof initial perchlorate. About 5% v/v of the enriched culture was added to the media to have final biomass concentration 100 mg/L. The

experiment was conducted for a maximum of 10 days at 28°C and the initial pH of 7.0. For analysis of various parameters 10 ml of treated samples were taken out daily.

3.2.6 Batch study on perchlorate bioreduction by the mixed consortium predominantly *Burkholderia*sp. at different initial succinate concentration

The influence of initial concentration of succinate on perchlorate biodegradation by the mixed consortium predominantly *Burkholderia* sp. was studied in a series of batch shake flasks of 250 mL capacity. The experiment was conducted at eight different concentrations of succinate between 300 and 1000 mg /L in the media. Initial perchlorate concentration in all the flasks was 500 mg/L; biomass was 100 mg/L and initial pH was7.0.

3.2.7 Enrichment, Isolation and identification of predominant perchlorate reducing strains

Biomass collected from batch flasks (degrading perchlorate using succinate and phenol as C-source) were analyzed to identify the major perchlorate reducing strains. Mixed consortium was incubated at 28°C and monitored by turbidity for growth. Growth was observed within 1 week. A 1% transfer was made into a fresh medium and this transfer process was repeated twice. Microscopic examination of the second transfer revealed that the enrichment consisted primarily of rod shape. The second transfer was plated on a solid medium and incubated in an anaerobic jar on solid agar plates containing the media with 1.5 g/L agar. These plates were incubated at 28°C.

The Bacterial genomic DNA was isolated using Merck Biosciences DNA purification kit.

PCR amplification of 16S rDNA was done using Merck B. PCR master Mix and forward and reverse primer. Cells from 2-ml cultures were harvested by centrifugation, resuspended in 40 ml of sterile water, and lysed by adding 5 ml of chloroform and incubating the preparations for 10 min at 95°C. Primers specific for bacterial 16S ribosomal DNA (rDNA) (primer 8F [59-AGA GTTTGATCCTGGCTCAG-39] and primer 1525R [59-AAGGAGGTGATCCAGCC-39]) were

used in 50-ml PCR mixtures that contained 10 mMTris-HCl (pH 9.0), 50 mMKCl, 0.1% Triton X-100, 1.2 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 75 ng of each primer, 0.5 ml of Taq polymerase (Gibco/BRL), and 1 ml of lysed cells. Amplification was performed by using the following conditions: 94°C for 3 min, followed by 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final step consisting of 10 min at 72°C. The amplification products were send to by Genie (Bangalore, India) for 16SrDNA sequencing.

3.2.8 Growing of isolated bacterial strains

The bacterial strains were grown and maintained in the specific media as discussed in the 4.2.2.4 in the liquid broth. The strains were maintained through subculturing in liquid media and in solid cultures in petriplates. The petriplates were grown in anaerobic jar and liquid cultures were purged with N_2 to maintain the anaerobicity of the strain to retain their perchlorate reducing property.

3.2.9 Optimization of the culture conditions for perchlorate bioreduction by the mixed consortium predominantly *Burkholderia*sp.

For preliminary studies pH and temperature was chosen for analyzing the optimum culture conditions for perchlorate degradation by the mixed consortium. The degradation efficiency (%) of perchlorate by the mixed consortium was investigated under different pH and temperature. Succinate was used as sole C-source for the preliminary study. The enriched culture was added to 800 mg/L of perchlorate in media set to five different pH ranging from 5.0 to 9.0. The pH of the solutions was adjusted by adding required quantity of 1M HCl and 1M NaOH. Effect of temperature on the reduction of perchlorate was investigated at five different values ranging from 20^{o}C to 40^{o}C. Samples were withdrawn at regular intervals, centrifuged (8000 rpm for 15 min) and filtered.

Based on the preliminary studies some more factors were selected for further analysis if optimization through initial screening. Screening and optimization of media constituents can be done by either varying one variable at a time or non-conventionally using statistical methods.

Conventional screening and optimization techniques involve varying factor and their levels by maintaining the other factors at an unspecified constant level, and by doing so, the combined effect of the factors is generally neglected; moreover, the technique is time-consuming and requires a sufficiently large number of experimental runs. These limitations of a classical method can be eliminated by screening and optimizing all the affecting factors collectively by employing statistical experimental design and empirical model building using regression analysis. To reduce

the number of factors to be used in an optimization study, screening of factors is normally performed by employing another statistical design such as Plackett– Burman. Plackett-Burman design, an efficient way to identify the important factors among a large number of variables (Stanbury et al., 1986), was used in the present study to screen the important variables that significantly influenced phenol degradation.

In this study, a 16-run Plackett-Burman design was applied to evaluate five factors. Each variable was examined at two levels: –1 for the low level and +1 for the high level. Table 3.2 illustrates the variables and their corresponding levels used in the experimental design. The values of two levels were set according to our previous preliminary experimental results. The Plackett-Burman design and the response value of phenol degradation are shown in Table 3.2. The effect of individual variable on perchlorate degradation was calculated by the following equation:

$E(X_i) = 2(i^+ - M_i^+)/N$

where, $E(X_i)$ is the effect of the tested variable (X_i) and M_i^+ and X_i^+ are responses of trials at which the variable is at its high or low levels respectively

The standard error (SE) of the effect was the square root of V_{eff} and the significance (p-value) of the effect of each variable on perchlorate degradation was measured by Student's t-test according to the equation:

$t(X_i) = E(X_i)/SE$

Where, $E(X_i)$ is the effect of variable X_i.

Taguchi design

The Taguchi method, one of the optimization, has good reappearance of experiments concerned only with the main effects of design parameters. In principle, the Taguchi's design of experiments is used to get information such as main effects and interaction effects of design parameters from minimum number of experiments. The objectives of Taguchi method for parameter design were to find out the best combination of design parameters and reduce the variation for quality.

Based on our previous work the main operational parameters and their levels were selected and showed in Table 3.5. The orthogonal array of L16 type was used, and is represented in Table 3.3. L and 16 mean Latin square and the replication number of the experiment, respectively. Five–four level factors can be positioned in an L16 orthogonal array table. The number in table indicates the levels of a factor (Kim et al, 2004).

Total five parameters of reduction of perchlorate utilizing succinate as sole C-source, temperature, pH, inoculum age, inoculums volume and ration of carbon source and perchlorate were chosen for the screening with Plackett-Burman method of screening (Table 3.2). Among the five three factors, inoculums age, temperature and carbon to perchlorate ratio were further anaysed for optimization with Taguchi design as shown in Table 3.3.

Table 3.2 Plackett-Burman screening to determine the significant factors affecting perchlorate reduction employing succinate as sole C-source.

Serial No	Temperature (^{o}C)	pH	Inoculum volume (mg/L)	Inoculum age (days)	Carbon: perchlorate ratio
1	24	6.5	3.5	2	1
2	32	7.5	6.5	2	2
3	24	7.5	3.5	2	1
4	32	7.5	3.5	4	1
5	28	7.0	5.0	3	1.5
6	32	7.5	3.5	4	2
7	24	7.5	6.5	2	2
8	24	7.5	6.5	4	1
9	28	7.0	5.0	3	1.5
10	32	6.5	6.5	2	1
11	28	7.0	5.0	3	1.5
12	28	7.0	5.0	3	1.5
13	32	6.5	6.5	4	1
14	24	6.5	6.5	4	2
15	24	3.5	3.5	4	2
16	32	3.5	3.5	2	2

Table 3.3: Taguchi design for analysis of significant parameters affecting perchlorate reduction employing succinate as sole C-source.

Serial no	Inoculum age (days)	Temperature (^{o}C)	Carbon: Perchlorate ratio
1	1	24	0.5
2	1	24	0.5
3	1	24	0.5
4	1	28	1.0
5	1	28	1.0
6	1	28	1.0
7	1	32	2.0
8	1	32	2.0
9	1	32	2.0
10	3	24	1.0
11	3	24	1.0
12	3	24	1.0
13	3	28	2.0
14	3	28	2.0
15	3	28	2.0
16	3	32	0.5
17	3	32	0.5
18	3	32	0.5
19	5	24	2.0
20	5	24	2.0
21	5	24	2.0
22	5	28	0.5
23	5	28	0.5
24	5	28	0.5
25	5	32	1.0
26	5	32	1.0
27	5	32	1.0

3.2.10 Optimization of the culture conditions for perchlorate bioreduction by the mixed consortium predominantly *Pseudoxenthomonas*sp.

As in the previous case (section 4.2.2.10), here also total five parameters of reduction of perchlorate utilizing succinate as sole C-source, temperature, pH, inoculum age, inoculums volume and ration of carbon source and perchlorate were chosen for the screening with Plackett-Burman method of screening as shown in Table 3.4. Among the five three factors, inoculums age, temperature and pH were further analyzed for optimization with Taguchi design as shown in Table 3.5.

Table 3.4: Plackett-Burman screening to determine the significant factors affecting perchlorate reduction employing phenol as sole C-source.

Serial No	Temperature (^{o}C)	Inoculum age (days)	Inoculum volume (mg/L)	pH	Carbon: perchlorate ratio
1	32	2	3.5	6.5	2
2	24	4	3.5	6.5	1
3	28	3	5.0	7.0	1.5
4	32	2	6.5	6.5	1
5	32	4	6.5	6.5	2
6	28	3	5.0	7.0	1.5
7	32	4	3.5	7.5	1
8	24	2	3.5	6.5	1
9	28	3	5.0	7.0	1.5
10	24	2	6.5	7.5	2
11	32	2	6.5	7.5	1
12	24	4	6.5	6.5	2
13	24	4	3.5	7.5	2
14	24	2	3.5	7.5	2
15	24	4	6.5	7.5	1
16	28	3	5.0	7	1.5

Table 3.5: Taguchi design for analysis of significant parameters affecting perchlorate reduction employing phenol as sole C-source.

Serial no	Inoculum age (days)	Temperature (^{o}C)	pH
1	2	21	5
2	2	21	5
3	2	21	5
4	2	28	7
5	2	28	7
6	2	28	7
7	2	37	9
8	2	37	9
9	2	37	9
10	3	21	5
11	3	21	5
12	3	21	5
13	3	28	7
14	3	28	7
15	3	28	7
16	3	37	9
17	3	37	9
18	3	37	9
19	4	21	5
20	4	21	5
21	4	21	5
22	4	28	7
23	4	28	7
24	4	28	7
25	4	37	9
26	4	37	9
27	4	37	9

3.2.11 Kinetics of perchlorate bioreduction by the mixed consortium

The degradation kinetics of perchlorate was observed in different initial perchlorate concentration using succinate and phenol as also using sole C-source separately in batch systems by mixed consortium. The culture which was found to be predominant with *Burkholderia* sp. was used to analyze the degradation kinetics utilizing succinate as sole C-source and the culture predominant with *Pseudoxanthomonas* sp. used to kinetics analysis utilizing phenol as sole C-source. Initial perchlorate concentration were varied from 100 mg/L to 800 mg/L keeping intervals of 100 mg/L for both sets of experiments using succinate and phenol as sole C-source. Initial concentration of C-source was kept 1000 mg/L in all the sets.

The degradation rate constants (k_d) of perchlorate degradation under different initial perchlorate concentration were estimated with the zero-order rate equation and pseudo-first-order rate equation is mentioned below,

$$\frac{dC}{dt} = -k_d C \qquad \text{Eq no.1}$$

$$\ln C/Co = -k_d t \qquad \text{Eq no.2}$$

Where, C_0 is the initial concentration of perchlorate, C is the concentration of perchlorate at time t, and $k_d$is the degradation rate constant of perchlorate in pseudo-first-order equation. Values of k_d were calculated plotting ln (C_0/C) versus time (t).

3.2.12 Batch study on perchlorate bioreduction by the mixed culture predominantly *Pseudoxanthomonas* sp. at different initial phenol concentration

Increasing concentration of phenol was added in batch shake flasks with a fixed amount of perchlorate (500 mg/L) to examine the capacity of the consortium to withstand phenol concentration. Phenol was added to the batch shake flask in the following concentrations, 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L and 600 mg/L with the fixed amount of perchlorate in synthetic media. The removal of perchlorate, phenol and COD along with the growth was measured for each of the initial phenol concentrations in replicates.

3.2.13 Batch study on perchlorate biodegradation by the mixed consortium predominantly *Pseudoxanthomonas* sp. from industrial wastewater

The microbial consortium containing predominantly *Pseudoxanthomonas* sp. was tested for removal of perchlorate from oil refinery wastewater. Wastewater was collected a nearby oil and refinery industry and was characterize. Composition of the wastewater is given in Table 3.7. As given in the table, the wastewater contains about 2-3 mg/L of phenol but no perchlorate. Therefore, it was spiked with 500 mg/L of perchlorate and required amount of phenol was added before batch experiments. Reduction of phenol was observed in 7 different initial concentrations (50, 100, 200, 300, 400, 500, and 600 mg/L) Operating conditions were maintained as per the results obtained in the optimization experiment. The removal of phenol, COD and growth of the culture were measured.

Table 3.6: Characteristics of the petroleum refinery wastewater used in the study.

Parameter	Value
Turbidity	337 NTU
Total dissolved solids	0.554 g/L
Conductivity	0.82 mS/cm
Dissolved oxygen concentration	0.95 mg/L
pH	8.2
Oxidation reduction potential	-201.8 mv
NH_4^+-N	9.529 mg/L
NH_3-N	0.753 mg/L
Cl^-	740 mg/L
NO_3^-	162 mg
COD	195.33 mg/L
Phenol	2-3 mg/L

3.2.14 Effect of co-pollutants on perchlorate bioreduction by the mixed consortium

The capability of the seed sludge, grown in succinate or phenol as carbon sources, on perchlorate bioreduction in presence of other oxyanions (co-pollutants) such as chlorate (0.5 g/L, nitrate (0.5 g/L), phosphate (0.5 g/L) and nitrite (0.5 g/L) were evaluated in batch shake flasks. Optimum conditions as determined through culture condition optimization experiments were maintained during the study. Succinate or phenol of 1000 mg/L (as COD) was used as the sole C-source for the bacterial species predominantly *Burkholderia* sp. or *Pseudoxanthomonas*sp. respectively. Seed sludge harvested from 2.0 L conical flasks was centrifuged at 5000 rpm for 15 min, the resulting precipitate together with media containing perchlorate were added into a 250 ml conical flasks. Initial pH (7.0) of wastewater was adjusted by 0.1 M HCl or 0.1 M NaOH solution.

The dissolved oxygen in the medium was removed by purging with oxygen free nitrogen gas for 5 min in each of the flasks. The flasks were sealed with butyl-rubber stoppers and finally incubated at 28^{o}C in static condition. The alternative e^- acceptors (ie., oxyanions) were supplied individually and also in mixed solution into the medium to evaluate the ability of the enriched mixed consortium on utilization of these alternative e^- acceptors in presence of perchlorate. The effect of initial concentration of nitrate on perchlorate reduction was also observed elaborately. Bioreduction experiments with perchlorate and nitrate were carried out in batch shake flasks of 150 mL Erlenmeyer flasks, containing 100 mL media with 5 mL of cell suspension (OD_{600} 0.15–0.25). The culture medium was incubated at 28^{o}C under shaking condition (120 rpm). The initial concentration of perchlorate and nitrate in the culture medium was varied from 100 mg/L to 800 mg/L with an interval of 100 mg/L.

3.2.15 Perchlorate bioreduction in continuous PBR by the mixed consortium

In the present study, 2 nos PBR were operated simultaneously with different seed sludge and sources of carbon. Reactor start-up of the two reactors was done using seed sludge enriched either with *Pseudoxanthmonas* sp. or *Burkholderia* sp. The reactors were fed with phenol (reactor PBR-1) and succinate (reactor PBR-2) and, respectively, as the sole source of carbon. Temperature of the content in the reactor was maintained at 28±1^{o}C by circulating warm water through plastic pipes wrapped around the reactor. pH was maintained between 6.9 and 7.1 using buffer solution. Before inoculation, respective seed sludge was grown in 4 L containers in a suspended growth mode. Respective seed sludge was transferred into the reactor using peristaltic pumps for biofilm development on the PUF inside the reactors.

Biofilm development:

The inoculum was prepared as described in the previous section. The active cells grown in MSM were centrifuged (5000g, 20min at 28^{o}C), washed 1x phosphate buffer saline (pH 7.4), and suspended in four liter specific media containing 500 mg/L of perchlorate to give a cell suspension was inoculated by pumping into the reactor packed with PUF piece by means of a

peristaltic pump . Any biomass washed out of the reactor along with the effluent was recycled back. The reactor was continuously fed with the above mentioned specific media spiked with perchlorate concentration, 500 mg/L and was operated for about one month at different flow rates to maintain different HRT of 11-10 days, 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.

Performance evaluation of PBR:

The reactors (PBR-1 and PBR-2) were operated in different conditions with respect to HRT, influent perchlorate and carbon sources which has been tabulated below. As given in the table, PBR-2 was operated continuously for a period of about 9 months fed with phenol as only external carbon source. PBR-1 was operated for about 5½ months with succinate as sole source carbon source and 3½ months with mixtures of succinate and phenol as sources of carbon.

Table 3.7 (a): Operation schedule of PBR-1 with phenol as carbon source and seed sludge predominantly *Pseudoxanthomonas* sp.

Time of run (day)	HRT (hydraulic retention time)	Influent perchlorate (mg/L)	Influent phenol (mg/L)
0 to 11	10 day	200	400
11 to 32	7 day	200	400
32 to 38	6 day	200	400
38 to 55	5 day	200	400
55 to 62	4 day	200	400
62 to 75	3 day	200	400
75 to 87	10 day	400	800
87 to 105	9 day	400	800
105 to 113	8 day	400	800
113 to 123	7 day	400	800
123 to 132	6 day	400	800
132 to 143	5 day	400	800
143 to 152	4 day	400	800
152 to 167	3 day	400	800
167 to 182	2 day	400	800
182 to 197	1 day	400	800
197 to 209	18 hrs	400	800
209 to 224	12 hrs	400	800
224 to 242	18 hrs	400	800
242 to 260	16 hrs	400	800
260 to 275	14 hrs	400	800

Table 3.7 (b): Operation schedule of PBR-1 with phenol as carbon source and seed sludge predominantly *Burkholderia* sp.

Time of run (day)	HRT (hydraulic retention time)	Influent perchlorate (mg/L)	Influent succinate (mg/L)	Influent phenol (mg/L)
0 to 15 day	10 day	200	400	-
15 to 30 day	7 day	200	400	-
30 to 38 day	6 day	200	400	-
38 to 50 day	5 day	200	400	-
50 to 58 day	4 day	200	400	-
58 to 70 day	3 day	200	400	-
70 to 85 day	10 day	400	800	-
85 to 92 day	9 day	400	800	-
92 to 100 day	8 day	400	800	-
100 to 112 day	7 day	400	800	-
112 to 118 day	6 day	400	800	-
118 to 128 day	5 day	400	800	-
128 to 135 day	4 day	400	800	-
135 to 148 day	3 day	400	800	-
148 to 163 day	2 day	400	800	-
163 to 173 day	2 day	400	750	50
173 to 188 day	2 day	400	700	100
188 to 218 day	2 day	400	600	200
218 to 238 day	2 day	400	500	300
238 to 258 day	2 day	400	400	400
258 to 278 day	2 day	400	450	350

3.3. Analytical methodologies

Solution pH was measured using a pH meter (Sartorius, BT224S). Optical density was determine using a UV-visible spectrophotometer (Model lambda-45, Perkin Elmer, USA). Various instruments / equipments used to measure different parameters are given in Table 3.1. Analytical procedures followed to determine some specific parameters are described in brief in this section.

Determination of biomass concentration:

Quantification of mixed microbial consortium 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). Aliquot samples in the amount of 2 ml were taken using a needle and sterile syringes, and centrifuged in 8000 rpm for 10 min and kept in a refrigerator at 4^{o}C if not being analyzed on the same day. Samples were centrifuged at 8,000 rpm for 15 min to separate the biomass. 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). For determination of biomass concentration as MLSS, 10 ml of samples at different optical density ranging from 0.1-0.8 OD_{600} were centrifuged for 10 mins at 10000g in ambient temperature (Remi C-24-BL Mumbai, India) in a previously weighed 15 ml centrifuge tube (Tarson, India). Obtained pellets were dried overnight at 105^{o}C and its final weight was measured. One unit of absorbance was found equivalent to 235 mg/L of MLSS.

Determination of perchlorate and other anions:

Perchlorate, nitrate, chlorate, sulfate, phosphate, succinate were measured using an Ion Chromatograph (Metrohm AG, IC 792, Herisau, Switzerland), equipped with a Dual 3 column (250 mm × 4 mm), a RP guard column, and a conductivity detector. The detection limit for perchlorate was 0.5 ppm. Sodium hydroxide (5 mM) served as the eluent, and sulfuric acid (2.0 mM) as the regenerant. Sample volume was 20 µl. Ion-meter equipped with perchlorate sensitive electrode (Thermoscientific, Singapore) was also used for the determination of perchlorate anion

in initial stages. The calibration curve were prepared with the perchlorate standards with sodium perchlorate salt for analysis with both the instrument, ion chromatography and ion meter. One hundred mg/L of perchlorate solution was used to prepare perchlorate calibration standards. Standards were made to prepare calibration curve for analysis perchlorate samples each day. Samples were filtered through the C-18 reverse-phase cartridge and then through 0.45µm filter to remove the contamination of organic substances from the perchlorate samples and as well as standards. Standards were made depending on the probable concentration of samples the range of standards were fixed. Everytime, five standards were prepared i.e., if the sample contains perchlorate within the range of 10mg/L to 100mg/L, standards of 10mg/L, 20mg/L, 40mg/L, 60mg/L, 80mg/L and 100mg/L. Ultra pure water was used for all analysis.

Determination of phenol concentration:

Phenol concentrations were ascertained using a spectrophotometer (Odyssey DR/2500, Hach) following 4- aminoantipyrine technique (APHA, 1992). Briefly, this method involved the reaction of phenols with 4-aminoantipyrine in the presence of potassium ferricyanide, to form a colored antipyrine dye. The dye was extracted from the aqueous samples with chloroform and the color intensity was measured at the weave length of 460 nm.

Determination of chemical oxygen demand (COD):

Besides measurement of different organics (acetate, succinate etc.) concentration in IC, their chemical oxygen demand (COD) was also measured following closed refluxed methods as suggested in standard methods (APHA, 2005).

Microscopic methods:

Fragments of polyurethane foam (PUF) containing the microbial bio-film were sampled at various sampling points and cut into small thin pieces. The sample was dehydrated overnight and mounted on aluminium stubs and then coated with gold using sputter coater. The stubs were then introduced into the specimen chamber of LEO 1430vp scanning electron microscope (SEM) for scanning.

CHAPTER 4: RESULTS AND DISCUSSION

Perchlorate (ClO_4^-) contamination is a significant concern in surface and ground waters over the years. The presence of perchlorate increases the toxic effects exerted on the environmental life as well as on the human owing to its recalcitrant properties. Perchlorate is widely used by many industries and found to be difficult to remove from water bodies due to its very high solubility in aqueous system. Therefore, efficient treatment of water and water and wastewaters has been a matter of concern now a day. In view of the current scenario the present study is focused on the bioreduction of perchlorate contaminated water by an indigenously isolated mixed microbial consortium.

4.1 Acclimatization of perchlorate reducing microbial consortium

Biosludge collected from treatment plant was treated for enrichment for reducing perchlorate. Enrichment of the mixed consortium was carried out by adding gradually increasing amount of perchlorate in synthetic wastewater. The experiment was done for one and half months. The detailed enrichment phase presented it shows that the perchlorate removal rate was improved at each and every stage of acclimatization as shown in Fig.4.1. Morphological characterization of the mixed consortium was done by SEM (scanning electron microscope). SEM photograph in the Fig 4.2 shows the cells were of various sizes and shapes, from small rods to large rods. Most of the rods were between 0.2 × 1.7 μm and 0.6 × 1.8 μm in size (Fig.4.2). The major rod strains were found to be growing as clusters. Majority of the reported perchlorate reducers are also rod-shaped ranging from 0.2–0.8×1.0–2.8 μm (Logan, 2001).

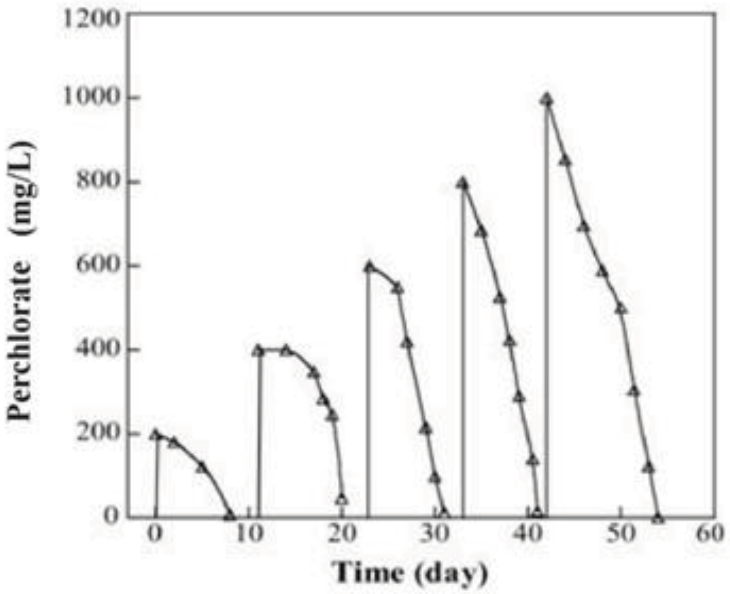


Fig 4.1: Acclimation results of mixed microbial consortium for perchlorate bioreduction (Temperature = 28^{o}C; pH = 7.0).

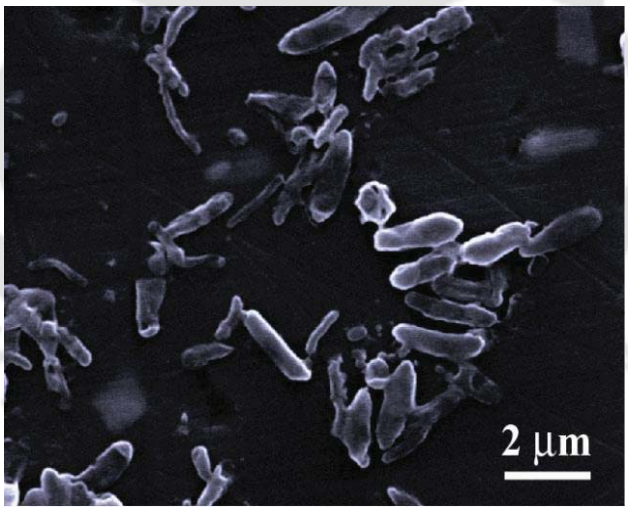


Fig 4.2: SEM (scanning elector microscopy) image of mixed microbial consortium capable of perchlorate bioreduction.

4.2 Effect of different C-sources on perchlorate bioreduction by mixed consortium

The mixed cultures of bacteria were capable to accept perchlorate as terminal electron acceptor with oxidation of several C-sources. Acetate has been used extensively as substrate for heterotrophic perchlorate reduction (Kengen and Rikken, 1999). The inoculation in each set was done as the biomass concentration becomes 100 mg/L as MLSS Therefore, the ability of the mixed consortium to utilize different organic acids during perchlorate degradation has been investigated in the present study. Fig 4.3 (a) shows the degradation profile of perchlorate by the mixed consortium in presence of five different organic acids such as oxalate, succinate, citrate, formate and acetate. Among these organic acids used, the mixed consortium was able to degrade perchlorate completely after six days by utilizing succinate as sole C-source. While using oxalate, formate, citrate or acetate as the sole C-source, the degradation was only ~68.1%, ~84.6%, ~98.4%, and ~94.4% respectively after six days.

The mixed microbial consortium was found to be able to reduce perchlorate using phenol as a sole C-source. When 300 mg/L of phenol was added to the synthetic media along with 500 mg/L of perchlorate the mixed consortium was able to reduce perchlorate upto 96.4 % within 5 days of experiment a shown in Fig 4.4. This was the first report of any mixed microbial culture reducing perchlorate while accepting phenol as sole C-source.

The probable reactions mechanisms of perchlorate degradation by the mixed consortium utilizing these five organic acids are given below,

for succinic acid,

$2C_4H_6O_4 + 4ClO_4^- = 4Cl^- + O_2 + 8CO_2 + 6H_2O$

$C_2H_4O_2 + 2ClO_4^- = Cl^- + 2O_2 + 2CO_2 + 2H_2O$

for citric acid,

$2C_6H_8O_7 + 5ClO_4^- = 5Cl^- + O_2 + 12CO_2 + 8H_2O$

for formic acid,

$2CH_2O_2 + 2ClO_4^- = 2Cl^- + 3O_2 + 2CO_2 + 2H_2O$

for oxalic acid,

$4C_2H_2O_4 + ClO_4^- = Cl^- + O_2 + 8CO_2 + 2H_2O$

Influence of succinate concentration on degradation of perchlorate was further examined by addition of different amount of succinate ranging from 300 to 1000 mg/L. With the increase in initial concentration, the removal efficiency (%) of perchlorate was increased and with 1000 mg /L of initial succinate concentration the mixed consortium was able to degrade the total amount of perchlorate within 10 days. The degradation rate constant (k_d) of perchlorate increased from 0.15 per day to 0.29 per day with the increase in initial succinate concentration from 300 to 1000 mg/L. These results showed that at lower

succinate concentration i.e. below 500 mg/L as COD, the mixed consortium could not achieve significant perchlorate removal.

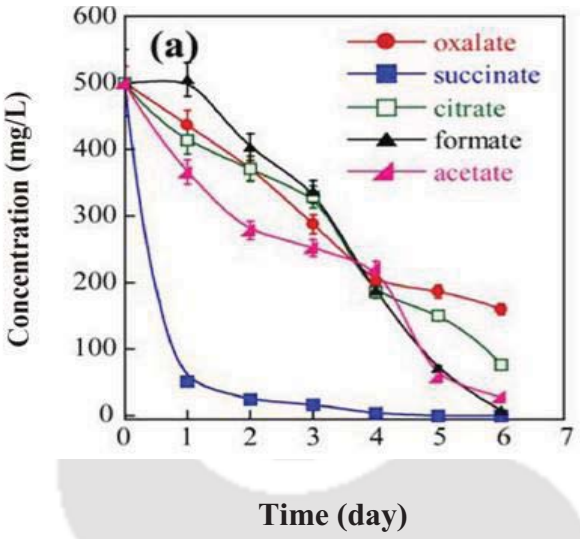


Fig 4.3 (a): Effect of different C-sources on perchlorate degradation by the mixed consortium (Temp = 28^0C and pH = 7.0)

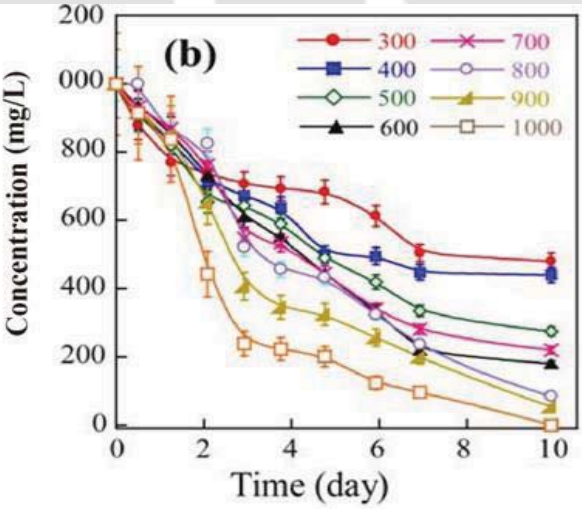


Fig 4.3 (b): Perchlorate reduction by the mixed consortium for different concentration of succinate in the mixed consortium (Temp = 28^0C and pH = 7.0)

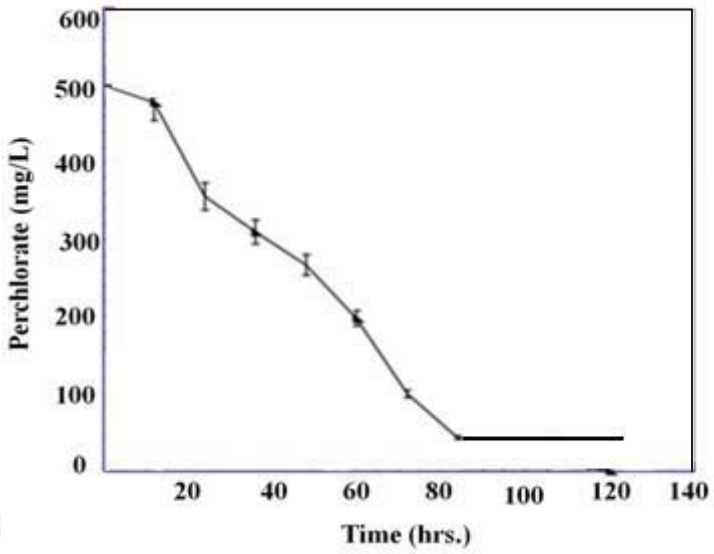


Fig 4.4: Degradation of perchlorate using phenol (300 mg/L) as sole C-source by the mixed microbial consortium.

4.3 Isolation and identification of perchlorate reducing strains from mixed consortium

The predominant perchlorate reducing strain was isolated from the enriched mixed consortium. The predominant strain for reduction of perchlorate using succinate as sole C-source and the one for using phenol as sole C-source has been designated as strain AG (Fig 4.3 a) and AG2 (Fig 4.3 b) respectively. Partial 16S rDNA sequencing result showed that strain AG had 1436 base pairs (bp). For *Pseudoxanthomonas*sp., the 16S rDNA was

isolated and PCR product of the gene was send for gene sequencing as described in the material methods section. The sequences were submitted to the Genbank and the accession number of the strain AG and AG2 were given as HM104637 and JX860406. Gene analysis by online BLAST tool indicates that the isolates contain sequences that are specific to the members of the family Proteobacteria. The phylogenetic trees (Fig. 4.6 a and b) were prepared using neighbor joining method based on near-full-length 16S rDNA gene sequences recovered from the isolated strain and other sequences obtained from the Genbank database (Genbank accession number has been indicated with the generic name in the respective trees in Fig 4.6 a and b).

The high bootstrap support of the tree shown in derived from the 16S rDNA analysis demonstrated that strain AG is a typical member of the genus *Burkholderia* sp. and it has closest relation (98%) to *Burkholderia*sp. ATSB16. This is the first report of the *Burkholderia* sp. involved in perchlorate degradation. The strain AG 2 has shown its closest homology with *Pseudoxanthomonasmaxicana* strain M. The thermodynamic properties as obtained by the online tool (BIOTOOL), indicated that strain AG has 54% GC content with 2399.1 kcal/mol Gibbs free energy (ΔG), 33093.3 kcal/mol enthalpy (ΔH) and 31576.1 cal/mol/K entropy (ΔS). The results of the biochemical tests done for both the strains have been enlisted in the Table 4.1 which confirmed the identification of both of the strains.

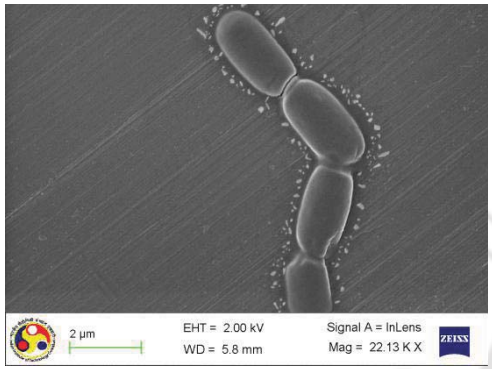


Fig 4.5 (a): SEM image showing cells of *Burkholderia*sp.(HM104637) isolated from the mixed microbal consortium

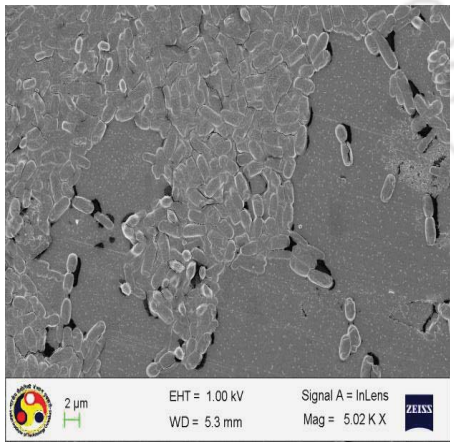
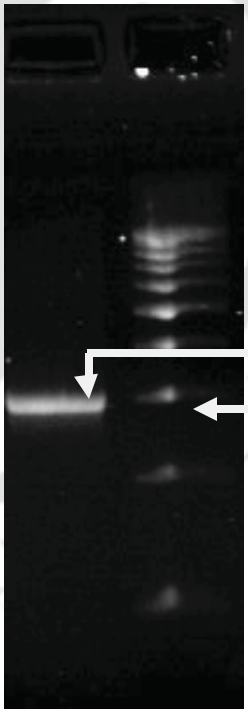


Fig 4.5 (b): SEM image showing cells of *Pseudoxanthomonas* sp. (JX860406) isolated from the mixed



Marker

Isolated 16SrDNA

Fig 4.5 (c): Agarose gel image showing the amplified PCR product of 16S rDNA isolated from *Pseduxanthomonas* sp.

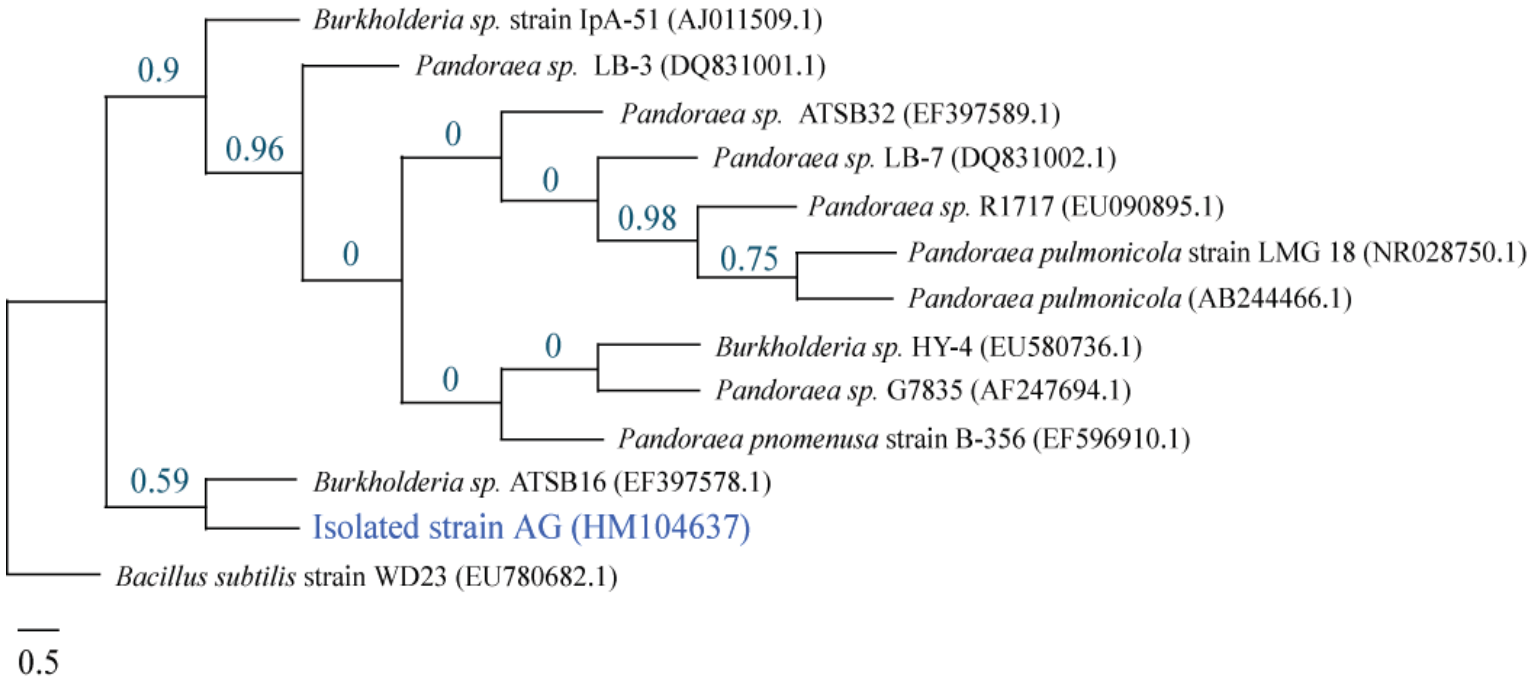


Fig 4.6 (a): Phylogenetic position of the strain AG (HM104637) in relation to other (per)chlorate-reducing bacteria. Bootstrap values (1 replicate) are indicated on the nodes. The scale bar indicates 0.5 substitutions per nucleotide position.

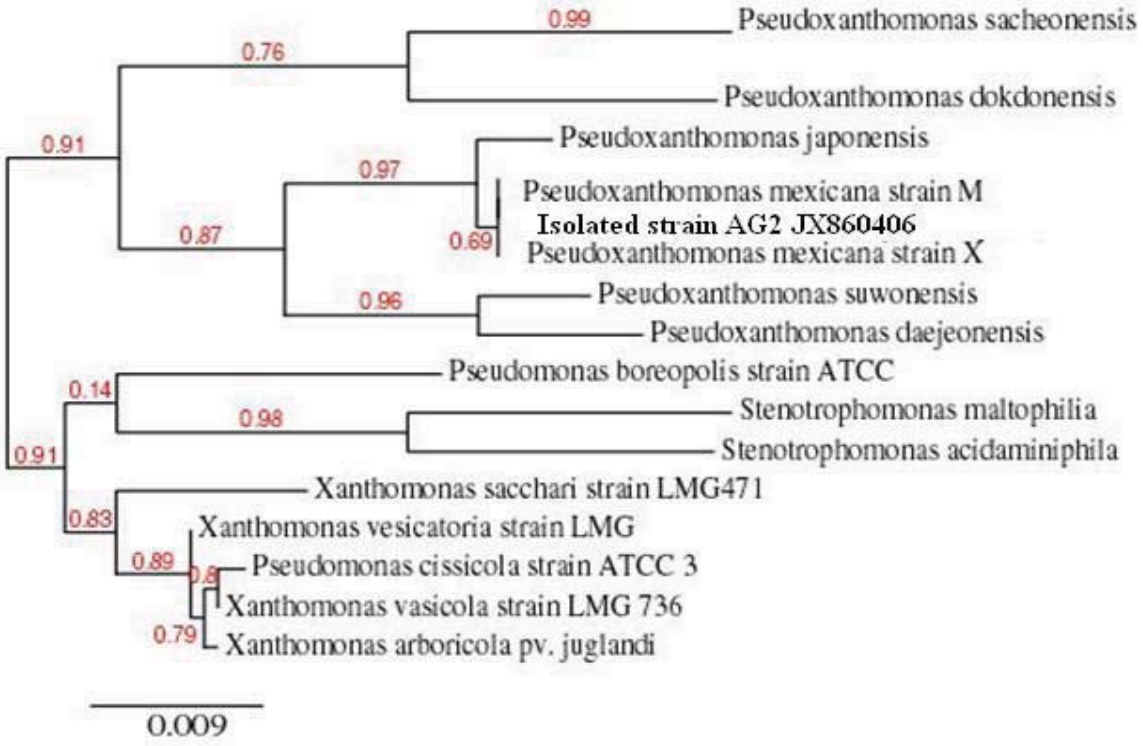


Fig 4.6 (b): Phylogenetic position of the strain AG2 (JX860406) in relation to other (per)chlorate-reducing bacteria. Bootstrap values (1 replicate) are indicated on the nodes. The scale bar indicates 0.5 substitutions per nucleotide position.

Chapter 4: Results and Discussions

Table 4.1: Morphological and biochemical characteristics of the bacterial starins*Burkholderia* sp. and *Pseudoxanthomonas* sp. predominantly present in the mixed microbial consortium.

Tests	***Pseudoxanthomonas* sp.** (JX860406)	***Burkholderia*sp.** (HM104637)
Configuration	Circular	circular
Margin	Entire	entire
Elevation	Convex	convex
Surface	Smooth	smooth
Pigment	Cream	cream
Opacity	Transparent	opaque
Gram's reaction	Gram-ve	Gram-ve
Cell shape	Rod	Ovoid or rod shaped
Size(µm)	0.5 x 1.5	1x1.2-1.5
Arrangement	Pairs	Singly or in pairs
Spore(s)	-	-
Motality	Motile	Non-motile
4°C	-	-
10°C	-	-
15°C	+	+
25°C	+	+
30°C	+	+
37°C	+	+
42°C	+	+
60°C	-	-
pH 4.0	-	-
pH 5.0	-	-
pH 6.0	+	-
pH 7.0	+	+
pH 8.0	+	-
pH 10.0	-	-

Tests	*Pseudoxanthomonas* sp. (JX860406)	*Burkholderia*sp. (HM104637)
Growth under anaerobic condition	-	-
Growth on McConkey	-	-
Indole test	-	-
Methyl red test	-	-
VogesProskauer test	-	-
Citrate utilization	-	-
H_2S production	-	-
Gas production From glucose	-	-
Gelatin Hydrolysis	+	+
Esculin hydrolysis	+	+
Starch hydrolysis	-	-
Urea hydrolysis	-	-
Nitrate reduction	+	+
Ornithine decarboxylase	-	-
Lysine decarboxylase	-	-
Arginine dihydrolase broth	-	-
Catalase test	-	-
Oxidase test	+	+
Tween 20 hydrolysis	-	-
Tween 40 hydrolysis	+	+
Dextrose	+	+
Lactose	+	+

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4. 4 Effect of pH and temperature on perchlorate degradation using succiante as sole carbon source by mixed consortium predominantly *Burkholderia* sp.

Perchlorate degradation was studied in five different temperatures ranging from 20^{o}C to 40^{o}C with initial perchlortae concentration 800 mg/L, succinate 1000 mg/L and pH 7.0. Maximum degradation was observed at 30^{o}C (Fig. 4.7 (b)), where the perchlorate concentration was reduced to 16.0 mg/L (~98%) from 1000 mg/L within 7 days with k_d value 0.27 day^{-1}. Results indicate that in 25^{o}C and 30^{o}C perchlorate degradation by the enriched mixed culture was sufficiently high, but in lower (20^{o}C) or higher (40^{o}C) temperatures the degradation efficiency was decreased in a considerable amount. In 30^{o}C, the perchlorate reduction was 100% within 6 days of experiment. Similarly, several studies have also reported increased perchlorate degradation in 30^{o}C by an enriched mixed consortium using acetate as sole C-source, initial pechlorate 500 mg/L and pH 7.0 (Urbansky, 2000).

Fig.4.7 (d) shows the perchlorate degradation profile by the mixed consortium at different pH between 5.0 and 9.0 (pH was adjusted with required amount of 0.1 M NaOH and 0.1 M HCl). Initial perchlorate concentration was 800 mg/L and succinate COD 1000 mg/L. The enriched mixed culture substantially degrades ClO_4^- in the pH range of 5.0 to 7.0 while the degradation efficiency was considerably reduced at pH 8.0 and 9.0 where only 65% and 55% removal was observed respectively.

Chapter 4: Results and Discussions

The maximum degradation of (~98%) ClO_4^- was observed at pH 7.0, where perchlorate was decreased substantially from an initial concentration of 800 to 16.6 mg/L within 6 days with degradation rate constant (k_d) 0.28 day^{-1} according to first order of reaction.

The pH in each set of experiments were measured to the same as initial values due presence of buffer in synthetic media (composition has been mentioned in the material methods section, 3.2.2.4). In a study by (Urbansky, 2000) reported an optimum pH of 8 for maximum removal of ClO_4^- by a mixed consortium from an indigenous source.

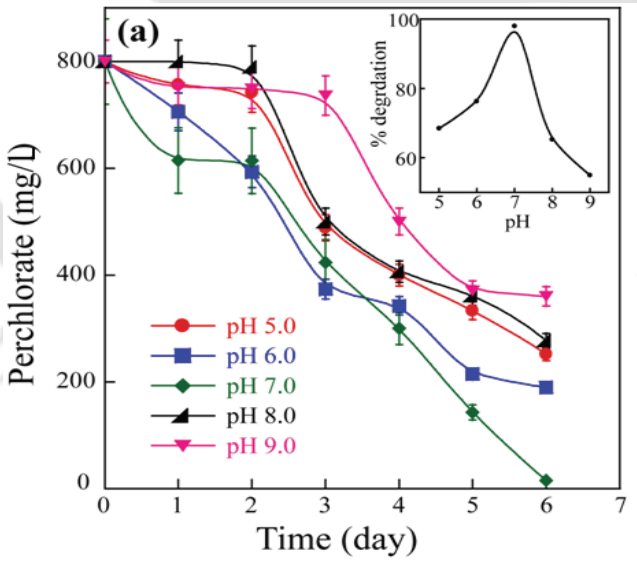

Fig 4.7 (a): Effect of different pH on perchlorate reduction by the mixed consortium. Initial conditions: perchlorate, 800 mg/L; succinate, 1 g/L; (Temperature = 28°C).

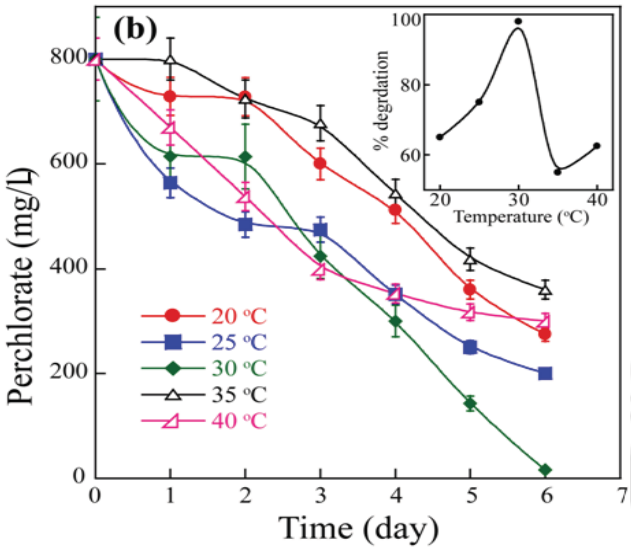

Fig 4.7 (b): Effect of different temperature on perchlorate reduction by the mixed consortium. Initial conditions: perchlorate, 500 mg/L; succinate, 1000 mg/L; pH = 7.0.

4.5 Optimization of the culture conditions for the bioreduction of perchlorate using succiante as sole carbon source by the mixed consortium predominantly *Burkholderia* sp.

Temperature, inoculum age, Carbon to ClO_4^- ratio were found to be more significant according to the results obtained by Plackett-Burman method of screening using succinate as a sole C-source for reduction of ClO_4^-In order to conduct an analysis of the relative importance of each factor more systematically, an analysis of variance (ANOVA) was applied to the data. The main objective of ANOVA is to extract from the results how much variations each factor causes relative to the total variation observed in the result. From the results of ANOVA in Table 4.3 and

4.4 the carbon to ClO_4^- ratio had the largest variance in case of succinate. In the previous section, 30°C was found to be the optimum, where 20, 25, 30, 35 and 40°C temperature were chosen. In this section 24, 28 and 32°C were the levels for temperature and 28°C was chosen as the medium value (Fig 4.8). These results shows similar pattern for effect of temperature on ClO_4^- reduction. 28°C is also reported to be the optimum culture condition for identified PRB (ClO_4^- reducing bacteria) responsible for ClO_4^- and chlorate respiration growing in anaerobic condition. The inoculum age was found to be optimum as 3 days in case of degradation using succinate which falls within the early log phase the isolated predominant strains observation is also similar to the previously reported studies on ClO_4^- degrading bacteria (PRB) where ClO_4^- has been found reduced in early and mid log phase.

Table 4.2: Estimated effects and co-efficients of perchlorate reduction by mixed consortium predominantly *Burkholderia* sp. using succinate as a sole C-source.

Terms	Effect	Co-efficient	SE Coefficient	T	P
Constants		27.533	1.863	14.78	0.000
Temperature	-16.00	-8.00	1.863	-4.29	0.002
pH	-5.667	-2.833	1.863	-1.52	0.163
Inoculum vol	10.2	5.100	1.863	2.74	0.023
Inoculum age	-7.267	-3.633	1.863	-1.95	0.083
Carbon	14.267	7.133	1.863	3.83	0.004
Ct Pt		-3.933	3.727	-1.06	0.319

R^2=84.16% R^2(adj)=73.59%

Table 4.3: Analysis of variance (ANOVA) of screening of perchlorate reduction by mixed consortium predominantly *Burkholderia* sp. using succinate as sole C-source.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main effects	5	1945.48	1945.48	389.10	9.34	0.002
Curvature	1	46.41	46.41	46.41	1.111	0.319
Residual error	9	375.03	375.03	41.67		
Lack of fit 0.671	6	219.91	219.91	36.65	0.71	
Pure error	3	155.12	155.12	57.71		
Total	15	2366.92				

Table 4.4: Analysis of varience (ANOVA) of perchlorate reduction by mixed consortium predominantly *Burkholderia* sp. for Taguchi experiment using succinate as sole C-source.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Temperature	2	11277.9	11277.9	5638.9	238.35	0.000
Inoculum age	2	9423.8	9423.8	4711.9	199.6	0.000
C/P ratio	2	277.3	277.3	138.6	5.86	0.01
Error	20	473.2	473.2	23.7		
Total	26	21452.1				

S= 4.86402 R^2= 97.79% R^2(adj)=97.13%

Table 4.5: S/N ratio of perchlorate reduction by mixed consortium predominantly *Burkholderia* sp.

Level	Temperature	Inoculum age	C/P ratio
1	24.26	25.56	30.21
2	37.10	30.58	30.42
3	31.58	36.79	32.31
Delta	12.84	11.23	2.10
Rank	1	2	3

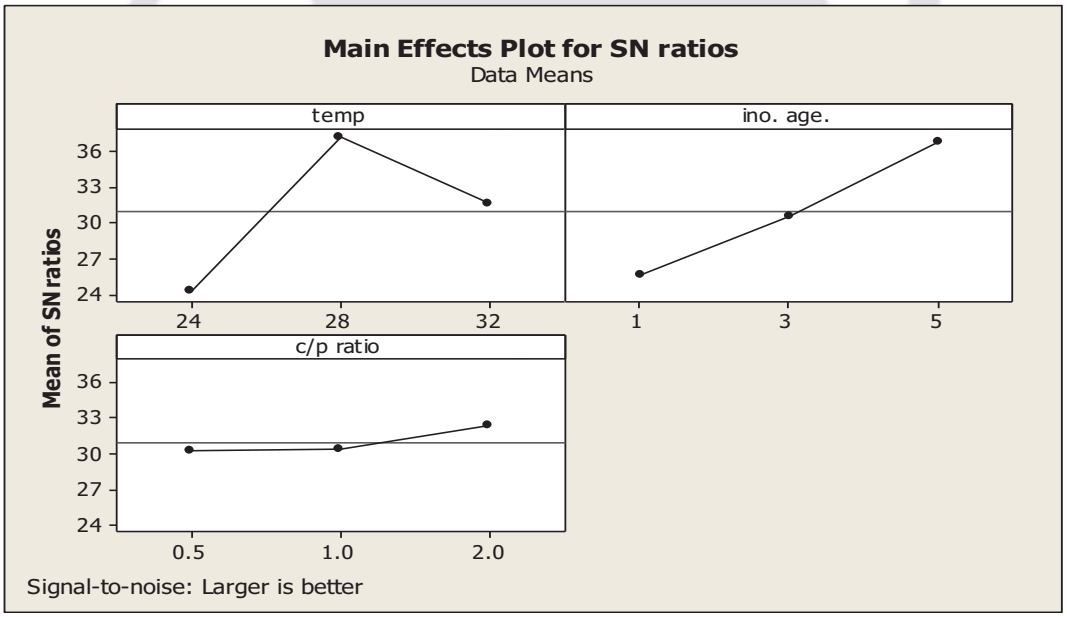

Fig 4.8: Effect of different parameters obtained from Taguchi experiment on perchlorate bioreduction using succinate as sole C-source.

Chapter 4: Results and Discussions

4.6 Optimization of the culture conditions for the bioreduction of perchlorate using phenol as sole carbon source by the mixed consortium predominantly *Pseudoxanthomonas*sp.

inoculum age, Temperature and pH were found to be more significant according to the results obtained by Plackett-Burman method of screening using phenol as a sole C-source for reduction of ClO_4^-. Whereas, temperature, pH and inoculum age were found to be significant among all the five factors when succinate was the sole C-source for ClO_4^- reduction.

As Fig 4.9, 28^oC is the optimum temperature i.e., ClO_4^- degradation in presence of phenol which is also reported to be the optimum culture condition for PRB (ClO_4^- reducing bacteria) responsible for ClO_4^- and chlorate respiration growing in anaerobic condition. The pH was found to be a significant variable in case of degradation using phenol which indicates some toxic effect on the organism due to the presence of phenol which was used as C-source.

The inoculums age was found to be optimum as 5 days in case of degradation using phenol which falls within the early log phase the isolated strain, this observation is also similar to the previously reported studies where it has been reported that ClO_4^- usually gets uptaken by the PRB in early and mid log phase.

Table 4.6: Estimate effects and co-efficients of perchlorate reduction by mixed consortium predominantly *Pseudoxanthomonas*sp. using phenol as sole C-source.

Terms	Effects	Co-efficient	SE Co-efficients	T	P
Constants		92.024	0.2790	321.89	0.00
Temperature	-2.228	-1.114	0.2790	-3.99	0.003
pH	1.382	0.691	0.2790	2.48	0.035
Inoculum vol	-0.052	-0.026	0.2790	-0.09	0.928
Inoculum age	-1.532	-0.816	0.2790	-2.92	0.017
C/P ratio	0.465	0.233	0.2790	0.83	0.426
Ct Pt		3.976	0.5581	7.12	0.000

R^2=90.12% R^2 (adj)=83.53%

Table 4.7: Analysis of variance (ANOVA) of perchlorate reduction by mixed consortium predominantly *Pseudoxanthomonas* sp. using phenol as sole C-source.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main effect	5	29.2671	29.2671	5.8534	6.27	0.009
Curvature	1	47.4218	47.4218	47.4218	50.76	0.000
Residual Error	9	8.4084	8.4084	0.9343		
Lack of fit	6	8.0884	8.0884	1.3481	12.64	0.031
Pure error	8	0.3200	0.3200	0.1067		
Total	15	85.0972				

Table 4.8: Analysis of variance (ANOVA) of perchlorate reduction by mixed consortium predominantly *Pseudoxanthomonas* sp. for Taguchi experiment using phenol as sole C-source.

Source	DF	SS	AdjSS	Adj MS	F	P
Inoculum age	2	4711.9	4711.9	4711.9	199.16	0.000
Temperature	2	27.3	27.3	27.3	5.86	0.048
pH	2	5417.6	5417.6	5417.6	238.35	0.000
Error	20	451.6	451.6	23.7		
Total	26	21452.1				

S = 5.6758 R^2 = 96.19% R^2(adj) = 96.23%

Table 4.9: S/N ratio of perchlorate reduction by mixed consortium predominantly *Pseudoxanthomonas* sp.

Level	Inoculum age	Temperature	pH
1	24.47	25.91	
47.1			
2	74.10	44.82	47.21
3	43.62	71.46	
47.88			
Delta	49.63	45.54	0.78
Rank	1	2	3

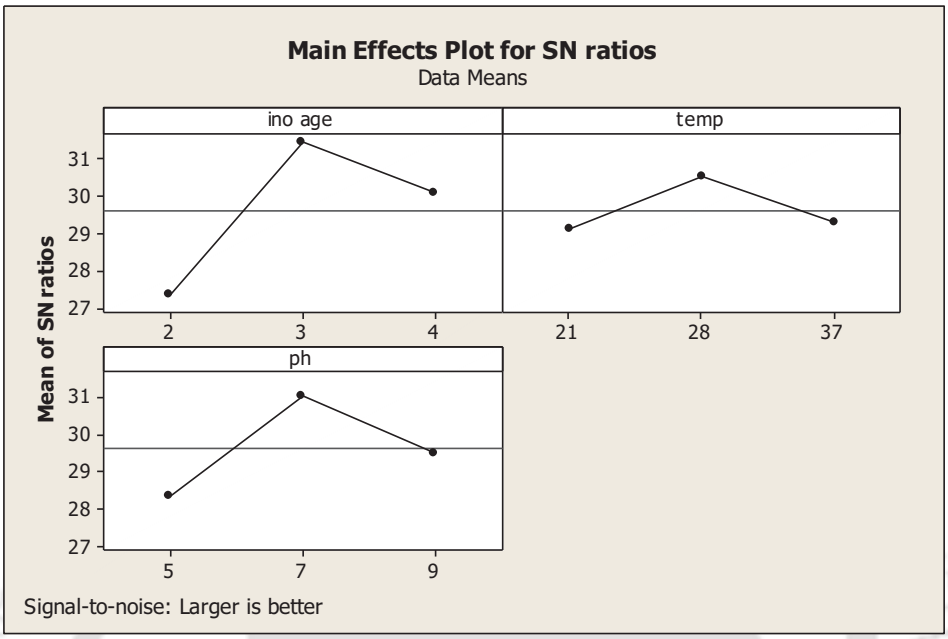

Fig 4.9: Effect of different parameter obtained by Taguchi experiment on perchlorate reduction using phenol as sole C-source.

4.7 Perchlorate bioreduction in batch system using succinate as sole carbon source by mixed consortium predominantly *Burkholderia* sp.

ClO_4^- reduction using succinate as sole carbon source was carried out with different initial concentrations starting from 100 mg/L to 800 mg/L as ClO_4^-. At higher concentration of 800 mg/L the microbial consortium has shown to degrade ClO_4^- perchlorate with decreased degradation rate. It was observed from (Fig. 4.10 (a)) that, the mixed consortium could effectively degrade ClO_4^- from the initial concentrations of 100, 200, 300, 400, 500, 600, 700 and

800 mg/L. However, the degradation efficiencies were significantly reduced in higher concentrations. There was 100% ClO_4^- removal in case of the initial ClO_4^- concentration of 100, 200 and 300 mg/L within 9 days of the experiment. For 400, 500, 600, 700 and 800 mg/L the removal of ClO_4^- were 88 %, 87 %, 90 % 75%, and 78 % respectively.

The reduction of ClO_4^- using succinate as sole C-source showed to follow more suitably zero order kinetics compaired to first order except for set where the initial ClO_4^- concentration was 100 mg/L. The rate of ClO_4^- reduction were found to be increased with increasing ClO_4^- concentration from 100 to 500 mg/L and then decreased with further increase in initial ClO_4^- concentration up to 800 mg/L (Table 4.10). The results indicated probable inhibitory effect of initial ClO_4^- concentration on its reduction, which has not been reported till date and needs further investigation which emphases on metabolic aspect of PRB (perchlorate reducing bacteria).

4.8 Perchlorate bioreduction in batch system using phenol as sole carbon source by mixed consortium predominantly *Pseudoxanthomonas* sp.

ClO_4^-reduction using phenol as sole carbon source was carried out with different initial concentrations starting from 100 mg/L to 800 mg/L as ClO_4^-. Earlier it was found that the mixed microbial culture was able to reduce ClO_4^- using phenol as a sole source as electron donor. The ClO_4^- reduction profile was studied in different initial ClO_4^- concentrations keeping the concentration of phenol same (1000 mg/L) at each set. In this case also, there was complete ClO_4^-

removal from initial ClO_4^- concentration 100 mg/L within 9 days of the experiment. Whereas it took 10 days to completely reduce all the ClO_4^- for the initial concentration of 200 and 300 mg/L.

For 400, 500, 600, 700 and 800 mg/L the % removal of ClO_4^- were 90 %, 90 %, 75 %, 78%, and 77 % respectively (Fig. 4.10 (b)).

The rate of the perclorate degradation was determined according to zero order and first order of reaction for each initial concentration. The R^2 values shows that the first order of kinetics suited better than zero order considering all the initial concentrations because with all the initial ClO_4^- concentrations, R^2 values for first order were above 90% (Table 4.10). For 500, 700 and 800 mg/L of initial ClO_4^-, the R^2 values were better in zero order reaction than the first order. This result differs from the previous experiment where the zero order reaction showed to be followed for ClO_4^- reduction with succinate as sole C-source.

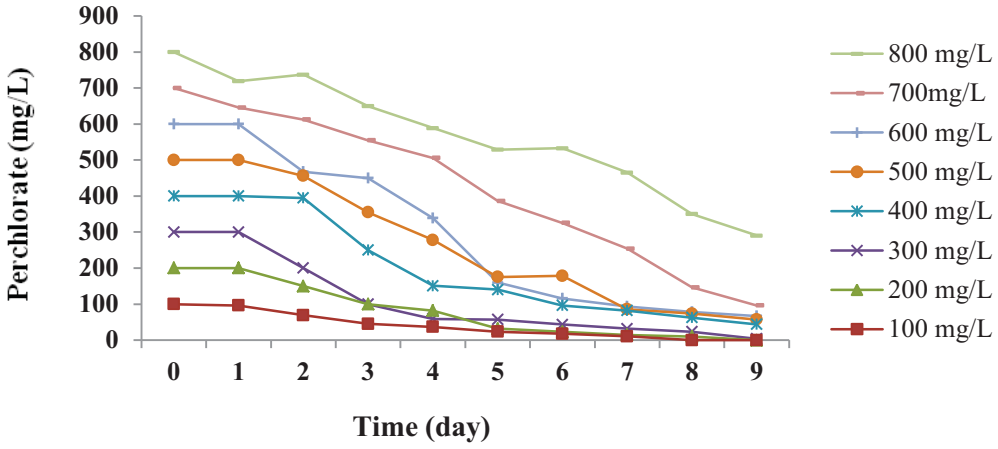

Fig 4.10 (a): Perchlorate reduction at different initial concentrations by the mixed consortium predominantly *Burkholderia*sp. using succinate as carbon source.

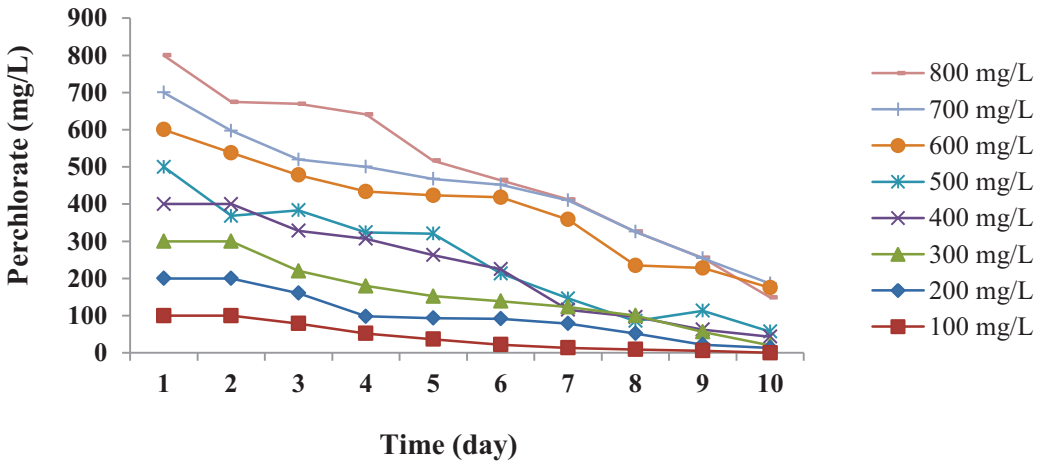

Fig 4.10 (b): Perchlorate reduction at different initial concentrations by the mixed consortium predominantly *Pseudoxanthomonas*sp. using phenol as carbon source.

Table 4.10: Estimated kinetic constant s of perchlorate reduction by the mixed consortium predominantly *Burkholderia* sp. and *Pseudoxantomonas* sp.

Initial ClO_4^- (mg/L)	Using Succinate				Using Phenol			
	Zero order		First order		Zero order		First order	
	k (mg $L^{-1}day^{-1}$)	R^2	k(mg $L^{-1}day^{-1}$)	R^2	k (day^{-1})	R^2	k (day^{-1})	R^2
100	21.0	.907	.44	.997	13.04	.95	.35	.992
200	21.1	.917	.263	.822	24.15	.885	.45	.98
300	29.98	.948	.284	.814	31.1	.766	.35	.95
400	46.53	.973	.307	.95	46.87	.87	.28	.98
500	49.66	.937	.277	.89	59.27	.95	.29	.92
600	45.58	.95	.121	.827	76.64	.915	.31	.92
700	43.52	.950	.129	.884	68.84	.97	.23	.90
800	35.27	.979	.17	.894	51.49	.96	.11	.91

4.9 Degradation of phenol at different initial concentrations by mixed consortium predominantly *Pseudoxanthomonas*sp. in synthetic and industrial wastewater

Phenol was added in different concentrations with fixed ClO_4^- concentration (500 mg/L) in the culture media. The microbial consortium has shown to degrade phenol upto 600 mg/L initial concentration. Beyond 600 mg/L, the microflora almost ceases to grow and stops degradation of phenol as well as ClO_4^-.

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The degradation rate was decreased with increasing initial phenol concentration (Fig 4.11 (a)). Phenol degradation efficiency from refinery wastewater by mixed consortium predominantly *Pseudoxanthomonas*sp. was also evaluated in batch shake flasks (Fig 4.11 (b)). As mentioned in the materials and methods chapter, the wastewater was spiked with phenol to get different phenol containing refinery wastewater. The overall degradation of phenol was faster in the industrial wastewater collected from petroleum refinery. Within 120 days of experiment 100 % phenol removal was observed in case of wastewater collected from refinery where as it took 140 days for total removal of phenol for synthetic wastewater.

The wastewater was collected from oil refinery and although the concentration of the phenol in wastewater was very low 2-3 mg/L it was likely to have phenol degrading indigenous microbe present in the media which would possibly enhance the degradation function of the microcosm. $ClO_4^-$was degraded in a substantial amount in both the cases i.e., using refinery wastewater and also the synthetic wastewater (Fig. 4.12 (a) and 4.12 (b)). In this experiment, the initial perchlorate concentration was 500 mg/L. In lower initial concentration of phenol (50 mg/L) only 60% of ClO_4^- was removed whereas at the highest phenol concentration (600 mg/L) 95% perchlorate was removed which was the maximum among all the cases. It is very clear from the figures that rate of degradation of $ClO_4^-$was increased with the increased phenol concentration.

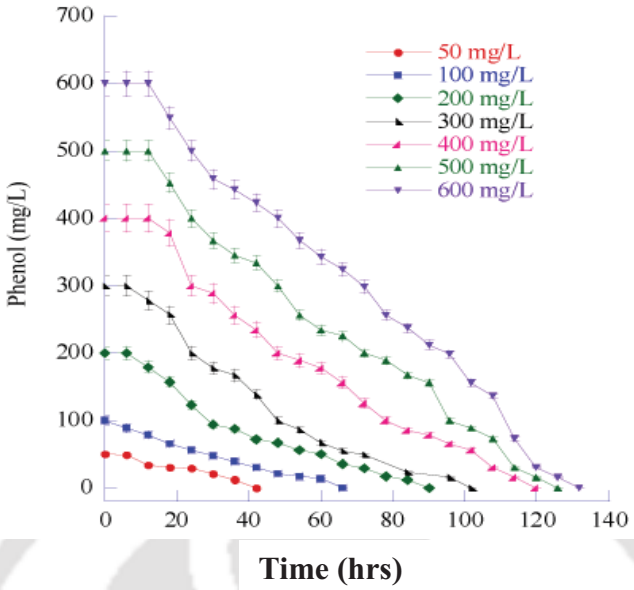

Fig 4.11 (a): Degradation phenol in different initial concentrations in synthetic wastewater by the mixed consortium predominantly *Pseudoxanthomonas*sp (Temperature = 28°C; pH = 7.0).

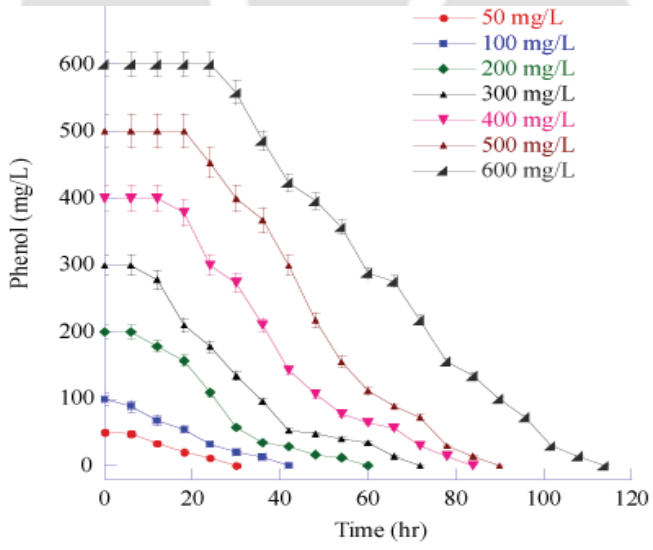

Fig 4.11 (b): Degradation phenol in different initial concentrations in industrial wastewater by the mixed consortium predominantly *Pseudoxanthomonas*sp (Temperature = 28°C; pH = 7.0).

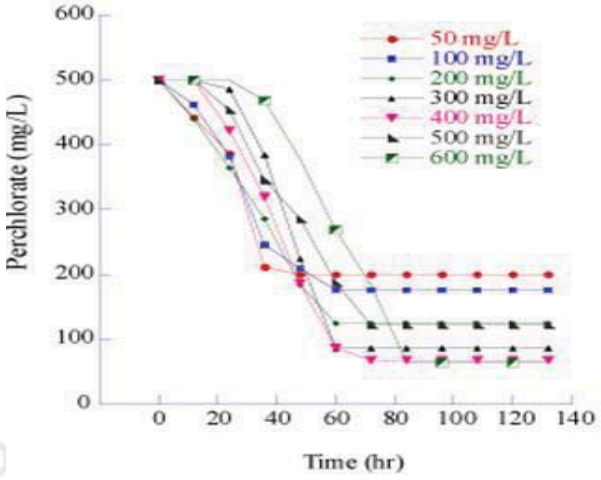

Fig 4.12 (a): Reduction of perchlorate in different initial phenol concentration present in synthetic wastewater by the mixed consortium predominantly *Pseudoxanthomonas*sp (Temperature = 28°C; pH = 7.0).

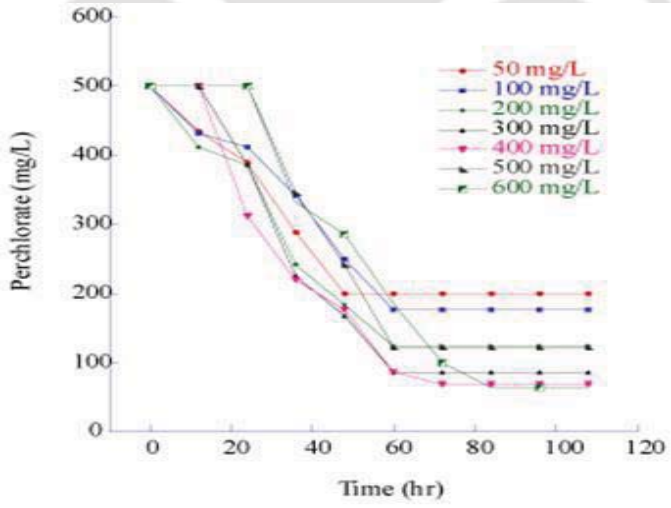

Fig 4.12 (b): Reduction of perchlorate in different initial phenol concentration present in synthetic wastewater by the mixed consortium predominantly *Pseudoxanthomonas*sp. (Temperature = 28°C; pH = 7.0).

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4.10 Effect of co-pollutants on perchlorate bioreduction using succinate as sole carbon source by the mixed consortium predominantly *Burkholderia*sp.

The anions like nitrate, chlorate and phosphate are used in several industries, especially army ammunitions, fireworks, fertilizer, electroplating and electro-polishing along with ClO_4^- and therefore are known to co-exist in waste streams originating from these industries. Among them nitrate is a common co-contaminant, and microbiological ClO_4^- reduction in many systems has been found to be affected in the presence of nitrate (Hackenthal, 1964; Kengen, 1999).The effect of different co-anions on ClO_4^- reduction by the enriched mixed consortium is depicted in Fig. 4.13 a,b& c which shows the ClO_4^-perchlorate degradation was affected by lower to higher extent in presence of these co-anions.

In case of the medium containing nitrate and ClO_4^- together, degradation started without any lag phase (Fig.4.13 (a)). The decrease in ClO_4^- concentration during the first 48 hrs was supported by an increase in the cell density (OD_{600}) from 1.0 (t = 1 hr) to 1.2 (t = 48 hrs). However, its degradation efficiency was only 47% at the end of six days and did not improve further. Several studies have reported the influence of nitrate on ClO_4^- degradation by denitrifying ClO_4^- reducers (Bruce et al.,1999; Hackenthal et al., 1964) and is attributed mainly due to the suppression of (per)chlorate reductase by nitrate (Batista and Liu, 2001). However, existence of separate pathways for the two e^- acceptors has also been proposed (Wallace et al. 1998). The preference of ClO_4^- to NO_3^- as electron acceptor likely to be associated with a different enzyme involved which lowered the activation energy (Nerenberg et al., 2008).

In presence of phosphate, both the anions simultaneously uptaken in substantial rate by the enriched mixed culture (Fig 4.13 (b)). For growth 1.5 OD_{600} in 6 days the enriched mixed consortium sufficiently utilized these two e^- acceptors. However, the ClO_4^- perchlorate degradation efficiency was low at ~54% (after 6 days) compared to media containing only ClO_4^-.

Reduction rate was sufficiently higher in presence of chlorate (ClO_3^-) (Fig 4.13 (c)). Unlike the previous cases, the consortium was able to degrade the total amount of ClO_4^- perchlorate (~100%) while only ~33% of chlorate was reduced (after 6 days of incubation). It has been reported that (Per)chlorate reducing bacteria (PCRB) use a single enzyme (per)chlorate reductase, for the degradation of ClO_4^- to chlorate (ClO_3^-), and chlorate to chlorite (ClO_2^-) (Herman and Frankenberger, 1998).

Chlorite (ClO_2^-) is then converted into chloride (Cl^-) and molecular oxygen by the enzyme chlorite dismutase (Bender et al., 2002; Dugan et al., 2009). Recent findings suggest that the enzyme chlorate reductase in chlorate reducing bacteria (CRB) does not react with ClO_4^- and therefore support the presence of a completely different enzyme in CRB versus those in PRB (Urbansky, 2002). Therefore observed effective degradation of ClO_4^- by the mixed microbial culture in the study in presence of chlorate is not unlikely.

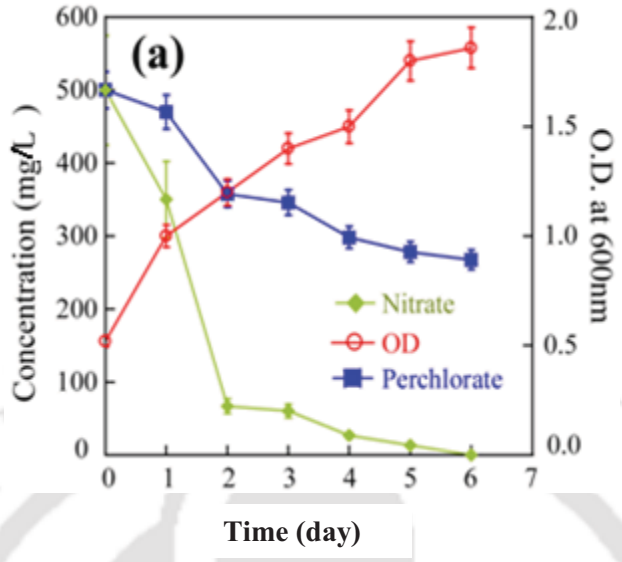

Fig 4.13 (a): Simultaneous reduction of perchlorate and nitrate by the mixed consortium predominantly *Burkholderia* sp. using succinate as sole C-source in a binary mixture along with microbial growth pattern (Temperature = 28°C; pH = 7.0).

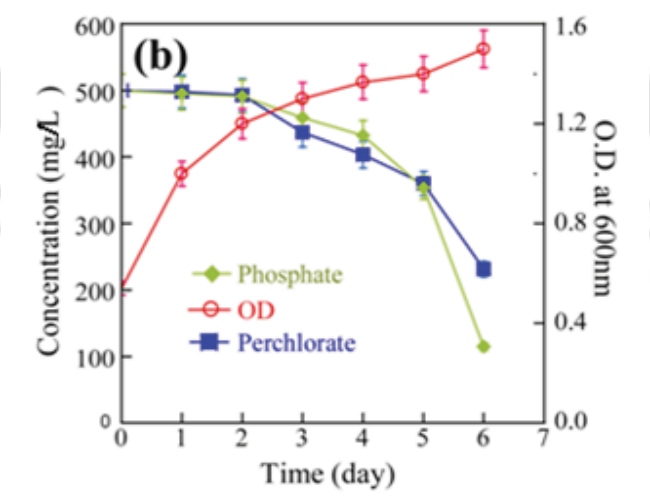

Fig 4.13 (b): Simultaneous reduction of perchlorate and phosphate by the mixed consortium predominantly *Burkholderia*sp. using succinate as sole C-source in a binary mixture along with microbial growth pattern (Temperature = 28°C; pH = 7.0).

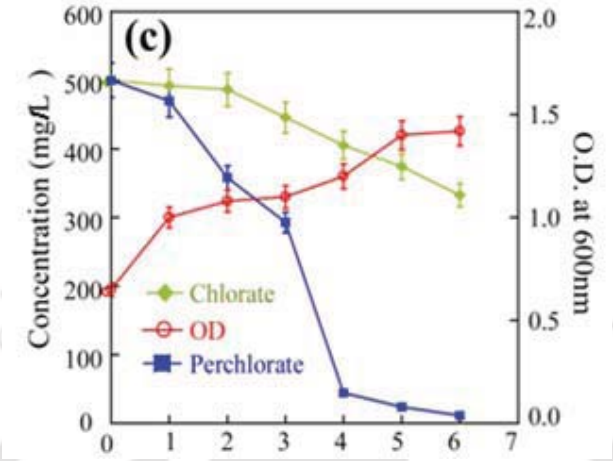

Fig 4. 13 (c): Simultaneous reduction of perchlorate and chlorate by the mixed consortium predominantly *Burkholderia*sp. using succinate as sole C-source in a binary mixture along with microbial growth pattern (Temperature = 28°C; pH = 7.0).

4.11 Simultaneous bioreduction of nitrate and perchlorate by mixed consortium predominantly *Burkholderia* sp.

The mixed microbial consortium was subjected to degrade ClO_4^- in presence of nitrate to observe the inhibition of ClO_4^- degradation (if any) in presence of nitrate as a competitive electron acceptor with ClO_4^-. It was noticed in the previous study that nitrate inhibited ClO_4^- bioreduction (section 4.10, Fig 4.13 (a)). The results showed that in presence of nitrate at equal concentration of ClO_4^-, the mixed microbial consortium almost ceased to degrade ClO_4^-.

Reduction profile of ClO_4^- and nitrate were measured adding eleven different initial nitrate concentrations (10 mg/L, 30 mg/L, 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500

mg/L, 600 mg/L, 700 mg/L, 800 mg/L) to fixed concentration of ClO_4^- (500 mg/L) in eleven separate sets of experiments in batch shake flasks. COD of the media was also measured to know the carbon source utilization during the degradation of both the pollutants. From the results of the varying initial concentration with initial ClO_4^- concentration, it was observed that the degradation of ClO_4^- was inhibited when the ratio of nitrate to ClO_4^- was more than 1:10. As shown in Fig 4.14 where initial ClO_4^- and nitrate concentration was 500 and 50 mg/L, degradation of perchlorate was not affected by presence of nitrate.

In higher nitrate to perchlorate ratio (> 1:10), perchlorate degradation had almost completely inhibited indicating the affinity of the mixed microbial consortium to uptake nitrate as electron acceptor than perchlorate. The carbon source (succinate) utilization profile by the mixed consortium using different ratio of perchlorate and nitrate were almost similar in every case.

Extra c-source was added freshly after the depletion of the c-source in the media, where perchlorate was almost unutilized. It was observed that once the c-source was totally utilized by the consortium further addition could not revive the culture and unutilized perchlorate was not uptake by the consortium.

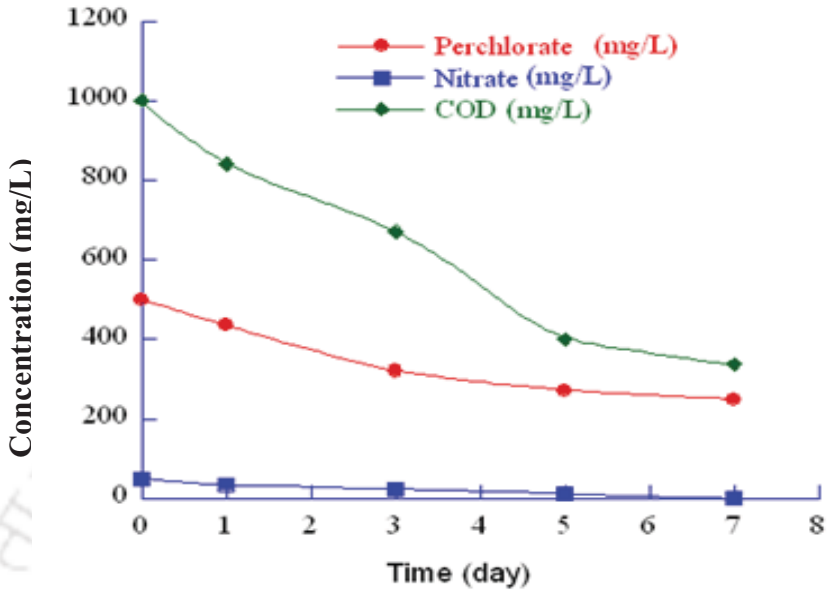

Fig 4.14: Perchlorate reduction in presence of nitrate by the mixed consortium predominantly *Burkholderia*sp. using succinate as sole C-source. Initial conditions: perchlorate (500 mg/L), nitrate (100 mg/L); Temperature = 28^{o}C; pH = 7.0.

4.12 Effect of co-pollutants on perchlorate bioreduction by mixed consortium predominantly *Pseudoxanthomonas*sp.using phenol as sole source of carbon

In case of the medium containing nitrate and perchlorate together at same concentration (500 mg /L), degradation started without any lag phase in a mixture containing perchlorate and nitrate

(Fig 4.15 (a)). The decrease in perchlorate concentration during the first 48 hrs was supported by an increase in the cell density ($O.D_{600}$). However, its degradation efficiency was only 47% at the end of six days and did not improve further. The preference of ClO_4^- to NO_3^- as electron acceptor

likely to be associated with a different enzyme involved which lowered the activation energy (Nerenberg et al., 2008).

No nitrite was found to accumulate in the media in presence of nitrate. Nitrate was completely reduced so it can be stated from the result that the mixed consortium was able to complete denitrification process starting from nitrate to nitrite and then to gaseous nitrogen. The higher rate of the nitrite reduction than nitrate reduction is also supported by the lower Gibb's free energy value required for nitrite reduction than nitrate reduction. It can also be inferred that the perchlorate reducing mixed consortium can withstand sufficiently high amount nitrogen, where as in a study by Bardiya and Bae (Bardiya and Bae, 2005) with indigenous mixed culture 100 mg/Lwas found to be toxic for the culture.

Reduction of perchlorate was not hampered much due to presence of nitrate (Fig. 4.15 (a)). Almost 95% of perchlorate reduction was observed where nitrate was reduced upto 98%. As shown in the previous section where succinate was used as sole C-source, nitrate hampered the degradation of perchlorate but here perchlorate degradation was not affected much in presence of nitrate.

Reduction rate was also not affected muchin presence of chlorate (ClO_3^-) (Fig 4.15 (b)). Chlorate was reduced in much faster rate than perchloarate. The consortium was able to degrade perchlorate up to ~96 % while chlorate was degraded up to ~98 % (after 6 days of incubation). It has been reported that (Per)chlorate reducing bacteria (PRB) use a single enzyme (per)chlorate reductase, for the degradation of perchlorate (ClO_4^-) to chlorate (ClO_3^-), and chlorate to chlorite

(ClO_2^-) (Herman and Frankenberger, 1998).Chlorite (ClO_2^-) is then converted into chloride (Cl^-) and molecular oxygen by the enzyme chlorite dismutase (Bender et al., 2002; Dugan et al., 2009). Therefore observed effective degradation of perchlorate with uptake of chlorate by the mixed microbial culture in the study is not unlikely. Although in presence of phosphate, the culture utilized both the anions (perchlorate and phosphate) simultaneously in substantial rate by the enriched mixed culture (Fig. 4.15 (c)).

The enriched mixed consortium sufficiently utilized these two e^- acceptors (perchlorate and phosphate) for growth (1.5 OD_{600} in 6 days). The decreased degradation of perchlorate in presence of other co-pollutants could be analyzed by the utilization of phenol in each system. From the results it was found that phenol was utilized almost fully by the mixed consortium after 6 days in each case, which affected the degradation of perchlorate. This insufficient amount of C-source hindered the further perchlorate degradation by the enriched mixed culture.

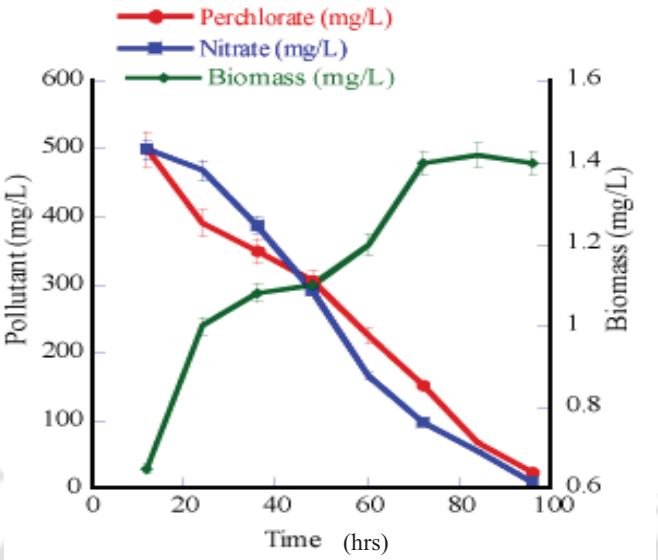

Fig 4.15 (a): Simultaneous reduction of perchlorate and chlorate by the mixed consortium predominantly *Pseudoxanthomonas*sp. using phenol as sole C-source in a binary mixture along with microbial growth pattern (Temperature = 28°C; pH = 7.0).

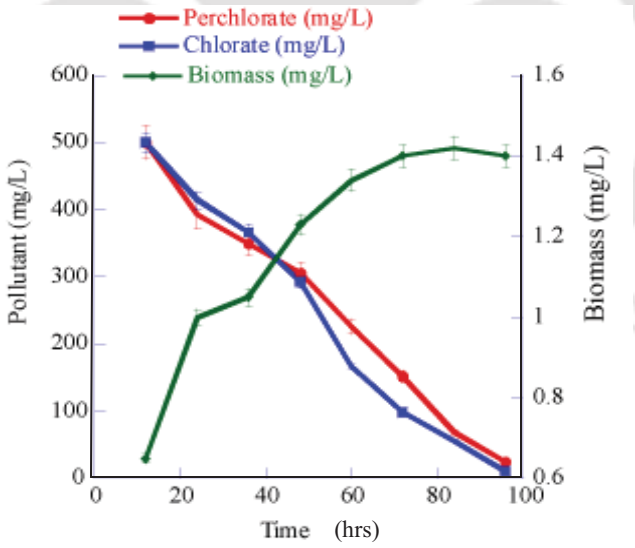

Fig 4.15 (b): Simultaneous reduction of perchlorate and chlorate by the mixed consortium predominantly *Pseudoxanthomonas*sp. using phenol as sole C-source in a binary mixture along with microbial growth pattern (Temperature = 28°C; pH = 7.0).

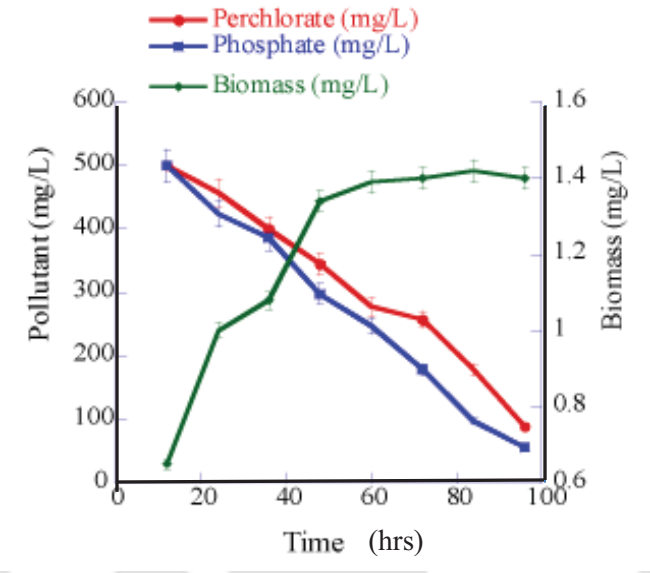

Fig 4.15 (c): Simultaneous reduction of perchlorate and chlorate by the mixed consortium predominantly *Pseudoxanthomonas*sp. using phenol as sole C-source in a binary mixture along with microbial growth pattern (Temperature = 28^{o}C; pH = 7.0).

4.13. Start-up period for perchloarte reduction in PBR (packed bed reactor)

Perchlorate reduction was studied in 2 nosupflow anaerobic PBR. One was to treat perchlorate using phenol as sole C-source (referred to as PBR-1) and another to treat perchlorate using succinate as C-source. In the start up phase 500 mg/L of perchlorate and C-source was added in the synthetic media. The media was passed through the peristaltic pump to maintain HRT of 10-11 days in the reactors to generate biofilm on the stationary media, PUF (Fig 4.16 (a)). The start up phase continued for one month when perchlorate concentration was measured in 2 to 3 days of intervals. The HRT kept unchanged until the perchlorate concentration in the effluent comes to more or less a constant value. The growth of biomass was examined under scanning electron

microscope (SEM) as shown in Fig 4.17 (b), the biomass (predominantly *Burkholderia* sp.) has grown on PBR-2 which was taken after 25-30 days of start-up phase.

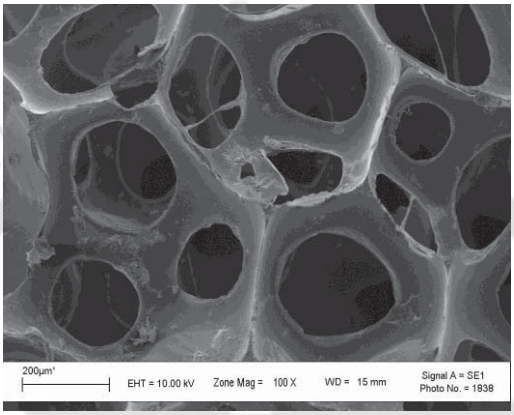

Fig 4.16 (a): Scanning electron microscopic (SEM) photograph showing the different sizes of PUF (polyurethane foam) used as packing media in the PBR (packed bed reactor).

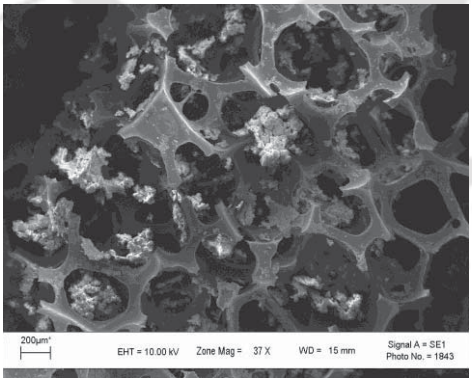

Fig 4.16 (b): Scanning electron microscopic (SEM) photograph biofilm growth on the surface of PUF (polyurethane foam) used as packing media in the PBR (packed bed reactor).

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4.14 Perchlorate bioreduction in continuous system succinate as sole alternative carbon source by the mixed consortium predominantly *Burkholderia*sp.

Degradation performances of PBR-2 were examined varying the hydraulic retention time (HRT). The reactor was subjected to start with 10 days HRT with 200 mg/L of perchlorate and 400 mg/L of succinate as sole C-source. Gradually the HRT was decreased from 10 days to 3 days. Flow rate was changed when the removal efficiency reached a steady state (Fig 4.17).

In this stage, the reactor has shown to reach a steady state within 5 to 6 days from the starting period when the HRT was set at 10 days. When the HRT was changed from 10 to 7 days the removal efficiency for perchlorate and COD came down to 20%. To decrease the HRT further from 7 days, the flow rate was adjusted to make HRT 6 days for 4 to 5 days and then HRT was adjusted to 5 days. Likewise, when further decreasing the HRT from 5 days to 3 days the reactor was run in HRT 4 days for 3 to 4 days. The reactor has shown upto 96% to 100% removal efficiency for perchlorate when steady state has reached.

In the next stage, perchlorate and COD concentrations were doubled. Unlike the previous stage,the reactor took 3 to 4 days to attain the steady state everytime when the HRT were changed. Steady state was achieved with 100% and 96% removal efficiency for perchlorate and COD. After that HRT was again reduced, phenol was gradually added increasingly with succinate keeping the HRT at 2days (Fig. 4.18). Reactor was run with same initial influent concentration,

400 mg/L of perchlorate and 800 mg/L phenol. When HRT was reduced to 2 days the steady state came after 11 days of running the reactor. After that unlike stage II, the effluent

concentration got increased and again decreased to reach the steady state within 10 days. In this stage the reactor was run with same HRT (2 days) and initial perchlorate 400 mg/L and succinate 750 mg/L and phenol 50 mg/L (Fig 4.18). Gradually succinate was replaced by phenol in following sets of operations. It was observed that the effluent concentration of perchlorate, phenol and succinate was increased and then decreased within 10 days after changing the loading rate. The phenol concentration was gradually increased from 50 to 100 and then to 200 mg/L (Fig 4.20). While increasing the phenol concentration upto 400 mg/L, the removal efficiency showed to be 10 to 15 % for both perchlorate and phenol (Fig 4.20). The effluent concentration was observed not to be decreased for 8 to 10 days. The phenol concentration was decreased upto 350 mg/L and the removal efficiency got increased within 3 to 4 days and reached a steady state for both perchlorate and phenol removal.

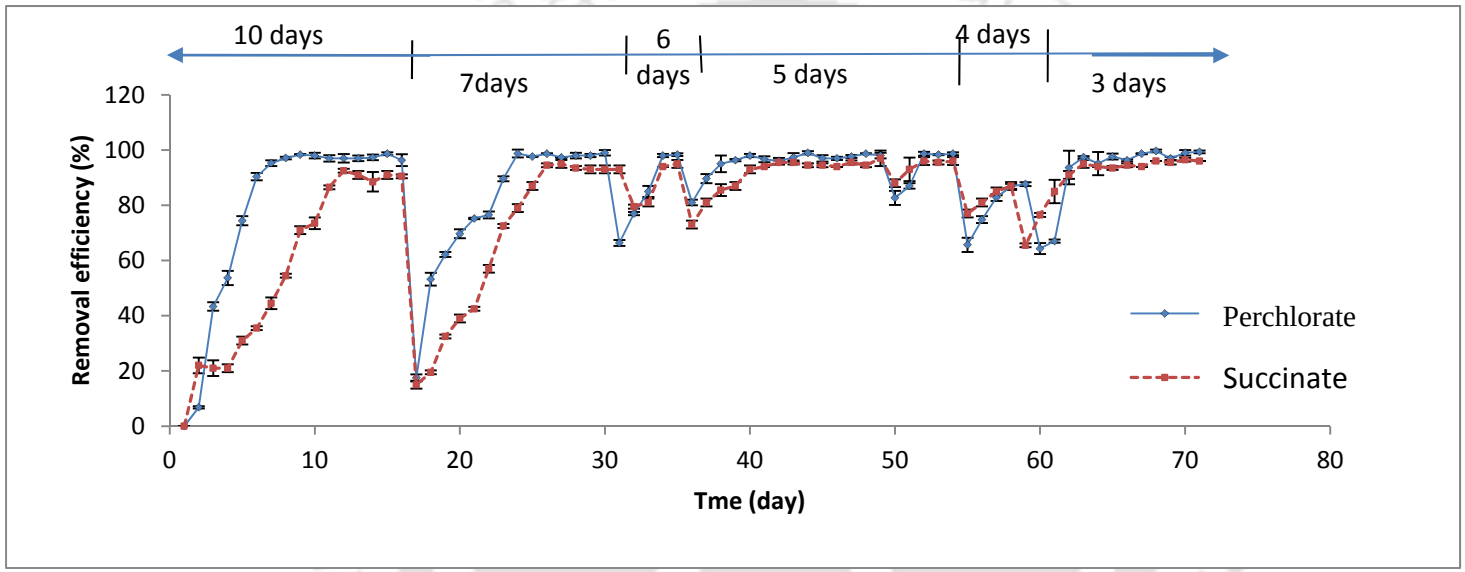

Fig 4.17: Performance of the PBR 1 showing removal of perchlorate and succinate at different HRT (initial concentration, perchlorate = 200 mg/L, succinate = 400 mg/L).

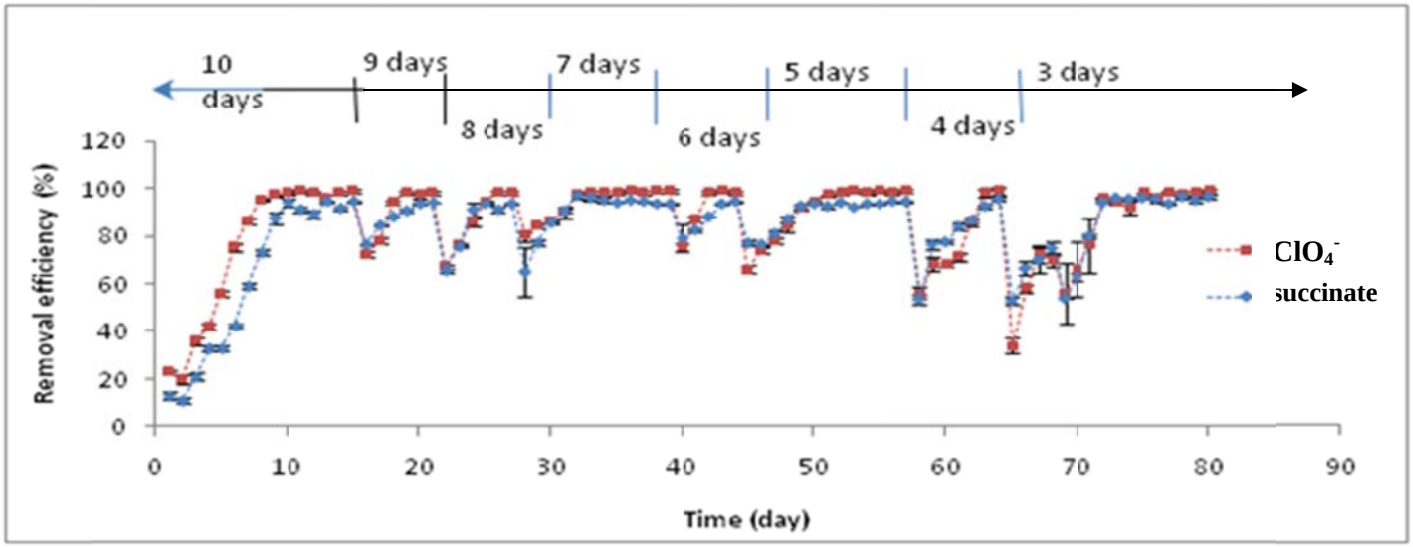

Fig 4.18: Effluent perchlorate and succinate concentration from the PBR 1 at different HRT (initial concentration, perchlorate = 400 mg/L, succinate = 800 mg/L).

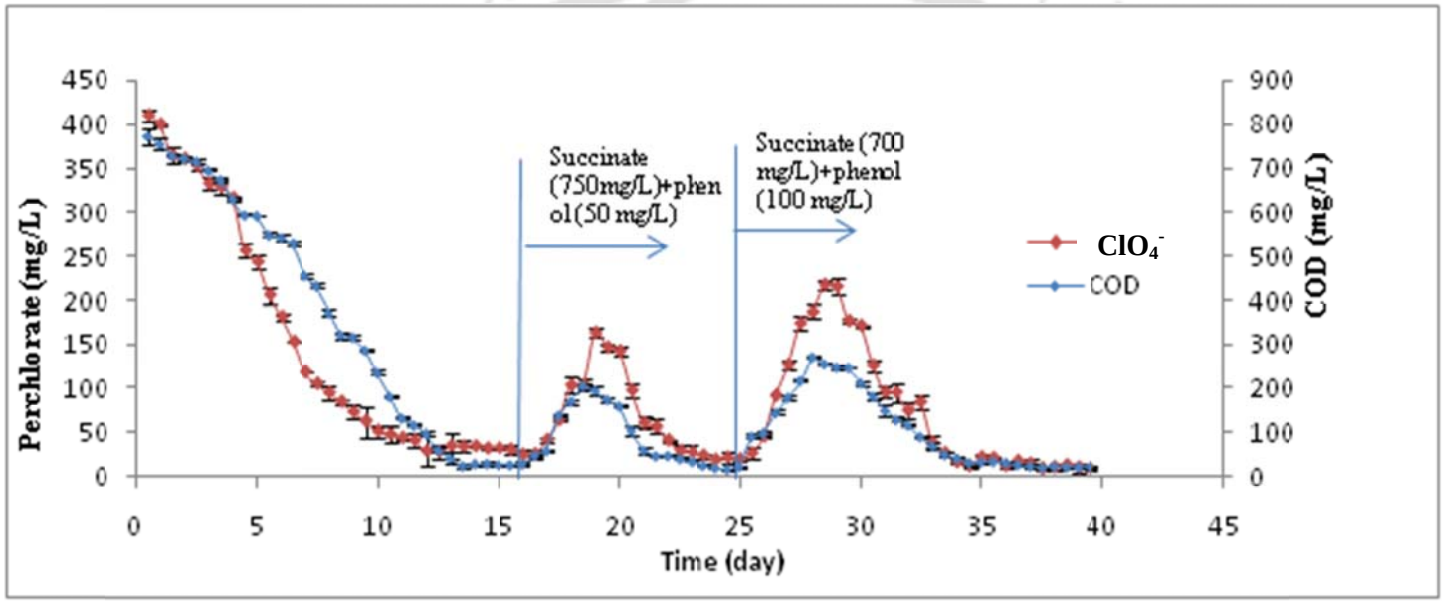

Fig 4.19: Effluent COD concentration from the PBR 1 at HRT = 2 days (initial concentration, perchlorate = 400 mg/L).

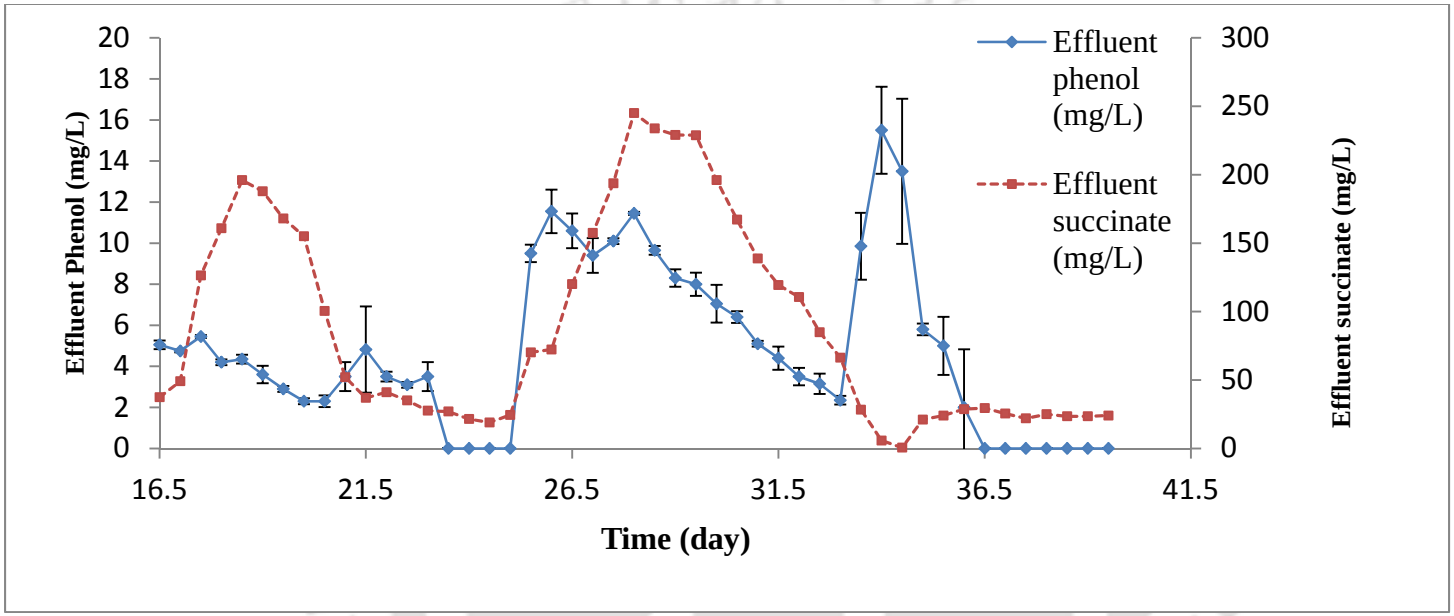

Fig 4.20: Effluent succiante and phenol concentration from the PBR 1 at HRT = 2 days (initial concentration, perchlorate = 400 mg/L).

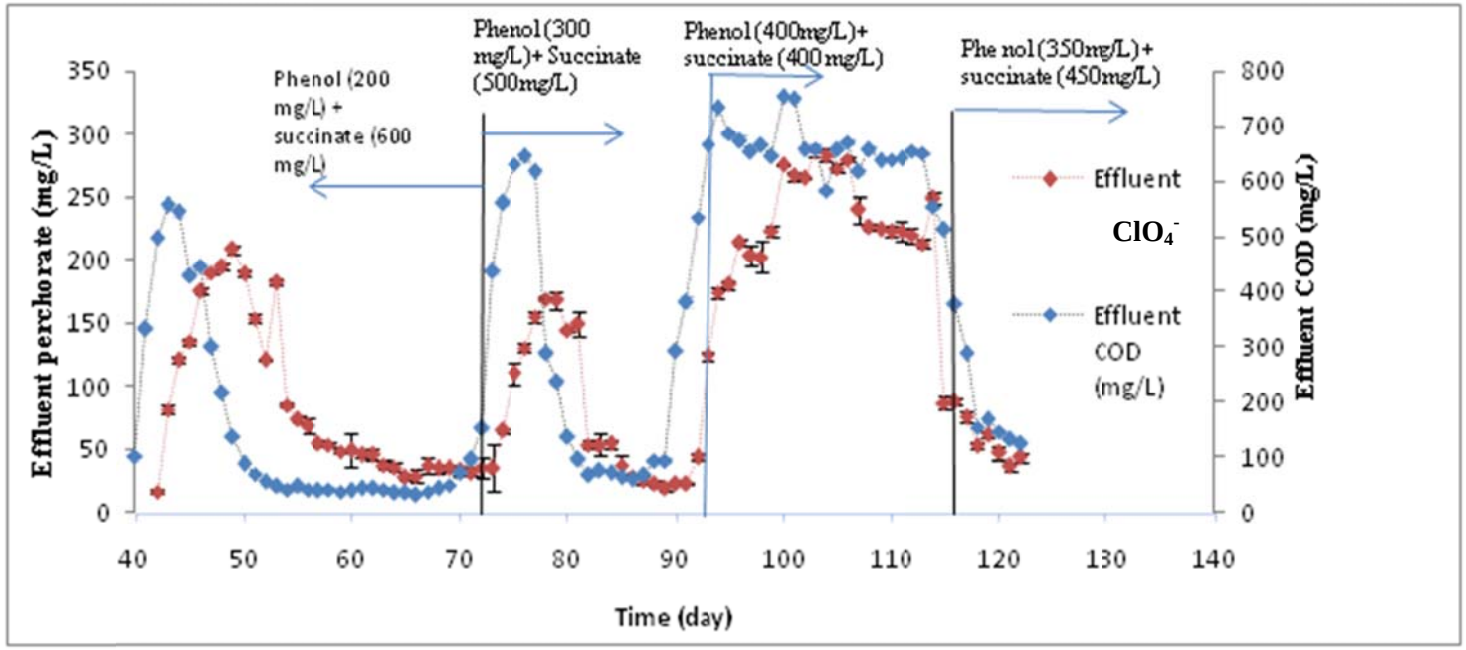

Fig 4.21: Effluent COD concentration from the PBR 1 at HRT = 2 days (initial concentration, perchlorate = 400 mg/L).

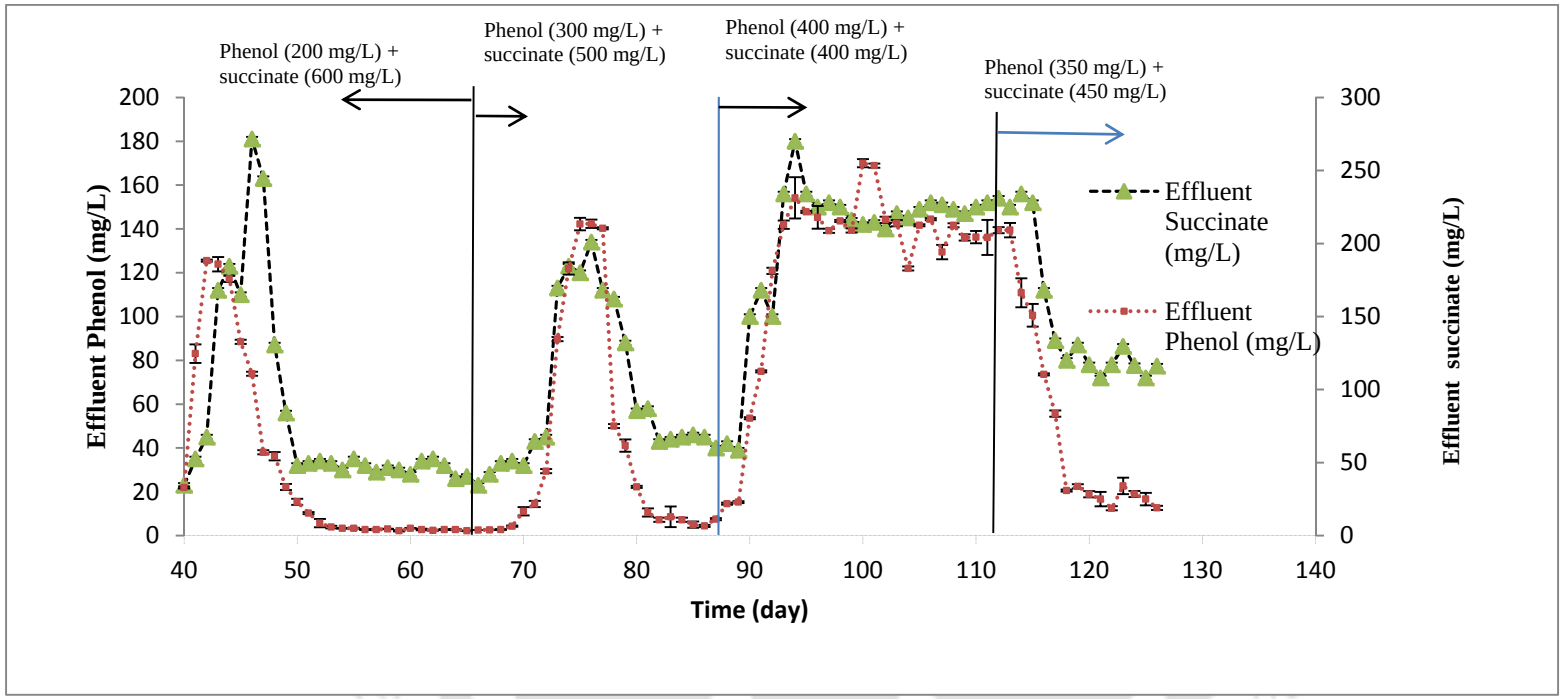

Fig 4.22: Effluent succiante and phenol concentration from the PBR 1 at HRT = 2 days (initial concentration, perchlorate = 400 mg/L).

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4.15 Perchlorate bioreduction in continuous system (PBR) phenol as sole C-source by the mixed consortium predominantly *Pseudoxanthomonas* sp.

As in PBR 1, the reactor subjected to start with 10 days HRT with 200 mg/L of perchlorate and 400 mg/L of C-source (phenol). Gradually the HRT was decreased for both the reactors from 10 days to 3 days. Flow rate was changed when the removal efficiency reached a steady state (Fig 4.21).

In the next stage reactor was run in 400 mg/L of perchlorate and 800 mg/L of C-source and again HRT was reduced from 10 days to 3 days, changing the flow rate after reaching a steady state (Fig 4.21). In this stage, the reactor has shown to reach a steady state within 5 to 6 days from the starting period when the HRT was set at 10 days. When the HRT was changed from 10 to 7 days the removal efficiency for perchlorate and COD in came down to 15 %. To decrease the HRT further from 7 days, the flow rate was adjusted to make HRT 6 days for 4 to 5 days for both the reactors and then HRT was adjusted to 5 days. Likewise, when further decreasing the HRT from 5 days to 3 days both the reactors were run in HRT 4 days for 3 to 4 days.

Both the reactors have shown upto 96% to 100% removal efficiency for perchlorate when steady state has reached. In HRT 10, 7, 5 and 3 days, flow rates were changed when steady states was observed for 6 to 8 days for both perchlorate and COD removal. In the following

stage perchlorate and COD concentrations were doubled and both the reactors operated at increasing HRT from 10 days to 3 days as in the first stage . HRT was gradually decreased from 10 to 9 and then 8 and then 7 days. As in PBR 1 the reactors took 3 to 4 days to attain the steady state everytime when the HRT were changed. When the HRT changed from 4 days to 3 days the steady state was reached after 10 days of operation. Steady state was achieved with 100% and 96% removal efficiency for perchlorate and COD as in stage III, HRT was again reduced in PBR2 (Fig 4.22). PBR2 was run with same initial influent concentration, 400 mg/L of perchlorate and 800 mg/L phenol. When HRT was reduced to 2 days the steady state came after 11 days of running the reactor. After that unlike stage II, the effluent concentration got increased and again decreased to reach the steady state within 10 days.

Again, when HRT was made 1 day same phenomenon was observed with less increase in effluent perchlorate and phenol concentration during the phase of acclimatization. When HRT was changed to 12hrs from 1 day the effluent pollutant concentration were increased and observed not to be decreased for 5 to 6 days. The HRT was increased upto 18 hrs and steady state was achieved within 10 days of operation. The removal efficiency was observed to be decreased when HRT was changed from 1 day to 12 hrs and so on (Fig 4.24). The same acclimation phase was observed before reaching the steady state when HRT was changed to 16 and 14 hrs (Fig 4.25) but the increase in effluent concentration for both perchlorate and phenol was observed to be reduced gradually, the efficiency remained same for perchlorate (97%) and phenol (96%).

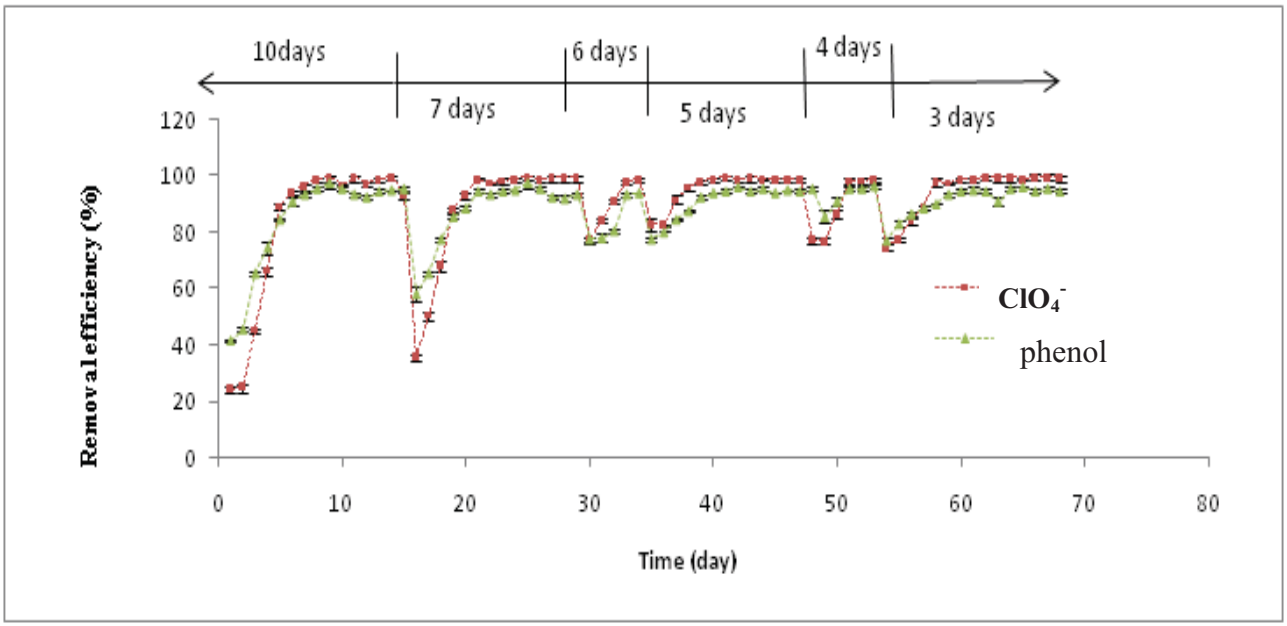

Fig 4.23: Performance of the PBR 1 showing removal of perchlorate and phenol at different HRT (initial concentration, perchlorate = 200 mg/L, phenol = 400 mg/L).

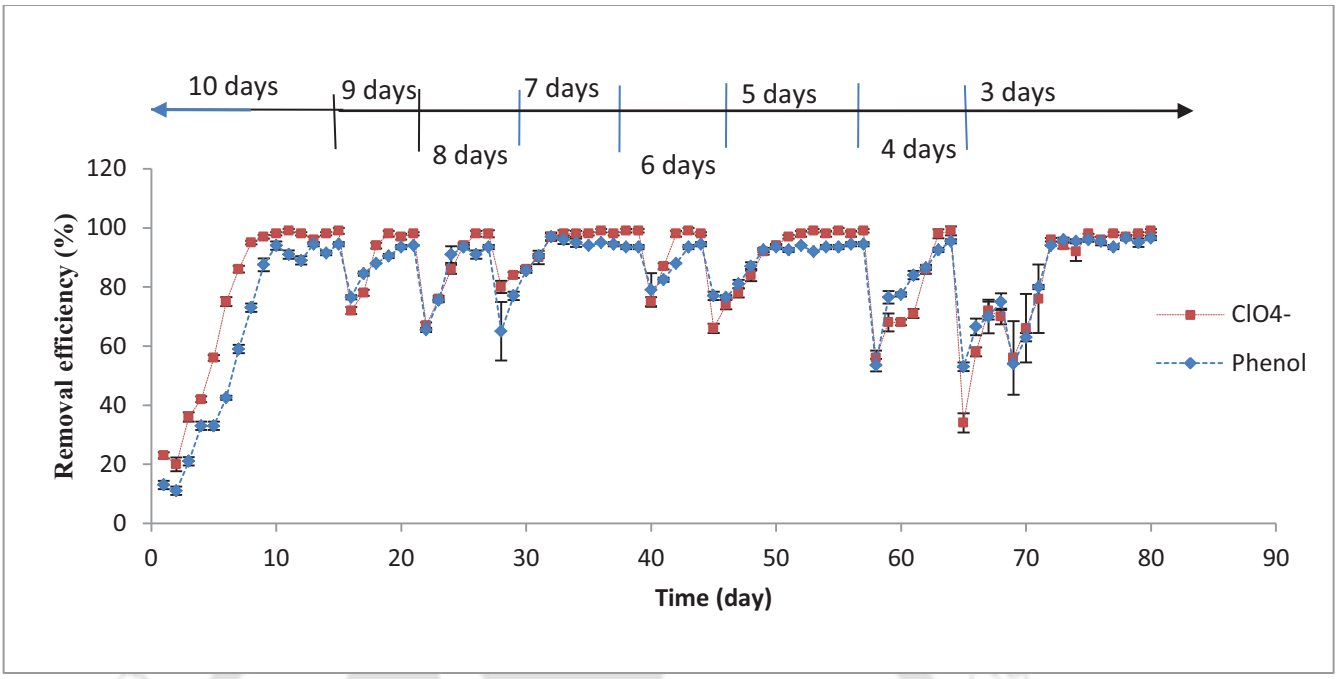

Fig 4.24: Effluent perchlorate and phenol concentration from the PBR 1 at different HRT (initial concentration, perchlorate = 400 mg/L, phenol = 800 mg/L).

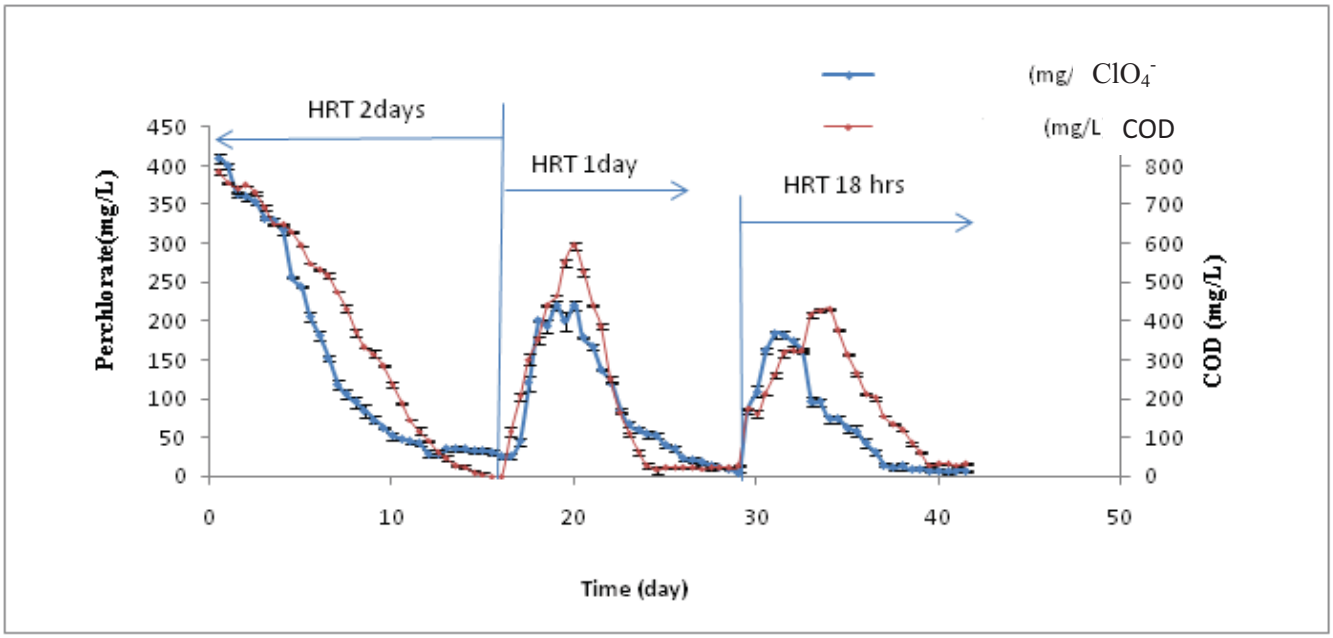

Fig 4.25: Performance of PBR2 showingeffluent perchlorate (400mg/L) and phenol (800 mg/L) in different HRT.

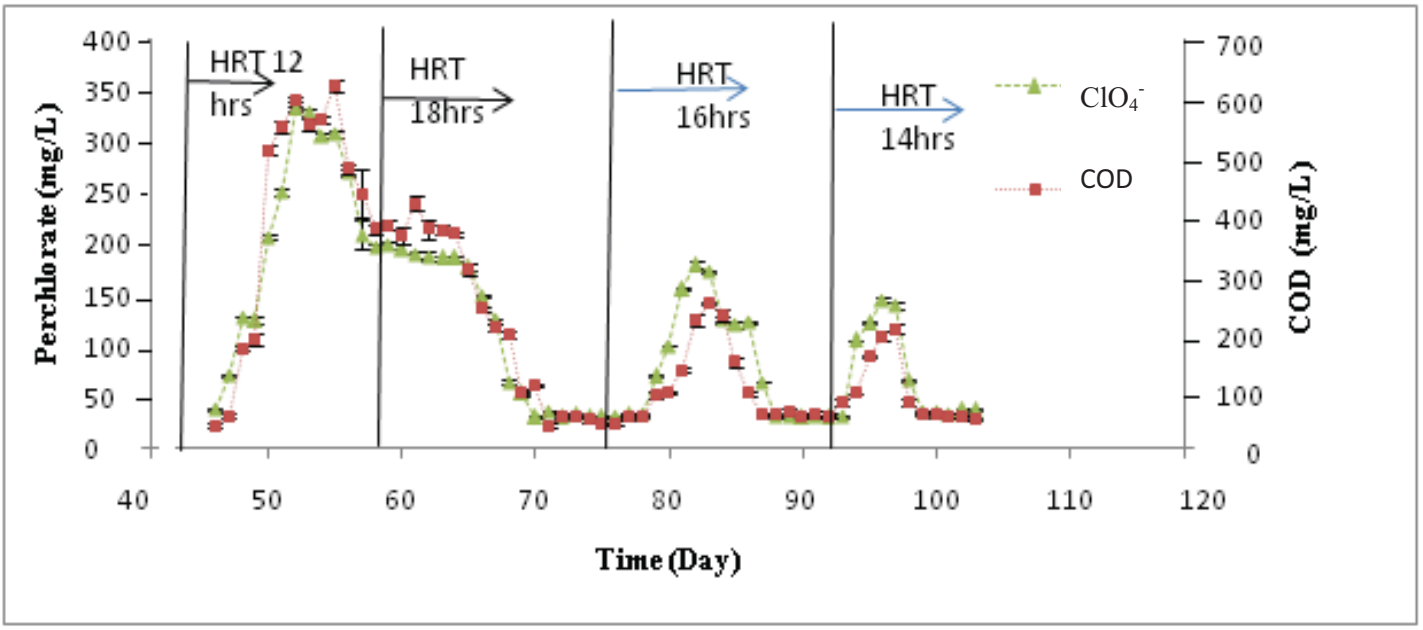

Fig 4.26: Performance of the PBR2 showing effluent perchlorate (400mg/L) and phenol (800 mg/L) in different HRT.

CHAPTER 5: CONCLUSIONS AND SCOPES OF FUTURE STUDY

Conclusion:

- Perchlorate has recently been identified as an environmental pollutant that emanates from various industries. An efficient treatment method for treatment of the pollutant is sought. Biological treatment using microorganism as compared to physico-chemical methods, are showing more promise for treating such wastewater. The mixed microbial culture used in this study was able to efficiently reduce perchlorate in both batch and continuous systems employing different C-sources.

- Five different organic carbon sources, viz.oxalate, succinate, citrate, formate and acetate were used to evaluate their effects on perchlorate reduction by mixed bacterial consortia. In presence of oxalate, formate, citrate and acetate the initial perchlorate was reduced up to 68.05%, 84.67%, 98.42%, and 94.44% respectively. In the presence of succinate, the mixed microbial culture predominantly *Burkholderia sp.* was able to completely reduce 500 mg/L of perchlorate, within 24 hours. The rate of perchlorate reduction was much quicker in presence of succinate compared to the other carbon sources. Different initial succinate concentration ranging from 300 to 1000 mg/L was tested to determine the optimum concentration. In case of 1000 mg/L initial succinate concentration, the mixed consortium was able to reduce 100% of perchlorate within ten days of incubation period.

- Theperchlorate reduction rate was high in case of 1000 mg/L of initial succinate. With an increase in the initial succinate concentration ranging from 300 to 1000 mg/L, the perchlorate reduction efficiency was also increased. No significant variation in the reduction rate of perchlorate with increasing initial succinate concentration was observed. The results suggested that a succinate concentration below 500 mg/L was not suitable to reduce 1000 mg/L of initial perchlorate by this enriched consortium. A decrease in the amount of microorganism due to lack of nourishment may be attributed to the observed lower rate of the perchlorate reduction.

- The microbial consortium was found to be capable of degrading perchlorate using various C-sources as electron donors. The microbial identification study with 16SrDNA shows that the mixed consortium was predominant with *Burkholderia* sp. when succinate was the C-source and with *Pseudoxanthomonas* sp. while using phenol as C-source.

- Among the various culture conditions studied for their effects on perchlorate reduction by the two predominant bacterial strains, inoculum age, pH and temperature were screened to be significantly important for *Burkholderia sp.*, whereas, .temperature, inoculum age and C/P ratio were found important for*Pseudoxanthomonas sp*. The results were further analyzed in the form of analysis of variance (ANOVA) for a better interpretation. From the results of ANOVA, the C/P ratio showed the largest variance in case of perchlorate reduction by predominantly *Burkholderia*sp. and in case of *Pseudoxanthomonas*sp. temperature was the most significant affecting the process. While the pH was found to be

a significant variable in case of only *Pseudoxanthomonas*sp.,but not for *Burkholderia*sp., probably due to the carbon source used, which was phenol in the case of

*Pseudoxanthomonas*sp. From the Taguchi optimization results, 28^{o}C was found the optimum temperature for both cases. Whereas three day old inoculum age was found to be optimum in case of perchlorate reduction by *Burkholderia* sp., for *Pseudoxanthomonas* sp., it was five days.

- The effect of different initial perchlorate concentration on its reduction by predominantly *Burkholderia sp.* was examined in the range 100–600 mg/L. The bacteria showed higher perchlorate reduction rate and efficiency at low concentration than at a high concentration (600 mg/L).

- The effect of different anions as co-pollutants on perchlorate reduction by predominantly *Burkholderia sp.*was observed. In the presence of nitrate at an equal concentration (500 mg/L), perchlorate reduction efficiency was minimum, which can be attributed mainly to the suppression of (per)chloratereductase by nitrate. However, existence of separate pathways for the two electron acceptors has also been proposed. The preference of ClO_4^- to NO_3^-as electron acceptor was also likely to be associated with a different enzyme involved which lowered the activation energy. In the presence of phosphate, the culture utilized both the anions simultaneously at a significantly high rate, which also resulted in for its growth (O.D. = 1.5). However, the perchlorate reduction efficiency was low at only ~54 % at the end of 6 days of culture compared with media containing only perchlorate.

- Continious perchlorate reduction by the mixed microbial consortium predominantly either *Burkholderia* sp. or *Pseudoxanthomonas*sp. was investigated in the continuously operated packed bed reactors using polyurethane foam maintained under different operational conditions mainly hydraulic retention time, perchlorate feed concentration.

- At different operating conditions of HRT and influent perchlorate concentration, both the reactors showed very good performance in continuous reduction of perchlorate. Maximum perchlorate reduction obtained in these reactors were 96% and 100 % for mainly *Pseudoxanthomonas sp.* and*Burkholderia sp.*,respectively.In general, perchlorate reduction efficiency in both the PBR showed decrease with a decrease in HRT, particularly at a value below 24 hrs. The perchlorate reduction efficiency was also slightly affected when succinate was replaced with phenol as the carbon source in the influent to the bioreactor with *Burkholderia sp.*

Scopes of future study:

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

- ✓ Scale up studies with the newly designed PBR for pilot scale operating system that can be used for field applications.

- ✓ Mathematical modeling for the perchlorate degradation and immobilized biomass growth in the continuously operated PBR for better understanding and predictability of the system performance.

- ✓ Feasibility of using sequential simultaneous degradation of perchlorate and phenol in the wastewater in anaerobic systems.

- ✓ Analysis of microbial community present in mixed consortium during continuous operation of the bioreactor system treating perchlorate as well as phenol in the contaminated water systems.

- ✓ Mechanism details of phenol removal using perchlorate as electron acceptor by the newly isolated microbial strain *Pseudoxanthomonas* sp.

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