Butanol Production from Rice Straw: Process Development and Optimization

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By

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to my dearest parents

Smt Shrida and Sri Ajay Srivastava

&

My loving husband

Pallav

and charming daughter

Anvesha

CERTIFICATE

It is certified that the work contained in the thesis entitled "**BUTANOL PRODUCTION FROM RICE STRAW: PROCESS DEVELOPMENT AND OPTIMIZATION**", by **Amrita Ranjan** (Roll No. 08615103), has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

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CHAPTER 1

GENERAL INTRODUCTION AND MOTIVATION OF THESIS

1.1 Introduction

The primary energy needs of a developing country like India with population over 1 billion, are in term of electricity and transport fuels like diesel & petrol. With a targeted 8% annual growth in GDP, the energy demands in various sectors such as agriculture, industry, transport & domestic are fast rising. As per the statistics published in BP Statistical Review [1], the rise in global primary energy consumption (including commercial renewable energy) grew by 5.6% in 2010. China was the largest global energy consumer (20.3%) followed by US (19%). India consumed 4.4% of the total energy, with fossil fuel demand growing by 7.8%. Due to growing economy, the per capita income of Indian household (especially the middle and upper-middle class) has increased significantly over past one decade that has resulted in increasing the burden on electricity and petroleum product demands. This is essentially consequence of increasing acquisition of electrical appliances in common households, and increasing vehicle ownership.

Table 1.1: Production of crude oil for year 2010-11 (in thousand Tonnes)

Item	2005-06	2006-07	2007-08	2008-09	2009-10*	2010-11*
(a) Onshore						
Gujarat	6251	6212	6177	5946	5960	5905
Assam/Nagaland	4474	4400	4357	4674	4740	4719
Arunachal Pradesh	104	109	102	102	131	116
Tamil Nadu	385	353	298	265	239	234
Andhra Pradesh@	216	252	279	289	304	305
Rajasthan	-	-	-	-	447	5149
Total (a)	11430	11326	11213	11276	11821	16428
Organization wise Production						
OIL	3234	3107	3100	3468	3572	3582
ONGC	8095	8058	7921	7565	7515	7446
JVC/Private	101	161	192	243	734	5400
(b) Offshore						
ONGC	16309	17993	18020	17801	17340	17002
JVC/Private	4451	4669	4885	4431	4529	4282
Total (b)	20760	22662	22905	22233	21869	21284
Grand Total (a + b)	32190	33988	34118	33508	33690	37710

Source: [2]

As far as the electricity sector is concerned, India's per capita electricity consumption stands at a meager 779 kWh per annum, while the global average is over 2000 kWh [3]. The scenario on the transportation fuels front is even poorer. The consumption of petroleum products in India during 2010-2011 was 141.785 million metric tons, while the production of crude oil was meager 37.71 million tones [2]. Table 1.1 and 1.2 give details of production of crude oil and consumption of petroleum products in India (Ministry of Petroleum, Basic statistics). This created a burden of import of 163.594 million tons of crude oil. This statistics published by Ministry of Petroleum and Natural Gas claim total reserves (proven and indicated) of 757 million metric tons of crude oil and 1241 billion cubic meter of natural gas in India as on April 2011 [2]. As the rise in oil production in India is not growing hand-in-hand with the consumption of oil, the burden on oil import is fast increasing. As per

Table 1.2: Consumption of petroleum products (2010-11, in thousand Tonnes)

Products	**2006-07**	**2007-08**	**2008-09**	**2009-10**	**2010-11***
Light Distillates Components	**37076**	**38557**	**39878**	**39086**	**41433**
LPG	10849	12165	12344	13121	14328
Mogas	9286	10332	11258	12818	14192
Naphtha	13886	13294	13911	10239	10691
Others	3055	2766	2365	2908	2222
Middle Distillates Components	**57595**	**62823**	**66378**	**71198**	**74949**
SKO	9505	9365	9303	9304	8928
ATF	3983	4543	4423	4627	5079
HSDO	42896	47669	51710	56320	59990
LDO	720	667	552	457	455
Others	491	579	390	490	497
Heavy Ends Components	**26078**	**27568**	**27343**	**27911**	**25402**
Furnace Oil @	9257	9469	9419	9105	8896
LSHS/HHS	3361	3248	3169	2484	1982
Lubes/Greases	1900	2290	2000	2657	2508
Bitumen	3832	4506	4747	4919	4566
Petroleum Coke	5440	5950	6166	6750	5487
Paraffin Wax	303	241	203	211	190
Other Waxes	65	65	65	78	73
Others	920	1799	1574	1707	1700
Total	**120749**	**128948**	**133599**	**138195**	**141784**
Refinery Fuel	10920	11751	11912	11607	15873
Grand total	**131669**	**140699**	**145511**	**149802**	**157657**

Notes: Consumption includes sales by all companies & direct private imports. SKO – Kerosene, LDO – Light Diesel Oil, LSHS – Low Sulfur Heavy Stock, HHS – High sulphur heavy stock, ATF – Aviation turbine fuel
Source: Petroleum Planning & Analysis cell, New Delhi**.**

statistics published by EIA [4], India ranked fifth globally in net oil import, with import of more than 2.2 million barrels a day (which amounts cumulatively to approx 163.594 million tons), and this is amounts to about 70% of the total consumption of gasoline and diesel. Most of the imported crude oil in India comes from Saudi Arabia (18%), Iran (11%), Africa (22%) and other middle east countries (34%) & western hemisphere (10%). The prices of petroleum products (diesel, kerosene, petrol, and jet fuel) have fluctuated significantly in past 5 years

with a net rise of over 50%. The above figures call for an urgent need for exploration of alternate & renewable source of liquid transportation fuel for reducing the burden on economic of the country & achieving sustainable growth.

Another issue is the GHG emission due to large consumption of diesel and petrol. India currently ranks among the top 5 contributors to carbon emission [5]. Typically, combustion of 1 liter of petrol causes emission of 2.31 kg CO_2, while combustion of 1 liter of diesel leads to 2.68 kg of CO_2. Replacement of petroleum-based fuel by renewable fuel will have an added advantage of gross reduction in the emission of CO_2.

1.2. Potential of Biomass Energy in India

India being an agricultural country, the major source of biomass in India is the waste and byproduct of agriculture. A comprehensive survey of this massive resource was first undertaken by National Productivity Council during 1985-86 [6, 7]. Simultaneously, an estimate of typical crop to residue ratio for different residue obtained from crops was also made, and on this basis an estimate of the crop wise residue generation was made. Since then this data has been updated regularly by Ministry of Agriculture of Government of India. In addition, forest residue is also a major source of biomass in majority of state in India. A survey conducted by Indian Institute of Science (IISC) Bangalore, as a part of project on biomass atlas of India sponsored by Ministry of New and Renewable Energy, Government of India, puts the estimate of surplus agro residue (for the year 2008-09) available for biofuels at about 125 MMTPA [8]. Table 1.3 gives a statewise account of the estimated agro and forest residue as per the survey of IISc Bangalore [8]. The estimated energy potential of this biomass is 16.25 GW of electricity production. The total biomass surplus in the form of agro and forest residue is estimated at 249 million tons with power potential of 33.29 GW.

Table 1.4 gives the data of cultivation area, production and yield (in per hectare) of

major crops in India between 2005-2010 [9]. It could be inferred from this table that the major food grain crop of India is rice with an average production of 94.02 MMTPA (million metric tons per annum) followed by wheat (77 MMTPA). India ranks second in the world after China in rice production, contributing to 20% of total global production. Figure 1.1 depicts of the history of rice production in India since independence in 1947 [9]. It can be inferred from Figure 1.1 that rice production in India has grown nearly 5 to 6 times in the last six decades. The major rice producing states of India are West Bengal, Punjab, Uttar Pradesh, Andhra Pradesh and Table 1.5 gives the data of area of cultivation, total production (MMTPA) and yield (in kg/hectare) of rice in these States during 2008-09 and 2009-10 [9].

The present estimate of agro residue available in India and its potential as renewable energy source can be made on the basis of the data on production of major crops during 2010-11, which is given in Table 1.6 [9]. Different types of crop residues are straw, husk, bagasse and top trash (for sugar cane). Table 1.6 gives an estimated account of production of agro residues on the basis of crop to residue ratio, which could possibility be used for bio energy generation [6,7,9]. Of course, not all of these residues are available for bioenergy production, as many conventional uses have to be met with this biomass such as cattle feed and domestic fuel. Nonetheless, about 15-20% of the biomass could be surplus, and is available for bio energy generation. The biomass can be converted to various type of biofuel through either thermo chemical or bio chemical route in either gaseous (biomethane through biomass digestion or producer gas, which is the mixture of H_2 and CO through biomass gasification) or liquid forms (mainly alcohols like methanol, ethanol and butanol), which could be further utilized for generation of electricity and liquid transportation fuels. This will not only help India to become self-reliant in terms of energy needs but will also help achieving a cleaner and pollution-free environment. It is in this spirit that the present thesis work was undertaken.

Table 1.3. State-wise biomass production (in the form of both agro-residues and forest & wasteland) for the year 2008-09 in India

State	Biomass Class	Area (kHa)	Crop Production (kT/Yr)	Biomass Generation (kT/Yr)	Biomass Surplus (kT/Yr)	Power Potential (MWe)
Andhra Pradesh	F & W	3623.9	--	5151.6	3484.4	487.8
	Agro	9983.2	21167.1	43893.2	6956.4	863.3
Arunachal Pradesh	Agro	208.5	251.1	400.4	74.5	9.2
	F & W	5467.4	--	8313.1	6045.3	846.3
Assam	F & W	2676.8	--	3674.0	2424.2	339.4
	Agro	3460.3	8250.6	11443.6	2346.9	283.9
Bihar	F & W	906.0	--	1248.3	831.9	116.5
	Agro	7348.7	18817.6	25756.9	5147.2	641.1
Chhattisgarh	Agro	4758.2	6636.6	11272.8	2127.9	248.5
	F & W	8762.1	--	13592.3	9065.8	1269.2
Goa	F & W	153.4	--	180.7	119.3	16.7
	Agro	154.2	489.5	668.5	161.4	20.9
Gujarat	Agro	8007.6	23895.7	29001.0	9085.5	1224.8
	F & W	9030.3	--	12196.3	8251.8	1155.2
Haryana	F & W	294.7	--	393.3	259.6	36.3
	Agro	5707.3	15226.2	29034.7	11342.9	1456.9
Himachal Pradesh	Agro	788.3	1504.0	2896.9	1034.7	132.6
	F & W	2259.8	--	3054.6	2016.0	282.2
Jammu & Kashmir	Agro	749.4	773.8	1591.3	279.6	37.1
	F & W	9838.0	--	11461.7	7564.7	1059.1
Jharkhand	Agro	1850.3	2459.5	3644.9	890.0	106.7
	F & W	3506.8	--	4876.6	3249.8	455.0
Karnataka	F & W	6993.7	--	10001.3	6600.8	924.1
	Agro	9683.6	43139.6	34167.3	9027.2	1195.7
Kerala	F & W	1235.4	--	2122.1	1429.1	200.1
	Agro	2306.8	5561.0	11644.3	6352.1	864.4

Table 1.3 continued……

State	Biomass Class	Area (kHa)	Crop Production (kT/Yr)	Biomass Generation (kT/Yr)	Biomass Surplus (kT/Yr)	Power Potential (MWe)
Madhya Pradesh	F & W	12802.2	--	18398.2	12271.2	1718.0
	Agro	13167.3	17951.7	33344.8	10329.3	1373.3
Maharashtra	F & W	13177.4	--	18407.1	12440.4	1741.7
	Agro	18851.5	64336.1	47624.8	14789.6	1983.7
Manipur	Agro	340.8	435.1	909.4	114.4	14.3
	F & W	1260.9	--	1264.0	834.2	116.8
Meghalaya	Agro	174.4	284.2	511.1	91.6	11.3
	F & W	1532.6	--	1705.9	1125.6	157.6
Mizoram	Agro	19.0	33.3	61.1	8.5	1.12
	F & W	1638.8	--	1590.9	1050.0	147.0
Nagaland	Agro	179.6	276.1	492.2	85.2	10.0
	F & W	786.4	--	843.8	556.9	78.0
Orissa	F & W	6265.0	--	9370.2	6084.8	851.9
	Agro	6667.6	12262.7	20069.5	3676.8	429.3
Punjab	F & W	229.1	--	398.5	263.0	36.8
	Agro	6993.5	35934.0	50847.6	24842.9	3172.2
Rajasthan	F & W	14135.0	--	9541.6	6297.5	881.6
	Agro	14851.4	16135.5	29851.3	8645.7	1126.7
Sikkim	Agro	58.0	69.1	149.5	17.8	2.29
	F & W	372.8	--	531.5	350.8	49.1
Tamil Nadu	F & W	3187.2	--	4652.4	3070.6	429.9
	Agro	4165.1	30415.4	22507.6	8900.0	1160.0
Tripura	Agro	9.5	3.70	40.9	21.1	2.94
	F & W	831.0	--	1035.5	683.4	95.7
Uttar Pradesh	F & W	3856.5	--	5478.4	3672.0	514.1
	Agro	15950.9	138945.4	60322.2	13737.9	1746.2

Table 1.3 continued……

State	Biomass Class	Area (kHa)	Crop Production (kT/Yr)	Biomass Generation (kT/Yr)	Biomass Surplus (kT/Yr)	Power Potential (MWe)
Uttaranchal	Agro	1015.7	7783.3	2903.2	638.4	80.9
	F & W	2885.5	--	4559.2	3055.3	427.7
West Bengal	F & W	1113.9	--	1430.7	949.0	132.9
	Agro	6090.2	22807.8	35989.9	4301.5	529.3

F & W – Forest and Wasteland

(B) Summary of Biomass Atlas of India

Biomass Class	Area (kHa)	Crop Production (kT/Yr)	Biomass Generation (kT/Yr)	Biomass Surplus (kT/Yr)	Power Potential (MWe)
Agro-Total	143540.9	495845.6	511041.0	145026.6	18728.7
F & W Total	118822.9	0.000	155474.0	104047.4	14566.6
Total	262363.8	495845.6	666515.0	249074.0	33295.4

Source: [8]

Table 1.4: Normal (average of 2005-06 to 2009-10) area, production and yield of major crops in India

Group of Crops	Crops	Season	Area	Production	Yield
	Rice	Kharif	39.36	80.38	2042
		Rabi	4.41	13.64	3092
		Total	**43.77**	**94.02**	**2148**
	Wheat	Rabi	27.75	77.04	2777
	Jowar	Kharif	3.43	3.54	1034
		Rabi	4.62	3.79	820
		Total	**8.05**	**7.33**	**911**
	Bajra	Kharif	9.26	8.29	895
	Maize	Kharif	6.96	13.04	1875
		Rabi	1.05	4.00	3813
		Total	**8.01**	**17.04**	**2128**
I. Food grains	**Total Coarse Cereals**	Kharif	21.97	27.32	1244
		Rabi	6.31	9.14	1449
		Total	**28.28**	**36.46**	**1290**
	Tur	Kharif	3.54	2.57	726
	Gram	Rabi	7.61	6.44	847
	Total Pulses	Kharif	10.65	4.99	469
		Rabi	12.27	9.32	760
		Total	**22.92**	**14.31**	**625**
	Total Foodgrains	Kharif	71.97	112.70	1566
		Rabi	50.74	109.15	2151
		Total	**122.71**	**221.85**	**1808**
	Groundnut	Total	6.06	6.93	1144
	Rapeseed & Mustard	Rabi	6.36	7.04	1108
II .Oilseeds	Soyabean	Kharif	8.83	9.59	1086
	Sunflower	Total	1.94	1.23	632
	Nine Oilseeds	**Total**	**26.92**	**26.92**	**1000**
	Sugarcane	Total	4.60	312.44	67929
III . Other Cash Crops	Cotton @	Total	9.35	22.66	412
	Jute & Mesta$	Total	0.92	11.10	2172

Area - Million Hectares, Production - Million Tonnes, Yield - kg/ Hectare
Source: [9]

1.3 Biofuels Endeavors of India

Government of India has a special Ministry for New and Renewable Energy established in 1993. This Ministry is dedicated for promotion and implementation of various renewable energy sources in India such as wind, solar (photovoltaic), solar (thermal), small and micro-hydro, biomass, biogas etc. The main focus of the renewable energy

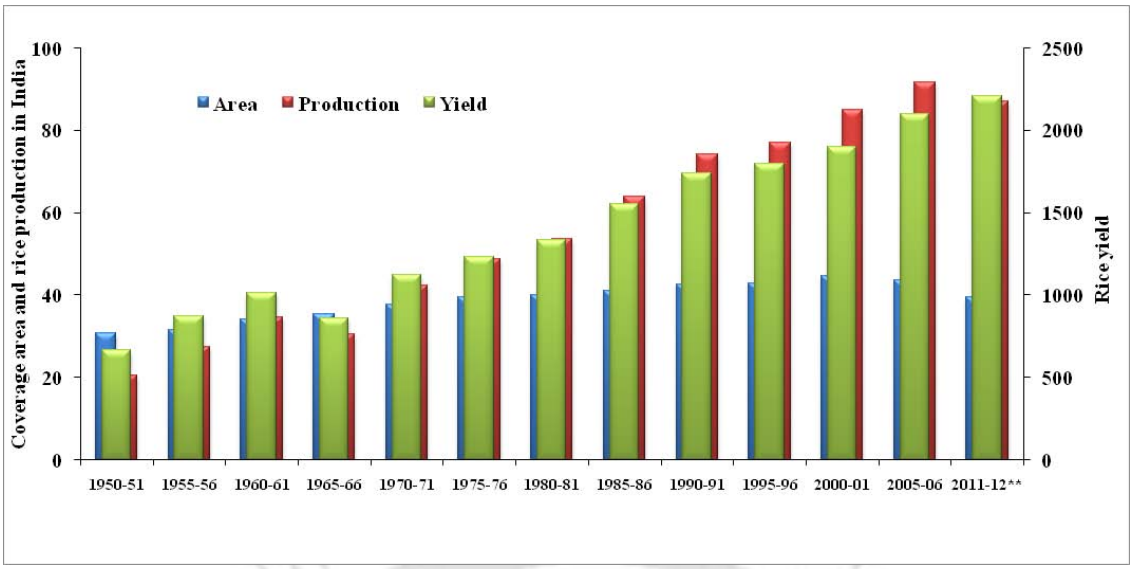

Figure 1.1. All-India Area, Production and Yield of Rice alongwith coverage under Irrigation (Area - Million Hectares, Production - Million Tonnes, Yield - kg/Hectare) Source: [9]

implementation in India has been for electricity. Table 1.7 gives the latest account of the achievements in electricity generation through renewable sources in India as of September 30, 2012 [10]. The Government of India has also undertaken effort to promote production and use of liquid biofuels, which are clean and carbon neutral, to seek solution to problem of rise oil imports and carbon emission. As mentioned in section 1, India is heavily dependent on crude oil import to meet the need of transportation fuels in various sectors. Rising prices of crude oil have caused enormous burden on Indian economy. Moreover, large growth of transportation sector have also raised environmental concern, as India's carbon emissions are growing at ~ 3.2% per annum, which ranks India among world top 5 contributors to carbon emission, as noted earlier. The main components of Government of India's strategy for promotion of liquid biofuels are [11]:

1. Ethanol blended gasoline (with ethanol derive from sugar industries).

2. Bio diesel blended diesel (with biodiesel derive from non edible oil and oil waste)

Table 1.5. Area, production and yield of rice during 2008-09 and 2009-10 in major producing states

State	2009-10					2008-09				
	Area	AI %	Production	AI %	Yield	Area	AI %	Production	AI %	Yield
West Bengal	5.63	13.43	14.34	16.10	2547	5.94	13.03	15.04	15.16	2533
Punjab	2.80	6.68	11.24	12.61	4010	2.74	6.01	11.00	11.09	4022
Uttar Pradesh	5.19	12.37	10.81	12.13	2084	6.03	13.25	13.10	13.20	2171
Andhra Pradesh	3.44	8.21	10.54	11.83	3062	4.39	9.63	14.24	14.36	3246
Orissa	4.37	10.41	6.92	7.76	1585	4.45	9.78	6.81	6.87	1529
Tamil Nadu	1.85	4.40	5.67	6.36	3070	1.93	4.24	5.18	5.23	2683
Assam	2.50	5.95	4.34	4.87	1737	2.48	5.46	4.01	4.04	1614
Chattisgarh	3.67	8.76	4.11	4.61	1120	3.73	8.20	4.39	4.43	1176
Karnataka	1.49	3.55	3.69	4.14	2482	1.51	3.32	3.80	3.83	2511
Haryana	1.21	2.87	3.63	4.07	3008	1.21	2.66	3.30	3.33	2726
Bihar	3.21	7.67	3.60	4.04	1120	3.50	7.68	5.59	5.64	1599
Maharashtra	1.47	3.51	2.18	2.45	1485	1.52	3.34	2.28	2.30	1501
Jharkhand	1.00	2.37	1.54	1.73	1546	1.68	3.70	3.42	3.45	2031
Gujarat	0.68	1.62	1.29	1.45	1903	0.75	1.64	1.30	1.31	1744
Madhya Pradesh	1.45	3.45	1.26	1.41	872	1.68	3.69	1.56	1.57	927
Kerala	0.23	0.56	0.60	0.67	2557	0.23	0.51	0.59	0.60	2519
Others	1.75	4.18	3.35	3.77	@	1.75	3.85	3.56	3.59	@
All India	**41.92**	**100.00**	**89.09**	**100.00**	**2125**	**45.54**	**100.00**	**99.18**	**100.00**	**2178**

% AI – Percentage of total area / production of All India. Area - Million Hectares, Production - Million Tonnes, Yield - kg/ Hectare

Table 1.6. Estimation of agro-residues from production of major crops in India (2010-11)

Crop	Main crop production (MMTPA)	Type of Residue	Crop to Residue Ratio	Residue Quantity (MMTPA)
Rice	95.32	Straw	1.3	123.92
		husk	0.3	28.6
Wheat	85.93	Straw	1.5	128.9
Coarse cereals	42.32	Straw and husk	1.8	76.176
Sugarcane	339.17	Bagasse	0.3	101.75
		Top	0.05	16.96
		Trash	0.07	23.74
Cotton	33.43	Stalks	3.0	100.29
		Gin Waste	0.1	3.34
Oilseeds	31.1	Straw and husk	1.1	34.21
Pulses	18.09	Straws	1.3	23.52
Jute	10.58	Stalks	2.0	21.16
GRAND TOTAL				**682.57**

Source: * Agricultural production data is for the year 2010-11 [9]. The residue ratio and conventional uses from reports of Taluka level studies by MNRE.

1.3.1 *Bioethanol Policy and Production*

Ministry of Petroleum and Natural Gas launched the program of ethanol blended petrol in 2003, with mandate of blending 5% ethanol in gasoline [11]. Table 1.8 gives the statistical account of production/supply of ethanol for the 5% ethanol blended petrol program [12]. However, the ethanol supply to petroleum companies soon declined after launch of this program due to drastic fall in sugarcane production in 2003-04 and 2004-05. With re-emergence of sugar industry in 2005-06, the Government of India announced the second phase of 5% ethanol blended petrol program. In November 2006, the petroleum companies in India entered an agreement for procurement of over 1.4 billion liter of ethanol over a period of 3 years at a guaranteed price of Rs. 21.5 per liter. However, as per industry sources, out of this agreement only 540 million liter of ethanol could actually be supplied to the petroleum companies. The major causes leading to the effect are fluctuation in availability of the sugar molasses and sugarcane juice for ethanol production, depending on the sugarcane cycle [11].

Table 1.7: Renewable energy in India at a glance*

Sl. No.	Source / System	Cumulative achievement (as on 30 September) (MW)
I.	**Power From Renewables**	
A	*Grid interactive renewable power*	
1.	Wind power	18191.85
2.	Small hydropower (≤ 25 MW)	3446.67
3	Biomass Power	1226.60
4.	Co-generation bagasse	3446.67
5.	Waste to Energy (Urban and industrial)	93.68
6.	Solar power (SPV)	1045.16
SUBTOTAL A		**26136.69**
B	*Off grid/Captive power*	
7.	Waste to energy	108.94
8	Biomass (Non-Bagasse) Cogeneration	412.61
9.	Biomass Gasifier (Rural and Industrial)	154.30
10.	Aero-Generators/Hybrid Systems	1.74
11	SPV Systems (>1 kW)	96.61
12.	Water Mills/Micro Hydel	2121 nos.
SUBTOTAL B		774.20
TOTAL (A + B)		**26910.89**
II.	**Remote Village Electrification**	
III.	**Decentralized Energy Systems**	
1.	Family type biogas plants (in lakhs)	44.45
2.	Solar Water Heating Systems - Collector Area (million sq m)	5.95

* Data as of November 30, 2012 (kW= kilowatt; MW = megawatt; Sq m = square metre)
Source: [10]

Lower availability of sugar molasses coupled with rising price of molasses affected the cost of production of ethanol and caused disruption in supply of ethanol at the pre-negotiated fix price over three years. Two possible solutions to the problem of interrupted supply of ethanol are: (1) The ethanol procurement price by petroleum companies should not be fixed, but should be linked to raw material prices, and (2) Government attempts to stabilize the sugarcane production. Government of India proposes to increase the blend ratio to 10% in the 3rd phase of ethanol blended gasoline program, and this would give a sharp boost to the demand of ethanol. To meet this demand, not only there would be a need to increase production of sugarcane, but also better and efficient technologies need to be adopted by sugar industries to produce ethanol directly from sugarcane juice.

Table 1.8: India's production & distribution of molasses and alcohol/ethanol in sugar

Item\Year	2006/07	2007/08	2008/09	2009/10
Total Molasses Production (Million tons)	13.11	11.31	6.88	8.60
Molasses Uses				
Alcohol Production (Million tons)	9.50	7.90	4.30	5.90
Animal Feed & Other Uses (Million tons)	3.61	3.41	2.58	2.70
Total Alcohol Production	2,280	1,890	1,032	1,420
Opening Stocks	700	1,269	1,254	706
Imports	39	5	200	200
Total Supply	3,019	3,164	2,486	2,326
Alcohol Use				
Industrial Use	650	700	700	720
Potable Liquor	800	850	880	950
Ethanol for Blended Gasoline	200	280	100	50
Other Use	100	110	100	110
Carryover Stock of alcohol	1,269	1,254	706	496
Total Distribution	3,019	3,194	2,486	2,326

Marketing Year (October/September) *
All units in Million Liters unless mentioned otherwise). Source [11]

Table 1.9. Diesel & biodiesel demand, area required under Jatropha for different blending rates

Year	Diesel Demand MMTPA	Biodiesel Demand @5% blend MMTPA	Plantation Area @5% blend (MHa)	Biodiesel Demand @10% blend MMTPA	Plantation Area @10% blend (MHa)	Biodiesel Demand @10% blend MMTPA	Plantation Area @10% blend (MHa)
2001-02	39.81	1.99	N.A.	3.98	N.A.	7.96	N.A.
2006-07	52.33	2.62	2.19	5.23	4.38	10.47	8.76
2011-12	66.9	3.35	2.79	6.69	5.58	13.38	11.19

Source: [12]

1.3.2 Biodiesel Policy and Production

In April 2003, the committee on biofuels under auspices of planning commission presented its report to Government of India, in which the committee use of biodiesel blended diesel in vehicles [12]. The statistics given by the committee for current and projected demand of petroleum/diesel, biodiesel required for 5%, 10% and 20% blend and the required

area for the plantation is depicted in Table 1.9. The committee recommended gradual increase of blend of biodiesel in diesel from 5% in 2006-07 to 20% by the end of 11th Plan (2011-12). The main recommendations / plans of this committee was as under [12]:

(1) The Ministry of Rural Development would be the nodal ministry that will launch the demonstration phase, which will aim at bringing 400,000 hectares area under Jatropha planting over a five-year period (2003-2008).

(2) The demonstration phase will also involve identifying suitable Jatropha cultivators, developing nurseries and providing subsidized planting material to farmers in various agro-climatic regions.

(3) The Indian Railways, a government owned entity, would launch an ambitious Jatropha plantation project on railway land adjoining railway tracks.

(4) The demonstration phase will be followed by a self-sustaining expansion of Jatropha cultivation on 11.2-13.4 million hectares. Several government, international and private research organizations would also involve in research and development of Jatropha – collecting and identifying elite germplasm; evaluation trials for growth, seed yields and oil content; hybridization; developing location specific agronomic practices; and farmers training.

In October 2005, the Ministry of Petroleum and Natural Gas announced a "bio-diesel purchase policy", in which oil companies would purchase bio-diesel and blend it with high-speed diesel (HSD) at a five percent blending ratio. 20 centers for procurement of biodiesel were set up across the country from January 2006, with a pre-determined procurement price of Rs. 26.5 (55 U.S. cents) per liter. However, this policy did not succeed, as the actual cost of production of bio-diesel was 20 to 50 percent higher than this purchase price [11].

Moreover, India's non-edible oil seed *Jatropha* based policy has encountered major hurdle like lack of good quality planting material, poor seeds yield, ownership issues, lack of sufficient financing etc. Considerable research is still required to identify appropriate seed variety suitable of agro climatic conditions. The government should also offer fiscal and financial incentive to Jatropha planters and train them to follow agronomic practices. The scenario on biodiesel production of India is rather grim in that India's commercial production of biodiesel is very small and unorganized. Small plants up to 1 ton per day have been established, but their product is sold locally for meeting energy demands of agricultural equipments. There are about 20 large capacity plants (up to 50 tons per day), and the products of these plants is mostly bought by R&D sections of automobile and transport companies. Although reliable production figures for biodiesel are not available, the estimated annual production of biodiesel is in the range of 100 to 200 million liters per day [11].

1.4 Aims and Scope of Present Thesis

In the preceding sections, we presented a brief overview of scenario on front of energy needs of transport sector in India. Moreover, we also presented discussion on potential of agro residue (mainly residue of rice crop) based biofuels generations, along with a brief history of India's policies and efforts for production of two main liquid biofuels, viz. bioethanol and biodiesel. It should however be noted that both ethanol and biodiesel can only be blended with gasoline and diesel and this cannot completely replace petroleum derives fuels. Moreover, there are also operational problem like separation of blend at low temperature and reduction in fuel economy due to low calorific values of ethanol.

Biobutanol has emerged as a new alternate alcoholic liquid biofuel, and it overcomes the demerits of bioethanol and biodiesel. As discussed in greater details in the next chapter, biobutanol has very similar properties as gasoline and thus, can be blended in any proportion

with it. This thesis work was undertaken with a goal of developing a process for production of biobutanol using the major agro residue of rice straw. Such a process will have high economic potential (due to abundant availability and low cost of rice straw). We have taken a step-by-step approach for process development, in which we have addressed both physical and physiological aspects of process. The thesis comprises of 8 chapters (including the present chapter), contents of which are briefly outlined below:

- In Chapter 2, a comprehensive review of literature on ABE fermentation is presented that addresses several important aspects of the fermentation process.
- In Chapter 3, we have given an overview of rice straw based processes for biofuels reported in literature. We essentially reviewed physical, chemical and enzymatic techniques, for pretreatment of rice straw prior to the process, and have also presented an account of production of different biofuels from rice straw.
- In Chapter 4, the results of study on screening and selection of substrate and microbial culture are presented.
- In Chapter 5, we have presented results on pretreatment of rice straw and have tried to identify and optimum technique (which is a combination of physical and chemical treatment) for this important step prior to main fermentation process.
- In Chapter 6, gives accounts of our results on development of rice straw based fermentation medium using the statistical approach.
- Chapter 7 presents the results on optimization of the process in term of physical and physiological parameters using statistical approach.
- Chapter 8 summarizes the major finding of the thesis and gives some suggestions for further research.

Abbreviations and Notations

GDP	Gross Domestic Product
OIL	Oil India Limited
ONGC	Oil and Natural Gas Corporation
JVC/Private	Joint Ventures Contracts
LPG	Liquid Petroleum Gas
SKO	Kerosene
LDO	Light Diesel Oil
LSHS	Low Sulfur Heavy Stock
HHS	High sulphur Heavy Stock
ATF	Aviation turbine fuel
EIA	Energy Information Administration
GHG	Green Houses Gas
IISC	Indian Institute of Science
MMTPA	Metric Million Tonnes Per Annum
F & W	Forest and Wasteland
HSD	High Speed Diesel

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CHAPTER 2

INTRODUCTION TO BIOBUTANOL: SCIENCE, ENGINEERING AND ECONOMICS

2.1 Introduction

The rapid depletion of fossil fuels with highly fluctuating market prices has made the quest for alternative fuels a high priority. Another growing concern has to do with greenhouse gas emissions and global warming. Therefore, an additional expectation for an alternative fuel is that it should be carbon neutral, with no net addition of green house gases into the atmosphere. Over the past two decades, several alternative liquid fuels have been investigated, which can either completely replace petroleum derived fuels (gasoline and diesel) or which can be blended with petroleum fuels at certain proportions, without requiring specially designed vehicle engines [1-5]. Biodiesel has been a popular biofuel, which is essentially composed of methyl or ethyl ester of fatty acids. However, due to considerable differences in the physical and chemical properties, biodiesel cannot completely replace petro-diesel, without significant changes in the engine configuration.

TABLE 2.1 Comparison of properties of different alternate liquid fuels [8]

Fuel	Energy Density (MJ L^{-1})	Air to Fuel Ratio	Heat of Vaporization (MJ/kg)	Research Octane Number	Motor Octane Number	Cetane Number
Gasoline	32	14.6	0.36	91-99	81-89	--
Butanol	29.2	11.2	0.43	96	78	--
Ethanol	19.6	9.0	0.92	129	102	54
Methanol	16	6.5	1.2	136	104	
Biodiesel	31 - 33	12.5	--	--	--	48-65

Biodiesel can be blended with petro-diesel up to 20% to be compatible with current engines. Another fuel is ethanol, which has also been employed as an oxygenate for gasoline and diesel [6-7]. However, ethanol suffers from limitations of low energy content (or heat of combustion) which impacts the economy of the blended fuel. Moreover, given its solubility characteristics, ethanol is also likely to separate from gasoline in the presence of water, which introduces operational problems. Butanol (or "bio-butanol" as it is more popularly known), overcomes most of the shortcomings mentioned above. In addition to being a potential biofuel, butanol is also a valuable C_4 feedstock for chemical synthesis (esters, ethers, acetates and plasticizers), as well as a solvent. The current international price of bulk grade butanol is approximately US $ 4 per gallon (Liquid Fuels, [8]) with a worldwide market of 350 million gallons per year. The conventional chemical processes for butanol is the oxo process, in which synthesis gas (a mixture of CO and H_2) is first reacted with propylene to yield buteraldehyde, which is subsequently hydrogenated to butanol. Another chemical process for butanol synthesis is through crotonaldehyde, in which two molecules of acetaldehyde undergo aldol condensation to yield the intermediate crotonaldehyde, which is then dehydrated and hydrogenated to give butanol.

Table 2.1 lists and compares some common properties of various alternative liquid fuels. It can be seen that the properties of butanol match more closely with gasoline than for

the other listed fuels. Substitution of gasoline by bio-butanol would result in a fuel consumption penalty of approximately 10%. Other distinct merits of butanol over ethanol and methanol are:

1. It can be blended to any ratio with gasoline as well as diesel directly in the refinery without the requirment for additional infrastructure for blending.
2. Easy transportation through pipelines due to low vapor pressure. Less corrosion to the pipeline through which it is transported.
3. The air to fuel ratio for butanol is close to that of gasoline. This is within the limits of the variation permissible in existing engines. Although complete replacement of gasoline by butanol would require an enhancement of the air-fuel ratio, blends of up to 20% butanol can be easily used in existing engines.
4. The heat of vaporization of butanol is slightly higher than that of gasoline. Therefore, vaporization of butanol is as easy as gasoline. An engine running on butanol-blended gasoline should not have a cold start problem. It should be mentioned that ethanol or methanol blended gasoline is known to cold weather issues due to higher heat of vaporization [9].
5. Low solubility of butanol in water reduces the potential for groundwater contamination.

Despite these merits of butanol, a noteworthy merit of biodiesel is its biodegradability, which the butanol lacks. Butanol can also be produced from various biological substrates such as sugars, starch and biomass through various different routes as depicted in Fig. 2.1. Biologically-produced butanol is popularly known as “Biobutanol”. Among the routes depicted in Fig. 2.1, the most popular and historical route to biobutanol has been the ABE (Acetone-Butanol-Ethanol) fermentation by means of solvent producing strains of *Clostridium sp* [11]. The conventional substrates for the ABE fermentation have been corn or molasses, however, as reviewed subsequently in this chapter, several alternative substrates have been considered.

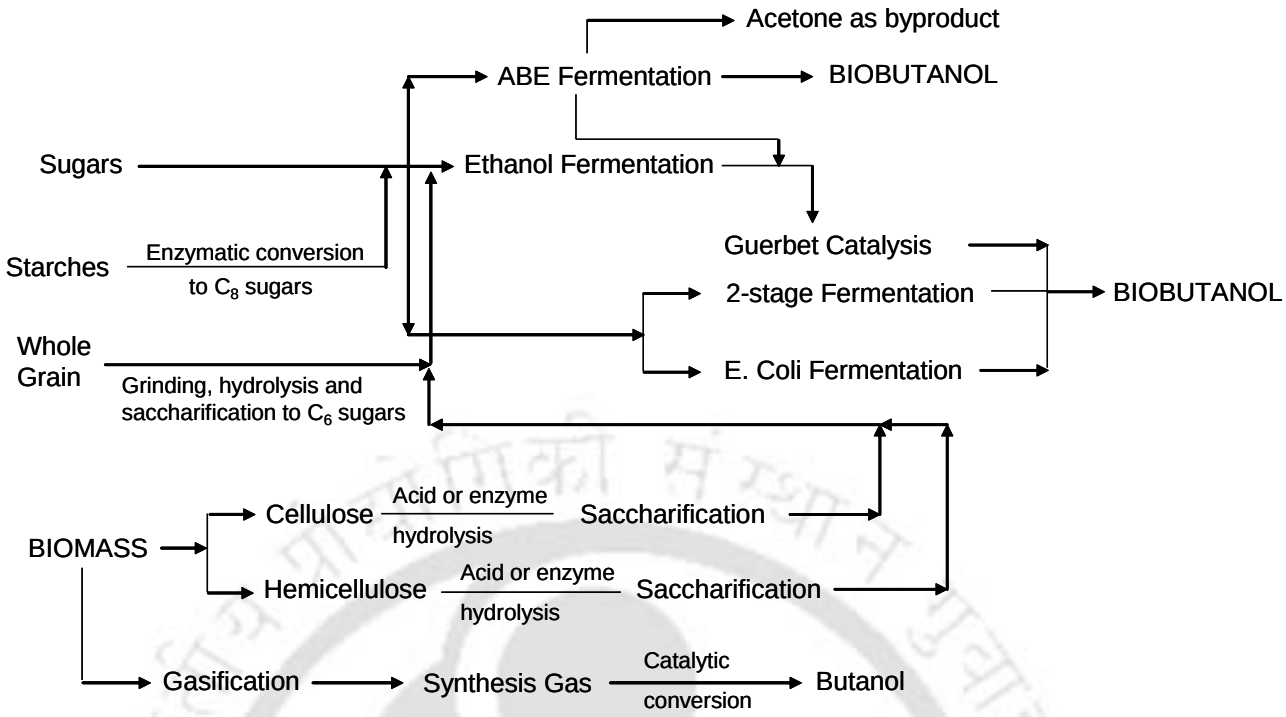

Figure 2.1 Various routes to Biobutanol (redrawn with modifications from ref. [10])

In the next section, we briefly review the history of the ABE fermentation (for additional details we refer the reader to Jones and Woods [12]).

2.1.1 Brief History of ABE Fermentation

The ABE fermentation is one of the oldest known industrial fermentations with a history of more than 100 years. Pasteur first reported butanol production through microbial fermentation in 1861. This was followed by Schardinger [13] reporting on the production of acetone by fermentation. In 1911, Fernbach fermentated biomass such as potatoes to produce butanol using an isolated culture. This research was promoted by the synthetic rubber industry, which used precursors such as butadiene and isoprene obtained from butanol [14-15]. Between 1912 to 1914, Chaim Weizmann working at Manchester University isolated cultures of C*lostridium acetobutylicum,* which had the capability of fermenting starchy substrates, with higher butanol yield than the cultures of Fernbach [16]. The era of World War I and II saw the largest growth of the ABE fermentation industry in Europe and USA, as

a source of acetone for manufacture of cordite, a smokeless powder used for production of ammunition. A change of substrate for the fermentation from maize to horse chestnuts [17] occurred due to the German blockade that caused scarcity of grain. Butanol was also an excellent solvent for quick drying lacquers used by automobile industry in the USA. In 1923, Commercial Solvents Inc. started production of butanol in a plant at Peoria (Illinois). By end of 1926, the production of acetone-butanol-ethanol solvents in the Peoria plant rose to 100 tons per day, most of which was utilized for production of lacquer [18]. At the end of World War II in 1945, 66% of the butanol and one-tenth of the acetone in USA was produced by fermentation [19]. In the former Soviet Union (USSR), large-scale production of acetone and butanol through ABE fermentation was implemented since 1929 [20]. The major substrates employed were maize, wheat, and rye. Till late 1980s, at least 8 industrial scale AB fermentation plants were operational in the USSR [21]. Other countries such as China, Japan, Australia, and South Africa also produced acetone and butanol through large-scale fermentation processes [21-25]. After World War II, the ABE fermentation industry suffered acute competition from the petrochemical industry that flourished at an unprecedented rate. Large quantities of much cheaper acetone and butanol were available through petrochemical processes, which rendered the ABE fermentation uneconomical. Molasses, the major feedstock for the ABE fermentation found an alternate outlet as cattle feed supplement. Most of the ABE fermentation industry in western countries ceased to exist by 1960. In South Africa, however, the fermentation was operational until 1982 due to abundant supply of cheap molasses, coal and the relatively small amount of acetone and butanol that was available from petrochemical industry due to import restrictions. However, severe drought in South Africa in early 80's resulted in a scarcity of molasses and consequently the plant had to be shut down. More than two decades later, the interest of the scientific community and industry in the process revived due to depleting oil reserves and a highly fluctuating crude oil

price. Basic research is now directed towards improvement of the complete process by use of genetically modified strains, alternate cheaper fermentation substrates, improved cultivation techniques, and more efficient product removal.

2.1.2 Aim and scope of the chapter

Most of the literature related to ABE fermentation originates from the microbiology community. For utilization of this literature for process design, development, scale up and optimization of the ABE fermentation, a critical analysis from engineering and economic perspective is necessary. This chapter is aimed at addressing this important issue. We would like to specifically mention that several reviews published previously have addressed various aspects of ABE fermentation. Some of the classical reviews published in 1980's focused on history, process design (batch/fed batch/continuous fermentation), microbial cultures, immobilized systems, biochemistry, product recovery and economics of ABE fermentation are by Haggstrom [26], Jones and Woods [12], Ennis et al. [27] and McNeil and Kristiansen [28], and Maddox [29]. Major reviews on genetic engineering and manipulation of the clostridia, cloning, DNA sequencing published in 1990s include those from Blaschek [30], Woods [31], Blaschek and White [32] and Girbal and Soucaille [33]. Park and Geng [34] and Belafi-Bako et al. [35] have reviewed the latest developments with respect to various techniques (vaccumization, gas stripping, liquid-liquid extraction, adsorption) used for simultaneous product removal and recovery from fermentation broth. Another review by Goma et al. [36] addressed the problem of product (butanol) inhibition in ABE fermentation. Maddox et al. [37] discussed the utilization of whey for the production of acetone, butanol and ethanol. Duerre and Bahl [38], Duerre [39] and Claassen et al. [40] also published reviews on various aspects of ABE fermentation process. Gapes and others [41-44] reviewed basic principles and economics of ABE fermentation. More recent reviews (published since 2000) eminated from researchers at the USDA (Peoria, IL) and the University of Illinois at

Urbana-Champaign [45-51] that specifically address issues such as substrates and reactor systems for fermentation, genetic strain improvement, immobilization techniques and solvent recovery techniques. Other reviews published in past 5 years are from Wu et al. [52] who have assessed life cycle energy and greenhouse gas emission effect using corn-based biobutanol, Karakashev et al. [53] who have given an overview of production of liquid alcoholic fuels such as ethanol and butanol, and Lee et al. [54] who have critically evaluated various strategies for strain improvement, fermentation processes and downstream processing.

This chapter will touch upon various aspects of ABE fermentation, including: (1) microbial cultures for fermentation and metabolic pathway; (2) substrates for fermentation; (3) fermentation protocols and reactor design (including immobilized systems); (4) solvent recovery techniques; (5) mathematical modeling; and (6) economics of the process. Genetic and metabolic engineering aspects of clostridial species is outside the scope of this review and reader is referred to the reviews by Blaschek and White [32], Woods [31], Ezeji et al. [51] and Lee et al. [54]. Moreover, this review mainly focuses on literature published since 1985; for earlier literature we refer the reader to Jones and Woods [12].

2.2 Biochemistry of the ABE Fermentation

The most commonly considered substrates for clostridial cultures include fibrous biomass containing hemicellolose and cellulose (e.g. wheat straw, rice straw), starchy biomass (such as ground corn and whey permeate) and fruits and vegetables containing fructose, glucose, xylose, etc. as basic components. Laboratory-based media for culture

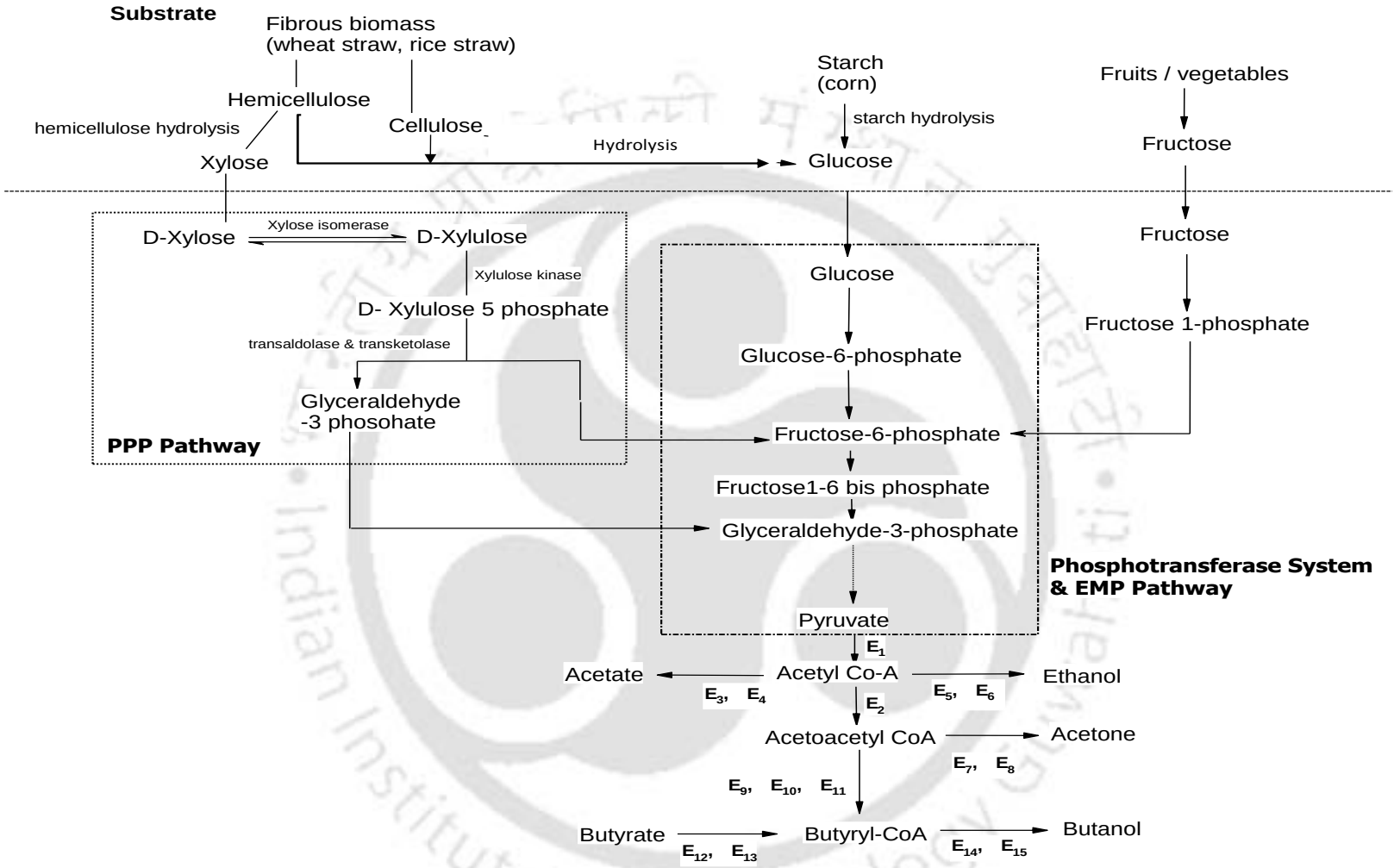

Figure 2.2: The metabolic pathway of ABE fermentation by *Clostridia*. Abbreviations: E_1 – pyruvate ferrodoxin oxidoreductase; E_2 – thiolase; E_3 – phosphate acetyltransferase; E_4 – acetate kinase; E_5 – acetaldehyde dehydrogenase; E_6 – ethanol dehydrogenase; E_7 – CoA transferase; E_8 – acetoacetate decarboxylase; E_9 – 3-hydroxybutyryl-CoA dehydrogenase; E_{10} – crotonase; E_{11} – butyryl CoA dehydrogenase; E_{12} – phosphate butyryl transferase; E_{13} – butyrate kinase; E_{14} – butyraldehyde dehydrogenase; E_{15} – butanol dehydrogenase

growth have normally been semi-defined and defined media in which the main carbohydrate source is supported by various vitamins and minerals depending upon the microbial strains. The optimum temperature for the ABE fermentation is between 30–40^{o}C. Two principal phases in the fermentation are characterized by: (1) an acid production phase or acidogenesis and (2) a solvent production phase or solventogenesis. The pH of the fermentation broth, initially at 6.8 to 7, drops to 4.5–5 during acidogenic phase. This phase is associated with the rapid growth of cells and the secretion of the carboxylic acids, acetate and butyrate. The switchover from acidogenesis to solventogenesis occurs at reduced pH. Bahl et al. [55] have suggested that the switchover is an adaptive response of the cells to the low pH of the medium, while Ballongue et al. [56] have suggested that acids produced in the acidogenesis phase act as inducers for the biosynthesis of solventogenic enzymes. We briefly describe below the metabolic pathway for the ABE fermentation employing the *clostridia*. Gheshlagi et al. [57] have recently reviewed in detail the metabolic pathway of the *clostridia*. For greater details on the enzymes involved in various steps of the metabolic pathway, we refer the reader to this review.

Fig.2.2 shows the metabolic pathway for clostridial cultures employing various substrates. Among the various nutrient transport mechanisms, anaerobes (obligate/facultative) accumulate sugars via the Phosphoenol Pyruvate (PEP) dependent Phosphotransferase system (PTS) [58, 59]. On the whole, hexose sugars are metabolized by the Embden Meyerhoff Pathway (EMP), while pentose sugars go through the Pentose Phosphate pathway (PPP) to produce pyruvate. Glycolysis utilizes Glucose (1mol) and produces pyruvate (2 mol), while PPP results in production of CO_2 (6 mol). During Glycolysis energy is stored as ATP and NADH (2 mol), while during PPP energy is stored as NADPH. The most common substrate for the ABE fermentation is starch, which is converted to glucose following acid/enzyme hydrolysis. Glucose (6C) is first phosphorylated to glucose-6-phosphate, which is

subsequently converted to pyruvate (3C) via EMP. Other fermentation substrates containing hemicellulose or cellulose (e.g. fibrous biomass such as rice straw or wheat straw) can be converted to xylose and glucose respectively, following hydrolysis [60]. Glucose enters the metabolic pathway in the same manner as stated earlier, while xylose (existing naturally in the form of D-xylose, an aldose) undergoes the "Isomerase pathway" in which, the enzyme xylose isomerase convert D-xylose to D-xylulose. Furthermore, D-xylulose undergoes phosphorylation to form D-xylulose-5-phosphate. D-xylulose-5-phosphate dissimilates by means of transketolase and transaldolase through non-oxidative pentose phosphate resulting in the production of glyceraldehydes 3-Phosphate and fructose-6-phosphate, which finally enters the EMP pathway for further conversion [12, 57, 61]. Fermentation of hexose sugar (1 mol) results in generation of 2 mol of ATP and 2 mole of reduced NADH, while fermentation of pentose sugar yields 5 mol of ATP and 5 mol of NADH [12]. ATP produced during the consumption of glucose results in the exponential growth of cells.

The pyruvate formed during glycolysis (or EMP pathway) is cleaved by pyruvate ferredoxin oxidoreductase in presence of co-enzyme A (CoA) producing CO_2, acetyl-CoA and reduced ferredoxin. Conversion of acetyl-CoA to acetate is achieved by the enzymes phosphate acetyltransferase and acetate kinase, while conversion of butyryl-CoA (formed through sequential conversion of acetyl-CoA, as explained later) to butyrate is catalyzed by the enzymes phosphate butyltransferase and butyl kinase. The pH of medium decreases as these conversions proceeds with accumulation of butyric and acetic acids in the medium. These acids can permeate the cell membrane and are involved in triggering the of solventogenic phase.

During the solventogenic phase, the products of the preceding acidogenic phase are reassimilated and converted to acetone and butanol. The enzyme catalyzing this conversion is Co-A transferase, which converts CoA from acetoacetyl-CoA (formed from acetyl-CoA by

action of thiolase) either to acetate forming acetyl-CoA or to butyrate resulting in butyryl-CoA. Out of these, acetyl-CoA can be converted to acetone, butanol and ethanol, while butyryl-CoA can only be converted to butanol, as explained below.

1. Acetyl-CoA can be converted to acetaldehyde (catalyzed by acetaldehyde dehydrogenase) and further to ethanol (catalyzed by ethanol dehydrogenase).

2. Alternatively, two moles of acetyl-CoA are converted to one mole of acetoacetyl-CoA by thiolase (as noted earlier). Removal of CoA from acetoacetyl-CoA by enzyme CoA-transferase yields acetoacetate, which is further converted to acetone by enzyme acetoacetate decarboxylase with release of CO_2.

3. Acetoacetyl-CoA can simultaneously undergo reduction to 3-hydroxybutyryl-CoA catalyzed by hydroxybutyryl-CoA dehydrogenase. Dehydration of 3-hydroxybutyryl-CoA catalyzed by crotonase results in crotonyl-CoA, which is reduced to butyryl-CoA by action of NADH and butyryl-CoA dehydrogenase. Butyryl-CoA can be further converted to butyraldehyde (via the action of NADH and butyraldehyde dehydrogenase), and further to butanol (via the action of NADH and butanol dehydrogenase).

4. The fate of butyryl-CoA is unique, as there is no metabolic pathway to regenerate acetyl-CoA from butyryl-CoA. It initially undergoes conversion to butyraldehyde by the action of NADH and butyraldehyde dehydrogenase, and further to butanol by the action of NADH and butanol dehydrogenase.

2.2.1 Inhibition of fermentation

The products of both phases of the ABE fermentation, i.e. acidogenesis and solventogenesis, cause inhibition of fermentation after reaching a certain concentration level in the medium. The presence of butanol in the cell membrane increases membrane fluidity causing destabilization of membrane [12]. For most clostridial cultures, the maximum amount of solvents (i.e. acetone, butanol and ethanol) that cells can tolerate is 20 g/L. Due to

this limitation, the maximum amount of sugar utilized in a batch fermentation is 60 g/L. An obvious solution to the problem of inhibition is continuous removal of solvents from the fermentation broth or development of new strains of *clostridia* that are more resistant and tolerant towards butanol. In the subsequent sections of this review, we deal with these issues in greater detail.

2.3 Microbial Cultures for ABE Fermentation

A large variety of clostridial cultures capable of producing acetone, butanol, isopropanol and ethanol, with varying yield and proportions have been known for more than half century [62]. A comprehensive review of these cultures has been given by Durre and Bahl [38] and Durre [39]. These cultures are capable of utilizing several substrates such as sugar and beet molasses, whey permeate, hydrolyzates of [60, 63] agricultural products such as wheat and rice straw, starch (corn, heat, rye, etc.). The "nomenclature" of the clostridial cultures has a history of over 100 years. Strains isolated by Louis Pasteur [64, 65] were named "Vibrion butyrique". Later, Albert Fitz isolated butyrate and butanol cultures [66, 67] and named these as *Bacillus butylicus*. Around same time, Dutch microbiologist *Martinus beijerinck* isolated butanol and isopropanol producing cultures which were named as *Granulobacter butylicus* and *Granulobacter saccharobutyricum* [68]. The name *Clostridium* was introduced by Adam Prazmowski [69]; However, official publication of the name *Clostridium acetobutylicum* was made in 1926 [70]. In the late 1930s and 1940s, a number of ne strains were developed for commercial use [71, 72]. These strains were able to utilize 7.5% fermentable sugar with solvent concentrations in the range 18–23 g/L and a yield of 30–33%. Hongo [73] patented cultures that produced up to 70% butanol. George et al. [74] published a comparative study of as many as 34 strains representing 15 species of *clostridia* for production of acetone, butanol, isopropanol and ethanol. The principle species that produced solvent in reasonable amounts (>1 mM) in a peptone yeast extract glucose medium

Table 2.2: Comparative analysis of 4 *Clostridia* species*

Microbial Strain	Maize Medium 6%		Molasses Mash Medium	
	Concentration (g/L)	ABE solvent Yield	Concentration (g/L)	ABE solvent Yield(%)
C. acetobutylicum				
NCIMB 619	19.6	24.5	7.8	13.0
ATCC 824	19.2	24	9.5	15.8
DSM 1732 or NCIMB 2951	17.9	22.4	4.1	6.8
C. saccharobutylicum				
NCP P262	11.3	14.1	17.9	29.8
NCP P258	10.8	13.5	18.3	30.5
C. saccharoperbutyl-acetonicum				
N1–4	14.2	17.8	4.9	8.2
N1–504	10.8	13.5	18.3	30.5
C. beijerinckii				
NRRL B592	16.2	20.8	11.1	18.5
NCP P260	11.3	14.0	18.9	31.5
NRRL B593	14.1	17.6	11.5	19.2

* Data taken from Shaheen et al. [80]

were *C. beijerincki, C. butylicum, C acetobutylicum, C. auranibuty-ricum, C. pasteurianum, C. sporogenes, C. cadeveris*. The strain *aurantibutyricum* produces acetone butanol and isopropanol, while *C. tetanomorphum* produces equal amount of ethanol and butanol.

Following a detailed taxonomic and phylogenetic study of all clostridial strains [75-79] the industrial strains of *clostridia* were categorized among four species namely *C. aceto-butylicum, C. beijerinckii (formerly C. butylicum), C. saccharoperbutylacetonicum* and *C. saccharobutylicum*. Shaheen et al.[80] have done comparative studies of fermentation by these four principal species (with different strains there of) using different substrates. A summary of their results is given in Table 2.2 for two media, namely molasses (with 6% fermentable sugar) and a maize mash medium. As seen from this Table, the performance of different strains of the four species was highly dependent on the substrates. On the basis of this study, Shaheen et al. [80] concluded that *C. acetobutylicum* was more suitable for starch based medium, while *C. saccharobuylicum* was suitable for molasses based medium. Strains

Table 2.3: Comparative study of utilization of various sugars in DDGS hydrolyzate by different *Clostridial* species*

Microbial Culture	Fermentation Time (h)	Solvent production (g/L)				Sugar utilization	Preference
		A	B	E	Total	G:M:A:X	
C. acetobutylicum 260	48	6.3	13.2	0.8	20.3	100:100:92:71	G > M > A > X
C. acetobutylicum 824	96	4.7	13.1	0.6	18.4	100:100:60:80	G > M > X > A
C. saccharo–butylicum 262	72	3.2	10.4	0.7	14.3	74:38:63:42	G > A > X > M
C. butylicum NRRL 502	60	6.7	12.5	0.5	19.7	100:100:89:86	G > M > A > X
C. beijerinckii 8032	60	3.4	10.5	0.7	14.6	81:42:49:64	G > X > A > M
*C. beijerinckii BA101***	84	3.5	13.9	0.5	18.0	100:54:65:78	G > X > A > M

* - Data taken from Blaschek and Ezeji [81]
** - A mutant strain of C. beijerinckii 8052 [82]

of *C. saccharperbutyl-acetonicum* had moderate yet consistent performance in terms of overall yield in both starch and sugar based media, and thus, these were versatile strains for mixed substrates. For species *C. beijerinckii*, only the NRRL B592 strain showed good performance on mixed substrate. (this is not consistent with the results in Table 2.2).

A similar comparative study of different strains for production of ABE solvents using DDGS (Distillers Dried Grains with Solubles) hydrolysates was done by Blaschek and Ezeji [81]. Principal constituents of DDGS hydrolysates are hexose sugars (glucose, mannose, galactose and fructose) and pentose sugars (xylose and arabinose), in addition to protein, fat, oil and lignin. Table 2.3 depicts the results of batch fermentation studies employing different strains with mixed sugar streams comprised of glucose:mannose:arabinose:xylose in the ratio of 25:5:10:20 g/L. Apart from these, comparative assessment of solvent formation by *C. saccharobutylaceto-nicum* (DSM 2152) and *C acetobutylicum* (DSM 792) has been made by Biebl [83]. This study reported much lower solvent production by the former strain.

Another related species of industrial solvent-producing clostridia is a *C. pasteurianum*.

This species was initially known as an acid producer that fermented carbohydrates to butyrate, acetate, CO_2 and H_2 [84]. However, Harris et al. [85] reported that the species could also produce significant quantities of acetone, butanol, and ethanol, when grown in media of high glucose content. Nakas et al. [86] have reported conversion of algal biomass supported with 4%w/v glycerol to butanol, 1,3–propanediol and ethanol. In another interesting study, Heyndrickx et al. [87] compared the fermentation characteristics of *C. butyricum* (LMG $1212t_2$) and *C. pasteurianum* (LMG 3285) with glycerol and a glycerol-acetae mixture as the carbon source. *C. butyricum* converted more than half of glycerol to n–butanol. Addition of acetate to glycerol resulted in less 1,3 propanediol and H_2 from *C. butyricum*, while fermentation products of *C. pasteurianum* remained unaffected. More recently, a few papers have appeared in the literature investigating conversion of crude glycerol resulting from transesterification process for biodiesel production by *C. pasteurianum* species [88, 89]. Dabrock et al. [90] have reported product ratios of 38 mol:18 mol:18 mol for ethanol:butanol:1,3 propanediol per 100 mol of glycerol fermented under phosphate limitation. Biebl [91] has also reported butanol (with concentration up to 17 g/L) as a major product of glycerol fermentation using *C. pasteurianum*. Taconi et al. [92] have reported butanol yields of 30 g/g on crude glycerol and 38 g/g with pure glycerol. Although these studies have shown high potential for industrial use of *C. pasteurianum*, especially for improvement of the economics of the biodiesel industry, significant research is yet to be done on process engineering aspect of *C. pasteurianum* based fermentation.

2.4 Substrates for ABE Fermentation

The conventional substrates for ABE fermentation have been molasses, corn, wheat, millet and rye [12]. The earliest ABE fermentation industry utilized batch fermentations with corn mash or molasses. For corn mash no additional or supplementary nutrients were necessary, while for molasses some supporting nutrients supplying nitrogen and phosphorous

were necessary [14, 18, 62, 93-95]. Molasses had many distinct advantages over corn mash such as a lower operational temperature and a higher solvent yield. As a result, many plants utilizing corn mash as substrate were switched over to using molasses, along with other substrates such as grains and corn cob hydrolyzates [19-20, 96-97].

After World War II, most of the conventional substrates were increasingly utilized as cattle feed, and the prices become unaffordable to the distiller industry. As the cost of substrate is a major factor determining the overall economy of ABE fermentation industry, significant research has been done on alternative cheap substrates. Initial research focused mainly on alternate sources of starch/carbohydrate. These include potatoes, rice, jawari, bajra, apple pomace, cheese whey, and Jerusalem artichokes [86, 95, 98-108]. Jerusalem artichokes require hydrolysis (acid/enzyme) prior to fermentation, while cheese whey requires the removal of casein by precipitation. Solvent production from Jerusalem artichokes was similar to that from molasses (23–24 g/L) [109-115], while cheese whey gave much lower productivity (5–15 g/L solvents). It was, however, observed that the relative yield of butanol (or the distribution of ABE products) was much higher as compared to when glucose was used. Voget et al. [106] investigated use of apple pomace as a substrate for butanol fermentation and reported a yield of 2% w/w. Nakas et al. [86] has reported algal biomass (sp. Dunaliella) as a substrate for fermentation with *C. pasteurianum*. Algal biomass supplemented with 4% glycerol produced a mixture (16 g/L) of butanol and 1,3 propanediol.

Another alternative substrate for fermentation is lignocellulosic biomass. Being most abundant and renewable, this source offers great promise for improvement of the economy of the ABE fermentation. However, effective hydrolysis of the hemicellulosic and cellulosic fractions of biomass is a major factor. Hydrolyzates of cellulosic fractions are mostly comprised of hexose sugars (glucose, fructose, mannose, sucrose), which are completely consumed; while hydrolyzates of hemicellulose are made up of pentose sugars (xylose,

galactose, arabinose and raffinose), which are only partially consumed. Extensive literature has been published since the 1920's that presents a comparative evaluation of fermentation of different sugars by clostridial cultures [20,116-121]. Fond et al. [122,123] have studied the kinetics of utilization of glucose, xylose, and a mixture of these in a fermentation employing *C. acetobutylicum* in batch and fed–batch mode. The principal findings of this study were as follows: (1) the fastest initial growth and transition from acidogenesis to solventogenesis occurs at a glucose concentration of 62 g/L. Relatively slower initial growth rate and transition from acidogenesis and solventogenesis is seen with xylose, which results in higher cellular concentration. (2) For the mixture of these two sugars in batch fermentation, glucose is fermented more rapidly. The highest fermented sugar concentration was 68 g/L for the mixture of glucose and xylose. (3) For the fed batch mode, the fermentation process is limited by the low sugar concentration feed rate. This has several consequences such as slower cellular growth, slower metabolic transitions, and higher accumulation of acids. At higher feed rates, results comparable with batch fermentation were obtained. Solvent production was triggered at a total acid concentration of 4.5 g/L, while final inhibition of fermentation occurred at a solvent concentration of 20 g/L. (4) For low glucose concentrations in the feed, both sugars are consumed at same rate, while at higher glucose levels, xylose utilization is inhibited as catabolic flux of glucose can alone satisfy metabolic activities of cell. (5) The acid concentration for the switchover from acidogenesis to solventogenesis, and final inhibition concentration of butanol was same for fed-batch mode as for the batch mode.

Hydrolyzates of lignocellulosic biomass are increasingly being used as substrates for the ABE fermentation. The hydrolyzates were prepared using either acid or enzyme hydrolysis. Lee et al. [124] have reviewed technological aspects and economic factors of acid hydrolysis of lignocellulosic biomass. Prospects of utilization of hydolyzates for ABE fermentation have been investigated for more than half a century. Earliest studies in this area

include those of Waksman and Kirsh [125], Underkofler et al. [126], Sjolander et al. [127], Leonard et al. [129], Langlykke et al. [130], Nakhmanovich and Shcheblykina [20].

Acid treated hydrolyzates are reported to contain inhibitory compounds such as salts, furfurals, glucuronic, p–coumaric acid, phenolics [130] etc. These need to be removed by techniques such as adsorption or solvent wash etc. On the other hand, no inhibitory compounds content has been reported in the hydrolyzates produced by enzymatic treatments. Another pretreatment for separation of lignocellulose biomass into cellulose, hemicellulose and lignin is steam cracking [131-134]. After this treatment, the hemicellulosic fraction is present in water (in solubilized form), while the cellulosic fraction is present in the insoluble substrate. Both of these fractions could be hydrolyzed (by acid/enzyme) to allow for further release of sugars. Although this pretreatment showed positive results for wood chips during a pilot scale process [135], Marchal et al. [136] reported that hydrolyzates obtained by enzymatic saccharification of wheat straw or corn stover, pretreated with steam explosion were non-fermentable. Heating of hydrozylates with alkaline compounds such as $Ca(OH)_2$ or $MgCO_3$ to restore neutral pH was necessary to make the hydrolyzates fermentable.

2.4.1 Cocultures

Utilization of cellulosic, hemicellulosic or lignocellulosic biomass as substrates for fermentation requires a hydrolysis step prior to fermentation. Enzymatic hydrolysis is relatively expensive, while acid hydrolysis yields hydrolyzates containing inhibitory compounds. Purification of hydrolyzates prior to fermentation adds additional cost component. A viable solution to this has been explored by some research groups in terms of examination of co-cultures of *Clostridium acetobutylicum* with microorganisms having enzymes capable of simultaneous hydrolyzing cellulose and hemicellulose, as the fermentation proceeds. Double et.al has briefly stidued the production cost of liquid fuels from varios biomass through fermentative and thermochemical route [137]. Literature in this

area, however, is limited. We give below a brief summary of some important publications in this area:

Soni et al. [138] used mixed cultures of cellulolytic fungi *T. reesei* and *A. wentii* to obtain hydrolyzates of agricultural wastes such as bagasse, rice straw and wheat straw. These hydrolyzates fermented either with cultures of *C. saccharoperbutylacetonicum* or *C. acetobutylicum* yielded 16 g/L and 17.3 g/L of butanol, respectively. Petitdemange et al. [139, 140] and Fond et al. [141] attempted use of mesophilic cocultures of cellulolytic (*C. cellulolyticum* H10) and glycolytic (*C. acetobutylicum*) clostridial strains for direct fermentation of cellulose. However, the major fermentation product was butyric acid, with a small amount of acetone, ethanol and butanol. Due to the relatively slow hydrolysis of cellulose by *C. cellulolyticum*, the level of glucose in the medium was low, which resulted in low solvent production. Yu et al. [142] attempted conversion of solka floc and aspenwood xylan (basically, a lignocellulosic substrate) with co-cultures of *C. acetobutylicum* (glycolytic bacteria) and *C. thermocellum* (cellulolytic bacteria). This co culture resulted in a 1.7–2.6 fold increase in total fermentation products with effective utilization of all hydrolysis products. However, the majority of the fermentation products were acids and not solvents. This result was similar to the earlier studies of Fond et al.[141] and Petitdemange et al. [139, 140], and was attributed to a rather slow rate of hydrolysis. These results suggest the importance of the development of mutant strains of cellulolytic microbes, which would give relatively faster rate of hydrolysis (at par with fermentation rates of glycolytic species). Two-stage fermentation process [143] with co culture *C. butyricum* and *C. pasteurianum* in the first stage, and *C. beijerincki* and *C. pasteurianum* in the second stage yielded 20% more butanol. This was a consequence of high level of butyric acid production in the first stage, which was effectively reduced to butanol in the second stage. A new process reported by Ramey [144] employs continuous immobilized cultures of *C. tyrobutyricum* and *C.*

acetobutylicuim with corn as substrate, at a productivity of 4.64 g/L–h and yield of 42%. *C. tyrobutyricum* maximizes production of hydrogen and butyric acid, while *C. acetobutylicum* effectively converts butyric acid to butanol. This new route has several advantages such as elimination of products such as acetic, lactic and propionic acids

Table 2.4 presents a summary of several papers published since 1985 that investigate viability of alternate substrates for ABE fermentation when using different cultures of clostridia.

2.5 Solvent Recovery Techniques

As noted in the section 2, the formation of ABE solvents in the fermentation broth leads to inhibition of fermentation. This is a consequence of the toxic effect of butanol on the culture. Most of the *clostridia* species can tolerate total solvent concentrations up to 20 g/L. For mutant strains (hypersolvent producers) such as *C. beijerinkii* BA101, this limit is raised to 32 g/L. Solvent toxicity leads to low productivity, low concentration of solvents in the fermentation broth, and most importantly, limits the solvent yield. An obvious solution to this problem is simultaneous or *in situ* removal of the ABE solvent from the broth, to prevent inhibition of the microbial culture. This subject has been intensively investigated by numerous research groups. Most common *in–situ* product recovery systems are: liquid–liquid extraction, gas stripping, pervaporation, membrane solvent extraction (perstraction), adsorption and reverse osmosis. We describe below salient features of each of this method [34, 179, 39, 180], and later present a review of literature on ABE fermentation employing various *in–situ* solvent recovery techniques.

2.5.1 Liquid–Liquid Extraction

In this method, the fermentation broth is continuously contacted with a water–immiscible extractant that selectively extracts acetone, butanol and ethanol from the broth, leaving all other components such as nutrients, cell etc. in the broth.

Table 2.4. Summary of literature on various alternate substrates for ABE fermentation

Ref. No.	Microorganism used	Substrate
[104]	*Clostridium acetobutylicum NCIB 8052*, *C. acetobutylicum IFP 904*	Enzymatic hydrolyzates of Jerusalem artichoke juices
[145]	*C. beijerinckii*	Sulfuric acid treated and enzyme treated corn fiber hydrolyzate
[146]	*C. beijerinckii* P260	Wheat straw hydrolyzate supplemented with glucose, xylose, arabinose, galactose, mannose
[147]	*C. beijerinckii* P260	Wheat straw hydrolyzate supplemented with glucose, xylose, arabinose, galactose, mannose
[148]	*C. beijerinckii* BA101	Saccharified liquefied corn starch; LCS,
[149]	*C. beijerinckii* BA101	Hydrolyzates of fiber-rich agricultural biomass [e.g., corn fiber, distillers dry grain soluble]
[150]	*C. beijerinckii* BA101	Degermed corn/ saccharified degermed corn based P2 medium
[151]	*C. acetobutylicum* P260	Corn fiber arabinoxylan (CFAX) and CFAX sugars (glucose, xylose, galactose, and arabinose)
[152]	*C. beijerinckii* BA101	Degermed corn mash containing corn oil
[153]	*C. acetobutylicum* P262	Gelatinized sago starch
[154]	*C. beijerinckii* BA101	Spray-dried soy molasses containing the sugars dextrose, sucrose, fructose, pinitol, raffinose, verbascose, melibiose, and stachyose.
[155]	*C. acetobutylicum* ATCC 824	Domestic org. waste (DOW)
[156]	*C. acetobutylicum* DSM 1731 *C. beijerinckii* B-592	Domestic org. waste (DOW)
[157]	*C. aurantibutyricum*	Palm oil factory effluent/wastes
[158]	*Clostridium*	Crude sago palm oil methylester
[159]	*C. acetobutylicum*	Hardwood (glucan and xylan)
[160]	*C. acetobutylicum* DSM 1731	Potato waste
[161]	*C. acetobutylicum*	Agricultural lignocellulosic biomass
[162]	*C. pasteurianum* I-53; *C. acetobutylicum* ATCC 4259; *C. pasterianum* VPI 2670	Dahlia inulin
[163]	*C. acetobutylicum*	Hydrolyzate of corncobs (enzymatic, steam exploded etc.)
[164] & [165]	*C. acetobutylicum*	Simultaneous sacchrificaion and fermentation with pretreated hardwood. Pretreatment methods: (1) supercritical CO2-SO2 treatment, and (2) monoethanolamine (MEA) treatment
[166]	*C. saccharoperbutyl-acetonicum*	enzymatic hydrolyzate of bagasse
[167]	*Clostridium isolate 8p-2*	Raw cassava
[168]	*Clostridium saccharo-perbutylacetonicum DSM 2152*	High-test or invert molasses
[169]	*C. acetobutylicum*	Grape pomace hydrolyzate (acid hydrolysis)
[170]	*C. acetobutylicum*	Glucose supported with iron
[171]	*C. acetobutylicum P262*	Hydrolyzates of pine, aspen and corn stover
[172]	*C. acetobutylicum KCTC 1037*	simultaneous saccharification and fermentation of rice straw (with cellulolytic enzyme from *Trichoderma viride*)
[173]	*C. acetobutylicum ATCC 824*	Glucose supported with two vitamins, viz. biotin and p-aminobenzoic acid
[174]	*C. saccharoperbutyl-acetonicum*	Glucose with inhibitors such as butyric acid and butanol
[175]	*C. acetobutylicum and C. butylicum*	Whey supplemented with corn steep and malt sprouts
[176]	*C. acetobutylicum P262* immobilized on calcium alginate beads	Cheese whey permeate
[177]	*C. acetobutylicum*	Glucose, Lactose, or glucose plus galactose (low-Fe glucose medium)
[178]	*C. acetobutylicum NRRL 594*	Sugarcane molasses, beet molasses and defatted rice bran

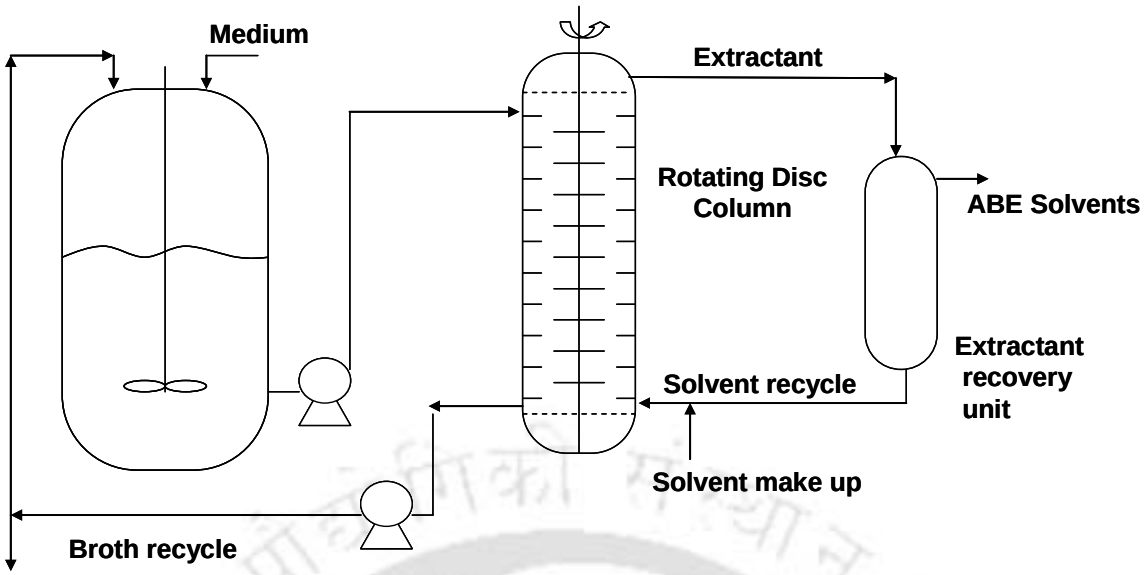

Figure 2.3. Fermentation process with product recovery using liquid-liquid extraction (redrawn with modifications from ref. [179])

A typical schematic of the fermentation process employing solvent recovery by liquid-liquid extraction using a rotating disc contactor is shown in Fig. 2.3. The ABE solvents are recovered from the extractant using distillation (usually steam stripping). The choice of extractant is the most important aspect of an integrated liquid–liquid extraction fermentation process. The extractant for the fermentation process should not be toxic to microorganisms. In addition to having high selectivity for the ABE solvents (and the highest selectivity for butanol among the three solvents), it must also have nearly zero partition coefficient for nutrients, substrates and intermediates of fermentation. Some authors [181-183] have reported removal of butyric acid (an important intermediate in the metabolic pathway of butanol) with extractant. A simple remedy to this problem would be to saturate the extractant with butyric acid prior to contacting with broth or to add supplemental acid directly to the broth. In addition, the extractant is also expected to have some obvious features such as high interfacial tension with water that would assist its easy separation, low viscosity, negligible

water solubility, high thermal stability, easy regeneration, biodegradability and high density difference which would assist countercurrent operation.

2.5.2 Use of mixed extractants

It has been observed that non–toxic solvents such as oleyl alcohol suffer from limitation of low partition coefficient. To overcome this problem, use of blended solvents is often recommended. In this technique, a low partition coefficient nontoxic solvent is mixed with relatively toxic solvent with high partition coefficient. However, these blended solvents have same limiting proportions, above which they become toxic. An example in this category is that of mixture of decanol (toxic) with oleyl alcohol (non toxic). Use of 20% decanol in oleyl alcohol allows effective extraction of ABE solvents, while keeping aqueous phase concentration below toxic limits [184]. Another motivation for use of mixed extractants is reduction in viscosity as compared to viscosity of single extractant. Some nontoxic, high partition coefficient extractants have high viscosity that has several undesired implications such as high power requirement for transportation and mixing, and low diffusion coefficient (resulting in slow mass transfer). These solvents can be mixed with other less viscous (may be relatively toxic) solvents, so as to reduce the viscosity. An example in this category is the mixture of oleyl alcohol with other solvents. Some common toxic solvents used for blending with oleyl alcohol (which is a non toxic but highly viscous extractant) are kerosene, benzyl benzoate and decane [185]. The toxicity characteristics and partition coefficients for some common extractant are listed in Table 2.5.

2.5.3 Pervaporation

This technique employs a membrane that is selectively permeable to the solvents in the fermentation broth. Thus, when contacted with fermentation broth, the ABE solvents in the broth first get solubilized into the membrane, followed by diffusion through the membrane and evaporation on the other side (or permeate side) at low pressure. These vapors

Table 2.5. Toxicity characteristics and partition coefficients for some common extractants in ABE Fermentation

Extractant	Toxicity **	Partition coefficient ***
Hexanol	Toxic	9.91–12
Hexanol	Toxic	10
Octanol	Toxic	7.33–10
Decanol	Toxic	6.2–8
Oleyl alcohol	Nontoxic	3.21–4
Castor oil	Nontoxic	2–6
Corn oil	Nontoxic	0.6–0.7
Hexadecane	Nontoxic	0.148
Kerosene	Non–toxic	0.127

** – For the cultures of *C.acetobutylicum* and *C.beijerinkii.*
*** – Data taken from [180, 184, 186]

can be recovered by either condensation or using a sweep gas that removes the vapors. A typical schematic of the fermentation process employing solvent recovery by this technique is shown in Fig. 2.4. This mechanism by which volatile solvents are removed from fermentation broth is known as solution–diffusion mechanism. The heat for evaporation of solvents is absorbed from the broth. Thus, as the pervaporation proceeds, the temperature of the broth reduces. Heat needs to be supplied to the broth to keep its temperature constant. The efficacy of a membrane for the separation of solvents is determined by two parameters: (1) selectivity of membrane, and (2) flux through the membrane (or rate of passage of volatile components per unit time per unit area).

The solvent selective (asymmetric) membranes available in market are made up of poly (dimethyl siloxane), poly (methoxy siloxane), polytetrafluoroethylene (PTFE), and silicone rubber as the skin of membrane (or the actual selective barrier) with supports made of poly (sulfone) or poly (acrylonitrile). Qureshi and Blaschek [187] have given

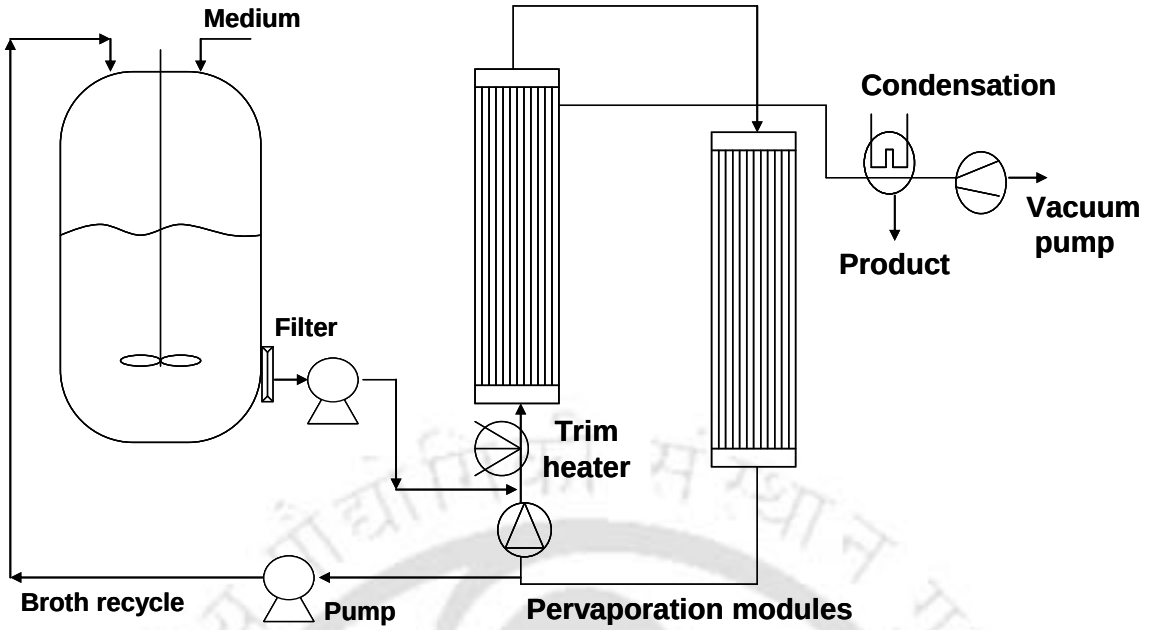

Figure 2.4: Fermentation process with pervaporative product recovery (redrawn with modifications from ref. [179])

comprehensive compilation of various membranes and their properties for recovery of butanol from fermentation broth/ model solution. In Table 2.6, we have listed some of these membranes. Overall mass transfer through the membrane has several features as follows:

(1) The mass flux through membrane is inversely proportional to the thickness of membrane.

(2) The resistance of mass transfer in liquid phase is determined by the flow rate and viscosity of the medium (Sherwood type correlation: Sh = *f*(Re, Sc)).

(3) The downstream side resistance to mass transfer (other side than fermentation broth) will be governed by pressure drop, due to transport of vapor. Large pressure drop would lead to reduction in concentration gradient and also the flux. In addition to these, temperature of fermentation broth is also a crucial factor governing flux through membrane. For every 10°C rise in the temperature of fermentation broth, the flux through the membrane doubles.

Table 2.6: Membranes for pervaporative removal of ABE solvents and their properties

Type of Membrane	Thickness (mm)	Total Flux (g/m^2–h)	Selectivity	Supplier
Poly(dimethyl siloxane) (PDMS) [188]	0.025	282–1000	15–35	GE Corporation
Poly (methoxy siloxane) PMS [188]	–	150–400	10–15	GFT Corporation, Germany
Polytetrafluoro ethylene (PTFE) [189]	0.025–0.040	35–2100	2.7–4.8	Biorecovery Inc, Hoboken
Poly(dimethyl siloxane) (PDMS) [190]	0.19	300	26.8	GFT, Germany
Zeolite filled PDMS	0.21	100–230	36.3	GFT, Germany
Silicone [191]	0.4	12.9–19.5	45–57	Rubber NV, Hilversum
Silicone [192]	0.25	–	13–30	Rubber NV, Hilversum
Silicone [193]	0.18	38–270	–	Fuji Polymer Ind., Japan
Liquid membrane [194]	0.025	25–450	180	Calanes Plastics NC, USA
Polypropylene [195]	0.4	3–10	4–5	Enka Wuppertal, Germany
Polypropylene [196]	0.4	3–10	3–6	Enka Wuppertal, Germany
Silicalite filled PDMS [197, 198]	0.306	90–237	55–105	–

Research on pervaporation integrated fermentation started in the 1980s, when Groot et al. [191, 192] from Delft University, Netherlands reported in–situ removal of solvents in IBE (isopropanol–butanol–ethanol) fermentation using 400 mm silicone tubing membrane. The productivity of continuous immobilized cultures increased by up to 70% by in-situ solvent removal, with butanol flux ~ 4 g/m^2–h and selectivity in the range 20–30. In some follow-up studies, from the same group, Groot and Luyben [199] reported butanol flux of 2.6 mL/h with selectivity of 11 at 30°C in ABE fermentation. Moreover, Groot et al. [200] carried out IBE fermentation in CSTR and fluidized bed reactors using 250 mm silicone tubing module with sweep gas for product recovery, with which the substrate consumption as well as yield increased by factor of 4. Other studies on pervaporative solvent recovery in late 1980s and

early 90s include those of Larrayoz and Puigjaner [201] and Gudernatsch et al. [202]. Friedl et al. [203] has also combined pervaporative recovery of solvent with continuous ABE fermentation using immobilized culture of *C. acetobutylicum* on a packed bed of bonechar. Maddox [204] has reviewed various membrane processes that have shown promising economics on lab scale. Liu et al. [205] have used Poly(ether block amide) (PEBA 2533) membranes for separation of acetone-butanol-ethanol mixtures by pervaporation, and have studied the effect of feed composition, operating temperature and membrane thickness on separation. A relatively thick membrane (100 microns) showed perm-selectivity in the order of n-butanol > acetone > ethanol. Liu et al. [205] have demonstrated that boundary layer effect becomes significant when a thinner (30 microns) membrane was used.

The composition of the membrane also determines the extent of mass flux through the membrane. This has been demonstrated by Qureshi et al. [197] with use of silicalite/silicone membrane. The idea underlying synthesis of silicalite/silicone membrane was to use adsorption capacity of silicalite for butanol [206, 207]. Inclusion of silicalite in silicone membrane would enhance the selectivity of membrane as silicalite adsorbs butanol onto it. The improvement in selectivity of the composite membrane was 5 fold. The composite membrane was found to be superior to other membranes with a life of 3 years [197]. Productivity of the ABE solvent in fed batch fermentation process (integrated with in–situ solvent recovery with composite membrane) was 155 g/L. High selectivity of composite membrane yielded up to 700 g/L of butanol concentration in permeate.

2.5.4 Perstraction

Perstraction operation essentially combines principles of pervaporation and liquid-liquid extraction in that it is a solvent extraction process combined with membrane extraction. Here a membrane contactor (preferably in the form of hollow fibers) provides a surface area with two immiscible phases (fermentation broth and the extractant) to exchange butanol. A

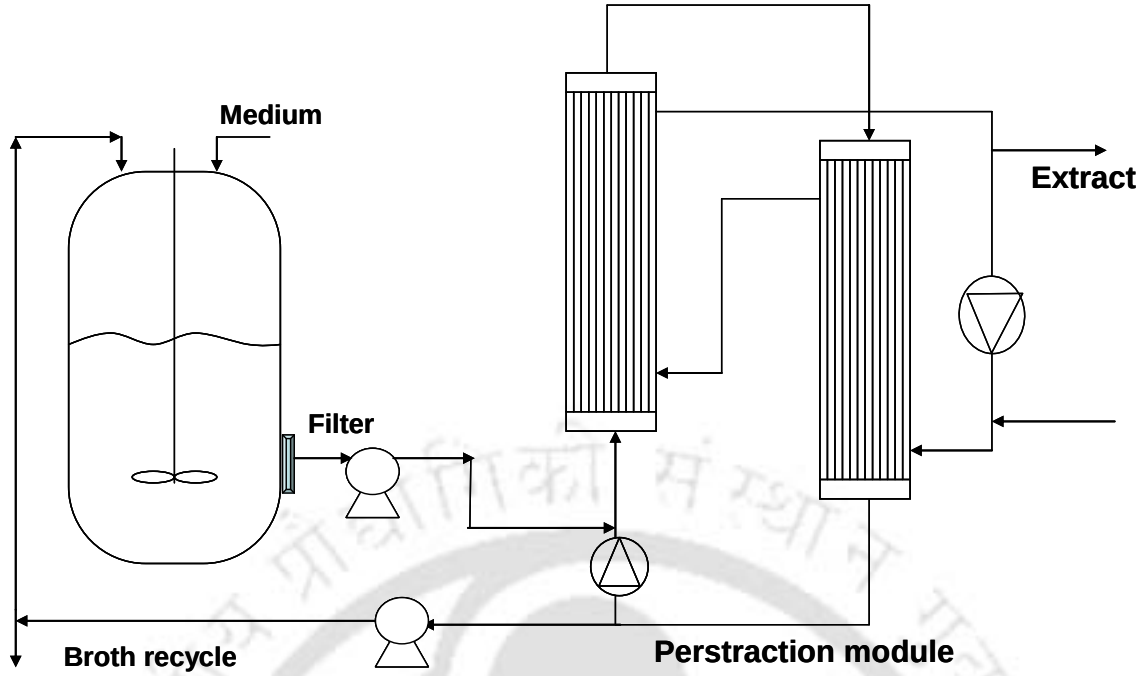

Figure 2.5: Fermentation process with solvent recovery using perstraction modules (redrawn with modifications from ref. [179])

typical schematic of the fermentation process employing solvent recovery by perstraction is shown in Fig. 2.5. This process overcomes several shortcomings of the liquid–liquid extraction process such as, solvent toxicity, emulsification, and cell aggregation at liquid–liquid interface. Another advantage is independent control over the flow rate of broth and extractant. Arrangement of loops of hollow fiber membranes in series for operation in countercurrent mode gives a true plug flow character to both phases, and an effective removal of butanol. Overall mass transfer in this case is determined by the individual mass transfer coefficient on aqueous (fermentation broth) and organic (extractant) side as well as the mass transfer coefficient of membrane. Although mass transfer coefficient of membrane is difficult to estimate, typical values are ~10^{-7} m/s can be assumed. Matsumara and coworkers [208, 209] and Groot et al. [186] have measured a value of mass transfer coefficient as 5.6×10^{-7} m/s. With this, the overall resistance to mass transfer is mostly contributed by the membrane itself (~86%). A possible solution to reduce the mass transfer resistance is to increase the

diameter of the hollow fiber membrane, with concurrent rise in the volumetric flow rate, so as to maintain constant velocities through the fiber; however, stability of the membrane at these conditions needs to be accounted for [179].

2.5.5 Liquid membranes

A possible solution to large mass transfer resistance offered by dense polymeric membranes is in term of liquid membranes. This process requires just a small amount of solvent to cover the support membrane. The fluidity of the organic film leads to the higher diffusion coefficient and high fluxes of solvent. However, the stability of the film is the major problem. Literature on use of liquid membrane for pervaporation is limited. Matsumara and Kataoka [193] have reported pervaporation through liquid membrane supported with hydrophobic microporous polypropylene flat sheet. The stability of membrane was found to depend on surface tension of feed solution. As long as it was less than critical surface tension of support membrane, the liquid membrane film stayed intact. The principle components of the liquid membranes were oleyl alcohol, di–n–butyl phthalate and tricresyl phosphate. Presence of oleyl alcohol in membrane imparted high selectivity to the membrane. In another study, Matsumara et al. [194] applied technique of pervaporation using oleyl alcohol liquid membrane for in-situ solvent removal in continuous butanol/isopropanol fermentation with immobilized *C. isopropylicum* on Na–alginate beads. The solvent recovery module was separated from fermentation unit. The support for liquid membrane was microporous polypropylene flat sheet (25 mm thick). The solvent recovery increased the productivity 2 times, with butanol concentration of 230 g/L in permeate (which is 30 fold higher than the fermentation broth). However, several practical problems were faced such as fouling of the membrane surface with some viscous impurities, and non-uniform distribution of culture

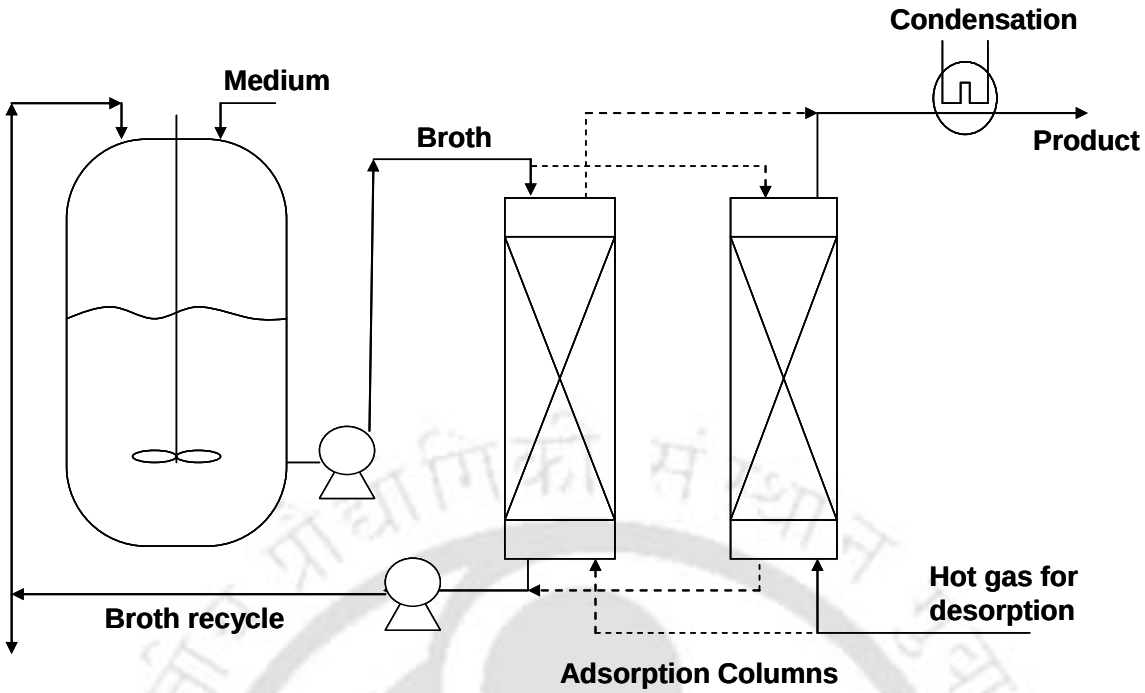

Figure 2.6: Fermentation process with integrated product recovery with adsorption (redrawn with modifications from ref. [179])

broth into each permeation cell of the membrane module. As a result, the experimental values of butanol flux were smaller than the theoretically estimated values.

2.5.6 Adsorption

Use of adsorbents that have high affinity for alcohol for removal of solvents from fermentation broth is a viable technique for in-situ product recovery. Being solid phase, the adsorbents have advantage of ease of separation in addition to other merits such as non-toxicity and ease of regeneration/reuse. Direct addition of adsorbents to the broth does not yield effective removal of solvents as other components in the broth may compete for adsorption sites. Moreover, the cells in the broth may form "biofilm" on adsorbent surface that would hinder effective and selective adsorption of butanol. These limitations necessitate an "off–line" or separate unit for adsorptive removal of solvents, where cell-free broth obtained by membrane filtration/centrifugal system can be contacted with the bed of

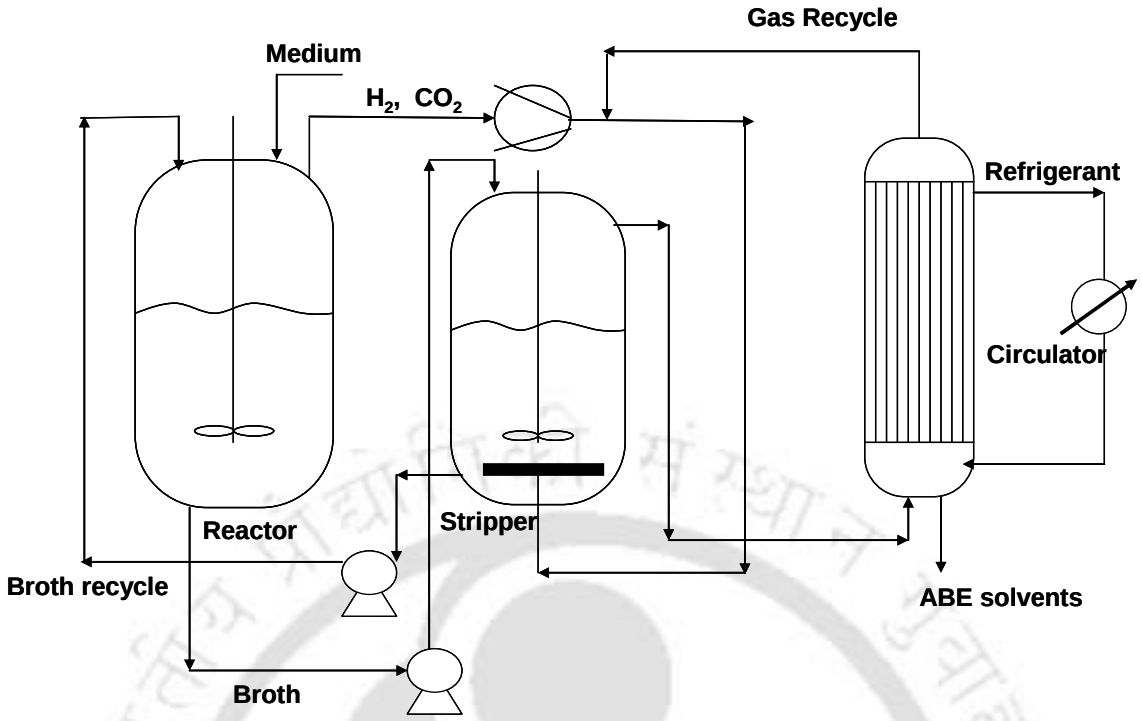

Figure 2.7: Fermentation process with integrated product recovery with gas stripping (redrawn with modifications from ref. [179])

adsorbent. A schematic of the fermentation process integrated with adsorption columns for solvent recovery is shown in Fig. 2.6.

The most popular alcohol selective adsorbents are activated carbon, silicalite, polymeric adsorbents such as polystyrene cross–linked resins, zeolite, and molecular sieves. Qureshi et al. [45] have done comparative assessment of various adsorbents such as silicalite, resins (XAD-2, XAD-4, XAD-7, XAD-8, XAD-16), bone charcoal, activated charcoal, bonopore, and polyvinylpyridine. In this study, use of silicalite (with adsorption capacity of 85 mg butanol/g silicalite) appeared to be more attractive as it could concentrate butanol solution from 5 g/L to 790-810 g/L. The adsorbed solvents could be completely desorbed. Regeneration of silicalite for reuse could be done using heat treatment. The energy requirement for butanol recovery by adsorption-desorption was calculated to be 1948 kcal/kg butanol, which is significantly smaller than energy requirement for other techniques such as

steam stripping distillation (5789 kcal/kg butanol), gas stripping (5220 kcal/kg butanol) and pervaporation (3295 kcal/kg butanol).

Other adsorbents such as activated carbon and polymeric resins suffer from fouling by cells and other media components [210]. Another problem with polymeric resins is adsorption of butyric and acetic acids due to which, solvent yield reduces [210-212] have reported ~ 4 fold reduction in adsorption of butanol from fermentation broth (~27 mg/g) as compared to pure aqueous solution (~83 mg/g) with amberlite XAD–4 resin. Similar reduction is seen for Bonopore (copolymer of divinyl benzene and styrene). The problem of adsorption of nutrients was severe for XAD–4 resins, in that no culture growth or butanol formation was seen in media treated with XAD–4 resin. On the other hand, Bonopore did not affect fermentation characteristics of the medium. These limitations render adsorption infeasible for larger scale operation. Modification in adsorbent characteristics such as increasing hydrophobicity and selectivity are possible means of improving the feasibility of adsorptive recovery of butanol.

2.5.7 Gas stripping

The technique of gas stripping involves removal of ABE solvents in the gas phase, followed by recovery of solvents through condensation. A schematic of the fermentation process integrated with adsorption columns for solvent recovery is shown in Fig. 2.7. The process is governed by vapor–liquid equilibrium. Oxygen free nitrogen or gases produced during fermentation (CO_2 and H_2) are sparged through fermentation broth for this purpose. These gases capture ABE solvents, which can be condensed. The gas can be recycled back. The principal advantage of this technique is its simplicity, which makes it most suitable for application on large scale. Other merits of this technique are:

1. Volatile products in clean form (as nonvolatile products such as organic acids , nutrients and cells are not removed).

2. The gas generated in the fermentation itself can be used for stripping of broth. No external gas is required.

3. Unlike pervaporation, the rate of removal of solvents is not limited by mass transfer resistances. The rate of mass transfer can be increased by improving gas liquid contact mode; however, gas stripping technique is not as selective as membrane based process, as it is governed by vapor-liquid equilibrium.

Countercurrent contact of fermentation broth with stripping gas on structured or random packing helps increase the contact area, and hence, rate of removal. This mode of contact is perhaps better than direct sparging of gas through broth, where it can be "short circuited" through the broth, without getting properly dispersed in the broth.

4. This technique was initially applied to ethanol fermentation [213] in an immobilized cell reactor separator. By countercurrent contact of liquid and fermentation gas in a trickle bed reactor, about 20% increase in productivity was achieved. The technique of gas stripping was applied to ABE fermentation in batch culture (*C. acetobutylicum* on whey permeate) by Ennis et al. [214]. Gas stripping resulted in selectivity for removal of butanol as 19.3, with concurrent ~ 50% rise in the yield. A summary of published literature employing various solvent recovery techniques is given in Table 2.7 in chronological order.

2.6 Fermentation Process Design

2.6.1 Batch fermentation

The simplest mode of fermentation is the batch fermentation. This is essentially a mechanically stirred vessel with other accessories such as gas sparger, heating/cooling jacket etc. To start with, the substrate and supplementary nutrients are charged in the vessel. Typical substrate concentration is 60–80 g/L (as mentioned earlier, this is limited by the product inhibition effect). The reaction mixture is autoclaved at approx. 120^{o}C followed by cooling to

Table 2.7: Summary of literature on solvent recovery techniques in ABE fermentation

Ref. No.	Mode of Fermentation and Culture & Substrate Employed	Solvent Recovery Technique
[148]	Batch and fed-batch fermentation with liquefied corn starch (60 g/L) and saccharified liquefied corn starch (225.8 g/L) as substrate and *C. beijerinckii* BA 101 culture.	Gas stripping
[159]	Simultaneous saccharification and extractive fermentation (SSEF), fed-batch, *C. acetobutylicum* with monoethanolamine pretreated Hardwood chips.	Semi permeable membrane
[160]	*C. acetobutylicum* DSM 1731 with potato waste as substrate.	Polypropylene perstraction system and an oleyl alcohol/decane mixture as extractant with microfiltration using hydrophilic membrane.
[193]	Simultaneous extraction-stripping process. *C. acetobutylicum*	Pervaporation through a liquid Membrane (oleyl alcohol) supported with a hydrophobic porous membrane
[201]	*C. acetobutylicum* with glucose as substrate.	Pervaporation
[207]	2-stage continuous reactor employing immobilized cultures of *C. acetrobutylicum* on bonechar utilising whey permeate as substrate	Gas strpping
[212]	Batch culture, *C. acetobutylicum*	Adsorbents for extractive bioconversion
[215]	Batch and continuous fermentation with broth recycle using *C acetobutylicum* DSM 2152 in a glucose based medium	Gas stripping
[216]	Packed bed reactor operated in vertical and inclined mode and Fluidized bed fermenter with immobilized costridias on bonechar using whey permeate as substrate.	Gas stripping
[217]	Trickle bed reactor using *C acetobutylicum* immobilized in polyester sponge strip in a glucose based medium.	Gas stripping
[218]	Continuous culture fermentation, *C. acetobutylicum* ATCC 824 with glucose (4 wt%) in minimal medium as substrate	Pervaporation using ionic liquid PDMS ultra-filtration membrane
[219]	Batch reactor with gas sparger and impeller, glucose (60 g/L) with P2 stock medium as substrate and *C. beijerinckii* BA 101 culture.	Gas stripping
[220]	Batch reactor with whey permeate supplemented with lactose as medium and *C. acetobutylicum* P262 as the culture	Perstraction with membrane and oleyl alcohol as perstraction solvent.
[221]	Fed-batch fermentation, *Clostridium beijerinckii* BA101 with glucose as substrate.	Gas stripping
[222]	Batch fermentation, *Clostridium beijerinckii* BA101 with glucose as substrate.	Gas stripping
[223]	Batch and continuous fermentation, crude palm oil	Extraction with biodiesel as extractant in batch and continuous mode
[224]	Fed-batch reactor**,** *C. acetobutylicum,*	Pervaporative recovery using silicalite-silicone composite membrane
[225]	Manually prepared ABE mixture in different proportions	Pervaporation with various membranes like Styrene Butadiene Rubber (SBR), Ethylene Propylene Diene Rubber (EPDM), plain Poly Di-Me Siloxane (PDMS) and silicalite filled PDMS.
[226]	Batch Fermentation, *Clostridium beijerinckii* BA101 in P2 and corn steep liquor CSL based medium.	Pervaporation membrane
[227]	Batch fermentation, *C. beijerinckii* BA101 with glucose as substrate.	Pervaporative recovery using silicone membrane.
[228]	Batch fermentation, *C. saccharo-perbutylacetonicum* N1-4 (ATCC 13564 with glucose as substrate.	Extractive acetone-butanol-ethanol fermentation using methylated crude palm oil as extractant.
[229]	Packed bed or fluidized bed reactor for continuous fermentation, *C. acetobutylicum*, with whey permeate as substrate.	Liquid-liquid extraction, extractant used: oleyl alcohol, benzyl benzoate and di-Bu phthalate
[230]	Repeated fed-batch fermentation with continuous product removal and cell recycle, *C. acetobutylicum.*	Extraction process using polyvinylpyridine (PVP) as an adsorbent.

[231]	Batch fermentation, *C. acetobutylicum* with glucose as substrate.	In situ adsorption using polyvinylpyridine
[232]	Extractive acetone-BuOH-EtOH (ABE) fermentation, *C. aceto-butylicum B18.*	Pervaporation module made of silicone membrane
[233]	Continuous fermentation, *C. acetobutylicum*	In situ Gas stripping
[234])	Continuous acetone-butanol fermentation, *C. acetobutylicum* ATCC 824	separation by reverse osmosis using polyamide membrane
[235]	Batch mode, C. acetobutylicum on glucose	Liquid-liquid extraction WITH 63 organic solvents (alkanes, alcohols, aldehydes, acids and esters)
[236]	Batch and continuous fermentation, *Clostridium* with glucose as substrate.	Coupled ultra filtration and distillation
[237]	micro porous hollow fiber based tubular fermentor-extractor, immobilized *C. acetobutylicum*	In situ dispersion-free extraction
[238]	Continuous fermentation, *C. acetobutylicum* with glucose as substrate.	Pervaporation
[239]	Continuous fermentation, *C. acetobutylicum* with glucose as substrate.	Membrane-assisted extraction
[240]	Batch fermentation, C. acetobutylicm with glucose	Liquid liquid extraction, Fermentation medium mixed with 1-octanol or 2-ethylhexanol
[241]	Fed-batch fermentation, *C. acetobutylicum* with glucose as subsrate.	Silicone membrane with oleyl alcohol and polypropylene glycol as extractant.
[242]	*C. acetobutylicum*, synthetic medium.	Supercritical extraction with CO_2.
[243]	Fed-batch extractive fermentation, *C. acetobutylicum* with glucose as substrate.	Oleyl alcohol extraction.
[244]	Extractive fermentation, *C. acetobutylicum* with P262, glucose, glucose-xylose mixtures, and hydrolyzates of lignocellulosic as substrates.	Extraction with: dibutyl phthalate.
[245]	Clostridium fermentation	Various solvents for extraction
[246]	Continuous fermentation, *C. acetobutylicum*	Hollow-fiber ultrafilter to separate and recycle cells.
[247]	Coupled continuous fermentation and ultrafiltration.	Continuous fermentation with Recycle
[248]	Dialysis fermentation, *C. acetobutylicum* ATCC 824 with corn, potato, and glucose as substrate.	Dialysis Fermentation.
[249]	Extractive fermentation, *C. acetobutyicum* IAM 19012.	Liquid – Liquid Extraction oleyl alcohol and C-20 guerbet alcohol

35–37^{o}C. It is then inoculated with seed culture. The anaerobic condition in the broth is maintained by sweeping oxygen free nitrogen or carbon dioxide over the surface of broth. As already discussed in section on substrate for fermentation, some substrates need pretreatment (or upstream processing) such as grinding, acid/enzyme hydrolysis, dilution, filtration, etc. The typical batch time for fermentation (depending on culture used and other operating condition.) is 48–72 h. As the concentration of solvent reaches 20 g/L (or 33 g/L for hyper–butanol producing culture), inhibition of cell growth occurs that stops the fermentation. At the end of fermentation, cell mass and other solids are removed by centrifugation and liquid portion is sent to product recovery unit, (which is basically a train of distillation column).

Typical volume of a batch fermeter ranges from few 100 liters to up to 800,000 L (or 800 m^3) [50].

2.6.2 Fed-batch fermentation

This mode of fermentation is applied when high concentration of substrate is toxic to microbial culture. The reactor is initiated in batch mode with low substrate concentration. Typically, broth occupies about half of the fermenter volume at the beginning. Later, as the substrate is consumed by the culture, additional substrate is added at a slow rate, so as to keep the total solvent concentration in the broth below toxic level. With this the total volume of fermentation broth rises, unless there is simultaneous withdrawal of the solvents. For no withdrawal of the product, the process can continue until the broth occupies about 75% of the fermenter volume. As far as ABE fermentation is concerned, the batch mode fermentation has to be coupled to a suitable solvent recovery technique to prevent product inhibition of Clostridial cultures. Results of several studies with different microbial cultures and substrates in fed-batch mode are summarized in Table 2.4 and 2.8.

2.6.3 Continuous fermentation

The technique of continuous fermentation is aimed at increasing the productivity of reactor. However, this advantage is compensated by relatively low product concentration, as compared to the batch process. Another problem pertaining to continuous culture systems is fluctuating levels of solvents. The solvent production may not be stable over time and reduce with time (with simultaneous rise in the acid concentration) Single stage continuous fermentation studies by Leung and Wang [277] produced 15.9 g/L of solvent with *C. acetobutylicum* with yield of 0.32 g/g and productivity of 1.5 g/L–h. Similarly, Monot and Engasser [278] reported productivity of 0.4 g/L–h at solvent concentration of 12 g/L. Two or multiple stage fermenter systems have been extensively investigated by several researchers.

Table 2.8: Summary of literature on bioreactors and fermentation processes

Ref. No.	Microorganism and Substrate	Details of Bioreactor and/or fermentation technique
[183]	*C. acetobutylicum* with glucose	Fermentation carried out in fluidized-bed bioreactor; Extraction of butanol from broth using oleyl alcohol.
[185]	*C. acetobutylicum* with glucose	Fed-batch extractive fermentation.
[243]	*C. acetobutylicum* with glucose	Batch extractive fermentation with six extractants, viz. kerosene, tetradecanol in kerosene, dodecanol in kerosene, oleyl alcohol, oleyl alcohol in decane and oleyl alcohol in benzyl benzoate.
[246]	*C. acetobutylicum*	Continuous fermentation with hollow fiber ultrafilter for separation and cell recycle.
[250]	*C. sachharobutylicum* N1-4; 150 g fresh grated potato with glucose	Solventogenic cells of the broth concentrated 10 times by membrane filtration; Continuous culture with cell recycling and bleeding.
[251]	*C. acetobutylicum* with glucose medium	Effect of fermentation conditions and role of additives on production of solvents studied.
[252]	*C. beijerinckii* NRRL B592 with 60 g/L of glucose with 5 g/L of yeast	Two phases of batch growth mimicked in two-stage continuous process. Cells grown in first stage acidogenically; transferred to second stage that mimicked solventogenic phase at acid break point.
[253]	*C. beijerinckii* NRRL B592 with glucose media with yeast extract	Series of 5 fermentors (lab scale stirred tank bioreactors) used without pH control
[254]	C. *acetobutylicum* with glucose medium in variable concentration	Bioreactor with in-situ spinning filter to achieve increasing cell density and continuous removal of inhibitory products.
[255]	*C. acetobutylicum*	Gas-sparged fluidized bed continuous fermentation with varying bead concentrations and gas sparging rate. Culture was immobilized in Ca alginate pellets.
[256]	*C. acetobutylicum* B18 on Supplemented YEM	Controlled pH batch Butanol- Acetone fermentation.
[257]	*C. acetobutylicum* with glucose	Gas-sparged reactor with calcium alginate-immobilized continuous culture.
[258]	*C. acetobutylicure* NCIB 8052, in RCM (Oxoid) with added glucose.	Continuous culture of alginate-immobilized Clostridias.
[259]	*C. acetobutyficum P262* on sweet potato with phosphate and nitrogen.	Response surface Methodology (RSM) applied for optimization of alcoholic fermentations.
[260]	*C. acetobutylicum* with saccharified and nonsaccharified starch	Continuous bioprocess of starch. A 2-stage process was developed for continuous ABE production from saccharified starch.
[261]	C. acetobutylicum with glucose	Optimization of pH and dilution rate is done with two stage continuous fermentor.
[262]	*C. acetobutylicum* with 20 g/L, 38.7 g/L and 60 g/L glucose.	Continuous fermentation in immobilized cell trickle bed reactor consisting of 2 serial glass columns packed with cells adsorbed on polyester sponge.
[263]	C. acetobutylicum with glucose	Conditions for maximum production of solvents determined with varying dilution rate to calculate yield and productivity in continuous culture.
[264]	*C. acetobutylicum* with glucose	Multiphase Fluidized-Bed Bioreactor (MPFB). A sufficiently active and stable biocatalyst developed by trapping bacterial cells in Ca alginate beads.
[265]	*C. acetobutylicum* with two salt based media (supplemented withyeast extract and peptone)	Immobilized cell trickle bed reactor consisting of 2 serial columns packed with Clostridium entrapped on the surface of natural sponge segments.
[266]	*C. acetobutylicum* with glucose.	Stable two stage continuous process with different dilution rates in 1st and 2nd fermentor.
[267]	*C. acetobutylicum* on glucose	Fed-batch operation; extraction in Karr reciprocating plate column using oleyl alcohol.
[268]	*C. acetobutylicum* ATCC 824	Continuous chemostat fermentation performed in a partial gas recycle system.
[269]	*C. acetobutylicum* NCIB 8052 with glucose	Chemostat under conditions of glucose, NH_4^+, PO_4^{3}, Mg^{2+}, and Fe^{2+} limitation
[270]	C. acetobutylicum P262 on whey permeate	Batch fermentation used. Effect of pH and lactose concentration on solvent production was studied.

[271]	*C. saccharoperbutyl-acetonicum* with glucose	Batch chemostat fermentation
[272]	*C. acetobutylicum*	Solvent production by batch culture in product limited chemostat. Influence of limiting nutrient studied through continuous process.
[273]	*C. acetobutylicum* ATCC 824 (DSM 792) in complex synthetic medium	Continuous culture of *Clostridium acetobutylicum* with cell recycling.
[274]	*C. acetobutylicum*	Fermentation observed at varied impeller speed and its effect on solvent production.
[275]	*C. acetobutylicum* (ATCC 824) on RCM supplied with oxoids.	Effect of increased hydrogen partial pressure on AB fermentation studied by pressurizing the head space of fermentor using ultra-pure hydrogen gas.
[293]	*C. acetobutylicum*	Batch fermentations at varied agitation rates and either pressurized or non-pressurized mode.

This system aims at separating the acidogenic and solventogenic phases of fermentation in cascade of 7–11 stages of fermenter [279, 280]. Some other studies published in the 1980's are those from Bahl et al.[55] and Afscher et al. [273]. Bahl et al. [55] studied two–stage phosphate limited fermentation of *C .acetobutylicum* in a chemostat and reported solvent concentration of 18.2 g/L with yield of 0.34 g/g and productivity of 0.55 g/L–h. The final solvent concentration in this system approached the limit of 20 g/L in a batch process. Afscher et al. [273] combined continuous fermentation with cell recycle, in order to overcome the problem of selection of acid producing cells and cells degeneration occurring at high solvent concentration. The productivity of solvents was ~ 2–3 g/L–h with total yield of 12–15 g/L.

Meyer and Papoutsakis [281, 282] have analyzed the solvent production in steady state continuous cultures of *C. acetobutylicum* and have deduced the effect of biomass recycle at various dilution rates and recycle ratios on product yield and selectivity under both glucose and non-glucose limited conditions. For glucose limited cultures, Meyer and Papoutsakis [281, 282] derived following relationships between butanol ($Y_{B/G}$) and butyrate ($Y_{BE/G}$) yields and the ATP ratio (R_{ATP}, which is an energetic parameter). The physical interpretations of these relations were as follows: 1. For non-glucose limited cultures, the

Table 2.9: Summary of literature on ABE fermentation using immobilized culture systems

Ref. No.	Microorganism and Immobilization Support	Substrate
[176]	*C. acetobutylicum* P262 entrapped in alginate beads.	Cheese whey permeate
[203]	*C. acetobutylicum* immobilized onto a packed bed of bonechar.	Lactose
[283]	*C. saccharoperbutylicum* N-1 4 (ATCC 13564). Active immobilization in alginate & passive immobilization with stainless steel rubber, nylon scrubber poly-urethane foam (uniform and non uniform pores) and palm oil empty fruit bunch fiber.	TYA Medium
[284]	*C. acetobutylicum* ATCC 55025 immobilized on a Fibrous matrix	Butyric acid medium containing of butyric acid and glucose
[285]	*C. acetobutylicum* immobilized on sodium alginate polyacrylamide, activated carbon and silica gel carriers.	Glucose
[286]	*C. acetobutylicum* on Calcium alginate	Cane sugar molasses.
[287]	Eight *C. acetobutylicum* strains (B-591, B-594 and P-262) immobilized in Ca alginate beads	De-fibered sweet potato slurry, supplemented with potassium phosphate and cysteine-HCl
[288]	*C. beijerinckii* BA101 adsorbed onto clay brick	Glucose
[289]	*C. acetobutylicum* adsorbed on bonechar.	Lactose
[290]	*C. acetobutylicum* NRRL-B-643 co-immobilized with membranes in κ-carrageenan gel beads.	Glucose
[291]	*C. acetobutylicum* immobilized on alginate and bonechar	Whey permeate
[292]	*C. acetobutylicum* DSM 792 (ATCC 824) immobilized on alginate, κ-carrageenan and chitosan.	Complex Glucose based medium.
[293]	*C. acetobutylicum* spores immobilized in Ca alginate beads.	Glucose-based medium

ratio $Y_{B/G}/Y_{E/G}$ is close to 8, which agrees well with experimentally observed ratio of 9 to 10; 2. Alcohol production depends on growth, but inversely; 3. No butanol or ethanol will be produced if the specific growth rate is higher than 0.36 h^{-1} (for butanol) and 0.48 h^{-1} (for ethanol). These relations also help choosing the operating conditions of continuous fermentation in order to achieve desired yield. The maximum possible molar yield of butanol in continuous culture, as predicted by these relations is 60%. Continuous culture systems have been extensively investigated for various substrate and cultures. We have given a summary of the literature in this area in Table 2.4 and 2.8. In addition, Table 2.8 also presents an overview of studies with various kinds of reactor designs for fermentation process.

2.6.4 Immobilized culture systems

The immobilized culture systems have severe distinct merits over free cell system as follows [12]: 1. Physical retention of cells on the matrix, 2. Ease of separation of cells from products, 3. High cell density per reactor volume, 4. High cell concentration, 5. Smaller reactor volumes, 6. Greater productivity, 7. Flexibility of reactor design (such as fixed bed, trickle bed, fluidized bed etc.) for continuous operation, 8. Control over reaction kinetics, 9. Minimum nutrient depletion and product inhibition, 10. Better mass transfer characteristics due to reduced viscosity of the medium. The demerits of the immobilized system are, however, mass transfer limitation of substrate and activity loss due to immobilization. Another operational problem is accumulation of bubbles of gas produced during fermentation within immobilization matrix, due to which contact between cell and substrate is hindered, resulting in improper utilization of substrate.

A summary of literature on use of different immobilized culture system for fermentation of various substrates is given in Table 2.9. Early contributions to this area are from research group at Delft University of Technology [26, 294, 295]. Cultures of *C acetobutylium* were immobilized over calcium alginate beads and used in both batch and continuous fermentation. The solvent yield was between 1.44–4.53 g/L. The major limitation was loss of activity of the immobilized cells with time, which was more pronounced in non–growth media. Another substrate for immobilization was beechwood shaving. Being a cheap adsorbent with relatively simple adsorption technique, this immobilization system had potential for large scale application. Ennis et al. [214] also investigated continuous production of cheese whey permeate using alginate–entrapped cells of *C. acetobutylium* in stirred tank as well as fluidized bed reactor. 6 fold increase of productivity (as compared to batch fermentation) was seen with 40% lactose utilization.

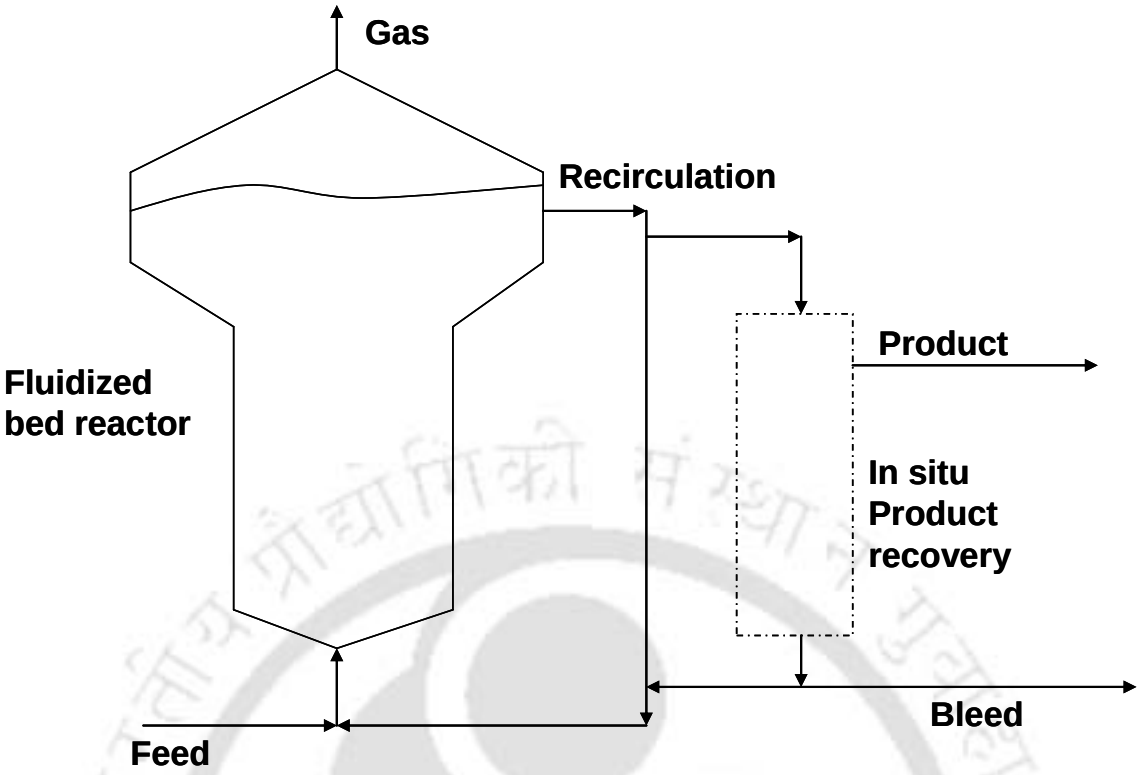

Figure 2.8: Fluidized bed reactor with integrated in-situ product recovery (redrawn with modifications from ref. [179])

In another study, Qureshi and Maddox [216] investigated performance of cells of *C. acetobutylium* immobilized by absorption onto bonechar for fermentation of whey permeates. Two reactor configurations were used, viz. packed bed and fluidized bed (as shown in Fig. 2.8). The productivity of packed bed (6 g/L–h) was higher than that of fluidized bed (4.8 g/L–h). However, fluidized bed gave more stable operation for 2000 h. Problem faced with packed bed reactor were blockage due to excess biomass production and gas holdup. More recently, several new immobilization supports have been attempted such as polyurethane foam, nylon scrubber, fibrous bed bioreactor, polyacrylamide, activated carbon, silica gel etc.

2.7 Mathematical Models of ABE Fermentation

In this section, we review the mathematical models developed during past two decades by various research groups. We give herewith summary of these studies. Nonetheless, we have described two models, by Paputsakis and Shinto et al., which have made notable contribution in greater detail.

2.7.1 Fermentation Equations of Papoutsakis

An important contribution to modeling of ABE fermentation was made by Papoutsakis [296], who derived fermentation equations for butyric acid bacteria. For anaerobic fermentation, the stoichiometric balance equation for carbon, hydrogen, oxygen and nitrogen is written in the form:

$$CH_mO_l + a\ NH_3 = y_c\ CH_pO_nN_q + z\ CH_rO_sN_t + b\ H_2O + c\ CO_2 \qquad (7.1)$$

where CH_mO_l, $CH_pO_nN_q$ and $CH_rO_sN_t$ denote elemental compositions of organic substrate, microbial biomass and extracellular products respectively. Out of these, numerical values of subscripts *m*, *l*, *r*, *s* and *t* are known from molecular formulae of organic substrates and products. The numerical values of subscripts *p*, *n* and *q* are related to the microbial biomass and can be determined by elemental analysis of biomass. The degrees of reductance (γ) of the above compounds, defined as number of equivalents of available electrons per carbon atom of compound are written as: γ_s (substrate) = $4 - m - 2l$; γ_b (biomass) = $4 + p + 2n - 3q$; and γ_p (extracellular products) = $4 + r - 2s - 3t$. The available electron balance for overall fermentation equation was: $\gamma_s = y_c\gamma_b + z\gamma_p$. Next, defining representative composition of microbial biomass as $C_4H_{4p}O_{4n}N_{4q}$, Papoutsakis [296] used two well established irregularities as follows: (1) weight fraction of carbon in the biomass, σ_b, and (2) reductance degree of biomass, γ_b are relatively constant with coefficients of variation 5 and 4% as: $\sigma_b = 0.462 \pm 0.023$ and $\gamma_b = 4 + p - 2n - 3q = 4.291 \pm 0.172$. The typical value of dry biomass produced

per mole ATP (in gram per mole) used in biosynthesis is 10.5 g/mol. From this, the ATP requirement for biomass synthesis per 2 mol glucose incorporated into biomass is 29.7 mol, while 1.75 mol $NADH_2$ are needed for every 2 mol of glucose incorporation into biomass that brings glucose to oxidation level of $C_4H_{4p}O_{4n}N_{4q}$. Thus, overall equation representing synthesis of biomass from glucose is:

$$2\text{Glucose} + 1.75\text{NADH}_2 + 29.7\text{ATP} \longrightarrow 3\text{C}_4\text{H}_{4\text{p}}\text{O}_{4\text{n}}\text{N}_{4\text{q}} \qquad (7.2)$$

On the basis of stoichiometric balance of 12 reactions in the EMP pathway (after multiplication with coefficients *1*, *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, *i*, *j* and *k*) the following comprehensive equation was obtained:

$$\begin{aligned}&(2+a)\,\text{glucose} \longrightarrow 3\text{C}_4\text{H}_{4p}\text{O}_{4n}\text{N}_{4q} + (2a-b-2k)\,\text{pyruvate} + \\ &(b-e-f-g-h-i)\,\text{AcCoA} + (2a-1.75+d-2f-2g-i-2j)\text{NADH}_2 \\ &+(b-c-d)\,\text{FdH}_2 + (2a-29.7+e)\,\text{ATP} + (e+g-f-h)\,\text{acetate} \\ &+(f-g)\,\text{butyrate} + cH_2 + (b+h+2k)\,\text{CO}_2 + g\,(\text{butanol}) + (h-i)\,\text{acetone} \\ &+i\,(\text{isopropanol}) + j\,(\text{ethanol}) + k\,(\text{acetoin})\end{aligned} \qquad (7.3)$$

This equation is known as fermentation equation for butyric acid bacteria. The relations between various coefficients of fermentation equation are as follows:

(1) The fermentation intermediates such as AcCoA, pyruvate, $NADH_2$ and FdH_2 do not accumulate in the fermentation broth and their coefficients could be set to zero. Thus, four equalities are obtained as: $2a-b-2k=0$; $b-e-f-g-h-i=0$;

$2a-1.75+d-2f-2g-i-2j=0$ and $b-c-d=0$.

(2) In addition to the above equalities, the coefficients of the products of ABE fermentation, which can accumulate in the broth, are set either equal to or greater than zero to yield following inequalities. ATP: $2a-29.7+e\geq 0$; Acetate: $e+g-f-h\geq 0$; Butyrate: $f-g\geq 0$; CO_2: $b+h+2k\geq 0$; Acetone: $h-i\geq 0$; Hydrogen: $c\geq 0$; Butanol: $g\geq 0$; Isopropanol: $i\geq 0$; Ethanol: $j\geq 0$; Acetoin: $k\geq 0$.

In addition to these, following inequalities are obtained on the basis that glucose, pyruvate and acetyl–CoA have non–zero concentrations. Glucose: $a > 0$; Pyruvate: $b > 0$; Acetyl–CoA: $e \geq 0$. In addition, the inequalities $f - g \geq 0$ and $h - i \geq 0$ also necessitate that $f \geq 0$; $h \geq 0$. Thus, the overall fermentation equation model of Papoutsakis [296] comprises of 12 equations and 16 variables. Obviously, if numerical values of 12 variables are known, rest 4 can be calculated. Papoutsakis [296] has validated this model against several experimental data reported by previous authors [297-300]. More recently, Chauvatcharin et al. [301] have applied fermentation model of Papoutsakis [296] for analyzing culture metabolism of AB fermentation to provide different physiological states of fermentation. Papoutsakis and Meyer [302, 303] have extended this theme to obtain fermentation equations of propionic acid bacteria and butanediol and mixed acid fermentation.

2.7.2 Model development by group at TU Delft

Research group at Technical University of Delft (Netherlands) has developed series of models for the continuous ABE fermentation by immobilized clostridia. The initial model reported by this group [304, 305] was based on 9 physical, kinetic and physiological parameters. However, this model did not take into account product inhibition, which is an important aspect governing overall yield of fermentation. Moreover, immobilization matrix was modeled as an inhomogeneous sphere with an inactive core. Thus, the biomass distribution within the matrix was not taken into account. In later publications [306, 307] improvements in the model were made to account for the above effects. The salient features of the model were as follows: (1) The bacterial species under consideration is C. beijerinckii. (2) The substrate (glucose) consumption was written in the form of Herbert–Pirt equation. (3) The relation between substrate concentration and specific growth rate was given in terms of Monod kinetics, while the effect of product inhibition was based on a maximum limit of butanol concentration. (4) The substrate consumption rate was also defined in terms of

dilution rate, concentration of substrate in feed, and reactor voidage. Identifying that biomass in the reactor is spread over two locations, viz. within immobilization matrix, and as freely suspended cells, the latter was used to determine the rate of substrate consumption. The effectiveness factor can be determined using generalized Thiele modulus [308, 309]. In a follow–up publication, Schoutens and Kossen [307] extended this model for a new strain, *Clostridium* DSM 2152 with mixture of glucose and whey permeate as substrate. Both fermentation models from TU Delft research groups are simple yet effective tools of designing large scale fermenters.

2.7.3 Physiological and kinetic model from McGill University

Research group at McGill University (Canada) have developed a "process kinetics model" and "physiological state model" for the acetone–butanol fermentation. The former model is a representation of acetone–butanol process dynamics, while the second model is aimed at elucidation of key process parameters and quantitative extent of their influence in control of solvent biosynthesis. The physiological model was based on the experimental observation of significance of mass transfer phenomena through the cellular membrane. The major culture parameters affecting solvent biosynthesis were permeability of cellular membrane and number of active sugar transport sites. A brief description of the features of these modes is given below.

Process kinetics model [310, 311]: Assuming that the batch fermentation is glucose limited, with no process limitation by nitrogen source, the model takes cellular RNA content as the basis. This approach, in turn, is based on the fact that dimensionless concentration of RNA (y) is related to specific growth rate (μ) as: $\mu = a(y-1)$ where a is numerical coefficient with value of 0.56 for most bacterial cultures [312]. μ is a function of concentration limiting substrate (S) and also those of inhibitory products such as butanol (B). The model uses a linear relation of y with respect to substrate concentration and hyperbolic relationship for

product inhibition. The biomass content of the broth is determined by two simultaneous processes, viz. consumption by the culture and inhibition by the butanol formed in the broth. $y = 1$ (at $t = 0$) characterizes the inoculum in the stationary phase. The exponential phase of growth is characterized by $y \leq 1$. The kinetics of butanol and butyric acid are related in that butyric acid is produced from limiting substrate with simultaneous inhibition by butanol. The principal merit of this model was its capability to reflect all phases of batch fermentation cultures using physiological state marker (y), indicative of cellular RNA content.

Physiological model: This model differs from the Process Kinetics Model in that it accounts for both intra– and extracellular culture conditions, and it also contains transport parameters for solvents on cellular level. The model comprises of set of 8 ordinary differential equations for 8 variables, viz. physiological state marker (y), biomass concentration (X), substrate or sugar (S) utilization rate, number of active sugar transport sites ($n' = kn$), internal concentration of butyric acid (BA_i) in wet biomass, external concentration of butyric acid (BA_{ex}), Internal concentration of butanol in wet biomass (B_i). The mass balance for butyric acid comprised of four components, viz. synthesis rate of butyric acid (which varies directly with sugar utilization rate and is inhibited by butanol); conversion of butyric acid to butanol (represented by Monod [313] kinetics); butyric acid transport out of the cell by diffusion, transport rate of butyric acid by electrical potential gradient and change of butyric acid concentration with changing volume of biomass during growth. Parametric sensitivity analysis done by Yerushalmi et al. [311] reveals that kinetics of butanol and butyric acid formation, and biomass growth showed the highest sensitivity towards performance of culture and solvent production. Another contemporary paper from this research group [314] reports a model for acetone–butanol fermentation in a cell retention reactor. The set of equations in this model is essentially similar to the process kinetics model, except that inhibition due to butyric acid / butanol is modeled with an inhibition function dependent on concentration of

butanol and butyric acid. An important prediction of this model was that for glucose feed concentrations greater than 52 g/L, the cell retention reactor cannot attain a steady state.

2.7.4 Physiological and kinetic models of Polish Academy of Science

Jarzebski and coworkers [315, 316] have proposed a kinetic and physiological model for continuous acetone–butanol fermentation. This model is based on two assumptions: (1) solventogenesis is triggered by attaining a given threshold concentration of intracellular butyrate (DBA_i); (2) Undissociated form of butyric acid (UBA) passes freely through cell membrane, and thus $(UBA)_i = (UBA)_o$. With these assumptions, the relation between UBA and DBA is: $UBA_i = UBA_o = \frac{DBA_i}{10^{(pH_i - pKa)}}$, and the extra–cellular concentration of butyric acid is given as: $[BA]_{ex} = DBA_i\left(1+10^{pH_o - pKa}\right)\Big/\left(10^{pH_i - pKa}\right)$. This model has been validated against experimental data and has also been compared with model of Mulchandani and Voleski [314]. This model correctly predicts the formation of acids in case of strong substrate limitation, and also effect of increased dilution rate on drop in solvent concentration. The model was found to predict performance of continuous as well as batch fermentation satisfactorily [317, 319].

2.7.5 Model for extractive fermentation from Nagoya University

Researchers at Nagoya University (Japan) have developed a general framework for extractive fermentation [320] which could be applied to batch, repeated batch and repeated fed–batch modes. The model consists of a set of ordinary differential equations for biomass concentration, substrate utilization and concentration of various products. The growth rate is taken as a product of mean growth rate defined on the basis of Monod kinetics and an inhibition coefficient. This coefficient based on production rate of an inhibiting product and the inhibition constant for that products. The production rate of i^{th} component in fermentation broth was given as: $P_i = \mu/Y_i\left(1+m_i\eta\right)$, where Y_i is the yield coefficient for the product, m_i is

the partition coefficient for the product and η is the volume ratio of fermentation broth to the extraction solvent. Using series solution of the differential equation, the relation between production rate of a particular product *i* (P_i) and production of biomass over batch operation time of τ was obtained as: $P_i(\tau) = P_i(0) + \frac{X(\tau) - X(0)}{Y_i(1 + m_i\eta)}$. Honda et al. [320] have extended this analysis to repeated–batch and repeated–fed batch operation. For repeated fed–batch, the cell concentration ($\overline{X}$) was assumed to be constant. The productivity of component *i*, $P_i(\tau)$, is expressed in terms of objective functions I_i (representing total productivity of i^{th} metabolite) and J_i (representing average concentration of i^{th} metabolite) as: $I_i = \eta J_i / (1+\eta)\tau$, where $J_i = m_i P_i(\tau)$.

In a subsequent paper from same research group Shi et al. [321] applied the above model for evaluation of acetone–butanol fermentation with addition of two solvents, viz. oleyl alcohol (an extractant for butanol) and benzyl benzoate (an extractant for acetone). The simulations results showed that significant performance improvement in terms of productivity and the product concentration is expected when two extractants are added simultaneously.

2.7.6 Kinetic model of Shinto and co-workers

A group of scientists from Japan (Kyushu University, Maebashi Institute of Technology and Saga University) have developed a kinetic model for acetone–butanol–ethanol fermentation on the basis of metabolic pathway of C. acetobutylicum ATCC 824 [322, 323]. Various reactions in the metabolic pathway and the corresponding rate expressions along with kinetic parameters and notation are summarized in Table 2.10. In a modified model that considered butanol inhibition to cell growth, the rate expressions for reactions 1, 17 and 19 in Table 2.10 were modified to include a factor *F* that considered the on–off mechanism of metabolic reactions in presence or absence of ATP and NADH; i.e. exhaustion of glucose. The kinetic parameters were calculated using time course of

experimental data. Simulations conducted with model with and without incorporation of *F*–factor (on/off mechanism) indicated that models with *F*–factors could best describe the experimental data. Models without *F*–factors predicted continued organic acid assimilation and solvent production even after glucose exhaustion, which is in contradiction with the experimental data. Sensitivity analysis of model parameters revealed that 5% increase in V_{max1} and K_{m1} had negative impact on butanol production, while 5% increase in V_{max17} had a positive impact. Furthermore, 5% decrease in V_{max15} had negative impact on acetone production. In a later publication, Shinto et al. [323] have extended the model for pentose–phosphate pathway for xylose utilization. Sensitivity analysis of the kinetic parameters in this model revealed similar results as in glucose model.

2.7.7 Models of Chang–Ho Park and co-workers

Park et al. [265, 262, 217] have modeled simultaneous fermentation and separation in packed column, i.e. an immobilized cell trickle bed reactor with gas stripping. The modeling approach has been that of equilibrium stage with steady state and isothermal operation, and surface reaction with no diffusion limitation in immobilization matrix. The reactor comprises of two sections, viz. enricher and stripper, stacked over one another. The gas and liquid flows are concurrent in the enricher section and countercurrent in the stripper section. The main model equations are essentially discretized mass balances for various components in the enricher and stripper section divided in discrete stages. The relation between gas and liquid phase compositions of a component is: $y = Kx$, where $K = \gamma P^o / P$, is calculated using Wilson equation for activity coefficients (γ) and Antoine equation for vapor pressure (P^o). With approximation that molar flow rates of liquid and vapor do not change from stage to stage and *K* value for various components stays constant, the rate of reaction $r_{i,j}$ is written in terms of Monod kinetics type expression. The above equation can be transformed in a tridiagonal matrix system.

Table 2.10: Reactions and their rate expressions in the fermentation model of Shinto et al. [322, 323]

Reaction	Rate Expression
Glucose $\xrightarrow{r_1}$ Fructose-6-P	$r_1 = V_{max1}[Glucose][Biomass] \times F/(K_{m1} + [Glucose])$
Fructose-6-P $\xrightarrow{r_2}$ Glyceraldehyde-3-P	$r_2 = V_{max2}[F6P][Biomass] \times F/(K_{m2} + [F6P])$
Glyceraldehyde-3-P $\xrightarrow{r_3}$ Pyruvate	$r_3 = V_{max3}[G3P][Biomass] \times F/(K_{m3} + [G3P])$
Pyruvate $\underset{r_4}{\overset{r_5}{\rightleftharpoons}}$ Lactate	$r_4 = V_{max4}[Lactate][Biomass] \times F/(K_{m4} + [Lactate])$ $r_5 = V_{max5}[Pyruvate][Biomass] \times F/(K_{m5} + [Pyruvate])$
Pyruvate $\xrightarrow{r_6}$ Acetyl-CoA	$r_6 = V_{max6}[Pyruvate][Biomass] \times F/(K_{m6} + [Pyruvate])$
Acetate $\underset{r_7}{\overset{\text{Acetyl-P}}{\rightleftharpoons}}$ Acetyl-CoA	$r_7 = V_{max7}[Acetate][Biomass] \times F/(K_{m7} + [Acetate])$
Acetate $\xrightarrow[\text{CoA, } r_8]{\text{Acetoacetyl-}}$ Acetyl-CoA	$r_8 = V_{max8}\left[\frac{1}{1 + K_{m8A}/[Acetate]}\right] \times \left[\frac{Biomass}{1 + K_{m8B}/[AACoA]}\right]$
Acetyl-CoA $\underset{r_9}{\overset{\text{Acetyl-P}}{\rightleftharpoons}}$ Acetate	$r_9 = V_{max9}[ACoA][Biomass] \times F/(K_{m9} + [ACoA])$
AcetylCoA $\xrightarrow{r_{10}}$ Acetoacetyl-CoA	$r_{10} = V_{max10}[ACoA][Biomass]/(K_{m10} + [ACoA])$
AcetylCoA $\longrightarrow$ Acetaldehyde $\xrightarrow{r_{11}}$ Ethanol	$r_{11} = V_{max11}[ACoA][Biomass] \times F/(K_{m11} + [ACoA])$
AcetylCoA $\xrightarrow{r_{12}}$ Biomass	$r_{12} = \frac{V_{max12}[ACoA][Biomass]}{\{K_{m12}(1 + [Butanol]/K_{ii12}) + [ACoA](1 + [Butanol]/K_{ii12})\}}$
Biomass $\xrightarrow{r_{13}}$ Products	$r_{13} = k_{13}[Biomass]$
Acetoacetyl-CoA $\xrightarrow{r_{14}}$ Butyryl-CoA	$r_{14} = V_{max14}[AACoA][Biomass] \times F/(K_{m14} + [AACoA])$
Butyrate $\longrightarrow$ Acetoacetyl-CoA $\xrightarrow{r_{15}}$ Butyryl-CoA	$r_{15} = V_{max15}\left(\frac{1}{1 + K_{m15A}/[Butyrate]}\right) \times \left(\frac{[Biomass]}{1 + K_{m15B}/[AACoA]}\right)$
Acetoacetate $\xrightarrow{r_{16}}$ Acetone + CO_2	$r_{16} = V_{max16}[Acetoacetate][Biomass]/(K_{m16} + [Acetoacetate])$
Butyrate $\longrightarrow$ Butyryl-P $\xrightarrow{r_{17}}$ Butyryl-CoA	$r_{17} = V_{max17}[Butyrate][Biomass] \times F/(K_{m17} + [Butyrate])$
Butyryl-CoA $\longrightarrow$ Butyryl-P $\xrightarrow{r_{18}}$ Butyrate	$r_{18} = V_{max18}[BCoA][Biomass] \times F/(K_{m18} + [BCoA])$
Butyryl-CoA $\longrightarrow$ Butyraldehyde $\xrightarrow{r_{19}}$ Butanol	$r_{19} = V_{max19}[BCoA][Biomass]/(K_{m19} + [BCoA])$

Notation: k_i – reaction rate constant for i^{th} reaction; K_{aj} – activation constant for activator for j^{th} reaction; K_{ij} – inhibition constant for inhibitor for j^{th} reaction; K_{isj} – inhibition constant for substrate for j^{th} reaction; K_{mj} – concentration of metabolite (m) when rate is half of maximum rate V_{max}; r_j – rate equation for metabolite reaction, j being the number of reaction; V_{maxj} – maximum reaction rate for j^{th} reaction; [XX] – concentration of the species XX in mM, XX = Glucose, Lactate, Pyruvate etc.

Park et al. [217] have studied the effect of in–situ product stripping on glucose conversion and product separation with variation of various factor such as proportion of gas / water mole ratio in the enricher and stripper, total gas flow rate, product inhibition kinetics, operating temperature and pressure and inlet glucose concentration. 54.6% improvement in glucose conversion was seen in stripper by application of in–situ product removal. Higher total gas flow rate increased glucose conversion and the product separation, while operation under vacuum improved glucose conversion when gas was circulated.

Park and Geng [324] have also proposed a model for fed batch butanol fermentation with simultaneous pervaporation. The system considered for the model comprises of a fermenter and a pervaporation module mounted inside the fermenter. Starting with Monod growth kinetics (with inhibition term modeled as $I = 1.33 - 0.083C_B$ when $C_B > 4.5$ g/L). The complete model for the fermentation system with pervaporative solvent recovery comprises of 8 ordinary differential equations. In order to simulate the switch over from acid uptake to solvent production (when butyrate concentration, C_{BA}, reaches a specified concentration, C_s) Park and Geng [324] made use of a term (*sig*) that indicates a physiological signal. The value of C_s was given as: $(0.48-0.176\,pH)\left(1+10^{(pH-pKa)}\right)$. The values of (*sig*) are (*sig*) = 0 when $C_{BA} < C_s$, and (*sig*) = 1 when $C_{BA} \geq C_s$. The fluxes of various components were written in terms of diffusivities as: $q_{sp} = DC/l$, where D is the diffusivity of component through silicone membrane, l is the membrane thickness and C is the concentration of component. The volumetric water flux across membrane was considered to be constant. Simulations done with the above model made following predictions: (1) Glucose concentration decreased slowly in the lag phase of biomass growth. With onset of solventogenesis, it decreased rapidly until cell growth was inhibited by high butanol concentration in the medium. (2) Effect of membrane area on glucose consumption rate revealed that after lag phase, the consumption rate increased almost instantly with onset of solventogenesis. The consumption

rate was also related to use of parameter (sig) that turned on glucose consumption for production of acetone and butanol simultaneously. (3) Variation of glucose consumption rate with membrane of varying thickness showed a "saw tooth" type behavior. The thinner the membrane, the greater was the glucose consumption rate.

2.7.8 Other models for ABE fermentation

Model for flash fermentative process: The most popular extractant for ABE fermentation is oleyl alcohol. The solvent extracted by oleyl alcohol are recovered by distillation. However, distilling the solvents under vacuum conditions by maintaining boiling point at mild temperature is both realistic and reasonable. On the basis of this, Shi et al. [325] have proposed a flash extractive fermentation system consisting of a fermenter, vacuum distillation unit and flash unit, and have assessed its performance with a mathematical model. This model comprises of six mass balance equations for the cells, substrate, product in solvent phase, product in aqueous phase and overall balance. The major results of simulations with the above flash extractive model are summarized as follows: (1) Highly concentrated feed resulted in most pronounced performance improvement; (2) Solvent distribution rate increases total productivity, but results in loss of energy utilization efficiency; (3) Two vessel flash system does not simultaneously satisfy the requirement of product purity and energy efficiency.

Application of KALMAN Filter for online states and parameter identification: Jahanmiri and Rasooli [326] have studied use of Extended Kalman Filter (EKF) for determination of outlet compositions and growth rate of ABE fermentation process from outlet CO_2 concentration measurement. The fermentation process has been modeled using the cell retention model of Mulchandani and Volesky [314]. The process noise covariance matrix Q and R were used tuning the filter. Various methods were used to process the covariance matrix such as Loan, Monte Carlo and continuous time case. The results of this study showed that predictions

made by the EKF were in good agreement with the fermentation product composition values measured off line, when covariance matrix was treated with Loan method.

Optimization of flowsheets for downstream processing: Liu et al. [315] have adopted an approach of generating complete flowsheets for downstream processing, i.e. separation and purification of products of fermentation. The flowsheets analyzed by Liu et al. [327] with Aspen software include conventional unit operations such as gas stripping, distillation and liquid–liquid extraction. The total cost of flowsheet (sum of present values annualized capital costs and operating costs) is taken to be the objective function to be minimized, with payback period of 3 years. The cost estimation was done on the basis of production of 200 million pounds of butanol per year and purities of 99.9 wt%, 99.5 wt% and 99 wt% for butanol, ethanol and acetone respectively. Cost analysis mainly features the separation equipment.

With various combinations of equipment, Liu et al. [327] have evaluated 14 different flowsheets. For the distillation separation of ABE solvents extracted with extractant such as 2–ethyl–1–hexanol, various combinations of distillation units have been used such as direct sequence, indirect sequence, complex–direct sequence and complex–indirect sequence etc. In these flowsheets, the extractant recovery and recycle system precedes the main distillation unit for ABE solvent separation. The most optimal flowsheet (complex–direct sequence) with total annual capital cost of $9.4 million is shown in Fig. 2.9. In the flowsheets for separation of gas stripped ABE solvents, azeotropic columns have been considered. These columns break ethanol–water–butanol ternary azeotrope, water–butanol binary azeotrope and water–ethanol binary azeotrope. However, these flowsheets were not among the 10 best ones. Analysis of Liu et al. [327] has given a very interesting result that although gas stripping, as considered individually, is a relatively simple technique of solvent recovery than liquid–liquid extraction, the entire process employing this technique (including downstream processing) is less economic due to relatively high costs of azeotropic systems.

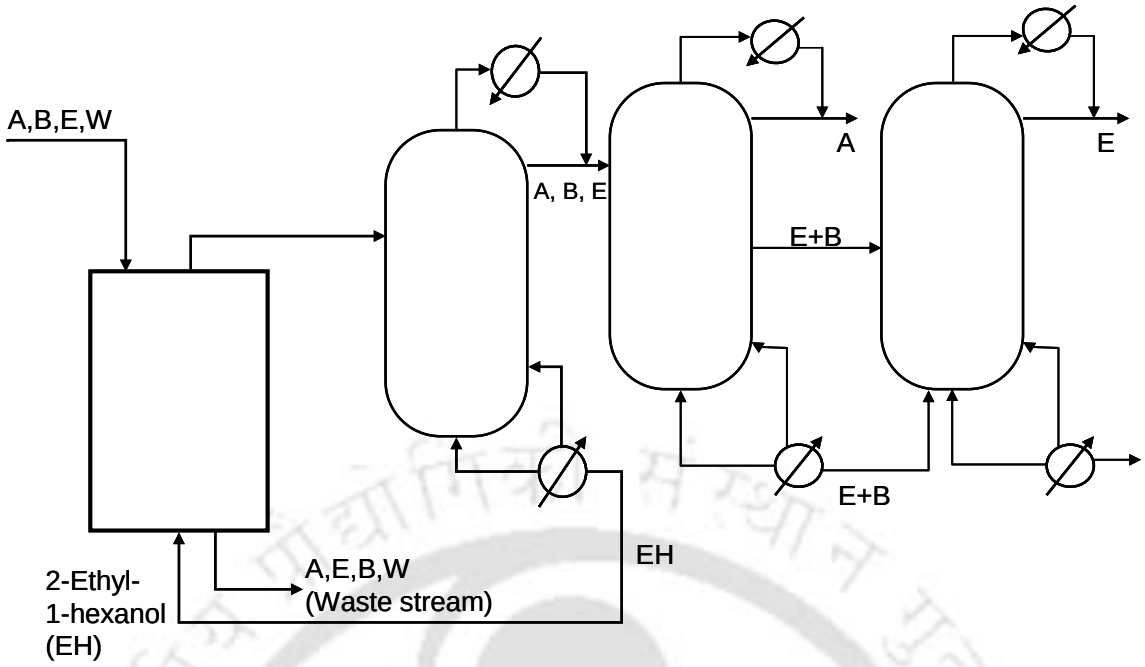

Figure 2.9: The most optimal flowsheet (Complex Direct Sequence; A – Acetone, B – Butanol, E – Ethanol, W – Water, EH – Ethyl hexanol) for downstream processing of ABE fermentation (redrawn with modifications from ref. [327]).

Inhibition kinetics model: Yang and Tsao [328] have presented a mathematical model of cell growth kinetics of the culture under the synergistic inhibition of multiple products / byproducts. The model is based on assumption that Monod equation could be used to express cell growth kinetics under product inhibition condition. The maximum specific growth rate under inhibited conditions was written as: $\mu_m^i = \mu_m F_x(I)$, where F_x(I) is the function of inhibitors. The ratio of specific growth rate (μ_m) to inhibited specific growth rate (μ_m^i) was given by following correlation on the basis of experimental data: $\frac{\mu_m^i}{\mu_m} = b_o + \sum_{i=1}^{5} b_i \chi_i + \sum_{\substack{i=1 \\ j=i-1 \\ (i \neq j)}}^{5} b_{ij} \chi_{ij}$, where χ_i represents individual effect of i^{th} compound, and χ_{ij} is the combined effect of interaction of i^{th} and j^{th} compounds. b_i and b_{ij} are regression parameters representative of the extents of these inhibition effects, respectively. The five components imposing inhibition effect are acetone, butanol, ethanol, acetate and butyrate. It was observed that inhibition effect of butanol augmented in presence of other inhibitors like

acetate and butyrate. Acetone and ethanol did not cause much inhibition individually nor did they interact with other products. The inhibition effect was also dependent on pH level due to dissociated and undissociated forms. Therefore, effect of pH on cell membrane and other physiological functions was taken into account by considering pH as an independent parameter.

Model for simultaneous fermentation – extraction with hollow fiber membrane: Shukla et al. [237] have reported experiments and a mathematical model for an integrated fermenter – extractor. In this system, a tubular fermenter was filled with 295 hydrophobic microporous hollow fibers running axially through the fermenter. The microbial culture was immobilized onto wood chips suspended in the broth. The extraction solvent was 2–ethyl–1–hexanol. The model proposed for this system comprises of 11 equations in which the fermentation model of Mulchandani and Voleski [314] has been coupled to simple mass balance equations for simultaneous solvent extraction. The model assumes that there is no mass transfer resistance in radial direction in the fermenter and steady state conditions exist. The inhibition function was based on total concentration of butanol and butyric acid (in exponential form below total concentration of 8 g/L and in linear form for total concentration between 8 and 13.9 g/L). With concurrent analysis of experiments and model simulations, Shukla et al. [237] obtained conclusions: (1) overall productivity can be increased up to 40% with extractant flow through fibers; (2) discrepancy in the theoretical and experimental rate of glucose consumption was attributed to inhibition due to butanol concentration built in the wood chip pores, which was not accounted by the model.

Stoichiometric model with nonlinear constraint: The stoichiometric fermentation model of Papoutsakis [296] suffers from singularity due to interacting pathways for some products. In

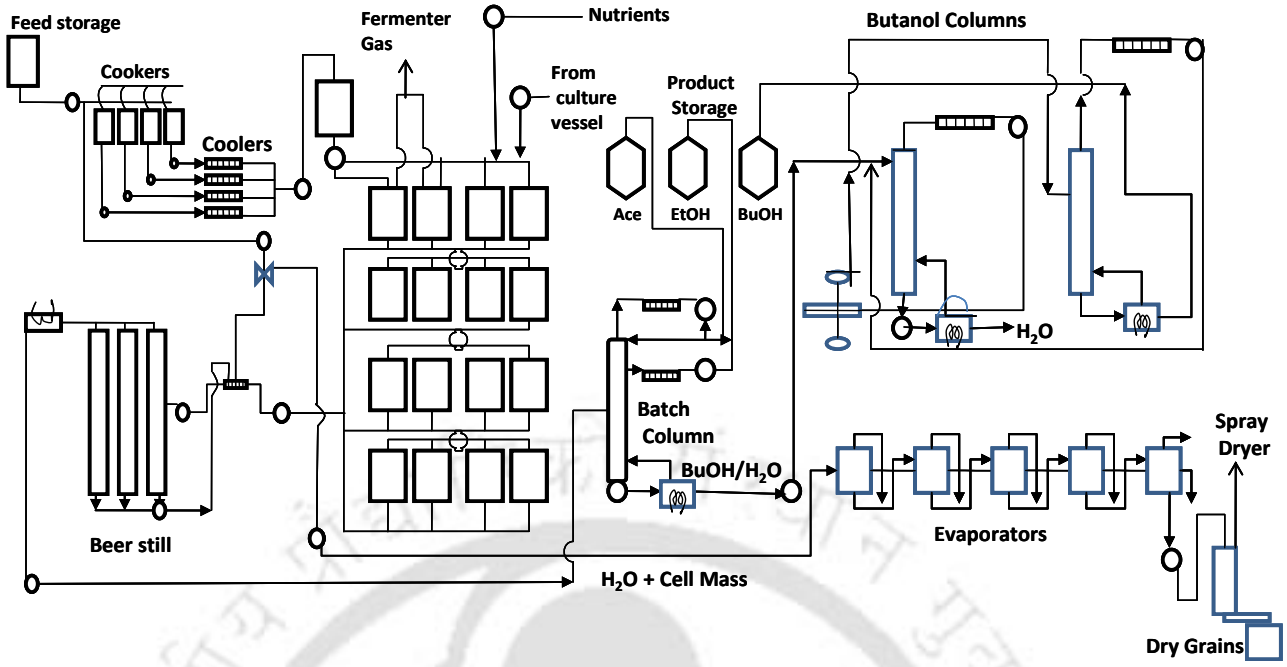

Figure 2.10: ABE batch fermentation process flowsheet considered by Lenz and Moreira [330] (redrawn with modifications)

this situation, the calculation of in–vivo fluxes becomes a non–trivial issue. To eliminate singularity, Papoutsakis [296] grouped together acetone pathways, with in–vivo fluxes replaced with net production rate of acetate, butyrate and acetone. However, this technique resulted in loss of information concerning physiologically relevant in–vivo fluxes. Several other techniques have been attempted to remove singularity such as measurement of one of the invivo fluxes, introduction of optimality principles (minimizing norm of flux vector) etc. Desai et al. [329] proposed a new method based on the fact that, rate of uptake of butyric acid and acetic acid is catalyzed by the same enzyme, and obtained the relation between invivo uptake through the acetone formation pathway of butyrate to that of acetate. However, as the acetate and butyrate concentrations are also a function of rate of formation of acetate and rate of formation of butyrate, this relation represented a nonlinear constraint. The stoichiometric balances of Papoutsakis [296] can now be used with above constraint to determine unique set

of invivo metabolic fluxes capable of describing the metabolism of solventogenic clostridia. Advantages of this technique are distinct: (1) It is a physiologically based nonlinear constraint to resolve singularity in the stoichiometric model. (2) It allows incorporation of nonlinear equations in the stoichiometric models. (3) With this technique, a single metabolic network is capable of describing the metabolism of a range of substrate mixtures without a priori determination of fluxes, which are insignificant at a given substrate mixture.

2.8 Economics of Acetone Butanol Fermentation

For butanol to be a viable diesel or gasoline substitute, the economics of the acetone butanol fermentation need to be assessed in comparison to the market prices of petroleum fuels as well as the prices of the butanol available from petrochemical industry. It is clear from the discussions presented in preceding sections that principal factors governing economics of butanol are mode of fermentation, solvent recovery and substrates. The first two factors govern the total fixed capital investment of the process, while the third factor governs the total production cost. Numerous studies have been published that evaluate the economics of ABE fermentation process with different modes (batch, fed batch, continuous), different solvent recovery techniques and different substrates. In this section, we review these studies and try to identify "niche" areas for the improvement of economics of biobutanol. We use two yard sticks to comparatively evaluate different ABE fermentation processes, viz. return on investment (ROI) and break-even price of butanol ($/kg or $/lb). Initial economic analysis published in 1980's including those from Lenz and Moreira [330], Marlatt and Dutta [331], Roffler et al. [332], Dadgar and Fouch [333], and Busche and Allen [324].

Lenz and Moreira [330] have compared the economics of a batch fermentation process using either molasses or liquid whey permeates as feedstock. The flow sheet of fermentation process considered by Lenz and Moreira is shown in Fig.2.10. The plant

capacity was 45 million kg or 45000 tons of total solvents (equivalent to 57 million liters annually) production, with 300 days/year of operation. The microbial culture was *C. saccharoacetobutylicum* and fermentation was carried out in a battery of 16 fermenters, each with capacity 1514 m^3. The cost data for the plant is given in Table 2.11(A). The product from molasses based process were acetone, butanol, ethanol, dry slops (or stillage), along with gases such as CO_2 and H_2. The net annual income from these products was estimated at $ 36 million. The profitability analysis of batch fermentation plant is given in Table 2.11(B). It can be perceived from this analysis that molasses based fermentation process is uneconomic. In order to make the process viable, Lenz and Moreira [330] have proposed the alternate substrate as, liquid whey permeate. The unit cost of whey permeate ($ 4.4 per ton) is much smaller than that of molasses ($ 84 per ton with 50% fermentable sugars). The substitution of molasses by whey permeates improves the economy of fermentation significantly, as seen from data presented in Table 2.11(A&B). This improvement is attributed to low cost whey permeate and more valuable byproduct of whey protein (20,000 tons at $ 0.46/Kg). The net return on investment is estimated at 30.8% and the break-even price of butanol is was $ 0.205. The market price of butanol in the 1980s was $0.53/kg, and hence, fermentation process with whey permeates as substrate was economically viable. Marlatt and Dutta [331] have analyzed the economics of a batch ABE fermentation process with capacity of 200 million tons of butanol. The microbial culture was *C. aceto-butylicum* and substrate was corn. The cost analysis is summarized in Table 2.11(C). Marlatt and Dutta [331] have compared the corn based process with oxo–process for hydro–formulation of propylene with H_2 and CO with soluble rhodium catalyst. For corn price of $ 3/bushel and propylene price of $0.19/lb, the breakeven prices for butanol from the two processes is same.

Table 2.11: Summary of economic analysis of ABE fermentation processes

(A) Analysis of Lenz and Moreira [330]

Plant Capacity (Annual)	45000 tons of ABE solvents
Substrate	Molasses
Total Capital Investment (TCI)	$ 19.89 million
Total Production Cost (TPC)	$ 39 million
Annual Income (AI)	$ 36 million
Gross Profit (AI – TPC)	**– $ 30,37,008***

* - The process is not economic as the profit is negative

(B) Analysis of Lenz and Moreira [330]

Plant Capacity (Annual)	45000 tons of ABE solvents
Substrate	Whey permeate
TCI	26,331,777
TPC	34,292,546
Profit after tax (tax @ 46%)	8,112,524
Breakeven price	0.205/kg butanol
ROI	30.5%

(C) Analysis of Marlatt and Dutta [331]

Plant Capacity (Annual)	200 million tons of butanol
Substrate	Corn
Total Capital Investment	$ 119.26 million
Total Production Cost	$ 94.4 million
Breakeven price	0.34/kg
Profit (after tax at 46%)	$ 43.85 million
ROI	36.7%

* Analysis of Marlatt and Dutta [331] did not take into account the depreciation and general expenses (for sales, administration and distribution)

(D) Analysis of Roeffler et al. [332]

Cost component	**Batch Fermentation Process**	**Extractive Fed Batch Process**
	$ million	**$ million**
Plant Capacity (Annual)	200 million tons butanol	200 million tons butanol
Substrate	Molasses	Molasses
Total Capital Investment	159.66	135.72
Raw material	86	84.6
Total Production Cost	143.27	120.37
Breakeven Price	1.518 (@ i = 18%, n = 15 yr)	1.215 (@ i = 18%, n = 15 yr)

(E) Analysis of Qureshi and Blaschek [338]

Mode of fermentation	Batch	Batch	Fed-batch	Immobilized continuous
Plant Capacity	153,000 tons of total ABE solvents with 122,000 tons of butanol			
Substrate	Corn			
Mode of product recovery	Distillative	Pervaporative	Pervaporative	Pervaporative
Cost Analysis				
Total Capital Investment	109.56	68.18	63.31	56.01
Raw material	37.22	42.28	42.28	44.28
Total production cost	78.98	66.69	65.83	66.19
Breakeven price	0.609	0.445	0.425	0.409

[1] includes maintenance, operating supplies and lab charges; [2] including operating and supervisory labor.

(F) Analysis of Liu et al. [327]

Plant capacity: 200 million metric tons of butanol, Substrate: Dry distiller's dried grains

Rank of Flowsheet	The description of flowsheet (type of sequence of column)	Total annualized cost \$ ($\times 10^3$)
1.	Complex direct sequence*	9.416
2.	Prefractionator (3–column)*	10.172
3.	Indirect sequence*	10.192
4.	Thermally coupled prefractionator*	10.211
5.	Direct sequence*	10.387
6.	Pasteurization column (recovery of middle distillate as liquid)*	11.58
7.	In direct sequence with side stream*	11.742
8.	Pasteurization column (recovery of middle distillate as vapor)*	11.842
9.	Direct sequence with side stream*	12.023
10.	Side stripper*	12.009

* Solvent recovery by liquid–liquid extraction. A distillation column for recovery of solvents from extractant with recycle of extractant precedes the main sequence in each flowsheet.

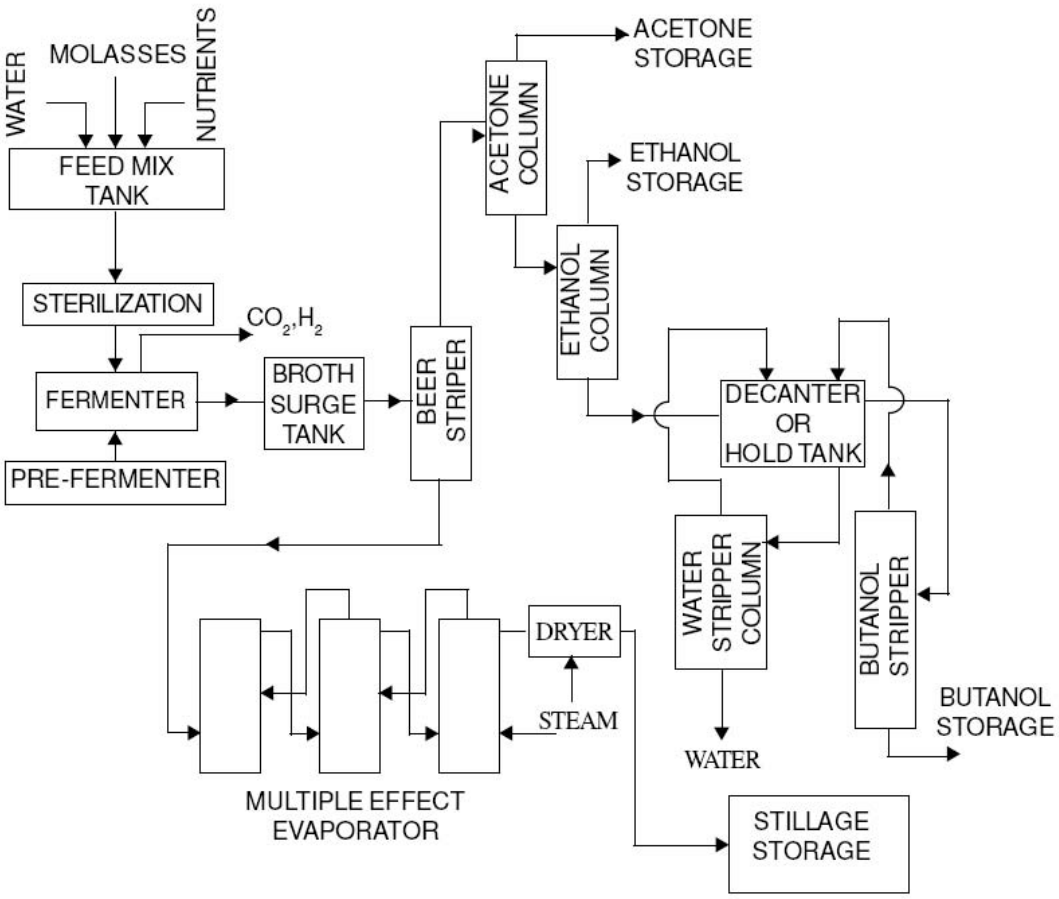

Figure 2.11: Process flow diagram for batch ABE fermentation with molasses as feedstock (redrawn with modifications from ref. [332])

In a notable contribution, Roffler et al.[332] have demonstrated as how design of the ABE fermentation improves the economy of process even with relatively expensive feedstock like molasses. Roffler et al.[332] have analyzed two processes; (1) batch fermentation and (2) extractive fed–batch fermentation with oleyl alcohol (diluted with n–decane) as the extractant the flow sheets for these two processes are shown Figs. 2.11 and 2.12, respectively. extraction using diluted oleyl alcohol (redrawn with modifications from ref. [332]). The substrate for fermentation was molasses containing 55 wt% sugar and 30 wt% non–fermentable solids at a price $ 100 per ton. The total plant capacity was 200 million tons of butanol. The total purchased equipment cost for the batch process was $ 37.28 million, while that for the extractive fed–batch process was $ 29.78 million.

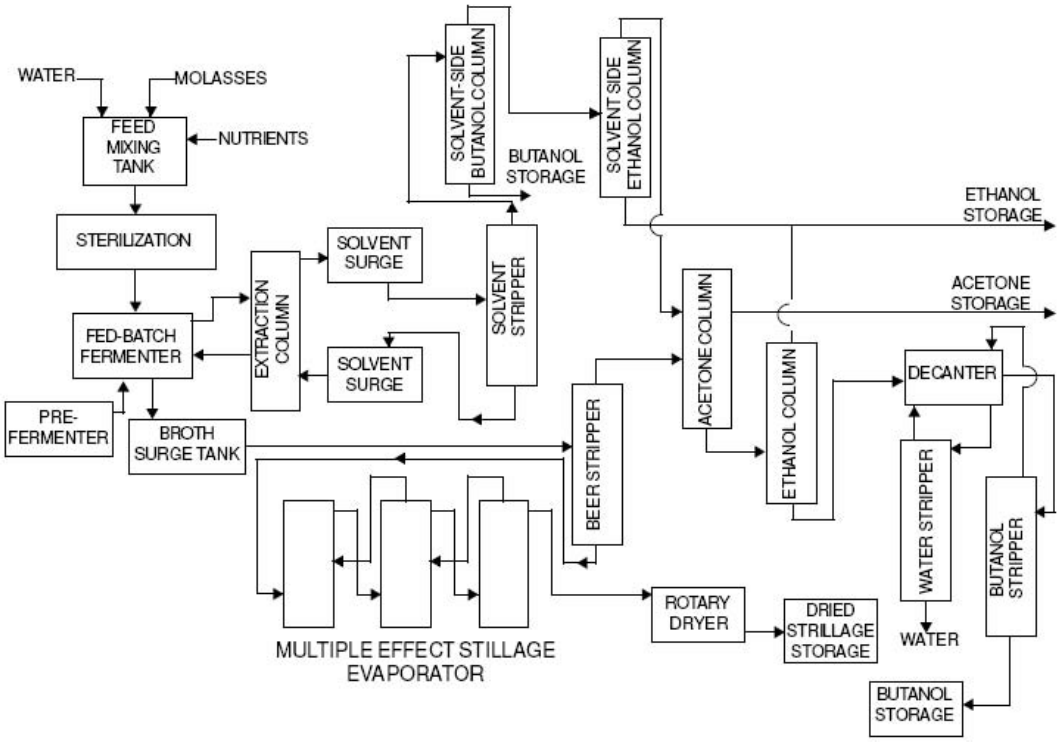

Figure 2.12: Process flow diagram for fed–batch ABE fermentation with solvent

The microbial culture used was *C. acetobutylicum*. Separation of the acetone, butanol and ethanol was achieved with trains of energy integrated distillation column. The major byproduct of the process was acetone, ethanol, hydrogen and dried stillage (i.e. solids in fermentation broth). The entire cost data of the two processes is summarized in Table 2.11(D). It could be inferred from Table 2.11(D) that capital cost of extractive fed batch fermentation is 20% lower than the conventional batch process. As a consequence the breakeven price of butanol produced from the two processes also differs by about 20%. This is a consequence of the two factors: (1) in situ removal of butanol (which is inhibitory to microbial culture) increases the solvent productivity, (2) extractive fermentation also permits treatment of broth with concentrated sugar solutions due to which the volume of fermentation broth and hence the number of fermentor reduces. Low volume of fermentation broth also brings down the size of equipment and the energy requirement on the downstream processing side of distillation columns, thus reducing the overall capital cost by 20%.

Dadgar and Foutch [333] have compared the fermentation process design and economics of Marlatt and Dutta [331] with actual extractive fermentation process using 2–ethyl–1–hexanol as extractant. The process uses corn and corn steep liquor as the substrate with *C. acetobutylicum* culture. The production capacity is 200 million pounds butanol per year. With an economic analysis along same lines as described before, Dadgar and Foutch [333] have estimated the breakeven price of butanol from extractive fermentation at $ 0.26/lb (as against $ 0.3/lb calculated by Marlatt and Dutta [331]. This result confirms that in situ product recovery from fermentation broth improves the economics of ABE fermentation process.

Another study published by Basche and Allen [334] compares the economics of a novel multiphase fluid bed fermenter (MPFB) with that of conventional batch process. This is an extension of the application of fluidized bed system (developed for coal gasification) for the purpose of fermentation. In this reactor, the fermentation broth is circulated in a fluidized bed reactor, in which coarse particles of immobilized cells are fluidized. The breakeven price for butanol in this process is $0.59/lb with ROI of 16% (discounted at interest rate of 13%)

Ladisch [41] has reviewed the economic potential of fermentation derived butanol. He has identified three niche areas for improvement of economics of biobutanol, viz. use of clostridial strains having higher tolerance to butanol inhibition, in-situ removal of butanol through recovery techniques like liquid–liquid extraction or adsorption, and design of new bioreactors (trickle bed reactor, hollow fiber fermentor–extractor).

Qureshi and Maddox [334] have analyzed the economics of a biobutanol production plant using fixed bed fermenter (with cells of *C. acetobutylicum* immobilized onto bonechar) coupled to product recovery unit comprising of silicone tubing's membrane module. The substrate for fermentation was concentrated whey permeate, containing upto 46–150 g/L of lactose. The productivity of reactor is 4.5 g/L–h with product yield of 0.38. The selectivity

and flux of membrane was 10 and 3–30 g/m^3–h, respectively. The ABE solvents are removed from the membrane module using a sweep gas and condensed at –3°C. The plant capacity was to treat 300 m^3/day of whey permeate. The total capital investment for plant was $ 1.2 million, with total production cost of $1.47 million, for annual butanol production of 1.94 million liters. The estimated breakeven price of butanol for this plant was $0.76 per liter. This figure would still look unattractive, however, Qureshi and Maddox [334] have pointed out the economy of scale, for reduction of breakeven price. If the plant capacity is increased to treatment of 6000 m^3/day of whey permeate, the breakeven price of butanol would reduce to $0.44/L, and for a plant capacity of treatment of 20000 m^3/day of whey permeate, it is further expected to lower at $0.37/L. Thus, cost of fermentation substrate and production scale are the major factors affecting the breakeven price of biobutanol.

Tanner et al. [336] has analyzed the profitability of an integrated fermentation system with product recovery using a variable yield function. This analysis begins with definition of a value function V (in $ per year) as follows: $V = N \cdot p - S \cdot s - N \cdot \gamma$, where N is total production of acetone, butanol and ethanol (kg/year), p is price of the product ($/kg), S is amount of raw material (kg/year), s is the cost of raw material ($ per kg) and γ represents fixed cost, variable costs and additional cost of membrane for pervaporative recovery. N and S are related through integral yield function Y as: $Y = N/S = \alpha \cdot N + \beta/N$, where α and β are proportionality constants depicting increase in production as number of membrane module are increased ($\propto N$) and, reduction in product concentration (and yield) as production increases ($\propto 1/N$). V is essentially the profit function that has to be maximized at a given value of N. Tanner et al. [336] evaluated function V for either variable Y or fixed Y (= 0.38) for two production rates: $N_1 = 1.6 \times 10^6$ kg/year and $N_2 = 32 \times 10^6$ kg/year. The cost term γ was fixed at $ 0.4 per kg ABE. Analysis of trends in V with N revealed that process was unprofitable in the production range of 2×10^6 kg/year to 18×10^6 kg/year, if Y was assumed to vary with N. However,

process was profitable for $N < 2\times10^6$ and $N > 18\times10^6$ kg/year. Moreover, for Y fixed at 0.38, the profitability was achieved for all production values, with linear rise with production capacity.

Literature published in the area of economic analysis of ABE fermentation since 2000 mainly focuses on genetically improved strains of *Clostridia*, such as *C. beijerinckii* BA101. Qureshi and Blaschek [337] have analyzed the economics of a batch fermentation plant using *C. beijerinkii* BA101 (capacity 121.6×10^3 tons of butanol per year) with corn as substrate (with estimated price of $71/ton). The total purchased equipment cost (including corn milling, fermentation unit and product recovery with distillation) was $\$33.2\times10^6$ using Lang factor of 3 for equipment installation. The total fixed capital cost was estimated at $ 99.6 million. Other cost components were: Working Capital: $9,960,000; Total Production Cost (inclusive of direct costs, indirect costs and fixed charges): $87.5 million; Byproduct cost: $34 million. With this, the breakeven price of butanol is estimated at $0.55/kg. This cost was found to reduce with increase in yield of process, reduction in cost of corn, rise in cost of byproducts and energy integration of process. Qureshi and Blaschek [337] assumed the Federal taxes at 35% and interest rate for discounting fixed capital at 20%. Any reduction in these rates would also reduce the breakeven price of butanol.

Gapes [43] has reviewed the economics of acetone–butanol fermentation from view point of theoretical and market considerations. In this review, Gapes [43] has shed light on some highly important practical aspects of the ABE fermentation process that influences its economic viability. We summarize these points below:

1. Batch process is often argued to be less economic than continuous production; however, maintenance of absolute sterility in the system is a crucial issue. There are extra costs involved in installing dedicated sterilization equipment but also install piping, valves and other fixtures capable of reliably supporting absolute sterility. Therefore, from viewpoint

of investment costs alone, it is unlikely that continuous operation is of great advantage. Sterility is the dominating factor governing investment in fermentation plant.

2. Plant designed for absolute sterile operation is unavoidably more expensive than non-sterile designs (as in case of ethanol). Compromising quality of equipment for ABE fermentation will leave the process susceptible to contamination by competing micro organisms and viruses.

3. Automation and instrumentation in the plant can bring down production costs significantly by reduction in manpower required and more precise control of the process.

4. Average market prices of butanol and acetone varied by ± 30% over a period of five years (1995-2000). The demand however is quite high consistently over this period. These fluctuations need to be properly accounted for in economic analysis. The breakeven substrate price lies between 0.09 and 0.13 €/kg for a new and existing plant, respectively. Above these prices, the production is not likely to be economically viable.

5. The sale of byproducts is unlikely to change the economics of biobutanol significantly. Main byproducts of the ABE fermentation are H_2, CH_4, CO_2 and stillage. H_2 and CH_4 need to be used in the plant itself to satisfy energy demands. If these are sold, then energy import for the process could increase production cost by 10–20%. Sale of dried stillage or slope as animal feed is not a warranted income as animal feed market is not reliable. CO_2 can be purified and sold, however CO_2 market may not be large enough to accommodate additional CO_2 available from fermentation plant.

6. Fermentation plants with substrate flexibility are highly economic, as substrate cost contributes 60% to the overall cost. The substrate range for a microbial culture should be broadened. Most exciting research in this field would be to activate the endogenous cellulase enzymes in *Clostridium acetobutylicum*, so that these bacteria can metabolize cellulose

directly. This would not only eliminate the expensive hydrolysis step but would also aid to "substrate flexibility" of the bacteria.

7. Online product removal by pervaporation membranes increases productivity; however, these membranes need to have high flux and selectivity. Moreover, high investment costs associated with membranes, salt accumulation in the fermentation medium, blockage of narrow capillaries due to contaminants should be taken into account. Pervaporative membranes are also associated with phase change, which is highly energy intensive phenomenon. Moreover, energy of phase change is a permanent loss as this energy cannot be recovered in any other form.

Qureshi and Blaschek [329] have compared the economics of four different processes for ABE fermentation, viz. batch fermentation with distillative recovery, batch fermentation with pervaporative recovery, fed batch fermentation with pervaporative recovery, and immobilized cell continuous fermentation with pervaporative recovery. The substrate for fermentation was corn with microbial culture being *C. beijerinckii* BA101. The fermentation media was one volume of corn steep liquor containing 100 g/L of solids added to five volumes of fermentation medium. The price of corn was $79.23 per ton (or $2.01 per bushel), and production capacity was 153,000 metric tons of ABE solvent, with butanol content of approximately 122,000 tons. The yield ($Y_{p/s}$) of the process was 0.42. The cost of silicone membrane used for pervaporation was $\$500/m^2$, with overall flux and selectivity of 5 L/m^2–h and 60, respectively and average life of membrane module is 3 years. For the immobilized reactor, the support for immobilization was clay brick, which gave the productivity of 15.8 g/L–h. In order to avoid reactor blockage due to excessive cell growth, a fluidized bed reactor was used. The overall cost data is summarized in Table 2.11(E). The breakeven prices of butanol resulted from this analysis are highly attractive, as they are far smaller than the current market price of butanol ($1.21/kg) from petrochemical industry. However, one must

keep in mind that the breakeven price of butanol is rather *onsite* or *ex–factory* price. Many other factors such as cost of transportation, sales tax, octroi contribute to it, and the actual market price could be double. Even with this estimate, the price of biobutanol is competitive with the price of butanol from petrochemical industry. A rise in the price of byproducts of the process may still bring the breakeven price down.

In a subsequent paper, Qureshi and Blaschek [339] have assessed economics of batch fermentation plants with capacity of 153,000 tons of the ABE production. Two types of plants have been considered for analysis, viz. a new (or gross–rooted) plant and expansion of an existing facility. The purchased equipment cost for the plant is $33.47 million. The total capital investment for fresh plant is estimated to be ~5 times the purchased equipment cost, i.e $167 million, while for expansion of existing facility, the total capital investment is expected at $110.46 million (~ 3.3 times the purchased equipment cost). The price of corn is $79.23 per ton and ABE yield is 0.42 (g ABE solvent/g glucose). The breakeven price of butanol for these conditions is estimated at $0.34/kg for an existing plant and $0.73 for a new or grass-rooted plant. If the price of corn rises to $197/ton, the breakeven prices for butanol are projected at $ 0.47/kg and $ 1.07/kg for existing and fresh plants, respectively. Moreover, if the ABE yield improves to 50% (or 0.5 g ABE solvents/g glucose), the breakeven price for existing plant is expected to drop to $ 0.29/kg.

Liu et al. [327] have analyzed economics of 14 different flowsheets for downstream processing of the ABE fermentation. These flowsheets essentially comprise of various options for separation of acetone, butanol and ethanol from the fermentation broth. The separation of ABE solvents from the broth is achieved with two techniques, viz. gas stripping and liquid–liquid extraction (with 2–ethyl–1–hexanol as extractant), followed by train of distillation columns for separation and purification of acetone, butanol and ethanol from solvent mixture. For the gas stripping technique, two options for downstream processing have

been tried: (1) A distillation column separating acetone as top product followed by azeotropic column with 3 products, i.e. ethanol (top), water (middle) and butanol (bottom), and (2) a distillation column splitting (acetone+ethanol) and (water+butanol) mixtures as top and bottom products respectively; followed by one azeotropic column splitting butanol–water azeotrope, and one simple column separating acetone and ethanol. For the liquid–liquid extraction technique, as many as 12 different sequences of columns have been assessed for separation and purification of acetone, butanol and ethanol, after recovery of solvent mixture from extractant using steam stripping. The 10 best flowsheets have been shortlisted on the basis of annualized costs. These flowsheets and the associated costs are described in Table 2.11(F). The complex direct sequence is found to be the best flow sheet with least annualized capital cost. Quite interestingly, none of the 10 best flowsheets contains the solvent recovery by gas stripping. This is a consequence of the fact that for separation of high purity acetone, ethanol and butanol from gas stripped ABE solvent mixture, azeotropic column(s) are required to break butanol–water and ethanol–water binary azeotrope and ethanol–butanol–water ternary azeotrope. High cost of the azeotropic column affects the economy of the process adversely, although gas stripping is a relatively simpler technique than liquid–liquid extraction. Analysis of Liu et al. [327] also reveals that under certain circumstances, mere retrofitting of an existing process can be economically far more viable than constructing an entirely new process. For example, direct sequence of distillation columns for separation of acetone, butanol, and ethanol mixture can be easily retrofitted to indirect sequence with modest effort of changing piping, which gives about 33% improvement in the economy.

Sustainable ABE fermentation process: Food vs Fuel has been a long debate. The substrates used for ABE fermentation in its initial years such as corn and molasses are no longer economic due to their food value. Especially in developing countries facing acute food shortage use of any food grade substrate is out of question. Secondly, the economic analysis

indicates that about 60% of the cost of butanol is contributed by the substrate itself. This means that use of a cheap and abundant substrate is key to improvement of the economy of biobutanol. Obviously, the cheapest substrates are the agro-residues and forest residues. Some examples are wood chips, rice straw, wheat straw etc. These are basically lignocellulosic biomass containing cellulose, hemi-cellulose and lignin. The microbes are not able to utilize these substrates directly. They need to be converted to fermentables sugars (wither hexoses or pentoses) by acid and/or enzyme hydrolysis. Therefore, although the substrate itself is cheap, the cost of pretreatment affects adversely the overall economics of the process. For a sustainable process employing cheap substrates in the form of agro-residues, it is essential that the cost of pretreatment is reduced. There are two possible solutions to this issue – coculturing of clostrial with cellulolytic fungi such as T. Reesei (in order to have simultaneous saccharification and fermentation) or development of genetically engineered strain with activated endogenous cellulose enzyme.

2.9 Overview

Although the process for production of biobutanol, ABE fermentation, is more than a century old, the interest of the scientific community in this process is renewed in the quest of alternate liquid fuels. In this review, we have attempted to give an overview of the developments in the area of ABE fermentation in past two and a half decades.

The biochemistry of the ABE fermentation is well established. Detailed information on intermediate enzymes involved in butanol formation from various substrates, the activity of these enzymes and their contribution to the metabolic pathway has also been extensively investigated. Metabolic engineering of clostridial strains for optimization of the metabolic fluxes for maximization of butanol production is a highly active research area [54].

Development of genetically engineered strains having higher tolerance to solvent inhibition is crucial to improvement of economics of large-scale fermentation process.

Another crucial aspect of ABE fermentation process that influences the economic viability is substrate for fermentation. Extensive research has been done in past three decades on cheap alternate substrate for fermentation. Lignocellulosic biomass (such as wheat straw, rice straw etc) is perhaps the cheapest alternative in the current context. However, hydrolysis of this biomass prior to fermentation is an additional cost component, which has been addressed by several researchers. A possible solution to avoid pre-treatment step is in terms of co-cultures of cellulolytic and glycolytic strains, where hydrolysis occurs simultaneously with fermentation. However, at present, these systems have been found to be limited by slow kinetics of the cellulolytic strains due to which production of acids instead of solvents is seen to prevail. Any improvement in kinetics of hydrolysis of the cellulolytic strains would be vital to the success of the co-culture systems.

Efficient in-situ recovery of solvents from broth to avoid product inhibition is an important feature of the ABE fermentation process. In this review, we have attempted to give a comprehensive account of various solvent recovery techniques and the published literature in this area. Conventional technique of liquid-liquid extraction with oleyl alcohol or 2-ethyl-1-hexanol has limitations such as toxicity of extractant towards microbial culture, low partition coefficients, requirement of large volume of extractant etc. However, major advantage is no water contamination of the extracted solvents, which makes downstream processing (i.e. separation and purification of acetone, butanol and ethanol) through distillation easy and economic. Among all alternate techniques for solvent recovery, gas stripping seems to be the easiest; however, water contamination of the extracted solvents is a major problem that outweighs this merit. As revealed in the analysis of Liu et al. [327], the downstream processing of the solvents extracted with gas stripping requires azeotropic

distillation columns, due to which the economy of the whole process is adversely affected. Pervaporative recovery of solvents through highly selective membranes is a viable solution. Significant research has been done in both academic institutions and industry to develop new membranes that have higher stability, selectivity and flux. The new silicalite filled membranes have shown greater promise due to high selectivity for butanol and high flux. Further research on pilot scale is needed on fermentation integrated with pervaporative solvent recovery to assess stability of this process for large-scale operation.

This review has also given an account of various fermentation processes developed in last two decades. Most of these processes are integrated with some kind of in-situ solvent recovery system. Considerable effort is also dedicated towards development of processes with cell recycle and cell immobilization on various supports. For large-scale continuous processes, immobilized systems have advantage of flexibility of design in terms of either fixed or fluidized bed operation. Although immobilized culture systems have demonstrated high potential for scale-up, an extensive pilot scale studies are essential to assess performance of these systems in terms of consistent productivity and selectivity. Dividing the fermentation process in stages to isolate the acidogenic and solventogenic phases has also been attempted by some groups with moderate degree of success.

Elaborate mathematical models of ABE fermentation are available in the literature, a review of which has been presented in this chapter. These models could be useful tool for design and optimization of the fermentation processes. Most of the literature in this area uses *C. acetobutylicum* as model strain, and the kinetic data available is for this strain. Now that better strains such as *C. beijerinckii* BA 101 are available, it is worthwhile to apply the present models for fermentation processes with new strains. Generation of kinetic data for the new strains could be a crucial input for design of fermentation processes with these strains.

Extensive economic analysis of the ABE fermentation process has been done by several authors since 1980s. This review has also given a comprehensive account of this literature. The results of these studies have clearly demonstrated that better strains, efficient in-situ recovery, cheap substrates and fermenter design are four pillars of the economy of the process. The costs of byproducts of the process also have significant influence on the production cost of butanol. However, one must be cautious in that these are theoretical predictions. Many other factors such as maintenance of sterility and stability of the process for consistent production, fluctuations in cost and availability of substrates, fluctuations in cost of byproducts may render actual production cost of butanol significantly higher than predicted by the theoretical analyses. Any subsidy given by the Government in terms of reduced sales tax, income tax, octroi duties for transportation etc. can help in improvement of the economy of biobutanol production.

2.9.1 Future of Biobutanol

Biobutanol offers a great promise as a diesel/gasoline substitute. The principal merit of biobutanol (over other liquid alternate fuels) lies in the fact that, butanol is a pure alcohol with longer hydrocarbon chain making it non polar, with an energy content similar to that of gasoline, and hence, can be directly used as fuel in existing internal combustion engines in either blended or pure form. Another merit of biobutanol is that its combustion occurs at a consistent temperature and pressure, as it is a solitary component fuel [9]. This is different from gasoline, as gasoline ignites over a broader temperature and pressure, resulting in incomplete combustion as it is made of many different kinds of molecules. This incomplete combustion results in lower competence for internal combustion engines. Designing a self tuned combustion engine to burn biobutanol can bring elevated gas mileage. Irrespective of its route of production (biomass or fossil fuel), the property of biobutanol remains the same unlike other alternate source of energy. It does not have to be stored in high pressure vessels

like natural gas, and can be blended (10 to 100 percent) with any fossil fuel. It can also be transported through existing pipelines for supply.

Major factor influencing the economics of biobutanol includes the cost and availability of feedstock. Rising end user acceptance and recognition for butanol as a carbon neutral fuel and an alternative to fossil fuels can motivate agriculture and industries. New government policies supporting the use of alternate energy resources may result in lowering of production costs. Developing new smaller biorefineries for small municipalities and underdeveloped area could commence state-of-the-art technologies at a faster rate than the traditional practices [3, 21].

Although voluminous literature has been published on various facets of ABE fermentation, most of the studies are on lab-scale, with very few studies on bench or pilot scale. There is an urgent need assess viability of the process designs on pilot scale that have been found promising on labscale. Development of an economic and efficient ABE fermentation technology is possible only through concerted efforts of microbiologists, biotechnologists and chemical engineers. Development of genetically modified strain having higher solvent tolerance and generation of metabolically engineered strains capable of utilizing varied carbon source and producing higher amount of butanol rather than other side products are the challenges set for scientific community [30-33]. As mentioned in previous sections, coculturing of clostridia and simultaneous saccharification and fermentation are few of the technologies, which on successful implementation can lead to economic acceptance of the process at large scale [34]. With that, it is beyond doubt that biobutanol has a bright future for commercialization. Recent advances in the fields of biotechnology and bioprocessing have resulted in a renewed interest in the fermentation production of chemicals and fuels, including n butanol. With continuous fermentation technology, butanol can be produced at higher yields, concentrations and production rates.

Nomenclature

a, b, c, d, e, f, g, h, i, j, k, y_c	Stoichiometric coefficients
Y	Marker of the physiological state of culture (dimensionless concentration of RNA)
S	Concentration of limiting substrate (section 7.3); amount of raw material (section 8)
S	Cost of raw material
B, C_B	Butanol concentration in fermentation broth
X	Biomass concentration (or concentration of cells)
n (or n′)	Number of active sugar transportation sites
BA_i	Intracellular butyric acid concentration
BA_{ex}	Extracellular butyric acid concentration
B_i	Intracellular butanol concentration
DBA_i	Intracellular butyrate concentration
UBA_i	Intracellular butyric acid concentration (undissociated form)
UBA_o	Extracellular butyric acid concentration (undissociated form)
pH_i	Intracellular pH
pH_o	Extracellular pH
P_i	Production rate of i^{th} component in fermentation broth
m_i	Partition coefficient for i^{th} component in the extraction solvent
X(0), X(τ)	Initial concentration of cells in broth and concentration at any time τ, respectively
I_i	Total productivity of i^{th} metabolite

J_i	Average concentration of i^{th} metabolite
$Y_{B/G}$	Yield of butanol per glucose consumed
$Y_{BE/G}$	Yield of butyrate per glucose consumed
$Y_{E/G}$	Yield of ethanol per glucose consumed
Y_i	Yield coefficient for product *i*
$\bar{X}$	Effective cell concentration for immobilized cells systems
R_{ATP}	Energetic parameter
b_i, b_{ij}	Regression coefficients (section 7.8)
C_{BA}	Concentration of butyric acid in broth
C_s	Critical butyric acid concentration required for solvent production
q_{sp}	Flux of any component through membrane
D	Diffusivity of a component through membrane
C	Concentration of a component in broth extracted with membrane
l	Thickness of membrane
y	Equilibrium mole fraction of a component in vapor phase
x	Equilibrium mole fraction of a component in liquid phase
K	Vapor-liquid equilibrium constant
P	Total pressure of the system
p	Price of a product
P^o	Vapor pressure of a component
V	Value function for biobutanol production
N	Total production of acetone, butanol and ethanol

Greek letters

γ_b, γ_p, γ_c	Degree of reductances for biomass, products and substrates
σ	Weight fraction of carbon in biomass
μ	Specific growth rate of culture
η	Volume ratio of fermentation broth to the extraction solvent
χ_i	Parameter representing effect of inhibitor *i*
χ_{ij}	Parameter representing combined effect of inhibitors *i* and *j*
μ_m	Maximum specific growth rate
μ_m^i	Maximum specific growth rate under inhibition
α, β	Proportionality constants for increase in production rate and reduction in product concentration, respectively
γ	Activity coefficient ,fixed and variable cost

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CHAPTER 3

RICE STRAW AS POTENTIAL FEEDSTOCK FOR BIOFUELS: ASSESSMENT AND REVIEW

3.1 Introduction

In Chapter 1, we outlined the scenario in the energy sector of India, and also discussed the potential of biomass based energy production. We have also given a quantitative account of the main source of biomass in India, i.e. agro residue. Rice being the major crop of India, is also a source of large biomass in the form of rice straw and rice husk, which could be potentially utilized for biofuels production – both gaseous as well as liquid. In this chapter, we have given an overview of the structure and properties of rice straw and different pre-treatment techniques reported in literature. We have also presented a brief review of the literature published in the area of rice straw base biofuels production.

3.2 Rice Straw: Structure, Composition and Properties

Rice is a grass (Gramineae) and belongs to the genus Oryzae (meaning oriental). *Oryza sativa* is grown in a wide range of environments from the equatorial tropics to sub tropical

mid-latitudes, from lowland paddy fields to high altitude terraces, and from swamps to upland rice fields. Rice straw is one of the most abundant residue available from the rice crop. Straws are almost entirely made of cell walls, and cell walls are generally lignified structural carbohydrates with small amount of structural proteins and minerals [1]. Straw is the only organic material available in significant quantities to most rice farmers. About 40% of the nitrogen (N), 30-35% of the phosphorus (P), 80-85% of the potassium (K), and 40-50% of the sulfur (S) taken up by rice remains in vegetative plant parts at crop maturity [2]. Rice straw is unique relative to other straws in having low lignin and high silica content. Silicon is involved in major roles in rice: carbohydrate synthesis, grain yield, phenolic synthesis and plant cell wall protection [3]. The high silica content, in addition to inner structure of rice straw and husks often hinders their combustion, and complete combustion of this material needs three items: fuel (the wastes), an oxidizer (the air) and energy (the heat). If one of these is missing, the combustion releases a larger amount of residue (ashes, toxic gas, carcinogenic compounds, etc.). The husks' shell shape prevents air from running through rice heaps, causing nitrogen oxide and carbon monoxide to be emitted, as well as releasing heavy ashes, thus polluting the environment [4-5].

Rice straw is mainly composed of three types of structural carbohydrate polymers: (1) cellulose, (2) hemicelluloses and (3) pectic polysaccharides (lignin). The layers of hemicelluloses and cellulose are densely packed by outer layers of lignin. Other components are residues of these storage polymers, viz. glucan, xylan, mannan etc. In straws, cellulose and hemicellulose are the dominant components. Cellulose chains bind together by hydrogen bond to form high tensile strength micro-fibrils, which are the fundamental structural unit of straw’s cell wall [6]. Lignin acts as cementing material, which protects these structural polymers. Lignin achieves this by acting as a physical barrier, and by suppressing the microbial activity due to the presence of phenolic compounds (*p*-coumaric acid, ferulic acid

Table 3.1: Mineral content of various cereal straw [1]

Mineral	Barley	Oat	Rice	Rye	Wheat
	(g/ kg dry mass)				
Ash	60	59	189	39	50-61
Silica	15	11	130	34	31-32
Ca	2.9	3.9	2.4	2.8	2.1-3.2
P	0.8	0.9	0.9	1.0	0.8
Mg	1.0	1.5	1.2	0.9	0.9-1.1
K	14.0	21.9	13.2	9.8	10.0-11.8
Na	-	-	-	0.5	0.5
Cl	7.7	8.1	-	2.5	3.5-6.1
S	1.4	2.5	1.3	1.2	1.4-1.6

etc.) as one of its component [1]. It has been reported that wheat straw is more lignified than rice straw. The crude protein content of rice straw ranges from 37-42 g/kg dry mass (DM) [7-8]. Carbohydrate, protein and mineral content of rice straw may vary with variation in soil conditions, weather, fertilization, harvest time, crop growth etc. A comparative account of mineral content of rice straw with other straws is given in Table 3.1.

In terms of chemical composition, the straw predominantly contains cellulose (32–47%), hemicellulose (19–27%) and lignin (5–24%) [9-12]. The pentoses are dominant in hemicellulose, in which xylose is the most important sugar (14.8–20.2%) [10,13]. The chemical composition of feedstock has a major influence on the efficiency of bioenergy generation. Rice straw as feedstock has the advantage of having a relatively low total alkali content (Na_2O and K_2O typically comprise < 15% of total ash), whereas wheat straw can typically have > 25% alkali content in ash [14].

Thus, from the above discussion it can be inferred that rice straw has a great potential to be utilized as feedstock for the production of renewable source of energy. Because of its structural constraints, it is necessary to pre-treat rice in order to break the lignin seal and to expose cellulose and hemi-cellulose for chemical and enzymatic action. Pre-treatment aims to decrease crystallinity of cellulose and hemi-cellulose fraction, increase biomass surface area,

remove hemi-cellulose, and break lignin seal. Pre-treatment makes cellulose more accessible to microbial, chemical or enzymes so that conversion of carbohydrate polymers into fermentable sugars can be achieved more rapidly and with more yields. Pre-treatment and hydrolysis includes physical, chemical, thermal, enzymatic and microbial methods and their combinations [3]. Pre-treatment has been viewed as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion [15]. In the next section, we have described various modes rice straw pre-treatment and hydrolysis reported in the literature.

3.3 Rice Straw: Pre-treatment/ Hydrolysis

Numerous techniques have been investigated in past few years to develop a cheap, efficient and environmental friendly pre-treatment technique for rice straw. In this section, we have grouped these techniques into four categories: (1) Physical, (2) Chemical, (3) Enzymatic, and (4) Biological, and are discussed below:

3.3.1 Physical Pre-treatment

Physical pretreatment increases the accessible surface area and size of pores, and decrease the crystallinity and degrees of polymerization of cellulose. Commonly used physical treatments to degrade lignocellulosic residues include steaming, grinding and milling, irradiation, temperature and pressure.

***Grinding and milling*:** Physical treatments such as crushing, grinding, steam and irradiation have been proven to be effective in creating accessibility of chemicals and enzymes [16]. Usually, grinding and milling are the initial steps of pre-treatment of any biomass, which reduces the particle size of straw, thus decreasing its crystallinity. Jin and Chen [17] attempted superfine grinding of steam exploded biomass, which yielded better results than ground residue on hydrolysis. But the process was observed to be energy intensive affecting

the overall economics of the process, which in turns effect its commercial applications. Hideno et al. [18] observed that for grinding rice straw, wet disk milling proved better than ball milling both in terms of glucose recovery as well as energy saving. Developments in this field provide a number of pre-treatments, which permit further saccharification, e.g. ball milling, roll milling, wet disk milling, and several type of grinding have been tried based on the biomass.

***Electron beam irradiation (E-Beam)*:** The cellulosic fraction of the lignocellulosic matter can be degraded by irradiation to fragile fibers, low molecular weight oligosaccharides and cellobiose [19]. Electron beam preferably dissociates the glycosidal bonds of the cellulose chains by irradiating in the presence of lignin. Jin et al. [20] irradiated milled dry rice straw using e-beam irradiation with accelerated electrons by a linear electron accelerator that had the capacity to produce electron beams. Enzymatic hydrolysis of electron beam irradiated rice straw indicated that in comparison to the untreated rice straw (22.6%), irradiated rice straw yield higher amount of glucose (52.1%). SEM and X-ray diffraction analysis of the irradiated rice straw confirms physical changes after electron beam irradiation. As these methods do not involve the use of extreme temperatures, the generation of inhibitory substances produced during acid or alkali pre-treatment can be either avoided or minimized, which is a major advantage of the process. On the other hand, irradiation methods are expensive, high energy demanding, and have difficulties in industrial application.

***Microwave Treatment*:** Xiong et al. [21] reported that microwave irradiation could change the ultra structure of cellulose, and can degrade lignin and hemicellulosic fractions of lignocellulosic biomass. It can also increase the enzymatic susceptibility of lignocellulosic materials [22]. Enzymatic hydrolysis of rice straw could be enhanced by microwave pre-treatment in presence of water [22-23], and also in glycerine medium with lesser amount of water [24]. Rice straw treated by microwave irradiation alone had almost the same hydrolysis

rate and reducing sugar yield compared to the raw straw [25]. Yang et al. [26] studied the effect of radiation on enzymatic hydrolysis of straw, and observed an increase in glucose yield with elevated irradiation doses, and achieved the maximum (13.4%) at 500 kGy. High heating efficiency and ease of operation are the major advantages of this process, while the demerits include high cost, scale-up limitations, security concern.

***Steam explosion*:** Steam explosion provides swift expansion of biomass, which opens up the structure of target biomass. Some researchers also refer it as "autohydrolysis". It has been reported to be more effective for hardwoods and agriculture residues than softwood [27]. The main factors affecting the results of steam explosion are residence time, temperature, particle size, and moisture content [28]. It is generally operated in a temperature range of 160-260^{o}C (corresponding pressure: 0.69-4.83 MPa) [29]. Several attempts have been made to increase the straw digestibility by steam and/or pressure. Guggolz et al. [30] reported that steam pressure results in solubilization of hemicellulose fraction of straws. Moniruzzaman [31-32] explored different exposure times and pressures, and observed 83% of glucose yield from cellulose (at 243^{o}C, 34 atm). To reduce or limit the damage to carbohydrates, Moniruzzaman has suggested pressure release after steam explosion. Steam explosion at very high temperature may lead to the production of inhibitors, hence a water wash is usually suggested in such extreme cases. *Advantage*: Shorter time, high efficiency, chemical free process, eco friendly. *Drawback*: Energy intensive process. Water wash leads to loss of some soluble sugars from the hydrolysate.

3.3.2 Chemical Treatment

Chemical hydrolysis mainly employs chemical agents like acids and alkali. Enzymes cannot effectively convert lignocelluloses to fermentable sugars without chemical pretreatment. They enhance hydrolysis and improve sugar recovery by removing lignin and hemicellulose

(Mosier et al. [15]).

***Alkaline hydrolysis*:** Alkali pre-treatment involves the application of alkaline solutions like NaOH or KOH to remove lignin and a part of the hemicelluloses, and efficiently increase the accessibility of enzyme to the cellulose [33]. The alkali pre-treatment can result in a sharp increase in saccharification yields. Alkali treatment is a time consuming process, and largely depends on the operational temperature. Pre-treatment can be performed at low temperatures, but with a relatively long time and high concentration of the base. The key mechanism of alkaline hydrolysis involves saponification of intermolecular ester bonds, which crosslink lignin, hemicellulose and cellulose. This increases the porosity and internal surface area of biomass matrix as well as decreasing the crystallinity of cellulose [29, 34]. Zhang and Cai [35] reported that alkaline pre-treatment of chopped rice straw with 2% NaOH with 20% solid loading at 85^{o}C decreased the lignin by 36%. The separated and fully exposed microfibrils increased the external surface area, and the porosity of the rice straw, thus facilitating enzymatic hydrolysis [36]. Besides NaOH, calcium hydroxide (lime) has also been explored as effective chemical agent for pre-treatment of lingo-cellulosic biomass. Chang et al. [37] suggested a loading of 0.1 g $Ca(OH)_2$/g dry biomass at 100-120 ^{o}C, and observed an increase of five times in sugar yield than that of untreated biomass. Chang and Holtzapple [38] have reported that lime treatment of biomass can result in sugar yield up to 80%. Effective hydrolysis of biomass is the major advantage of this process. Currently, most alkali pre-treatments use a large amount of chemical solution and water to soak substrates, which need recycling of chemicals, disposal of waste solution, and sometimes high temperature, and thus, could result in high facility investment, high treatment cost, and potential environmental pollution, which is a major demerit of this technique. Formation of unrecoverable salts with in feedstock is also a drawback.

Ammonia treatment: As a pre-treatment reagent, ammonia has number of desirable

characteristics. It is an effective swelling reagent for lignocellulosic materials. It has high selectivity for reactions with lignin over those with carbohydrates. Its high volatility makes it easy to recover and reuse. It is a non-polluting and non-corrosive chemical. One of the known reactions of aqueous ammonia with lignin is the cleavage of C–O–C bonds in lignin as well as ether and ester bonds in the lignin–carbohydrate complex [39]. A flow-through process called Ammonia Recycle Percolation (ARP) has been developed, in which ammonia is pumped through a bed of biomass maintained at 170^{o}C. It has been reported that it yields 85% delignification and almost theoretical yield of glucose in enzyme hydrolysis [40]. The effectiveness of the SAA process is strongly dependent on the pre-treatment temperature. The ammonia fiber/freeze explosion/expansion (AFEX) process uses anhydrous ammonia instead of aqueous ammonia, and this can also be recovered and recycled due to its high volatility. After treatment, the only exit stream is a gas mix containing ammonia and water vapor. All biomass components remain with the treated solids. Thus, there is no loss of any carbohydrate fraction. Since all of the ammonia will quickly evaporate, there is no need for pH adjustment of the treated material over a wide range before it can be used in subsequent enzyme hydrolysis and fermentation. This results in greater than 90% of theoretical glucose yield and 80% of theoretical xylose yield, with no formation of inhibitory compounds [40]. AFEX is reported as an effective pre-treatment process for rice straw, as it resulted in just 3% sugar loss during pretreatment [41]. Ferrer et al. [42] carried out pre-treatment of rice straw by a process called Ammonia Pressurization and Depressurization (PDA) using a laboratory-scale ammonia reactor unit consisting of a 4-L reactor with appropriate support equipment. Pre-treatment followed by enzymatic hydrolysis resulted significant increase in sugar yield. Major advantage of this process as compared to other alkalis such as sodium hydroxide or lime is that ammonia is highly selective for lignin removal, and shows significant swelling effect on lignocellulose. Also, it is easily recoverable due to its high volatility [43].

Acid Hydrolysis: Acid pre-treatment has received considerable attention over years [15]. Pre-treatment of lignocellulose with acids at ambient temperature enhances the anaerobic digestibility. Dilute acid pre-treatment predominantly affect hemicellulose with little impact on lignin degradation. Acid pre-treatment effectively removes the hemicellulose sheathing over cellulose. This technique increases accessibility of cellulose to enzymes, while at the same time loosening the structure of lignin and decreasing the crystallinity of cellulose. Mild acidic conditions are preferred for hydrolysis, as application of higher concentration of acids leads to the formation of furfurals, which acts as inhibitor for fermentation process. Acid pre-treatment is usually carried out using mineral acids like HCl and H_2SO_4, because of its comparatively less cost and high efficiency (up to 90 %) for both hemicellulose and sugar yield [43]. Yu et al. [44] reported hydrolysis of amorphous hemicelluloses in hot acid solution reaction, leaving larger perfect cellulose, and thus increasing of crystalline size of RS. Dilute acid pre-treatment can be a simple single-stage process, in which biomass is treated with dilute sulfuric acid at suitable acid concentrations, and temperatures for a period of time. To reduce enzyme requirements, a two-stage process was developed at the National Renewable Energy Laboratory (NREL) in Golden, Colorado. Besides sulfuric acid, phosphoric acid and carbonic acid have also been tested in a number of studies [45, 46]. Lower cost and high efficiency for sugar release are the advantages of this process, while production of inhibitors and toxins at high acid concentration, need for anticorrosive equipment, maintenance of pH, and acid recovery are some of the demerits.

3.3.3 Enzymatic Treatment

Enzymes are the biological catalysts. In this section, we specifically discuss the enzymes capable of digesting plant cell wall polysaccharides. These enzymes are introduced into the system either in pure form or as a constituent of microbial cell. Bacteria like *Cellulomonas*

fini and *Thermomonospora fisca*, and fungi like *Trichoderma* and *Aspergillus* are extensively utilized as source of biomass degrading enzymes. Cellululase is a complex enzyme containing endoglucanases, exoglucanases and β-glycosidases. These enzymes cleave the glycosidic linkages, along with reducing and non-reducing ends of polysaccharides (cellulose) generating sort chain oligos, which are further cleaved into glucose units by breaking the glycisidic linkages of sugar molecules [47]. Enzymes like cellulases, cellobiases, hemicellulases under optimum physical conditions reportedly avoid end product inhibition, and increase the final product yield [48-49]. Enzyme action mainly depends on properties of substrate, enzyme activity, reaction condition (pH, temperature, time etc). Yield of the whole process is generally enhanced by cumulative application of physical, chemical and enzymatic pre-treatment of biomass. Although use of enzymes gives an effective and efficient processing, high cost of enzymes is a major drawback of the process.

3.3.4 Biological pre-treatment

Biological pre-treatment exploits microbial metabolism to remove lignin or hemicellulose components from lignocellulosic biomass. Simultaneously it involves hydrolysis of cellulosic fraction in to soluble sugars. The most promising micro-organisms for biological pretreatment are white-rot fungi (*Phanerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora, and Pleurotus ostreatus*) that belong to class Basidiomycetes [50]. Of these white-rot fungi, *P. ostreatus* selectively degraded the lignin fraction of rice straw rather than the holocellulose component. The biological pre-treatment induces structural loosening of cells with a simultaneous increase in porosity. Patel et al. [51] performed a preliminary study on the microbial pre-treatment and fermentation of the agricultural residues like rice straw. A combination of five different fungi, viz. *Aspergillus niger, Aspergillus awamori, Trichoderma reesei, Phenerochaete chrysosporium, Pleurotus*

Table 3.2: Summary of pre-treatment techniques for rice straw

Reference	Mode of Pre-treatment	Fuel	Mode of Production	Product Titre
[52]	Microbial digestion	Biodiesel	Microbial conversion using BSFL* and microbes (Rid-X)	43.8 g L^{-1}
[53]	Alkali and Enzyme (cellulase)	Butanol	*Clostridial* Fermentation (SHF#)	1.41 gL^{-1}
[54]	Chemical hydrolysis	Biogas	Anaerobic digestion	327.5 ml/gVS
[57]	Lime pretreated and CO_2 neutralized	Ethanol	SSF	21.1 gL^{-1}
[58]	Microwave	Bio oil	Pyrolysis	38%
[74]	Alkali	Ethanol	SSF	24.25 gL^{-1}
[83]	Steam explosion, enzyme and fungus	Ethanol	SSF##	13.2 g L^{-1}
[84]	Solid acid catalyst	Bio-oil	Pyrolysis	44-48%

*****Black Soldier Fly Larvae
Simultaneous Hydrolysis and Fermentation
Simultaneous Saccharification and Fermentation

sajor-caju obtained from screening were used for pre-treatment and Saccharomyces cereviseae (NCIM 3095) was used for carrying out fermentation. Among the different fungi employed, *A. niger* and *A. awamori* yielded highest amount of ethanol (2.2 g L^{-1}). Low chemicals and energy requirement (making it environment-friendly) are the major advantages of this process, while slow kinetics is the major drawback.

The following Table summarizes various pre-treatment techniques for rice straw employed in production of different types of biofuels.

3.4 Biofuel Production from Rice Crop Residue: A Literature Review

As noted earlier, the major residue of rice crop is rice husk and rice straw. Past two decade have seen significant research activities in the field of rice straw and rice husk based energy production. We present in this section a review of some recent studies published in the

area. This review (comprising of some recent representative papers) is, by no mean, an extensive account of rice based biofuels, but is intended to give the reader an idea of potential of rice crop residue for production of diverse biofuels such as microbial oil, producer gas (mixer of CO and H_2), methane and biogas, and liquid biofuels such as biodiesel, bioethanol, and biobutanol.

- Zheng et al. [52] have demonstrated a co-conversion process using BSFL (black soldier fly larvae) to convert rice straw and restaurant solid waste (RSW) into larval grease of black soldier fly. About 43.8 g biodiesel was produced from 2000 BSFL grown on 1000 g mixed feed of rice straw (30%) and RSW (70%) in 10 days. This process resulted in digestion of 65.5% cellulose, 56.3% hemicelluloses, 8.8% lignin, 91.6% protein and 71.6% lipid in the feed. The result showed that grease from BSFL fed on rice straw and RSW was suitable for biodiesel production.
- Cheng et al. [53] have reported production of biobutanol from rice straw using an efficient butanol producing bacterial microflora (containing mainly *clostridial* species) obtained from hydrogen producing sewage sludge. The rice straw was alkali pretreated and then hydrolyzed using cellulases obtained from pseudomonas and *clostridial* species. The hydrolyzate obtained from this treatment was fermented using either simultaneous saccharification and fermentation (SSF) or separate saccharification and fermentation. In the separate saccharification-fermentation process, rice straw yielded maximum butanol concentration of 2.92 g L^{-1}, with productivity of 1.41 g L^{-1} day^{-1}, yield of 0.51 mol butanol per mol reducing sugar, and ABE ratio 0.19:1:0.1. For SSF process, the same values were 2.93 g L^{-1}, 0.86 g L^{-1} day^{-1}, and 0.49 mol butanol per mol of reducing sugar.
- Song et al. [54] have demonstrated used of rice straw for biogas production by anaerobic digestion. Pretreatment of rice straw with ammonium hydroxide and hydrogen peroxide increased the degradation of content of rice straw such as lignin, cellulose and

hemicelluloses, which was finally manifested in enhanced biogas production.

- Wang et al. [55] have reported performance of the pilot plant for cellulosic ethanol production (with capacity of 1 ton dry biomass per day) with feedstock as rice straw. The process for bio ethanol production consisted of a series of steps like acid hydrolysis, high solid to liquid ratio hydrolysis, xylose fermentation and distillation and dehydration process.
- Yang et al. [56] have reported production and characterization of bio-oil from fast pyrolysis of rice straw. The production system for bio-oil comprised of a bench scale fluidized bed reactor, char removal system, and oil collection system and oil recycling spray condenser. The optimum temperature for bio-oil production was 450^{o}C with pH value of bio-oil being 4.1 and viscosity 9 cP at 25^{o}C.
- Li et al. [57] have reported a novel technique of sequential use of *S. cerevisiae* and *P. stiptis* for bioethanol production from rice straw. The cells of *S. cerevisiae* were deactivated before addition of *P. stiptis.* In the SSF of lime pretreated and CO_2 neutralized rice straw, the inactivation of *S. cerevisiae* cells enabled full conversion of glucose and xylose. 21.1 g L^{-1} ethanol was produced from 10% w/w of pretreated rice straw, and ethanol yield of rice straw was 72.5% of theoretical value.
- Du et al. [58] have studied fast pyrolysis of rice straw and saw dust under microwave irradiation with 2 ionic liquids, viz. 1-butyl-3methyl imidazolium tetra fluoro borate as catalysts. For microwave heating of 20 min, yield of bio-oil from rice straw was 38%, while that from saw dust was 34%. The main components of bio-oil were furfural, acetic acid, 1-hydroxy-2-butanol. The composition of the bio-oil was found to depend mainly on the source of biomass and type of ionic liquid use.
- Okamoto et al. [59] have reported direct ethanol production from starch, wheat, bran, and rice straw by white rot fungus *Trametes hirsuta*. This fungus is capable of hydrolyzing biomass to fermentable sugar, and directly converting them to ethanol (without acid or

enzyme hydrolysis). The yield of ethanol from variety of hexose sugars was: glucose 0.49 g/g, mannose 0.48 g/g, cellobiose 0.47 g/g and maltose 0.47 g/g. The xylose fermentation yield was 0.44 g/g. This fungus during its growth in media containing 2 0 g L^{-1} rice straw yielded 2 g L^{-1} of ethanol, which was 57.4% of theoretical yield.

- Li et al. [60] have reported a novel process of growing high oil containing microalga *Chlorella pyrenoidosa* in rice straw hydrolyzate, and its in-situ trans-esterification. Enzymatic hydrolysis of rice straw yielded 13.7 g L^{-1} of sugar, and the species *C. pyrenoidosa* showed rapid growth (2.83 g L^{-1} in 48 h) with lipid content as high as 56.3%. In situ trans-esterification with 4 mL methanol and 0.5 M sulphuric acid resulted in 95% biodiesel yield.

- Lou et al. [61] have investigated pyrolysis products from rice straw using a pyrolysis tube furnace system under nitrogen atmosphere and temperature range of 450 to 900^{o}C. The pyrolyzate was classified in three groups, viz., bio-oil, light gas, bio-char. Due to high char generation from lignin pyrolysis, the volatile from rice straw pyrolysis exceeded that from lignin. The gaseous products mainly composed of H_2 and CO with other components like CO_2 and light hydrocarbons. The bio-oil mainly comprised of aromatic hydrocarbons, change hydrocarbons, mono-aromatic and minor amount of ketone.

- Silva et al. [62] have conducted optimization studies in fermentation of rice straw hemicellulosic hydrolyzate for ethanol production by *Pischia stipitis* NRRL Y-7124. The influence of initial xylose concentration, agitation and aeration on ethanol production was assessed through 22 full factorial design. Initial xylose concentration of 50 g L^{-1} was more suitable, while the lowest aeration and highest agitation level were found to give the highest cell yield factor. These results pointed out the importance of aeration in the process of fermentation of rice straw hemicellulosic hydrolyzate.

- Binod et al. [33] have reviewed the technical aspects of bioethanol production from

rice straw. Although high cellulose and hemicelluloses content of rice straw makes it a potential substrate, the demerits are presence of high ash and silica. Binod et al. have emphasized on the importance of pre-treatment method to enhance the efficiency of enzymatic saccharification.

- Dominguez-Escriba and Porcar [63] have reviewed the strategies of rice straw management. Globally the production of rice (grain) is estimated at 660 million tones that also produces 800 million tons (dry) of residue. The waste is managed either by burning (that creates pollution) or soil incorporation (that produces methane). Using the rice straw for production of bioethanol seems to be a feasible option, but significant technology developments in pretreatment, hydrolysis and fermentation techniques are required for commercial scale implementation of rice straw base technology.
- Chou C-S et al. [64] have reported preparation and characterization of solid biomass fuel made from rice straw and rice bran. The solid fuel was in the form of briquettes. The method for preparation of briquettes comprises of two steps, viz. smashing of the rice straw in pieces and compressing the smash rice straw pieces and rice bran into the briquettes. These briquettes were characterized for air dry density, compressive strength and heating value. As the percentage of rice bran increases, the compressive strength and heating value of biomass briquettes increases. The thermo-energy, which is used to compress the fuel briquettes from rice straw, will be minimized if the certain percentage of binder was mixed with smashed rice straw.
- Huang et al. [65] have reported enhanced inhibitor-tolerant strain of *Pichia Stipitis* through adaptation of acid treated rice straw hydrolyzate. The ethanol production obtained by fermentation of NaOH neutralized hydrolyzate without detoxification using the adapted strain was comparable to fermentation of overliming-detoxified hydrolyzate. The ethanol yield with adapted strain was 87% of the maximum; while addition of 3% sulfate and 1.3 g L^{-1} furfural

brought down the yield to 60%.

- Chang et al. [66] have reported establishment of semi-solid biohydrogen producing system using brewery yeast waste and micro flora from rice straw compost. For suitability of process for large scale implementation, a combination of aerobic and anaerobic microbial strains was used for production of biohydrogen and biofuels. The aerobic strain or *Bacillus thermoamylovorans* was suitable for pre-saccharification of substrate. Among the anaerobic strains, *C. beijerinckii* L9 showed highest H_2 producing ability. Authors mentioned that combination of aerobic *Bacillus* and anaerobic *Clostridium* can play key role in developing industrial scale process for biofuel production.
- Jung et al. [67] have studied production of bio-oil from rice straw and bamboo saw dust under different reaction condition. The process was carried out in a bubbling fluidized bed equipped with char separation system. The pyrolysis temperature was varied in the range of 415-540^{o}C for rice straw pyrolysis. The optimum temperature range for production of bi-oil from rice straw was 440-500^{o}C. Use of product gas as fluidizing medium enhanced production of bio-oil, which comprised of phenolics, furfural, acetic acid, levoglucosan, guaiacol and alkyl guaiacol. Low content of alkali alkaline earth metals in the bio-oil (which increased its quality) was attributed to excellent performance of the char separation system.
- Li et al. [68] have studied subcritical liquefaction of rice straw to bio oil with different kinds of solvents (basically mixtures of water and 1,4-dioxane in different proportions) in 500 ml autoclave at 573 K and 5-9.9 MPa. The maximum liquefaction conversion of rice straw is 89.05% with 1,4-dioxane-water mixture. Different compositions of mixtures of 1,4-dioxane-water were used, and it was revealed that yield of oil increases with increasing ratio of 1,4-dioxane:water in the range of 0:10 to 5:5, and again decreases further when the ratio goes from 5:5 to 10:0.
- Singh et al. [69] have presented a case study of utilization of rice straw/rice husk from

the agricultural fields of Indian state of Punjab for power generation. The fluidized bed combustor is ideally suited to burn such fuels, since uniform mixing condition ensure efficient combustion, even at low temperatures of 873 to 923 K. The thermal power plant is of capacity 10 MW located in Jalkheri (Fatehgarh Sahib Punjab). The CO_2 emissions from the plant are nearly zero, and SO_x and NO_x are below emissions from conventional coal based power plant.

- Nakagewa et al. [70] have discussed biomethanol production and CO_2 emission reduction from forage grasses & crop residues like rice bran, rice straw and rice husk. The rice bran gave a yield of 55 wt%, while rice straw and rice husk gave a yield of 36% and 39%, respectively. The net heat yield of methanol production of full scale commercial plant was 40%.
- Tsai et al. [71] have investigated fast pyrolysis of rice straw, sugarcane bagasse and coconut shell in an induction heating reactor to produce valuable products. The effect of the process parameters such as pyrolysis temperature, heating rate and holding time on the yield of pyrolysis products, and their chemical compositions was also investigated. The maximum yield of 50% on the pyrolysis liquid product could be obtained at proper process conditions.
- Lee et al. [72] have reported production of bio-oil from rice straw by fast pyrolysis in a fluidized bed in a bench scale plant, and also the influence of reaction temperature pre-treatment and char removal system comprising of a cyclone and hot filter. The experiments revealed that pre-treatment of rice straw (washing) reduced the content of alkali metals in the bio-oil and the optimum reaction temperature range for production of bio-oil was 410-510^{o}C.
- Pattiya and Suttibak [73] have reported experimental result of rapid of fast pyrolysis of rice straw and rice husk in a fluidized bed reactor unit incorporated with a hot vapour filter. The experimental results showed that the optimum pyrolysis temperatures for rice straw and rice husk are 405 and 452^{0}C, which gave a maximum bio-oil yield of 54.1 and 57.1

wt% on dry biomass basis. Use of hot filter led to reduction of 4-7 wt% bio-oil yield. However, glass wool hot filtered bio oil was found possess better properties.

- Oberoi et al. [74] have reported investigation in ethanol production from alkali treated rice straw via simultaneous saccharification and fermentation using a new thermo-tolerant species *Pichia Kudria-vzevii* HOP-1. A mixture of cellulose, β-glucosidose and pectinose was used for saccharification. The new strain could assimilate glucose, sucrose, galactose, fructose and mannose, but could not utilize xylose, arabinose, cellobiose and raffinose. Ethanol concentration of 24.25 g L^{-1} corresponding to 82% yield with productivity of 1.1 g L^{-1} h^{-1} was achieved. An insignificant amount of glycerol and no xylitol was produced during SSF.
- Calvo et al. [75] have reported gasification of rice straw in a fluidized bed gasifier for syngas application in close-coupled boiler-gasifier system. The gasification temperature was 700 and 850^{o}C. The stoichiometric air/fuel ratio for rice straw was 4.28 and actual air supplied was 7-25% of this ratio. For these experimental conditions, the higher heating value (HHV) of the producer gas was 5.1 MJ/Nm^3 with hot gas efficiency of 6% and cold gas efficiency of 52%.
- Kim et al. [76] have studied two stage pre-treatment of rice straw using aqueous ammonia and dilute acid. The aqueous ammonia in the first stage removed liquid selectively, but left most of cellulose and hemicelluloses. Dilute acid was applied in second stage, which removed most of hemicelluloses, while partially disrupting the crystalline structure of cellulose, thus, enhancing enzymatic digestibility of cellulose in the remaining solids. Under optimal pre-treatment conditions, the enzymatic hydrolysis yields of two-stage treated samples were 96.9% and 90.8%, with enzyme loading of 60 and 15 FPU/g of glucose.
- Park et al. [77, 78] have reported a simple DiSC process (direct-saccharification-of-culms) to produce ethanol from rice straw culms that accumulate significant amount of soft

carbohydrates such as glucose, fructose, starch and β-1,3-1,4-glucan. This study focused on fully mature culms containing 69.2% (w/w of dried culms) hexoses from soft carbohydrates and cellulose. A culm rich fraction (with recovery of 83.1% w/w soft carbohydrates) was prepared from rice straw flakes (54.1% w/w of rice straw). After an initial suspension in water (20% w/w) for starch liquefaction, the suspension was subjected simultaneous saccharification and fermentation with yeast, yielding 5.6% w/v ethanol (86% of theoretical yield) after 24 h fermentation. DiSC process has merit of yielding concentrated ethonal from rice straw without use of harsh thermo-chemical pre-treatment.

- Yadav et al. [79] have studied bioethanol fermentation of concentrated rice straw hydrolyzate using co-cultures of *Saccharomyces cerevisiae* and *Pichia stipitis*. The rice straw was acid hydrolyzed with initial sugar content of 16.8 g L^{-1}. The hydrolyzate was concentrated to 31 g L^{-1} by vacuum distillation prior to fermentation. Ethanol concentration was found to be 12 g L^{-1} with volumetric productivity of 0.33 g L^{-1} h^{-1}, yield of 0.4 g/g and fermentation efficiency of 95% with the co-cultures.
- Park et al. [80] have developed a novel lime pre-treatment process for bioethanol production from rice straw, which does not require a solid-liquid separation step. After pre-treatment lime was neutralized by carbonation, with final pH of solution being 6. Although $CaCO_3$ produced in the process was kept in reaction vessel, it did not give any inhibitory effect. Simultaneous saccharification and fermentation of pre-treated rice straw with co-cultures of *S. cerevisiae* and *P. stipitis* yielded 19.1 g L^{-1} ethanol (equivalent to 74% of theoretical yield from glucose and xylose).
- Lei et al. [81] have used rice straw particles a direct substrate for anaerobic digestion with acclimated sludge under room temperature and different phosphate levels. The average biogas and methane production rate constants were 0.027 to 0.03 L day^{-1} to 0.028 to 0.033 L day^{-1}. The results indicated that adequate phosphate addition (465 mg P L^{-1}) could accelerate

bio-gasification process.

- Zhang and Zhao [82] have reported conversion of lignocellulosic biomass into furans in the ionic liquid based medium in presence of microwave irradiation and $CrCl_3$ as catalyst. Common biomass like corn stalk, rice straw and pine wood produced HMF and furfural in yields of 45 to 52% and 23 to 31%, respectively. The conversion was quite fast, achieved in 3 min. This method, thus, represents an energy efficient and cost effective conversion of biomass into biofuels and basic chemicals.

3.5 Conclusion

The literature review presented above clearly demonstrates the potential of rice crop residue in the form of husk as well as straw for biofuels production. The most widely investigated biofuels from rice crop residue are bioethanol through fermentation and producer gas through biomass gasification. The former is a potential liquid transportation fuel while the latter could be utilized for electricity through producer gas generator sets. Rice husk can be used directly for gasification, while rice straw needs to be pretreated and hydrolyzed prior to fermentation for release of sugars. This adds a cost intensive step to the process.

Biobutanol synthesis through ABE fermentation based on rice straw as feedstock is not as widely investigated as bioethanol. This thesis tries to address this important issue. Taking this theme ahead, in the next chapter, we try to evaluate the potential of rice straw with respect to other alternate feedstocks for fermentation. Selection of a suitable microbial strain for fermentation is also an important step in the process development of rice straw based ABE fermentation. This aspect is also treated in the next chapter. Pretreatment of rice straw through various physical, chemical and enzymatic techniques and its subsequent optimization has been dealt with in the fifth chapter of the thesis.

Abbreviations

DM	Dry Mass
E-Beam	Electron Beam
SEM	Scanning Electron Microscopy
ARP	Ammonia Recycle Percolation
AFEX	Ammonia Fiber/Freeze Explosion/Expansion
PDA	Ammonia Pressurization and Depressurization
NREL	National Renewable Energy Laboratory
BSFL	Black Soldier Fly Larvae
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
RSW	Restaurant Solid Waste
DISC	Direct-Saccharification-of-Culms
ABE	Acetone Butanol Ethanol

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CHAPTER 4

SELECTION OF SUBSTRATE AND MICROBIAL STRAIN & UNDERSTANDING ABE FERMENTATION

4.1 Introduction

In the previous chapters, we outlined the potential of agro–residues as a substrate for biofuels production. Rice is a major crop in India and the residue of this crop in the form of husk and straw is a potential feedstock for gaseous and liquid biofuels [1–5]. The emphasis of this thesis is on development of the fermentation process for biobutanol production using rice straw as the feedstock. The first step of such a process is to select a suitable microbial strain that has capability of effectively fermenting sugars in the hydrolyzate obtained from rice straw. We would like to specifically mention that we are focusing on Separate Hydrolysis and Fermentation (SHF) process for biobutanol. Some earlier authors have also attempted Simultaneous Saccharification and Fermentation (SSF), which is also a process alternative [6–8]. Instead of isolating and characterizing a clostridial strain of our own, we have relied upon the microbial cultures that were available in various culture banks in India. In this chapter, we present a comparative assessment of these cultures for fermenting sugars released from rice straw for acetone–butanol–ethanol production. In addition, to prove the potential of

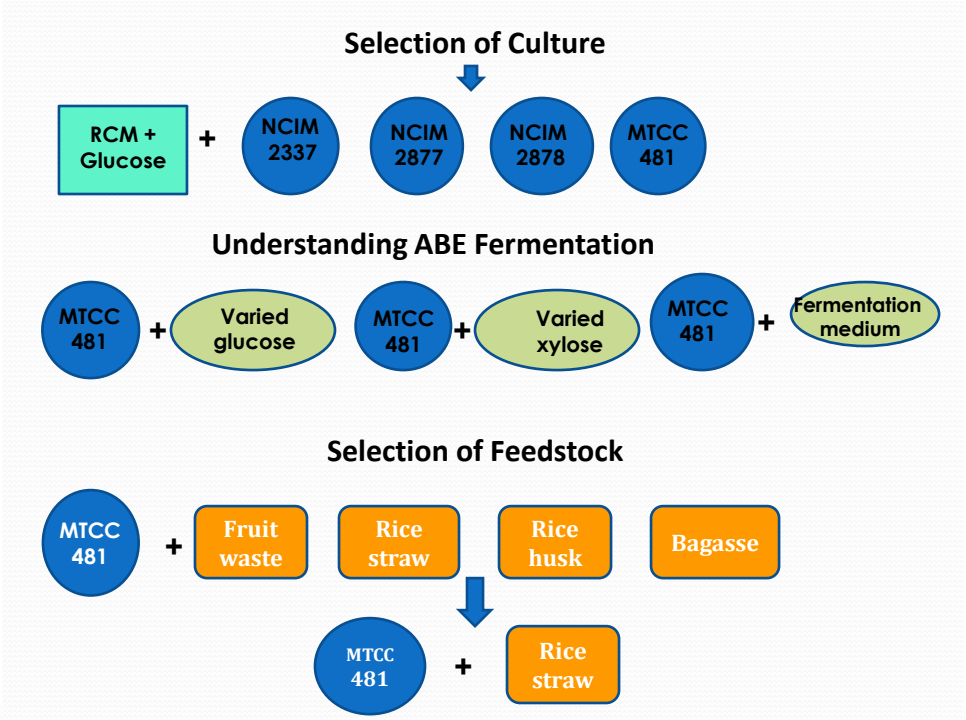


Figure 4.1: Schematic of methodology adapted in chapter 4

(i) (ii) (iii)

Figure 4.2: Anaerobic culturing of clostridial species.
(i) Anaero bag, (ii) Anaero indicator tablet, (iii) Anaerogas Pack

rice straw for fermentation, we have also done experiments with other cheap alternate feedstocks and compared the results with the fermentation of rice straw hydrolyzate.

Figure 4.3: Custom fabricated flask with a bottom spout and screw cap for anaerobic clostridial cultures

4.2. Material and Methods

4.2.1. Culture maintenance and growth

Lyophilized cells of *Clostridium acetobutylicum* MTCC 481 were procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology (Chandigarh, India). These cells were maintained as spore suspension in sterile water. Three strains of *Clostridium acetobutylicum* NCIM 2337, 2877 and 2878 in sand have been procured from NCIM Pune (India) in dried spore form. The cells were revived anaerobically inside an anaerobic culture bag system (Himedia) in RCA (Reinforced Clostridial Agar), and RCM (Reinforced Clostridial Medium: Broth) culture media at 37^{o}C (Fig. 4.2). The inoculums were prepared in RCM containing following components (with concentration mentioned in g L^{-1}): glucose, 5.0; yeast extract, 3.0; starch, 1.0; beef extract, 10.0; peptone, 10.0; sodium chloride, 5.0; sodium acetate, 3.0; Agar, 0.5; cysteine hydrochloride, 0.5. The pH of medium was 6.8 ± 0.2. 100 mL of media was autoclaved at 121^{o}C, 15 lb pressure and inoculated in a custom fabricated 250 mL screw capped Erlenmeyer flask (Fig. 4.3). In addition, CMM (Cooked Meat Medium) was also used for the maintenance of clostridia. Anaerobic condition in broth culture was maintained by adding 0.05% of cysteine hydrochloride, and regular sparging of nitrogen

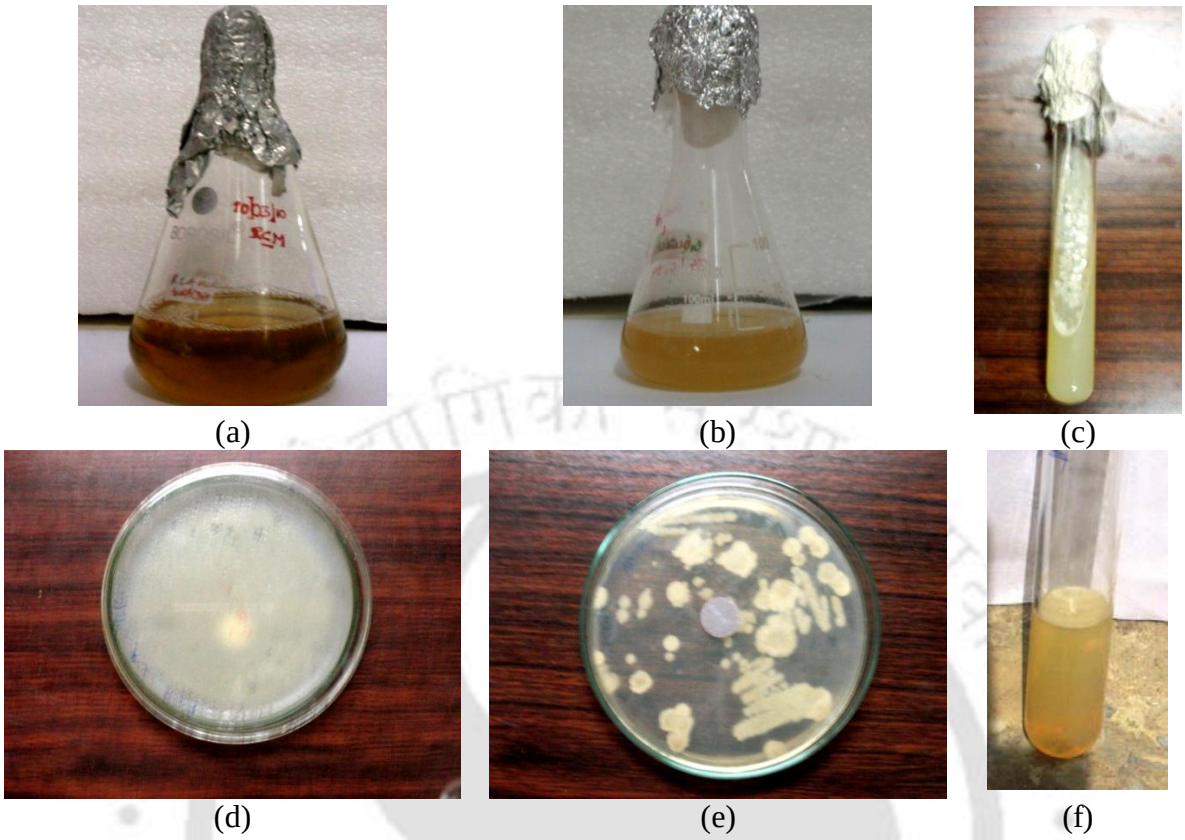


Figure 4.4: Maintenance and culturing of clostridial species. (a) RCM Broth, (b) Cultured RCM broth, (c) clostridial slant (d) bacterial lawn (spread plate). (e) streak plate of clostridia, (f) clostridial stab culture

through fermentation broth. All chemicals were of analytical grade procured either from Merck (Germany), Sigma Aldrich (Germany) or Himedia (India). The revived cells were maintained on RCM broth, RCA plates (bacterial lawn and streaks) and slants at 4^{o}C, and were used as a stock (Fig. 4.4). The cells were sub–cultured every month.

***Microscopic staining of clostridias via Grams stain and Malachite green* (*spore staining*)**

Sterilized microscopic slides, crystal violet dye, distilled water, Lugols iodine, ethanol, acetone tissues, safranin, oil immersion, microscope (Carl Fischer, Germany) and fresh Clostridial cultures were required for staining. All the dyes and chemicals were procured from Himedia, Germany. There are four basic steps of the Gram staining as follows: (1) application of a primary stain (crystal violet) to a heat–fixed (death by heat) smear of a

bacterial culture, (2) addition of a trapping agent (Gram's iodine), (3) rapid decolorization with alcohol or acetone, and (4) counter–staining with safranin.

Malachite staining requires sterilized microscopic slides, saturated solution of Malachite green dye, distilled water, hot plate/oven/water bath, tissues, safranin, oil immersion, microscope and old clostridial cultures (10 days +). Staining initiates with making a smear of the *C. acetobutylicum* species by air–drying and heat–fixing. Smear was then steamed, and flooded with the primary dye, malachite green, and left for 15 min. Extra moisture was removed with help of paper towel. The slide was steamed over boiling water for some time and again washed thoroughly with water and finally counter–stained with safranine.

4.2.2. Preparation of biomass hydrolyzate

Four biomasses, viz. fruit waste, bagasse, rice straw and rice husk were procured from local areas of Guwahati. All four biomasses were initially washed with water to remove the impurities, and then were allowed to dry at 50^{o}C for 48 h in a hot air oven (JSJW, India). Dried biomass was further chopped and grinded into small sizes using a mixer grinder (Sumeet, India). 3% w/v solutions of all four processed biomass were hydrolyzed using 0.5% v/v of sulfuric acid, and was agitated at 150 rpm at 60^{o}C using a shaker incubator (Sciegenics, India). Resulting hydrolyzate was filtered using a sterile muslin cloth, and filtrate was used as feedstock for fermentation processes.

4.2.3. Preparation of fermentation broth for selection of Clostridial strain

Reinforced Clostridial Agar medium (broth) was prepared as stated in section 5.2.1. Before autoclaving, broth was supplemented with 2.0% of glucose. All four strains of *C. acetobutylicum* were allowed to undergo fermentation in this glucose supplemented synthetic medium. Culture was selected on the basis of its ability to yield high amount of solvents, with greater selectivity towards butanol.

4.2.4. Preparation of fermentation broth with varied glucose concentration

Experiments were performed in 4 set of flasks, each containing 2, 4, 6 and 8% w/v of glucose solution. All reagents were of analytical grade. The concentrations of all other components of RCM medium as mentioned in section 4.2.1 (except glucose) were kept constant, and the glucose concentration was varied as 2, 4, 6 and 8% w/v. Experiments were conducted in this medium with MTCC 481 strain incubated at 37^{o}C at 120 rpm for 12 days.

4.2.5. Preparation of fermentation broth with varied xylose concentration

Experiments were performed in 4 set of flasks, each containing 2.5, 4, 5, and 6% w/v xylose. All reagents were of analytical grade. The concentrations of all other components of RCM medium as mentioned in section 4.2.1 (except xylose) were kept constant, and the xylose concentration was varied as 2.5, 4, 5 and 6% w/v. Experiments were conducted in this medium with MTCC 481 strain incubated at 37^{o}C at 120 rpm for 12 days.

4.2.6. Fermentation

Batch fermentation experiments were carried out with 100 mL of working volume of fermentation broth in custom fabricated 250 mL screw–capped Erlenmeyer flasks (Fig. 4.4). These flasks had a bottom port for sample withdrawal and nitrogen sparging in order to minimize oxygen contamination and maintain strict anaerobic conditions. Anaerobic condition in the flask was generated by addition of 0.5% w/v cysteine hydrochloride to the fermentation medium. An initial sample (0 h) was taken immediately after pretreatment for sugar analysis. Regular samples were withdrawn from the broth to study the growth, sugar release and utilization and solvent production by *Clostridium acetobutylicum*. All flasks were sparged with nitrogen at the start, and after every 24 h of fermentation to maintain anaerobic conditions. The samples of fermentation broth were withdrawn at constant intervals upto a period of 12 days. Each experiment was conducted in duplicate to assess the reproducibility of the results.

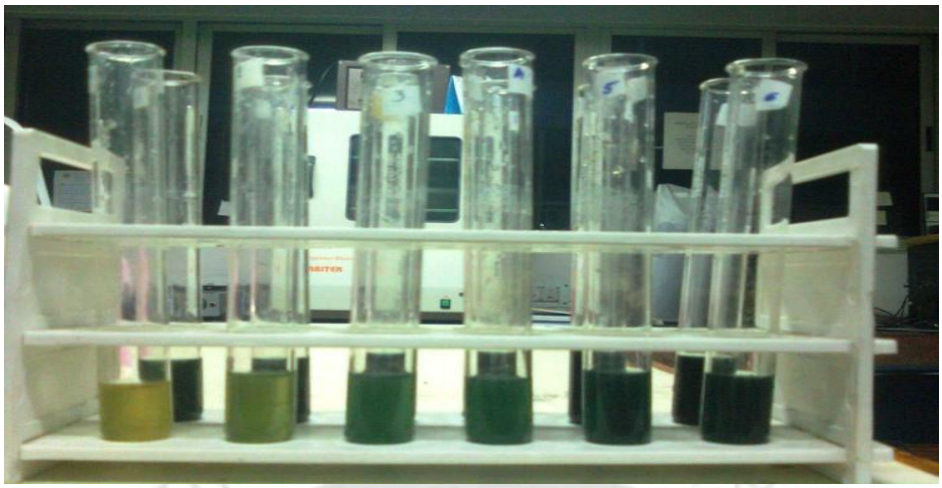


Figure 4.5: Anthrone test for quantitative analysis of total sugar

Figure 4.6: Glucose oxidase assay for quantitative estimation of glucose

4.2.7. Analysis

The optical density of the cells in the fermentation broth was measured using UV–Vis spectrophotometer (Thermo Fischer) with absorbance at 600 nm after appropriate dilution in water. Quantification of total sugar was done by Anthrone test (Fig. 4.5) as directed by Hedge and Hofreiter [9]. Glucose was analysed using Glucose (GO) assay kit procured from Sigma Aldrich, USA (GAGO20–1KT) (Fig. 4.6). All the samples were filtered with 0.2 μm filter and diluted appropriately for the qualitative and quantitative determination of the

fermentation products, viz. acetone, butanol and ethanol. Solvent production in the fermentation broth was monitored on a gas chromatograph (Varian) using a CP Wax 52CB (250 mm × 0.25 mm × 0.39 mm) capillary column, and a Flame Ionization Detector. The injector and detector temperatures were 230 and 250^{o}C, respectively. The oven temperature was programmed from 45 to 100^{o}C with an increment of 3^{o}C/min, and after 100^{o}C, an increment of 5^{o}C/min up to 200^{o}C.

Standard curve plot for quantitative estimation of acetone, butanol and ethanol using gas chromatograph

Standard curve for quantitative estimation of acetone, butanol and ethanol solvent was plotted using GC grade standard acetone, butanol and ethanol solvents from Sigma Aldrich. Standards plots achieved for all the three solvents are mentioned in Fig 4.7 to 4.9.

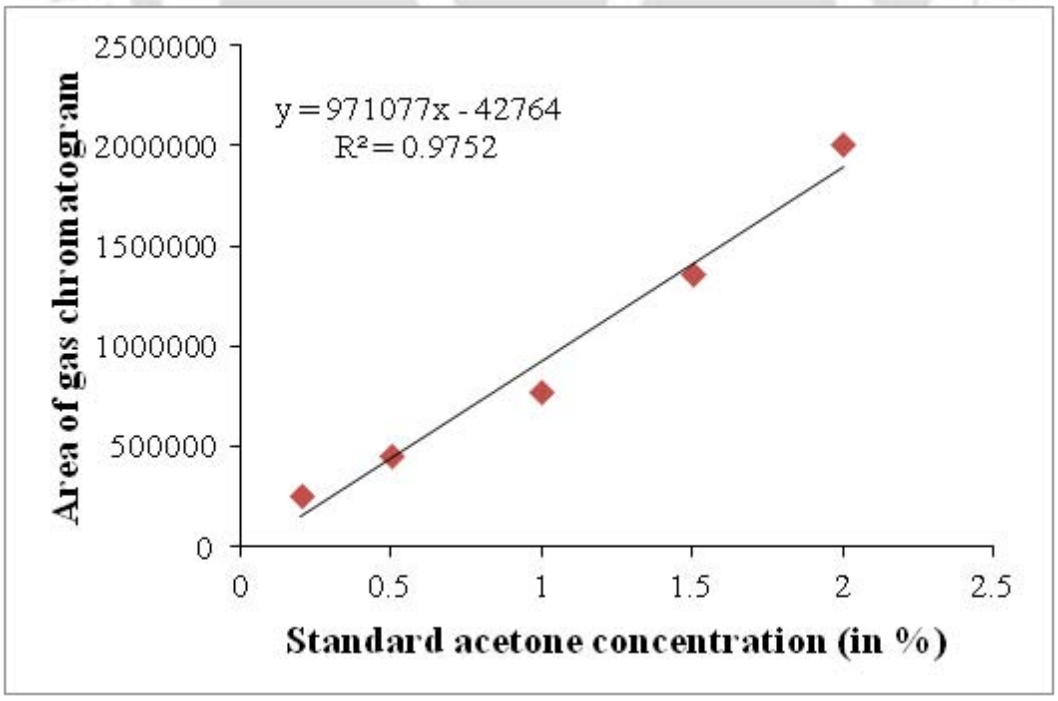


Figure 4.7: Standard plot for quantitative estimation of acetone

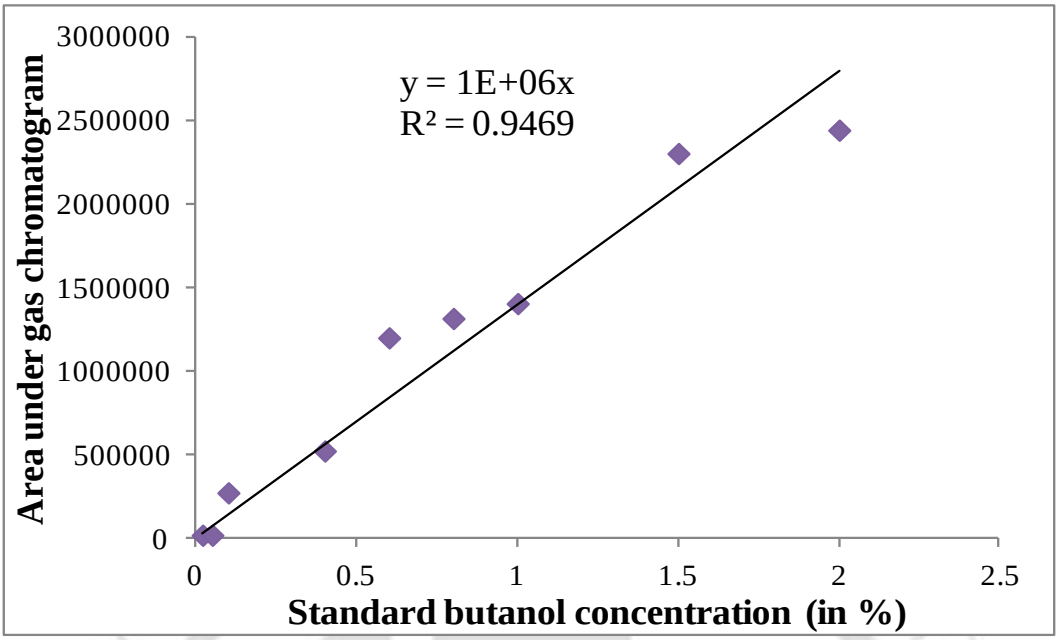


Figure 4.8: Standard plot for quantitative estimation of butanol

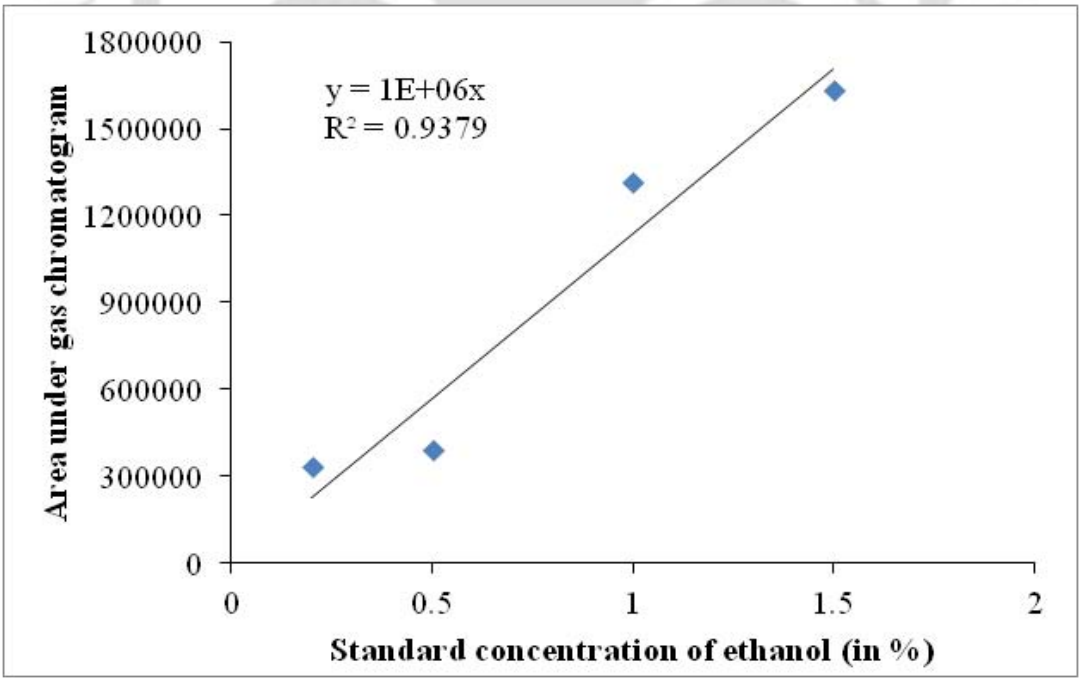
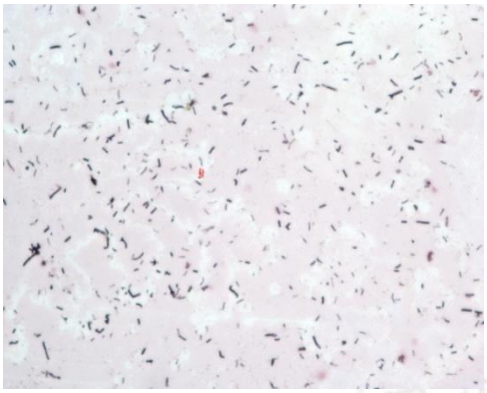
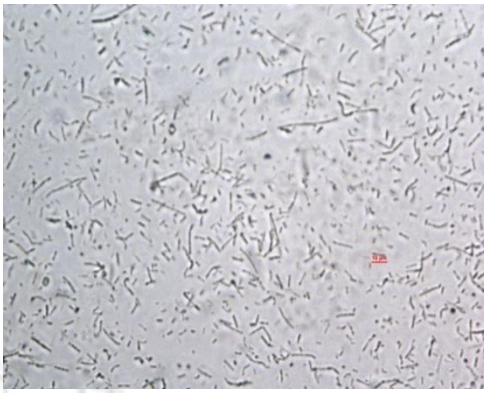


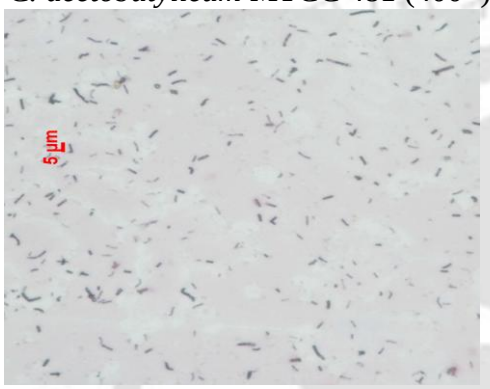
Figure 4.9: Standard plot for quantitative estimation of butanol



C. *acetobutylicum* MTCC 481 (400×)



C. *acetobutylicum* NCIM 2337 (400×)



C. acetobutylicum NCIM 2877 (400×)

C. acetobutylicum NCIM 2878 (1000×)

Figure 4.10: Gram staining of four clostridial strains

4.2.8. Estimation of yield and selectivity

Solvent yield was calculated as gram per liter of solvent produced per gram litre of total sugar added (g/g). Butanol selectivity was calculated as mol of butanol produced per mol of total solvent (ABE) production.

4.3. Results and Discussion

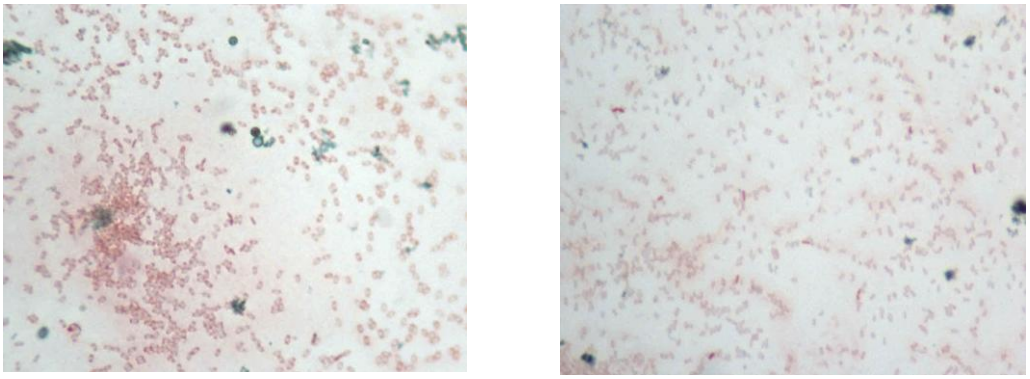
4.3.1 Selection of Microbial Culture

Cell structure and development

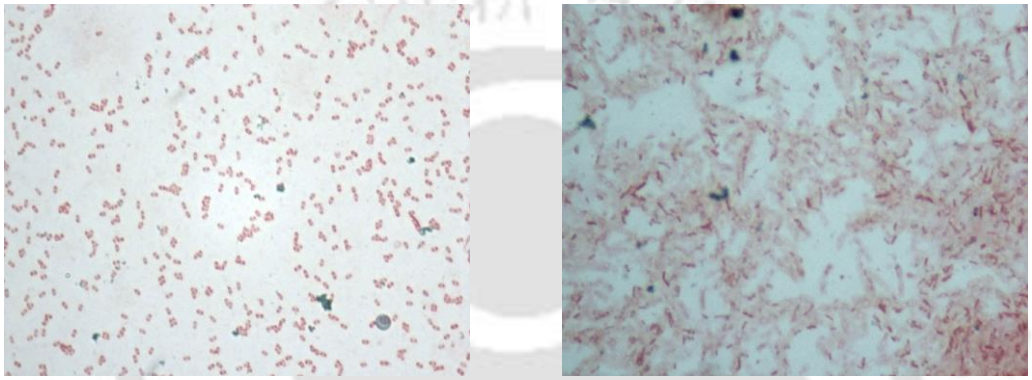
Clostridium species are rod shaped, Gram positive, often pleomorphic anaerobic bacteria. *C. acetobutylicum* is most often soil dwelling, although it has been found in a number of

different environments. It is mesophilic, exist in temperatures range of 30–65°C [10]. In addition, some of the clostridia are saccharolytic (can break down sugar), and most of them are capable of producing a number of different commercially useful products; most notably acetone, ethanol, dihydroxy acetone, propanol and butanol etc. [11]. *Clostridium* species vary considerably in their oxygen tolerance. Some species such as *Clostridium novyi* and *Clostridium haemolyticum* are among the strictest of obligate anaerobes, and may require extended incubation on pre–reduced or freshly prepared plates, and total handling in an anaerobic chamber [12]. Conversely, *Clostridium tertium, Clostridium histolyticum* and *Clostridium carnis* are aerotolerant and can grow in an atmosphere of air with 5–10% added CO_2. *Clostridium acetobutylicum* produces no toxins, unlike other members of this genus and poses minimal health risk [13]. *C. acetobutylicum* requires anaerobic conditions in order to grow in its vegetative state. It can only survive up to several hours in aerobic conditions, in which it will form endospores that can last for years even in aerobic conditions.

Gram staining of freshly inoculated 24 h old (at early cell development stage) culture of *C. acetobutylicum* MTCC 481, *C. acetobutylicum* NCIM 2337, 2877 and 2878 exhibited Gram positive nature (Fig. 4.10). However, they stained as Gram–negative as the culture ages (12 day old culture). During vegetative growth, microscopic view of all the four strains of *Clostridium* using oil immersion (100×) shows the cell's motility. Increased motility of clostridia is reported in increased solvent production due to chemotaxis [14] Attractants include butyric acid and sugar. Notable repellents include acetone, butanol, and ethanol. This mechanism is logical in allowing the cell to find nutrients, and move away from byproducts produced by its own metabolism [15–16]. In addition, different byproducts are produced at different phases of growth in *C. acetobutylicum*. During exponential growth phase, primary products are acetate and butyrate. Once the cell enters stationary phase, the production of solvents starts with acetone being the initial product.



(a) *C. acetobutylicum* MTCC 481 (1000×) **(b) *C. acetobutylicum* MTCC 2337 (1000×)**



(c) *C. acetobutylicum* MTCC 2877 (1000×) **(d) *C. acetobutylicum* MTCC 2878 (1000×)**

Figure 4.11: Spore staining of mature clostridial strains

The major stage of cell development is characterized by the formation of an endospore. An endospore is the most resistant cell type known. Upon other environmental cues, such as an anoxic environment, the cell germinates and begins the vegetative cycle again [17]. Spore formation begins when the cells are exposed to unfavorable conditions. Aerobic conditions, formation of organic byproducts, nutrient depletion, and dissipation of the proton gradient outside the cytoplasmic membrane, all lead to sporulation. Fig. 4.11 shows the Malachite green staining of cells, which are in their late stationary phase. Green structures are the cells, which have undergone endospore formation to protect themselves from adverse conditions [18].

Study of Solvent production from four strains of C .acetobutylicum

All four strains of *C. acetobutylicum*, viz. MTCC 481, NCIM 2337, 2877 and 2878 were allowed to undergo fermentation in a glucose (2%) supplemented synthetic medium (RCM), after an initial lag phase. All cells produced solvents; acetone, butanol and ethanol in different ratios. Although the total solvent produced from all four strains were comparative, MTCC 481 produced the maximum solvent (2.72 g L^{-1}). NCIM 2337 produces 2.17 g L^{-1}, NCIM 2877 produces 2.25 g L^{-1} and NCIM 2878 produces 2.27 g L^{-1} of total solvents. Butanol (which is the targeted product) was produced with the highest selectivity by MTCC 481 strain (0.69 g L^{-1}), while other strain produced only 0.23, 0.05, 0.047 g L^{-1} of butanol. Acetone to butanol ratio observed for different strains was as follows: MTCC 481 ~ 3:1, NCIM 2337 ~ 8:1, NCIM 2877 ~ 43:1 and NCIM 2878 ~ 47:1. These ratios clearly indicate that NCIM 2877 and 2878 produce very high amount of acetone in comparison to butanol, while MTCC 481 and NCIM 2337 produce comparatively higher amount of butanol. Fig. 4.12 illustrates the comparative study of solvent production by four clostridal strains.

Table 4.1 compares the solvent yield and productivity achieved by four clostridial strains after fermentation of 2% w/v of glucose supplemented reinforced clostridial agar medium. Total solvent yield achieved with all the three cultures from NCIM exhibited a uniform trend of 0.11 g/g. All cultures produced negligible amount of ethanol. MTCC 481 culture demonstrated the highest selectivity (0.21 mol/mol) towards butanol, while selectivity of NCIM cultures were observed to be less than 0.08 mol/mol. In addition to these results, our initial trial experiments have revealed that NCIM cultures are prone to degeneration. Especially, NCIM 2337 culture, which demonstrated high solvent production in initial experiments, was observed to lose its activity after 4–5 cycles of sub–culturing. Degeneration of clostridial cultures (i.e. loss of efficiency to produce solvents) is a common phenomenon, which generally occurs due to the loss of plasmid in bacterial genome, which encodes gene

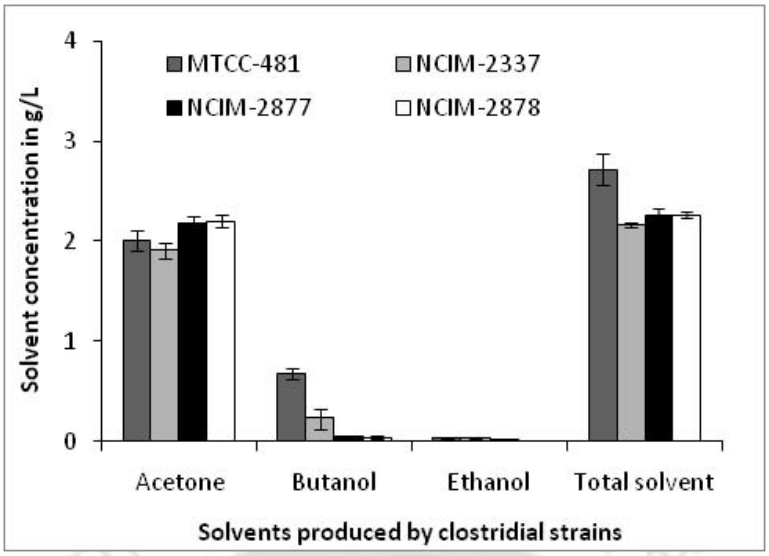


Figure 4.12: Solvent production by four *Clostridium* strain by fermentation of glucose supplemented with RCM

Table 4.1: Comparative study of solvent yield and butanol selectivity achieved from fermentation of four strains of clostridia

***Clostridium acetobutylicum* Strains**	**Solvent Yield (g/g)**				**Butanol selectivity mol/mol**
	Acetone	**Butanol**	**Ethanol**	**Total Solvent**	
MTCC–481	0.100	0.034	0.002	0.14	0.21
NCIM–2337	0.096	0.011	0.001	0.11	0.08
NCIM–2877	0.109	0.003	0.001	0.11	0.02
NCIM–2878	0.110	0.002	0.001	0.11	0.02

responsible for solvent production [19–22]. MTCC 481 strain was observed to be genetically more stable than NCIM strains.

Thus, on the attributes of higher stability and higher solvent yield and butanol selectivity, MTCC 481 was selected as the microbial strain for further process development.

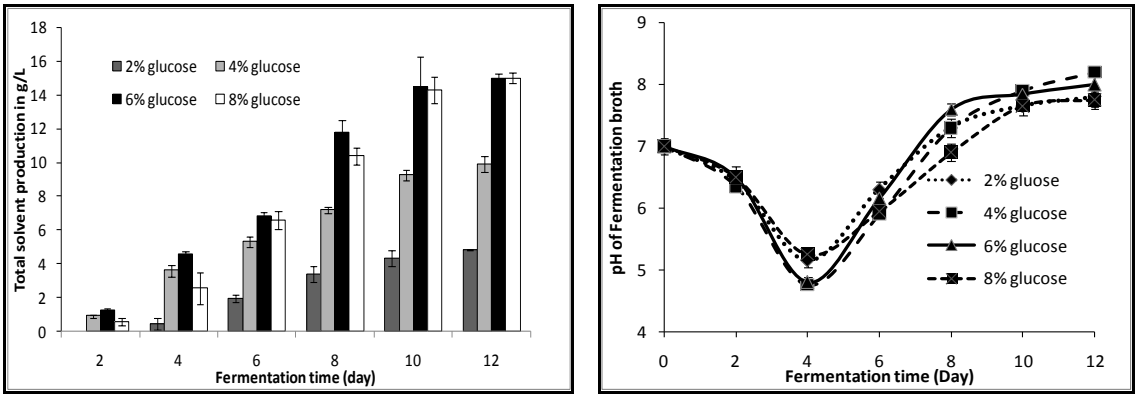


Figure 4.13:a) Solvent production by *C. acetobutylicum* MTCC 481 under varied glucose concentration, b) Time history of pH trend followed by *C. acetobutylicum* MTCC 481 under varied glucose concentration

Table 4.2: Comparative study of solvent yield and butanol selectivity achieved from fermentation of varied concentration of glucose by *C. acetobutylicum* MTCC 481

Varied Glucose Concentration (in % w/v)	Yield (g/g)		Butanol Selectivity
	Total solvent	Butanol	
2.0	0.24	0.13	0.46
4.0	0.25	0.13	0.48
6.0	0.25	0.13	0.47
8.0	0.19	0.09	0.40

4.3.2 Study of Effect of Varied Glucose Concentration on Solvent Production

C. acetobutylicum MTCC 481 was selected as the strain for the development of the ABE fermentation process utilizing a suitable biomass feedstock. All bacteria utilize the substrate from their environment in order to generate energy, mainly in the form of ATP. All bacterial biosynthetic processes, especially reproduction and respiration, require ATP [23]. Depending upon the cellular enzyme production, specific bacteria uses specific substrate (carbohydrates, glucose, lactose, xylose) as its energy source [24].

Clostridial strains are well known for their capability to utilize variety of sugars. One of the most abundant sugars, available in major quantity in most of the biomass, is glucose [25]. In this section, we have attempted to study the effect of varied amount of glucose on clostridial fermentation. Four concentrations of glucose (w/v), viz. 2, 4, 6 and 8% were supplemented to RCM medium. Fermentation was performed for 12 days. Fig. 4.13a and Fig. 4.13b illustrates the time history of solvent production and pH trend by *C. acetobutylicum* MTCC 481 strain under varied concentration of glucose. Experimental flasks containing 4–8% w/v of glucose showed solvent production from very 2nd day of fermentation cycle, while fermentation with 2% w/v of glucose displayed solvent production from 4th day. Fermentation of 2% w/v of glucose produces 4.85 g L^{-1} of total solvents, containing 2.13 g L^{-1} of acetone, 2.55 g L^{-1} of butanol and 0.18 g L^{-1} of ethanol. Total solvent yield achieved from this experiment is 0.24 g/g with a butanol yield of 0.13 g/g and butanol selectivity of 0.46.

Fermentation of medium containing 4.0% w/v of glucose resulted in production of 9.91 g L^{-1} of total solvent, containing 4.31 g L^{-1} of acetone, 5.32 g L^{-1} of butanol and 0.29 g L^{-1} of ethanol. Similarly, fermentation of medium containing further enhanced glucose concentration of 6% w/v produces 14.98 g L^{-1} of total solvents (acetone: 6.64 g L^{-1}, butanol: 7.89 g L^{-1}, ethanol: 0.46 g L^{-1}). Fermentation of 4 and 6% w/v of glucose resulted in total solvent yield of 0.25 g/g with a butanol yield of 0.13 g/g. In all three set of experiments (viz. 2, 4, and 6% w/v of glucose), a uniform butanol selectivity of 0.46–0.48 mol/mol was observed. On the contrary, when glucose concentration of fermentation broth was further enhanced to 8% w/v, comparatively lesser amount of solvent was produced, i.e. 15.01 g L^{-1} of total solvents, having higher proportion of acetone (7.57 g L^{-1}), and lesser amount of butanol (6.88 g L^{-1}). This resulted in a reduced total solvent yield of 0.19 g/g, butanol yield of 0.09 g/g and butanol selectivity of 0.4 mol/mol (Table 4.2).

D–glucose is a simple sugar and an important carbohydrate of biological sciences.

Cells use it as a source of energy and a metabolic intermediate. Glucose is one of the main products of photosynthesis, and starts cellular respiration. Glucose supplemented to complex bacterial growth media is expected to affect the production of a certain protein under the temperature control, which should further affect the production of ABE solvents [26–28]. Batch fermentation of 2 to 8% w/v glucose by *Clostridium acetobutylicum* MTCC 821 displayed an optimal glucose concentration range of 4–6% w/v, which favors solvent production (specifically butanol). It was also observed that high sugar concentration (≤ 8% w/v) resulted in reduction of solvent production. This was mainly due to the substrate inhibition exhibited by clostridias at elevated concentration of sugar. Maddox et al. [29] reported that in batch fermentation the maximum product concentration achieved by a clostridial sp., restricts the utilization of substrate in fermentation broth to a defined level. Sugar concentration greater than 6% w/v is observed to exhibit this effect. From our experiments, it could be inferred that a maximum solvent concentration of 1.5% w/v in fermentation broth, through shake flask scale batch studies with *C. acetobutylicum* MTCC 481 exhibits a profound effect on substrate utilization. It also restricts the sugar concentration in fermentation broth to 6% w/v only, as concentration higher than 6% w/v will result in the reduction of solvent yield due to the inability of bacteria to produce more solvents due to solvent inhibition [23].

4.3.3 Study of Effect of Varied Xylose Concentration on Solvent Production

Clostridial growth and metabolism under varied xylose concentration

C. acetobutylicum is a chemo–organotroph. It obtains energy via substrate phosphorylation during fermentation. As with all fermentations, the substrates are organic molecules, which act as electron donor and acceptor. It follows that it is heterotrophic with its source of carbon coming from organic molecules [30–31]. In particular, *C. acetobutylicum* requires a

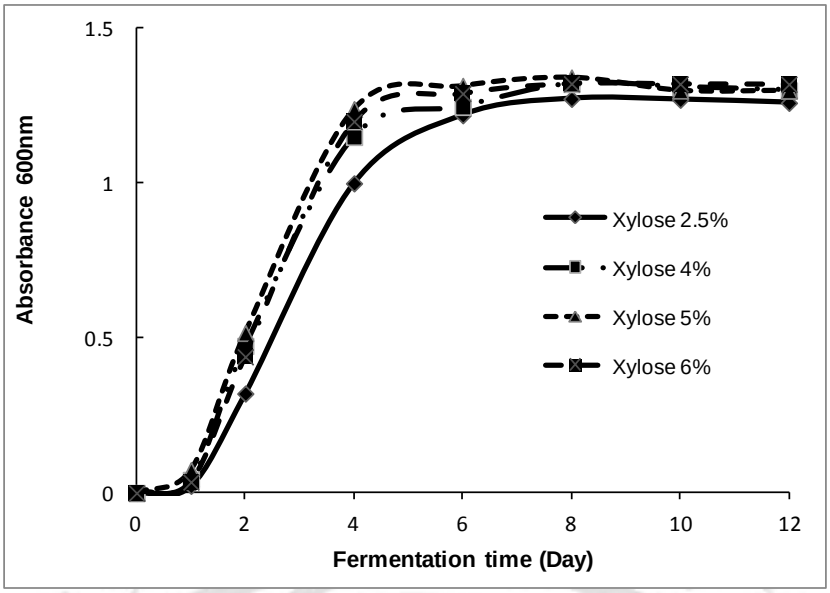


Figure 4.14: Growth cycle of *C. acetobutylicum* MTCC 481 under varied xylose concentration

carbohydrate source capable of undergoing fermentation to survive. *C. acetobutylicum* is able to use a number of different fermentable carbohydrates as an energy, as well as carbon, source. Considerable research has been done in establishing the metabolic pathways of *Clostridium acetobutylicum* in order to improve industrial fermentation operations [32].

Glucose and xylose being the primary hexoses and pentoses, which are reportedly utilized by *C. acetobutylicum* cultures, were maintained at neutral pH (6.8 ± 0.2) at which the solventogenesis prevails. However, it has also been reported that the *C. acetobutylicum* from a given blend of sugar, preferably utilizes glucose [33]. Xylose supplemented to complex bacterial growth media is expected to affect the production of a certain protein under the temperature control, which should further affect the production of ABE solvents. Fig. 4.14 illustrates the growth cycle of *C. acetobutylicum* MTCC 481 strain under the batch fermentation conditions with 2.5, 4, 5 and 6% of xylose in a complex RCM culture medium keeping initial pH of RCM at 6.8 ± 0.2 with no further pH control.

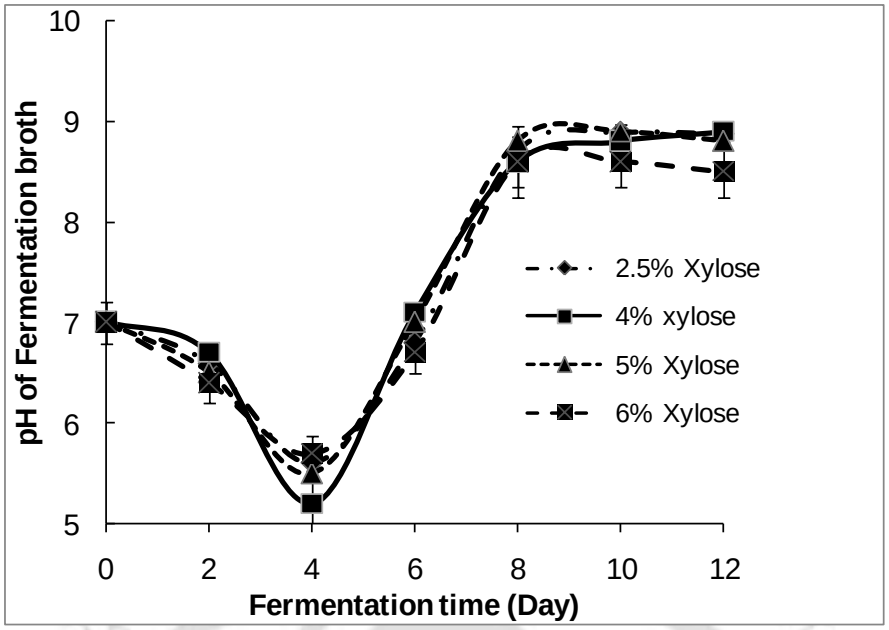


Figure 4.15: Time history of pH trend followed by *C. acetobutylicum* MTCC 481 under varied xylose concentration

Clostridial growth cycle observed under xylose rich fermentation medium was observed to have a comparatively longer lag phase (than glucose) of 24 h. This could be possibly due the reason that EMP pathway is dominant route to utilize hexose sugar, and pentose sugars like xylose are consumed via the PPP pathway [34]. Adaptation of clostridial cells to an environment rich in glucose was comparatively similar than adapting to an environment of pentose sugar. Once the cell enters exponential phase, a rapid growth cycle, which lasts for nearly 48 h, was observed. Low xylose concentration of 2.5% w/v exhibited a comparatively lesser bacterial cell density. As the cells enter stationary phase, primary metabolites produced during log phase start converting to solvents through a series of enzymatic reaction. This phase lasts till 10–12 days of fermentation cyle. The solvents acetone, acetate, butanol, butyrate, and ethanol are all derived from the common precursor, acetyl–CoA. In addition to these products, CO_2 and H_2 are produced [35].

Study of pH trend under varied xylose concentration

As mentioned earlier, fermentation was carried out with an initial pH of 6.8 (± 0.2). As the process proceeds, and enters acidogenesis phase, a sharp fall in the pH of fermentation broth was observed. Recently, numerous efforts have been made to study the regulatory mechanism prevailing the physiological shifts in pH during clostridial growth cycle [37–39]. The evidence suggests that the switch to solventogenesis is triggered not only by the fall in external pH, but also by the increased acid concentration in the medium, which occurs during acidogenesis [40]. Both factors were correlated with a critical role of the level of the un–dissociated acidic products, especially butyric acid, which were shown to be able to penetrate the cell membrane [41]. Therefore, it may be suggested that perturbations in intracellular pH caused by the production of acids would lead to modification of cell metabolism, and initiation of solvent formation [20]. The change in the fermentation products produced by *C. acetobutylicum*, as the culture pH was lowered (Fig. 4.15), indicated the presence of two different fermentation patterns, with the changeover point lying between pH 5.3 and 5.5.

Fig. 4.15 shows the pH trend followed by *C acetobutylicum* MTCC 481 grown in an oxoid (RCM) medium with xylose as main energy source. Four concentrations of xylose in the fermentation medium, viz. 2.5, 4, 5 and 6% w/v have been investigated. The minimum pH fall for strain MTCC 481 with 4 and 5% w/v xylose was observed to be 5.3. 2.5 to 6% w/v xylose has shown a steady increase in the pH dip from 5.4 to 5.5. The final pH achieved in all the sets of experiments was observed to be in the range of 7.2–7.5. Steep fall of pH from 6.8 to 5.2–5.5 signifies acidogenesis, and further increase in pH of fermentation broth signifies solventogenesis.

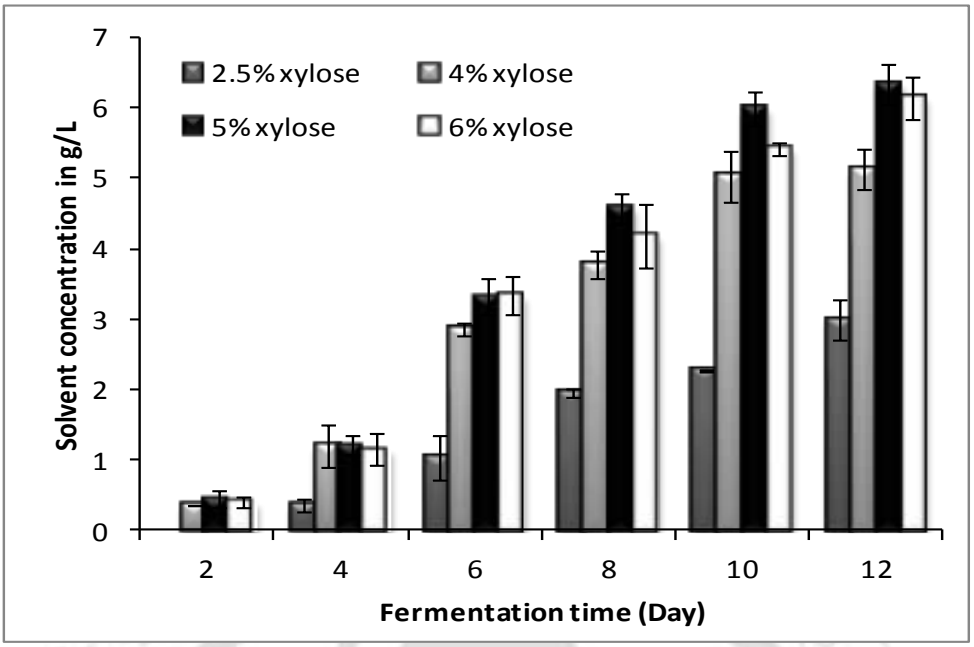


Figure 4.16: Solvent production by *C. acetobutylicum* MTCC 481 strain under varied xylose concentration

Table 4.3: Comparative study of solvent yield and butanol selectivity achieved from fermentation of varied concentration of xylose by *C. acetobutylicum* MTCC 481

Initial xylose concentration (In % w/v)	Yield (g/g)		Butanol selectivity
	Total Solvent	Butanol	
2.5	0.12	0.05	0.36
4.0	0.13	0.06	0.38
5.0	0.13	0.06	0.38
6.0	0.10	0.04	0.34

Solvent production at varied xylose concentration

Fermentation of synthetic medium supplemented with xylose as primary carbon source was performed in a set of four flasks, with each flask having different concentration of xylose, viz. 2.5, 4, 5 and 6% (w/v). Results shown in Fig. 4.16 indicate that all flasks reportedly produced ABE solvents, although concentration of ethanol in the flasks was observed to be negligible, and fraction of acetone was observed to be higher than butanol. Experiments with

2.5% w/v initial xylose concentration resulted in production of 3 g L^{-1} of total solvents, containing 1.8 g L^{-1} of acetone and 1.7 g L^{-1} of butanol. This resulted in total solvent yield of 0.12 g/g with a butanol yield of 0.05 g/g. For fermentation carried out with an initial xylose concentration of 4% w/v, enhanced total solvent concentration of 5.13 g L^{-1} was noted with acetone concentration of 2.82 g L^{-1}, and butanol concentration of 2.23 g L^{-1}. Further enhancing the xylose concentration of fermentation broth to 5% w/v produces 6.34 g L^{-1} of ABE, with higher acetone (3.49 g L^{-1}) and butanol (2.78 g L^{-1}). Interesting experiments with 6% w/v xylose concentration resulted in reduced solvent production of 6.14 g L^{-1}, with acetone concentration of 3.68 g L^{-1} and butanol concentration of 2.42 g L^{-1}.

Table 4.3 compares the solvent yield and selectivity achieved in for all four sets of xylose concentration. A uniform solvent yield of 0.12–0.13 g/g, and butanol selectivity of 0.36–0.38 was observed with 2.5–5% w/v of xylose. While fermentation of 6% w/v xylose yielded 0.1 g/g of total solvent, with butanol yield of 0.04 g/g. This resulted in reduced butanol selectivity of 0.34. Thus, from these studies it could be inferred that xylose concentration ranging from 2.5–5% w/v in fermentation broth yields best results in terms of solvent production. For effective utilization of substrate rich in xylose sugar, many reviewers have suggested supplementation of glucose to broth for achieving better yield and productivity, as addition of glucose is required for rapid activation of metabolic cycles of clostridia and it also helps in reducing the lag phase [42–47].

4.3.4 Selection of Substrate

Till this stage, we have selected the suitable strain for production of liquid biofuels, specifically butanol. We have also developed an understanding of the trend of hexose and pentose sugar utilization by *C. acetobutylicum* MTCC 481.

(a) **(b)** **(c)** **(d)**

Figure 4.17: Physically processed biomass to be used as feedstock for ABE fermentation. (a) Fruit waste, (b) Rice straw, (c) Rice husk, (d) Sugarcane bagasse

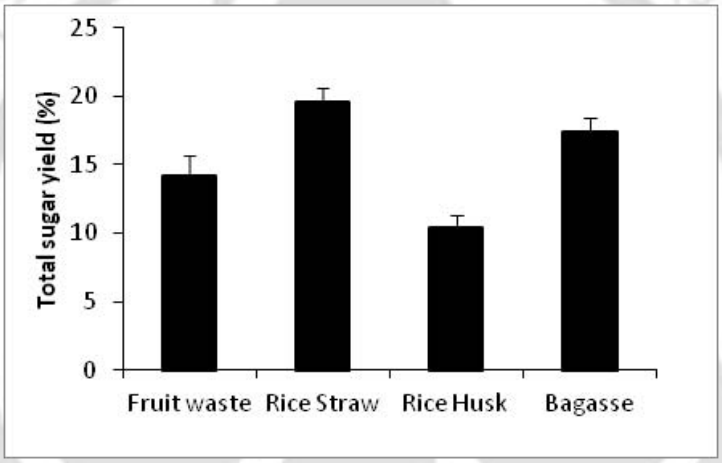


Figure 4.18: Total sugar yield achieved after pretreatment of fruit waste, rice straw, rice husk, and bagasse

We have also observed growth cycle and trend of pH and solvent production of *C. aceto–butylicum* MTCC 481 for different initial concentrations of sugar in the broth. The major objective of this thesis is to utilize a renewable source of carbohydrate (Biomass), which could successfully undergo clostridial fermentation and can produce butanol. In this section we have attempted to select a suitable biomass available in local region of Guwahati, Assam.

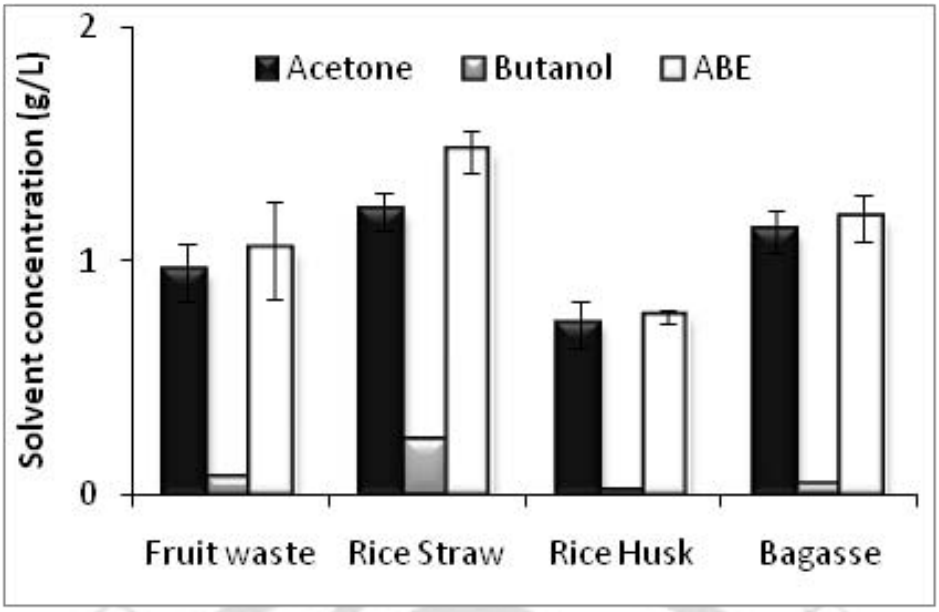


Figure 4.19: Solvent production by fermentation of hydrolyzate of fruit waste, rice straw, rice husk, and bagasse

Table 4.4: Comparative study of solvent yield and butanol selectivity achieved from fermentation of hydrolyzate of fruit waste, rice straw, rice husk and bagasse by *C. aceto–butylicum* MTCC 481

Substrate	Yield in terms of total sugar (g/g)		Butanol selectivity in terms of total solvents (mol/mol)
	Butanol	Total solvent (ABE)	
Fruit waste	0.011	0.147	0.057
Rice Straw	0.024	0.150	0.129
Rice Husk	0.004	0.147	0.019
Bagasse	0.005	0.135	0.029

Fig. 4.17 shows four substrates, viz. fruit waste, rice straw, rice husk and bagasse procured from local region. 5% w/v solutions of four substrates, fruit waste, rice straw, rice husk and bagasse, were acid pretreated and the feedstock obtained was allowed to undergo clostridial fermentation. All four extracts were analyzed, and Fig. 4.18 shows the percent composition of total sugar yield achieved from the pretreatment of different biomasses. Fig. 4.19 illustrates the comparative study of solvent production by *C. acetobutylicum* MTCC 481, utilizing biomass hydrolyzates as feedstock.

Fruit wastes on acid hydrolysis yielded 14.23 wt% of total sugar, which on fermentation produces 1.04 g L^{-1} of ABE with 0.95 g L^{-1} of acetone, and 0.075 g L^{-1} of butanol, while pretreated rice straw produces 1.47 g L^{-1} of total solvents containing 1.21 g L^{-1} of acetone, and 0.24 g L^{-1} of butanol. Similarly, rice husk on hydrolysis produces 10.4 wt % of total sugar, which on fermentation produces 0.76 of ABE solvents (acetone: 0.73 g L^{-1}, butanol: 0.018 g L^{-1}). On the other hand, hydrolysis of bagasse resulted in release of 17.4 wt% of total sugar, which on fermentation produces 1.18 g L^{-1} of total solvents containing 1.12 g L^{-1} of acetone and 0.05 g L^{-1} of butanol. All four feedstocks produced negligible amount of ethanol.

Table 4.4 compares the solvent yield and butanol selectivity achieved from the fermentation of all the four substrates. Solvent yield achieved from all four set of experiments was comparative (0.135 to 0.15 g/g), with a maximum solvent yield of 0.15 g/g from rice straw. The order of the highest solvent yield achieved by fermentation of these biomass is determined as: rice straw (0.15 g/g) > rice husk (0.147 g/g) > fruit waste (0.146 g/g) > bagasse (0.13 g/g). However, butanol yield from these biomasses follow a different trend: rice straw (0.024) > fruit waste (0.01) > bagasse (0.004) > rice husk (0.004). Thus, these experiments revealed that the highest butanol selectivity was observed with rice straw (0.13 mol/mol), while butanol selectivity observed with other three substrates was less than 0.05 mol/mol. Acetone to butanol ratio achieved from fermentation of different biomasses was: fruit waste = 12.6:1, rice straw = 5:1, rice husk = 39:1, bagasse = 25:1.

In addition to these quantitative tests, some other problems associated with use of feedstock like fruit waste and sugarcane bagasse have also been noticed during experiments. Drying and maintenance of fruit waste was a tedious job due to its high moisture content. Dried fruit waste was having high tendency to absorb atmospheric moisture. Because of the same reason, fruit waste was highly susceptible to microbial contamination. In addition to

these, as source of fruit waste was local juice shop; its sugar composition varies with every lot. This adds to instability to the expected outcome of process in terms of solvent production. Major problem with usage of sugarcane bagasse was unavailability of biomass throughout the year. In comparison to these two biomasses, Northeast India being a major producer of rice in India, has a constant supply of rice straw and rice husk throughout the year at a very cheap price. From all these results experiments we can conclude that among tested four set of biomass, Rice straw was observed as the most promising feedstock for the formative production of butanol.

4.4 Conclusion

In this chapter, we have presented the results of our studies on selection of suitable microbial culture for ABE fermentation process for biobutanol production. We have also assessed the comparative potential of rice straw as cheap and renewable biomass feedstock for ABE fermentation. Using the criteria of highest overall yield of solvents, and selectivity towards butanol, in addition to stability during the fermentation process, *Clostridium acetobutylicum* MTCC 481 was selected as the most suitable microbial strain for the process. Similarly, on the basis of overall solvent yield and selectivity for butanol, rice straw showed greater potential as a biomass feedstock for the ABE fermentation process. Based on these results, we have proceeded our study on design and optimization of biobutanol production through ABE fermentation process using rice straw as the biomass feedstock, and *Clostridium acetobutylicum* MTCC 481 as the microbial strain. In the next chapter, we shall address the matter of pre–treatment of rice straw, which is an important upstream step of the ABE fermentation process.

Abbreviations

SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
ABE	Acetone Butanol Ethanol
CMM	Cooked Meat Medium
CTCC	Microbial Type Culture Collection
NCIM	National Collection of Industrial Micro-organisms
RCA	Reinforced Clostridial Agar
RCM	Reinforced Clostridial Medium
RS	Rice Straw
RSH	Rice Straw Hydrolysate

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CHAPTER 5

OPTIMIZATION OF RICE STRAW PRETREATMENT

5.1 Introduction

Rice is a major crop of India and also a major source of agro–residue in the form of husk and straw, which forms potential feedstock for biofuels production [1–8]. In the previous chapter, we assessed the potential of rice straw as a substrate for ABE fermentation for biobutanol production, in comparison to other substrates. In addition, we also evaluated and compared different *Clostridial* strains available in Indian culture banks for fermentation of rice straw hydrolyzate. The pretreatment of rice straw is an important step in the ABE fermentation process as it governs the release of sugars from cellulose and hemicelluloses in rice straw, which is further gets converted to acetone, butanol and ethanol solvents [9–17]. Moreover, this process is also energy intensive, and contributes significantly to the production cost of the overall ABE fermentation process[18–23]. Therefore, careful optimization of rice straw pre–treatment is an important aspect of the ABE fermentation process. In this chapter, we address this matter and present our studies in optimization of pre–treatment of rice straw through physical, chemical and enzymatic techniques and combinations thereof.

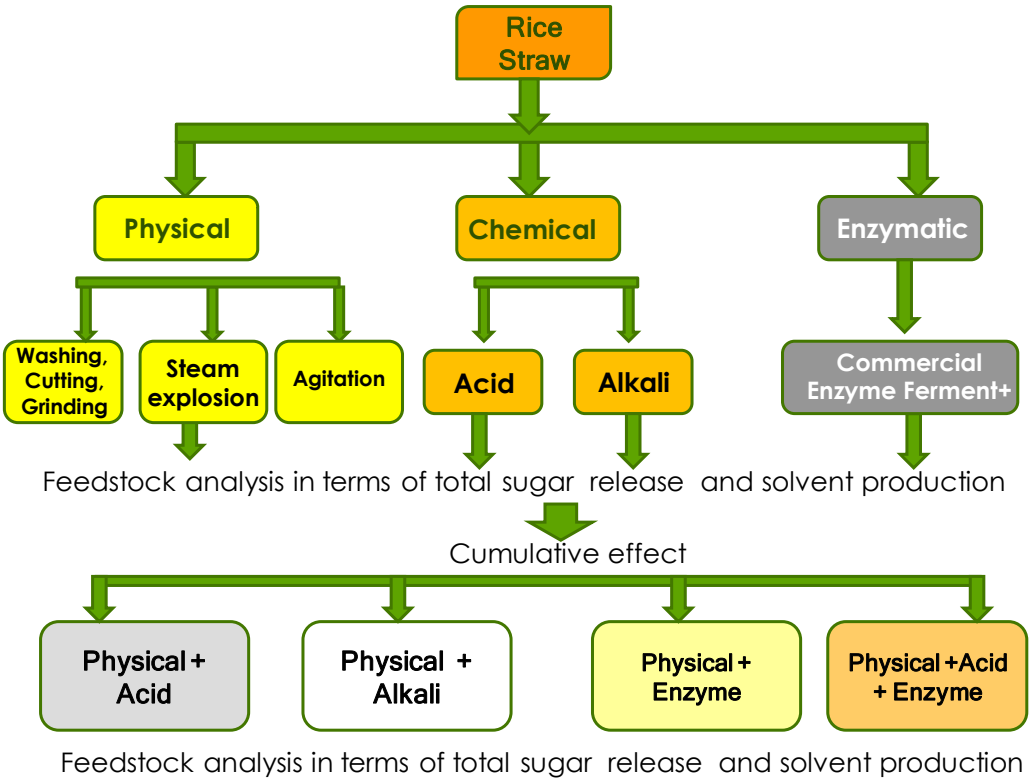

Figure 5.1: Schematic of methodology adapted for pretreatment of rice straw

Previous authors have addressed the subject of use of rice straw and similar agro–feedstock (such as wheat straw, corn straw, bagasse) after hydrolysis for alcoholic fuel production via fermentation [24–26]. Roslan et al. [27] has reported production of bioethanol from rice straw sacchharified with crude cellulase to release 90% of glucose in solid state fermentation. Rivers and Emert [28] have analyzed the effect of substrate concentration, cellulose crystallinity and particle size on the yields of enzymatic hydrolysis for the agro–residues, viz. bagasse and rice straw. This study revealed that nature of lignocellulose matrix is a major limiting factor in enzymatic hydrolysis. Hsu et al. [29] has studied effect of dilute acid treatment of rice straw prior to enzyme hydrolysis on the yield of hydrolysis. Acid pretreatment was found to increase the pore volume of the solids due to release of acid soluble lignin, which enhanced the yield of enzymatic hydrolysis. Marchal et al. [30] reported that hydrolysates obtained by enzymatic saccharification of wheat straw and corn stover, pretreated by steam

explosion (in neutral or acidic conditions) were non fermentable by *Clostridium*. Qureshi et al. [31] has compared use of acid and enzyme hydrolyzed corn fiber for butanol fermentation. Detoxification of hydrolyzate obtained from acid pretreated biomass increased yield with simultaneous reduction in fermentation time.

5.2. Material and methods

5.2.1. Culture maintenance and growth

All chemicals were of analytical grade procured from either from Merck (Germany) or Himedia (India). Lyophilized *Clostridium acetobutylicum* MTCC 481 has been procured from microbial type culture collection, IMTECH, Chandigarh, India. It was maintained as spore suspension in sterile water. This culture has been rejuvenated in RCA (Reinforced Clostridial Agar) and RCM (Broth) culture media at 37^{o}C. The inoculums were prepared in RCM containing (g L^{-1}): glucose, 5.0; yeast extract, 3.0; starch, 1.0; beef extract, 10.0; peptone, 10.0; sodium chloride, 5.0; sodium acetate, 3.0; Agar,0.5 and cysteine hydrochloride, 0.5; pH 6.5 ± 0.1. 100 mL medium were autoclaved at 121^{o}C and inoculated in 250 mL screw capped Erlenmeyer flasks, and then incubated for 72 h at 37 ± 0.5 ^{o}C at 120 rpm. In addition, Cooked Meat Medium (CMM) is also used for the growth and maintenance of *clostridia*. These were incubated anaerobically inside an anaerobic culture bag system (Himedia) till active growth was seen (72 h). Actively growing cultures (after lag phase, 18–20 h) of the *Clostridia* were added subsequently to experimental flasks.

5.2.2. Rice straw (RS)

Rice straw was obtained from local farmer (irrigated location; Guwahati, Assam, India). The crude composition of rice is observed as; cellulose: 42–47%; hemicellulose: 20–29%; lignin: 4–6%; ashes: 16-18% and sugar composition as; Total sugar: 35-38%, reducing sugar: 30-32 containing glucose: 24-30%; xylose: 10– 15%; arabinose: 2.7–4.5%; mannose: 1.8%;

galactose: 0.4% [32]. Strips of rice straw were cut into small pieces of length 4–5 cm. These were washed with water and then dried at 80°C in a hot air oven (JSJW, India). Size of dried RS was further reduced by in a mixer grinder (Sumeet, India).

5.2.3. Rice straw hydrolyzate (RSH)

Hydrolyzate of RS was prepared using methods as described below:

***Physical treatment*:** 5% w/v mixture of RS, i.e. 12.5 g RS added to 250 mL double distilled water, was taken in a 500 mL screw–capped Erlenmeyer flask. The flask was autoclaved at 121°C for 30 min at 15 lb pressure, and was then allowed to cool to room temperature. RS suspension was filtered using a sterile cotton cloth. pH of the hydrolyzate thus obtained was 1.0.

***Preparation of RSH by autoclaving and agitation*:** 1, 3, 5, and 7% w/v mixtures of rice–straw in distilled water were taken in 4 autoclavable 500 mL Erlenmeyer flasks. Two sets of experiments were performed with this set of 4 flasks. The flasks were heated at 60°C using a hot water bath (JSJW, India) with simultaneous stirring at 200 rpm for 24 h. 3 mL aliquots of solution were collected every 6 h. Finally, rice straw suspension was filtered using a clean muslin cloth. Same process was repeated at 80°C.

***Preparation of RSH by steam under pressure*:** 1, 3, 5, and 7% w/v mixtures of rice–straw in distilled water were taken in 4 autoclavable 500 mL Erlenmeyer flasks. Three sets of experiments were performed with this set of 4 flasks. One set of flasks was steam exploded at 121°C for 15 min at 15 lb pressure using a semi automated autoclave (Tuttnaeur, model 3870 ELV). After autoclaving, bottles were allowed to cool and filtered using a clean muslin cloth. 3 mL aliquots of solution were collected for the analysis of sugars. Process was repeated with 30 and 45 min of autoclaving.

***Preparation of RSH by Autoclaving and Agitation*:** Rice straw was steam exploded as stated in previous chapter. This was followed with agitating rice straw at elevated temperatures of 60°C at 200 rpm for 24h.

***Acid pretreatment*:** Four set of flasks containing 1, 3, 5 and 7% w/v mixture of RS in distilled water, i.e. 12.5 g RS added to 250 mL 0.5 % v/v H_2SO_4 (equivalent bulk concentration: 0.08 N) distilled water, was taken in a 500 mL of screw–capped Erlenmeyer flask. The flask was kept in an orbital shaker incubator for 24 h at 60^oC at 200 rpm. RS suspension was filtered using a sterile cotton cloth and filtrate was used as feedstock. Same experiment was repeated with 1% v/v of H_2SO_4 (equivalent bulk concentration: 0.16 N).

***Alkali pretreatment*:** The experimental procedure was same as stated in previous chapter. Instead of acid, alkaline solution of 0.5 (equivalent bulk concentration: 0.125 N) and 1.0 % v/v (equivalent bulk concentration: 0.25 N) of NaOH was used for hydrolysis.

***Enzyme assisted hydrolysis*:** A commercial enzyme blend, **Ferment+**, was generous gift from Varuna Biocell, Varanasi, India. Four concentrations of enzyme blend viz., 2, 4, 6 and 8 ppm were used to hydrolyze 1, 3, 5 and 7% w/v solutions of rice straw. These solutions were incubated at 60^oC for 24 h at 100 rpm in an orbital shake incubator. RS suspension was filtered using a sterile cotton cloth and filtrate was used as feedstock.

***Cumulative effect of physical, chemical and enzymatic pretreatment*:** Experiments were performed in 4 set of flasks, each containing 3 and 5% w/v aqueous solutions of rice straw. Procedure followed for each of these sets is as follows: **(Set I)** An acidified rice straw mixture was produced using 1% v/v H_2SO_4. This was then autoclaved at 121^oC for 15 min at 15 psi pressure, which was further agitated at 200 rpm and 60^oC. **(Set II)** An alkaline rice straw mixture was produced using 1% v/v NaOH. This was then autoclaved at 121^oC for 15 min at 15 psi pressure, which was further agitated at 200 rpm and 60^oC. **(Set III)** Aqueous mixture of rice straw was autoclaved at 121^oC for 15 min at 15 psi pressure. This was further hydrolyzed using 6 ppm of the commercial enzyme (Ferment+) with incubation and agitation at 60^oC for 24 h at 200 rpm using a mechanical stirrer. **(Set IV)** An acidified (1% v/v H_2SO_4) mixture of rice straw was autoclaved at 121^oC for 15 min at 15 psi pressure. This was further hydrolyzed using 6 ppm of commercial enzyme (Ferment+) with incubation and agitation at 60^oC for 24 h at 200 rpm

using a mechanical stirrer. Rice straw hydrolyzate obtained from above set of experiments was filtered using a sterile cotton cloth and filtrate was used as feedstock.

5.2.4. Fermentation

Batch fermentation experiments were carried out in custom–fabricated 250 mL screw–capped Erlenmeyer flasks with bottom port for sample withdrawal and nitrogen sparging in order to minimize oxygen contamination and maintain strict anaerobic conditions. Anaerobic condition in the flask was generated by addition of 0.5% cysteine hydrochloride to the RS hydrolyzate. An initial sample (0 h) was taken immediately after pretreatment for sugar analysis. Regular samples aliquots were taken to study the growth, sugar release and utilization and solvent production by *Clostridium acetobutylicum*. All flasks were sparged with nitrogen at the start, and after every 24 h of fermentation to maintain anaerobic conditions. The samples of fermentation broth were withdrawn at constant intervals up to a period of 10 days. Each experiment was conducted in duplicate to assess the reproducibility of the results.

5.2.5. Analysis

The optical density of the cells in the fermentation broth was measured using UV–Vis spectrophotometer (Thermo Fischer) with absorbance at 600 nm after appropriate dilution in water. Quantification of total sugar was done by Anthrone test as directed by Hedge and Hofreiter [33]. All the samples were filtered with 0.2 µm filter and diluted appropriately for the qualitative and quantitative determination of the sugars and fermentation products. Solvent production in the fermentation broth was determined using a gas chromatograph (Varian) using a CP Wax 52CB (250 mm × 0.25 mm × 0.39 mm) capillary column and a Flame Ionization Detector. The method was developed by keeping injector and detector temperatures at 230 and 250^{o}C, respectively. Column temperature was programmed at 45–100^{o}C with an increment of 3^{o}C per min. between the temperature intervals of 100–200^{o}C, increments was 5^{o}C per min; split ratio was set at 70 and carrier gas (nitrogen) flow rate was maintained at 2 mL/min.

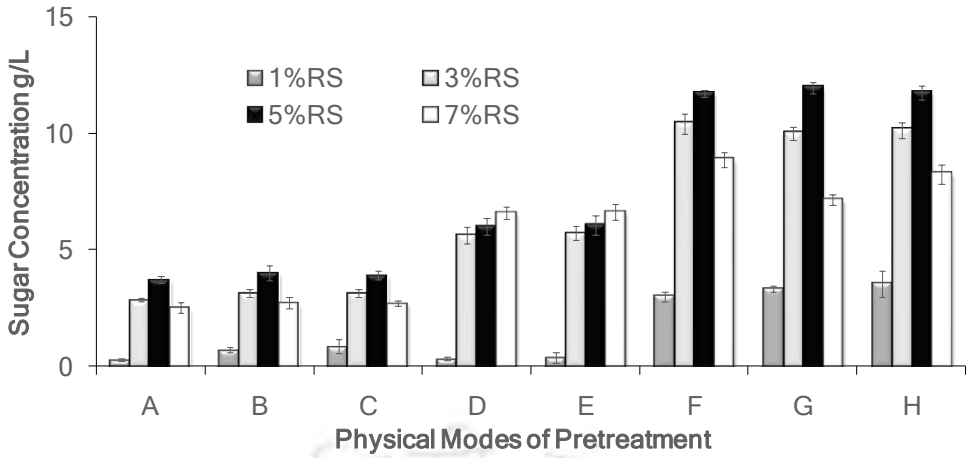

Figure 5.2: Total sugar release after physical pretreatment of rice straw (A) Autoclave for 15 min, (B) Autoclave for 30 min, (C) Autoclave for 45 min, (D) Agitation at 60^{o}C, 200 rpm, 24 h, (E) Agitation at 80^{o}C, 200 rpm, 24 h, (F) 15 min autoclave and stirring at 60^{o}C, (G) 30 min autoclave and stirring at 60^{o}C, (H) 45 min autoclave and stirring at 60^{o}C

5.2.6. Estimation of yield and selectivity

Solvent yield was calculated as gram per liter of solvent produced per gram per liter of total sugar utilized (g/g). Butanol selectivity was calculated as mol of butanol produced per mol of total solvent (ABE) production.

5.3. Results and discussion

Pretreatment of rice straw proved to improve the physical and chemical properties making it suitable to undergo fermentative processes.

5.3.1 Effect of physical treatment on sugar release and solvent production.

Chemical or enzymatic hydrolysis of lignocellulosic biomass with no physical pretreatment is reportedly not so effective because of the high stability of material to chemical and enzymatic attacks. The major parameters influencing pretreatment of rice straw are: crystallinity, accessible surface area, and protection by lignin and hemicelluloses. Physical treatment of rice straw essentially deals with breaking of lignin jacket and hemicelluloses sheathing [34]. In this section, we have attempted three modes of physical treatment: (1) Autoclaving through autoclaving, (2) Agitation at elevated temperature and (3) Cumulative effect of agitation and

autoclaving.

All experiments were performed at four initial concentrations of rice straw (w/v) in aqueous solution, viz. 1, 3, 5 and 7%. Fig. 5.2 illustrates the total sugar release after a given set of physical pretreatment. As stated in Chapter 3, the lignin content of rice straw is minimum in comparison to other straws [35]. Because of this, the suitable physical treatment of rice straw yields good results in terms of high sugar release. Autoclaving of rice straw was performed for 3 exposure periods, viz. 15, 30 and 45 min at 15 psi pressure and 121^{o}C temperature. Autoclaving was performed for all three exposure periods resulted in release of similar amount of soluble sugar. In all the three set of experiments, with initial 5% w/v of RS, only 4 g L^{-1} of total sugar was observed, while same experiment with 1, 3 and 7% w/v of initial RS concentration yielded less than the 3 g L^{-1} of total sugar. When the concentration of RS was enhanced to 7%, due to the high concentration of physically processed rice straw, effect of steam on the inner core of the vessel was observed less probably due to diffusion limitation. Working with low concentration of rice straw, makes fiber more exposed to the extreme conditions making the process more efficient at low concentration. As autoclaving is energy intensive process and increasing the exposure time adds more energy consumption with nearly no effect on sugar yield, among the 3 sets of experiments with 15 min of autoclaving (i.e. the minimum period) proved to be the best alternative.

In next phase, we have assessed the effect of stirring (200 rpm) at an elevated temperature of 60 and 80^{o}C. Stirring results in efficient loosening of straw structure.

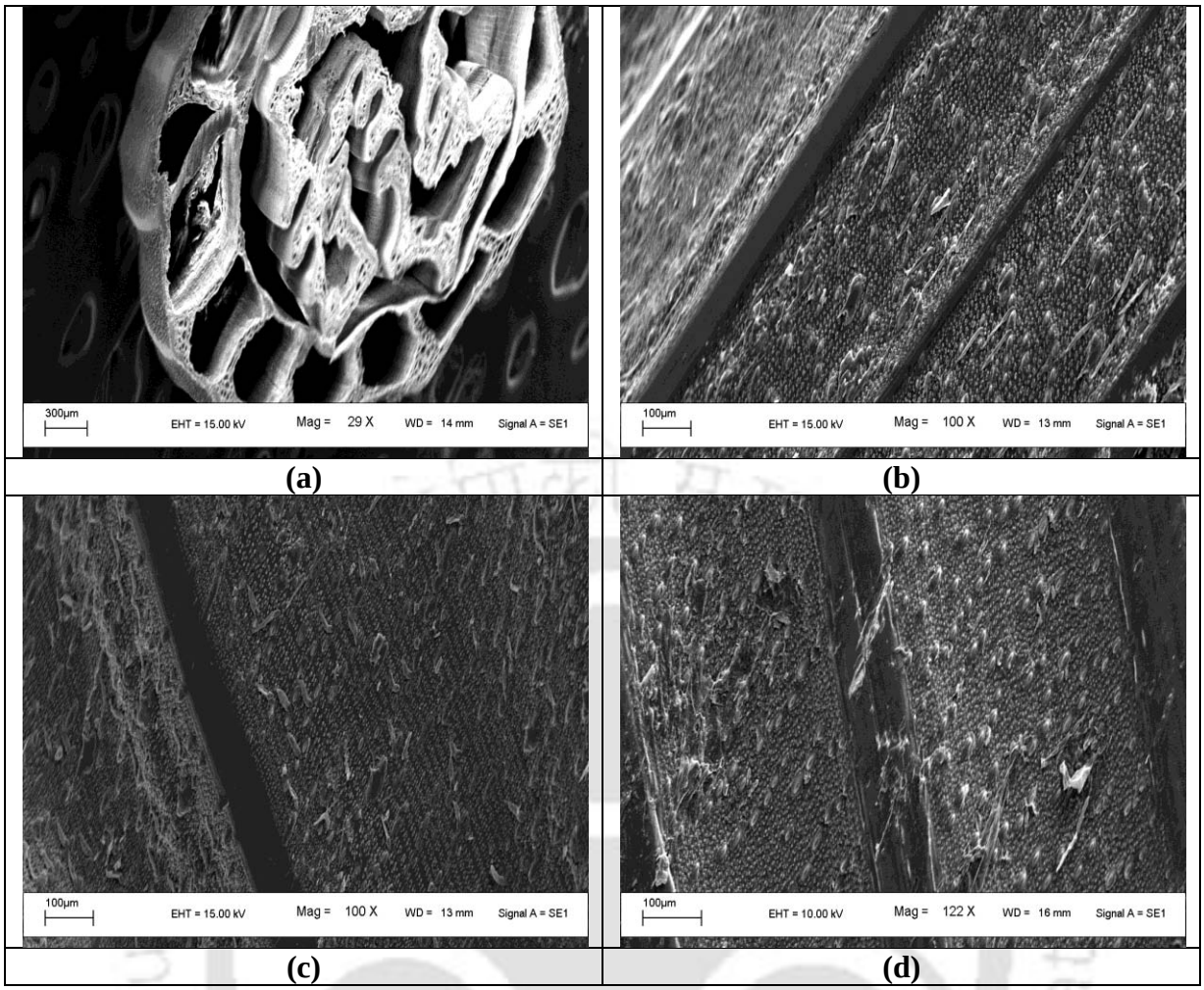

Figure 5.3: SEM Micrographs of: (a) & (b) untreated RS, (c) agitated RS, (d) agitated and steam exploded RS

For 3 to 7 % w/v solutions of rice straw nearly 8–10% of total sugar yield was observed, while for 1% w/v solution of rice straw, only 4% of total sugar yield was noted. The results achieved from agitating rice straw at elevated temperature of 60 and 80^{o}C yielded nearly similar amount of sugar release. Further experiments were conducted to check the cumulative effect of autoclaving and agitation at elevated temperature. In all four experimental sets, agitation was performed at 60^{o}C and 200 rpm for 24 h. Before agitation, all flasks were steam exploded for exposure time of 15, 30 and 45 min. Combined effect of autoclaving and agitation yielded comparatively much higher amount of total sugar in the hydrolyzate. Maximum sugar yield was observed to be 24% for 5% w/v solution of rice straw. Thus, 4 experimental sets of physical pretreatment of rice revealed that both autoclaving and agitation have profound effect sugar

release from rice straw. Fig. 5.3 shows the effect of physical pretreatment on rice structure. Combination of both the mode of physical treatment results in generation of smooth slurry of rice straw, which corresponds to increase in surface area that assists further chemical and enzymatic hydrolysis of straw.

5.3.2 Effect of chemical hydrolysis on sugar release and solvent production

It is reported that 1 kg of rice straw will contain nearly 390 g cellulose [3]. Hydrolysis is directly affected by porosity (available surface area) of lignocellulosic biomass, cellulose fiber crystallinity, and lignin and hemicellulose content. Pretreatment of fibrous feedstock is required in order to remove lignin and hemicelluloses fraction. Hydrolysis with dilute–acid solution gives elevated reaction rates and higher yields of cellulose hydrolysis [4, 34–37]. It is reported that depending on the feedstock, and the physical and chemical conditions used in hydrolysis, up to 95% of the hemicellulosic sugars can be recovered by dilute–acid hydrolysis of lignocellulosic feedstock [5]. The results of the experiments are presented in Figs. 5.4 and 5.5. As noted earlier, aqueous solutions of rice straw at 4 concentrations, viz. 1, 3, 5 and 7% (w/v) were used to study the effect of chemical hydrolysis (acid and alkali) for enhanced sugar release. Fig. 5.4 depicts the total sugar release achieved from treatment with aqueous solutions of 0.5 and 1 % v/v of H_2SO_4 (acid hydrolysis) and 0.5 and 1% v/v of NaOH (alkaline hydrolysis).

Chemical hydrolysis of 1% w/v of RS solution resulted in the highest total sugar yield of nearly 41% (5 g L^{-1}) with 1% of acid treatment. However, 0.5% v/v of acid and 1% v/v alkali treatment resulted in a comparatively lower yield of 32%. Similar results were observed with chemical treatment of 3 and 5% w/v of RS solutions, with the highest total sugar yield of 40–42% (1% v/v acid) and 35–36% yield with 0.5% acid and 1% alkali (v/v). On the contrary, chemical hydrolysis of 7% w/v RS solution resulted in reduced sugar yield in all the set of experiments. A maximum of 34.6% of totals sugar yield was observed with 7% of RS, when treated with 1% v/v of acid, while other sets of experiments, viz. 0.5% v/v acid and 0.5 and 1% v/v alkali resulted in

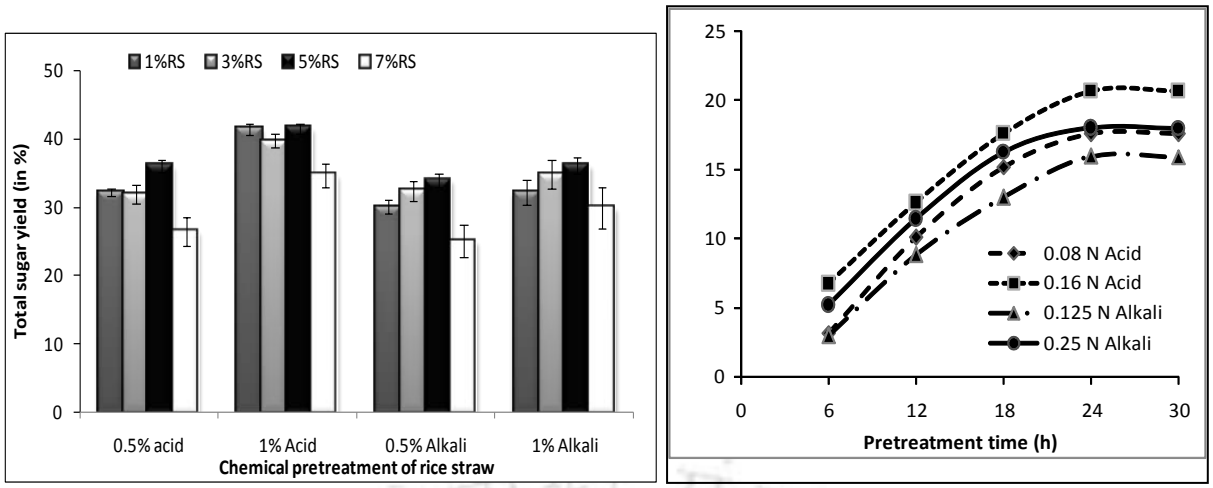

Figure 5.4: (a) Total sugar release after chemical hydrolysis of RS, (b) trend of sugar release after chemical hydrolysis

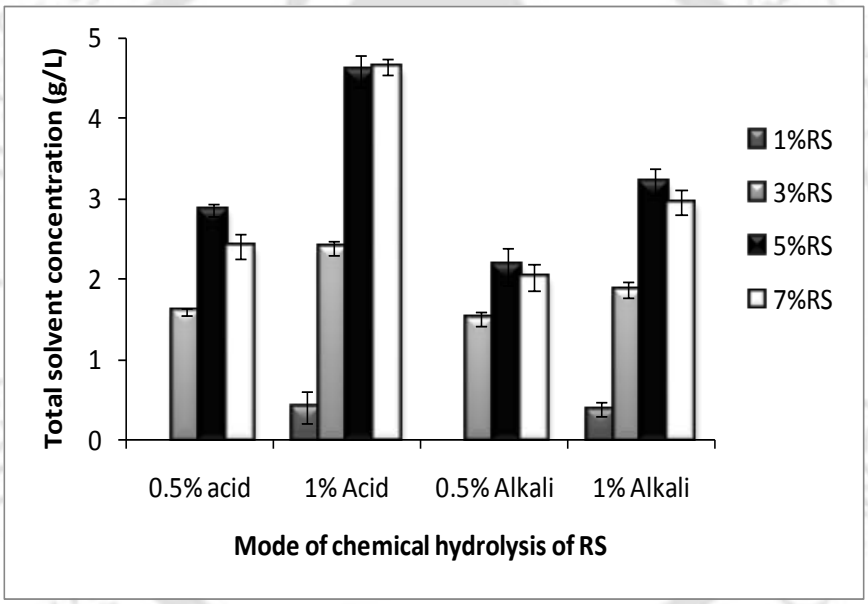

Figure 5.5: Total solvent produced after fermentation of chemical hydrolyzed RS

further reduced yield (less than 30%). This reduced sugar yield at 7% w/v concentration of RS was attributed to poor mixing, and hence, a reduced surface area of straws during hydrolysis. Second major objective of the work was the production of ABE solvents from fermentation of rice straw hydrolysates. Fermentation of hydrolyzate obtained from chemical hydrolysis of 1% of RS resulted in negligible production of solvents. RS solutions treated with 1% v/v acid produced 0.4 g L^{-1} of acetone, while RS solutions treated with 1% v/v of alkali produced 0.38 g

L^{-1} of acetone. None of the four set of experiments employing 1% w/v solution of RS could produce butanol and ethanol. While hydrolyzate obtained after pretreatment of 3% w/v RS solution resulted in an elevated solvent profile, with a total solvent concentration of 1.59, 2.46, 1.18 and 2.03 g L^{-1} for 0.5% and 1% v/v acid and alkali treatment, respectively. The highest butanol concentration achieved from these experiments was 1.97 g L^{-1} with RS treated with 1% v/v H_2SO_4. Similar experiments performed with 5% w/v RS solution resulted in more encouraging results. High total solvent concentration was achieved in all the set of experiments. 0.5% v/v acid treatment resulted in production of 2.87 g L^{-1} of ABE with 0.6 g L^{-1} of butanol, while 1% v/v acid treatment produced 4.59 g L^{-1} of total solvent with 1.85 g L^{-1} of butanol. Treatment of 5% w/v RS solution using 0.5% and 1.0% v/v alkali produced 2.17 and 3.21 g L^{-1} of total solvents with 0.13 and 0.58 g L^{-1} of butanol. Fermentation with hydrolyzate obtained from hydrolysis of 7% w/v RS solution resulted in reduced solvent concentration, obviously due to the less sugar release during the pretreatment process, as stated before.

Table 5.2 gives a comparative account of solvent yield, butanol yield and selectivity obtained from chemical hydrolysis of rice straw (at different concentrations) using dilute acid and alkali solutions. Overall conclusion of above experiments is that dilute acid treatment of rice straw was more potent mode of hydrolysis than dilute alkali treatment. In all sets of experiments, acid hydrolysis resulted in release of greater amount of sugar, which was manifested in higher solvent yield during subsequent fermentation. Among all concentrations of rice straw, viz. 1, 2, 5 and 7% (w/v), 5% w/v RS solution yielded the highest sugar release and solvent production.

Table 5.1: Comparative study of solvent yield, butanol yield, and butanol selectivity achieved after fermentation of chemically hydrolyzed rice straw at different concentrations

Rice Straw (%)	Total solvent yield (g/g)				Butanol yield (g/g)				Butanol Selectivity (mol/mol)			
	0.5% v/v Acid*	1% v/v Acid**	0.5% v/v Alkali#	1% v/v Alkali##	0.5% v/v Acid*	1% v/v Acid**	0.5% v/v Alkali#	1% v/v Alkali##	0.5% v/v Acid*	1% v/v Acid**	0.5% v/v Alkali#	1% v/v Alkali##
1	–	0.1	–	0.12	–	–	–	–	–	–	–	–
3	0.17	0.2	0.12	0.19	0.03	0.08	0.01	0.02	0.14	0.3	0.06	0.09
5	0.16	0.22	0.13	0.18	0.03	0.09	0.01	0.03	0.17	0.3	0.05	0.15
7	0.13	0.19	0.12	0.14	0.03	0.06	0.01	0.02	0.16	0.28	0.05	0.09

* - equivalent bulk concentration: 0.08 N, ** - equivalent bulk concentration: 0.16 N, # - equivalent bulk concentration: 0.125 N, ## - equivalent bulk concentration: 0.25 N

Table 5.2: Comparative study of solvent yield, butanol yield, and butanol selectivity achieved after fermentation of enzyme hydrolyzed rice straw at different concentrations

Rice Straw (%)	Total solvent yield (g/g)				Butanol yield (g/g)				Butanol Selectivity (mol/mol)			
	Enzyme concentration (in ppm)											
	2.0	4.0	6.0	8.0	2.0	4.0	6.0	8.0	2.0	4.0	6.0	8.0
1	–	–	–	–	–	–	–	–	–	–	–	–
3	–	0.08	0.09	0.09	–	0.01	0.01	0.01–	–	0.07	0.1	0.1
5	0.04	0.08	0.11	0.11	0.01	0.01	0.02	0.01	0.1	0.08	0.12	0.10
7	–	0.04	0.04	0.04	–	–	–	–	–	0.03	0.04	0.02–

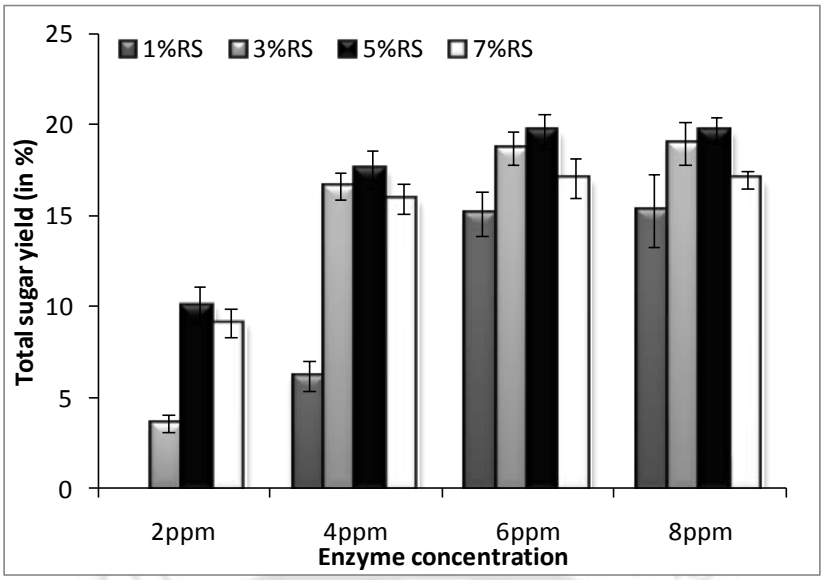

Figure 5.6: Total sugar yield achieved from enzymatic hydrolysis of varied concentration of RS

5.3.3 Enzymatic hydrolysis of rice straw

Enzymatic hydrolysis of lignocellulosic biomass like rice straw essentially involves cleaving the cellulosic and hemicellulosic polymers into simple sugar. Cellulose cleavage yields glucose whereas, hemicellulose degrades into mannose, xylose, galactose, glucose and arabinose etc. [38–40]. In this section, we have attempted to use a commercial grade enzyme known as Ferment+ (Varuna Biocell, India), which is a blend of enzymes constituting cellulose, glycosidases, cellobiases etc. However, being a proprietary product, the actual composition of Ferment+ was not revealed by the supplier. Varied concentration of rice straw, viz. 1, 3, 5 and 7 % w/v was treated with different concentrations of enzyme Ferment+ (2, 4, 6 and 8 ppm). 1% w/v RS solution on enzymatic hydrolysis gave the best sugar yield (~ 15%), after treatment with 6 and 8 ppm of enzyme concentration. 3% w/v RS solution resulted in increased sugar yield on enzyme hydrolysis for all four enzyme concentrations employed, viz. 3.6% (2 ppm), 16.62% (4 ppm), 18.74% (6 ppm), and 19.0% (8 ppm).

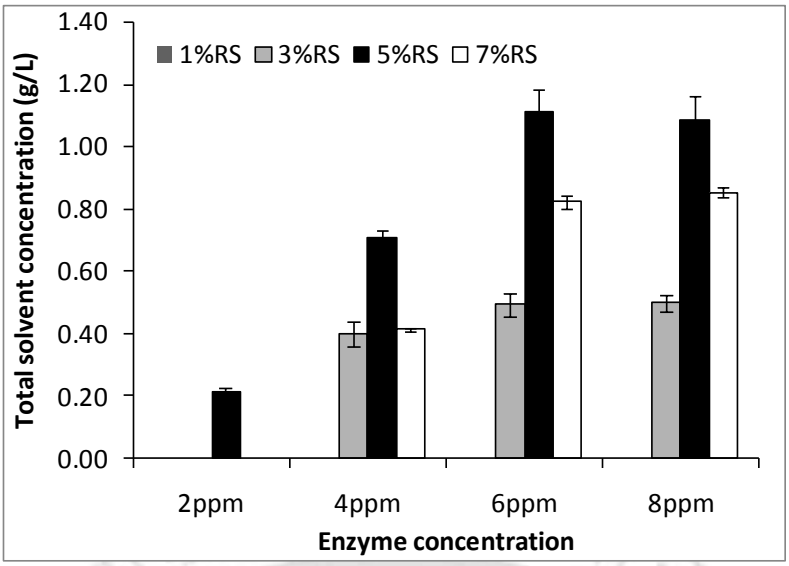

Figure 5.7: Total solvent production achieved from enzymatic hydrolysis of varied concentration of RS

Enzyme hydrolysis of 5% w/v RS solution gave elevated sugar yield of 10.1, 17.53, 19.66 and 19.69%, after treatment with 2, 4, 6 and 8 ppm of enzyme. Slightly reduced total sugar yield was observed with enzymatic hydrolysis of 7% w/v solution of RS. The trends of sugar release for varied enzymatic concentration for different concentrations of RS are compared in Fig. 5.6. Increasing the enzyme concentration from 2 to 6 ppm led to increasing in sugar release. Further increase of enzyme concentration to 8 ppm exhibited nearly negligible effect on sugar yield. Hence, among the four set of enzyme concentrations tested, viz. 2, 4, 6 and 8 ppm, 6 ppm was observed to be the most optimum concentration for hydrolysis. Although, it should to be noted that the sugar yield achieved from enzymatic hydrolysis is far less than that achieved from chemical hydrolysis of RS.

Fermentation of RS hydrolyzate obtained from enzyme hydrolysis resulted in production of solvents (acetone, butanol and ethanol). Fig. 5.7 depicts the total solvent production from fermentation of hydrolyzate obtained from enzyme treatment of RS solutions of different concentrations. No solvent production was observed for hydrolyzate obtained from treatment of 1% w/v RS solution. Moreover, among all experimental sets, hydrolyzate obtained with 2 ppm

enzyme concentration resulted in no production of solvent. Only negligible amount of acetone was observed with hydrolyzate of 5% w/v RS solution. Two possible causes leading to this effect are: (1) very low concentration of enzyme, and (2) very low concentration of rice straw, both of which result in low release of sugar. Hydrolyzate obtained from enzyme treatment of 3% w/v solution of RS produces 0.4 to 0.5 g L^{-1} of total solvents. Maximum solvent yield of 0.09 with a butanol selectivity of 0.1 was achieved with 6 and 8 ppm enzyme solution. Enzymatic hydrolysis of 5% w/v of rice straw results in increased solvent production with maxima ABE of 1.12 and 1.09 g L^{-1} for hydrolyzate obtained with 6 and 8 ppm enzyme concentration. On the contrary, enzyme hydrolysis of 7% w/v RS solution produces a reduced amount of total solvent ranging from 0.42 (4 ppm) to 0.85 g L^{-1} (6 and 8 ppm). The overall comparison of solvent yield and selectivity with fermentation of enzyme hydrolyzed RS (for different w/v concentrations of RS and different concentrations of enzyme in ppm) is illustrated in Table 5.2.

These results revealed that chemical hydrolysis of rice straw resulted in better sugar and solvent yield than sole enzymatic hydrolysis. Moreover very low (1% w/v) and very high (7% w/v) concentrations of rice straw gave poor sugar yield after hydrolysis. Hence, in the next stage of experiments, we have used only the intermediate concentrations of rice straw (3 and 5 % w/v of RS) for pretreatment. In next section, we have also attempted to study the influence of combined effect of physical, chemical and enzymatic treatments on sugar release and subsequent solvent production during fermentation.

5.3.4 Pretreatment with combined techniques

In previous sections, we have studied the individual effect of physical, chemical and enzymatic treatment on rice straw hydrolysis. In this section, we have presented results on treatment of rice straw with combined physical, chemical and enzymatic treatments, and solvent production from fermentation of the hydrolyzate obtained from the pre–treatment. Five sets of experiments have

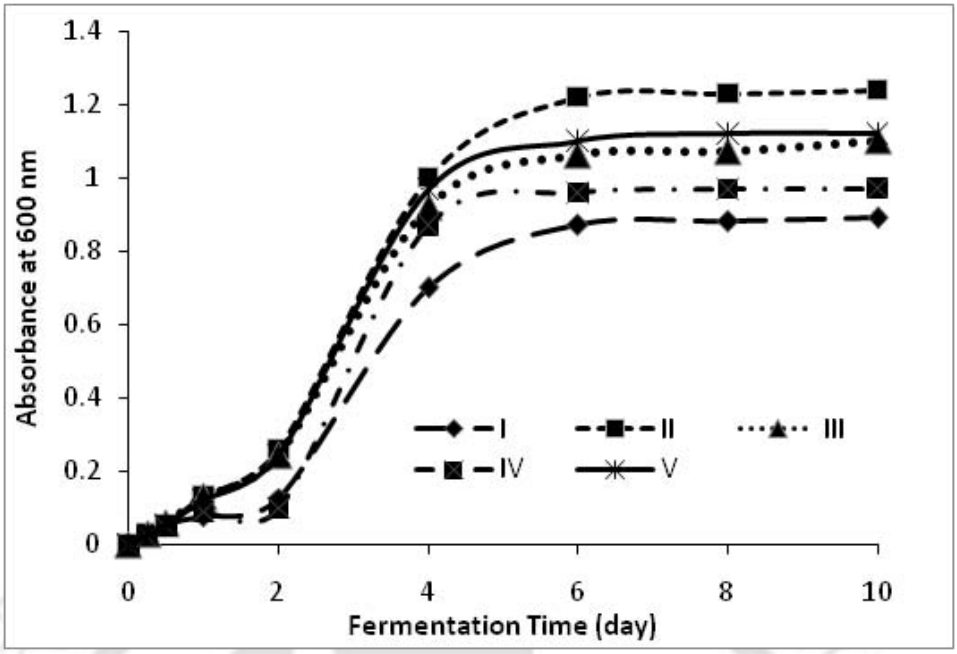

Figure 5.8: *Clostridial* growth in rice straw hydrolyzate obtained from different pretreatments. (I) autoclaving + agitatation; (II) autoclaving + agitation + dilute acid treatment; (III) autoclaving + agitation + dilute alkali treatment; (IV) autoclaving + agitation + enzyme treatment, (V) autoclaving + agitation + dilute acid treatment + enzyme treatment

been preformed: (I) Autoclaving + Agitation; (II) Autoclave + Agitation + 1% v/v H_2SO_4 (acid) treatment; (III) Autoclave + Agitation + 1% v/v NaOH (alkali) treatment; (IV) Autoclave + Agitation + Enzyme treatment; (V) Autoclave + Agitation + 1% v/v H_2SO_4 (acid) treatment + Enzyme treatment. From our previous sets of experiments, we have observed that autoclaving and agitation of rice straw results in better sugar yield, as it makes a smooth slurry of rice straw after pretreatment. Hence, in all the set of experiments, physical pretreatment of autoclaving and agitation of rice straw has been kept uniform.

***Study of Clostridial growth cycle*:** The hydrolyzates of rice straw obtained with five techniques described above were allowed to undergo anaerobic fermentation for 10 days (refer to Fig. 5.8 that depicts *Clostridial* growth cycle with different feedstocks), and samples of fermentation mixture were withdrawn at regular intervals to study the ABE production at various growth stages of *C. acetobutylicum*. A varied *Clostridal* growth pattern was observed in the hydrolyzates obtained from five experimental sets described above. Set I resulted in minimum

sugar release and also showed a minimal cell density in comparison to other four sets. The highest cell growth was observed in set II, which contained acidified RSH. Comparative growth of *clostridial* cultures was seen in RSH obtained with acid + enzyme hydrolysis and alkali + enzyme hydrolysis. Although a considerable difference in the microbial density was observed in these two hydrolyzates, the overall trend of *clostridial* growth curve remained the same. For the first 48 h (2 days) no solvent (acetone or butanol or ethanol) production was observed. Besides control flasks (set I) and enzyme treated flasks (set IV), all the other set of experiments exhibited a lag phase of 22–30 h, during which cells adapted themselves to their new growth environment. During this phase, *clostridial* cells remain least active. After this, cells from all flasks enter in log phase, during which cells grow exponentially and produce primary metabolites. During this phase *clostridial* cells utilize the carbon source from medium and convert them into acid (acetate, butyrate etc.), and other precursors required for the production of solvents [3]. This phase lasts for 48–80 h, and is generally termed as "acidogenesis". During this phase, cells are metabolically highly active and the cell density increases exponentially to a maximum. Optical density of cells during this phase of growth cycle is observed in range of 0.7 to 0.8. After this, the cells enter the long stationary phase, during which cells remain metabolically active, but cell growth is reduced. In this phase, solvent production starts. All the acids produced during acidogenesis get converted into solvents (acetone, butanol and ethanol), and hence, this phase is known as solventogenesis. Solventogenesis stage starts with the sole production of acetone. For 5–6 days, only acetone production has been observed. The samples taken during 10^{th} day of growth cycle reported to contain acetone, butanol and ethanol in considerable amount. The optical density of cells observed during this phase is above 0.95. *Clostridial* cell density was observed to be the least with control experiments due to the presence of least amount of sugar in the hydrolyzate. Cells could grow more effectively during other set of experiments due to comparatively higher amount of sugar in hydrolyzate (37–44%).

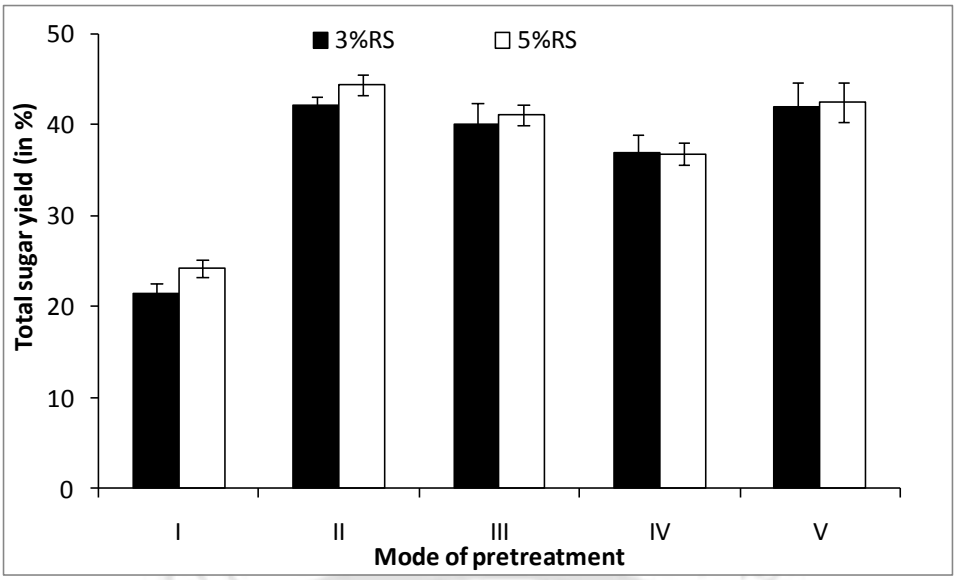

Figure 5.9: Total sugar yield achieved from different mode of rice straw hydrolysis with varied concentration of RS (3 and 5% w/v)

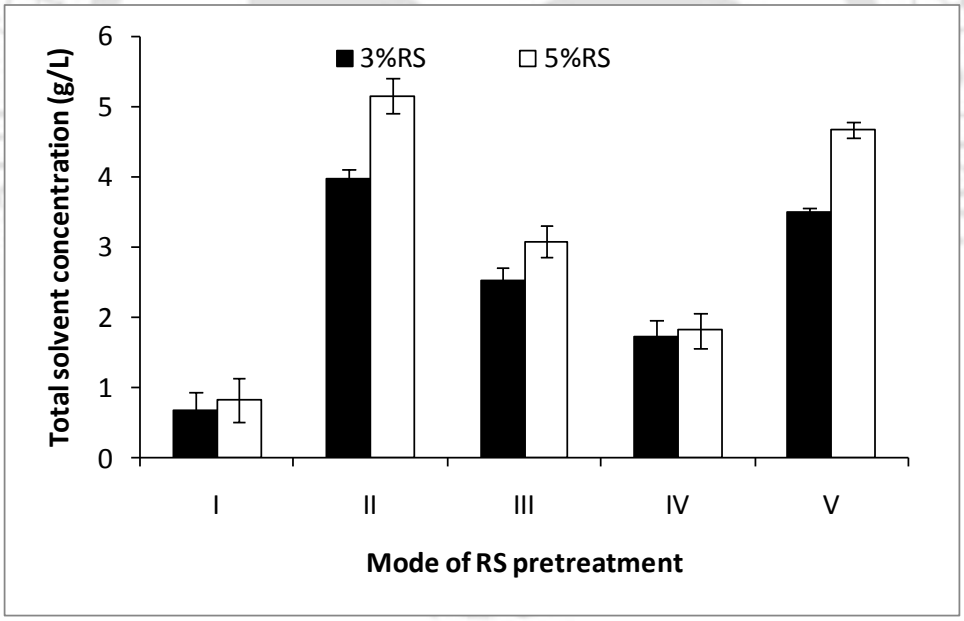

Figure 5.10: Total solvent produced fermentation of RSH achieved from different mode of rice straw pre–treatment with varied concentration of RS (3 and 5% w/v)

***Sugar release and solvent production*:** Among the five set of experiments, Set I (comprising of only physical treatment of autoclaving and agitation) was considered as control (or reference), so as to assess the additional effects of application of chemicals and enzyme for hydrolysis.

Table 5.3: Comparative study of solvent yield, butanol yield, and butanol selectivity achieved after fermentation of hydrolyzed rice straw through various mode and at different concentrations

Rice Straw (%)	Total solvent yield (g/g)		Butanol yield (g/g)		Butanol Selectivity (mol/mol)	
	3% RS	5% RS	3% RS	5% RS	3% RS	5% RS
I	0.11	0.07	0.02	0.01	0.17	0.18
II	0.24	0.22	0.09	0.09	0.32	0.35
III	0.20	0.16	0.06	0.06	0.26	0.31
IV	0.15	0.11	0.04	0.04	0.21	0.29
V	0.25	0.21	0.07	0.07	0.22	0.28

In Set I, physical pretreatment of rice straw by agitation and autoclaving resulted in release of nearly 20% of sugar from the pretreated RS (3 and 5% w/v solutions). Further assistance of autoclaving and agitation with 1% H_2SO_4 v/v treatment (Set II) resulted in an enhanced sugar yield of 42–44%. Fermentation of hydrolyzate in Set II (with 3% w/v of RS) resulted in production of 3.56 g L^{-1} of total solvents, with a butanol concentration of 1.13 g L^{-1}. Performing same experiment with a higher initial RS concentration of 5% w/v resulted in production of 4.87 g L^{-1} of total solvents, with butanol concentration of 1.96 g L^{-1}. Thus, Set II experimental conditions of hydrolysis (for both 3 and 5% w/v concn of RS) resulted in a solvent yield of 0.22 to 0.24 g g^{-1} and butanol yield of 0.9 g g^{-1}. Five methods of hydrolyzate preparation used in the present study have resulted in release of varied amount of total sugar from the processed rice straw. Figs. 5.9 and 5.10 gives a comparative illustration of sugar release (after pretreatment) and solvent production (after fermentation of hydrolyzate) in the five experimental sets. Table 5.3 gives a comparative account of solvent yield and butanol selectivity achieved in five sets of experiments.

For hydrolysis in set III, slightly reduced amount of total sugar was released (40–41%) as compared to set II, resulting in comparatively lesser solvent production, viz. 2.32 g L^{-1} (3% w/v RS) and 3.25 g L^{-1} (5% w/v RS). Fermentation of hydrolyzate in set III resulted in solvent yield of 0.16–0.2 with a butanol yield of 0.6 g g^{-1}. However, cumulative effect of physical treatment and enzyme hydrolysis (Set IV) resulted in poor total sugar yield of 37%. Fermentation of

hydrolyzate obtained from physical treatment and enzyme hydrolysis of 3% w/v solution of rice straw resulted in production of only 1.7 g L^{-1} of ABE solvent, with a butanol concentration of 0.44 g L^{-1}. For a higher initial RS concentration of 5% w/v, 1.98 g L^{-1} of ABE solvents, with 0.6 g L^{-1} of butanol was observed resulting in a solvent yield of 0.4.g g^{-1}. In Set V, we have attempted to study the cumulative effect of physical, acid and enzymatic treatment on RS hydrolysis. Hydrolyzate in this set contained 42–43% sugar, which was comparable to set II. Enzyme hydrolysis was observed to have negligible effect on overall process. It could be due to activity of enzyme under acidic conditions. In addition to this, our previous set of experiments (employing single technique) have revealed that the commercial grade enzyme (Ferment+) was less efficient in releasing sugar from rice straw. Solvent yield achieved after the fermentation of hydrolyzate obtained from set V was 0.25, with a butanol yield of 0.7 g g^{-1}.

Thus, from the above set of experiments, we have seen that autoclaved or steam exploded (15 psi, 121^{o}C, 15 min), acid treated (1% H_2SO_4 w/v) rice straw, agitated at 200 rpm (60^{o}C) yielded maximum of sugar, which further resulted in maximum solvent yield and butanol selectivity.

Many previous authors have mentioned that enzymatic hydrolysis of RS associated with acid hydrolysis yields best results. Major drawback of treatment with pure enzymes is the high cost of enzyme itself, longer hydrolysis time (few days) and additional techniques for enzyme recovery and maintenance. To overcome this shortcoming, we attempted to use a low cost commercial grade enzyme, which unfortunately showed poor sugar and solvent yield. Rice straw is composed of heterogeneous complex of carbohydrate polymers. Cellulose and hemicellulose content of RS are densely packed by layers of lignin, which protect them against hydrolysis. So it is necessary to have an initial physical pretreatment step to break lignin seal to expose cellulose and hemicelluloses for further hydrolysis [46–51]. Physical pretreatment of RS via autoclaving and agitation results in the removal of lignin layer and decreases the crystalline structure of sugar

Table 5.4: Comparative assessment of various alternative feedstocks in terms of total sugar and glucose release

Reference	Substrate	Pretreatment	Total Sugar ($g\ L^{-1}$)	Glucose ($g\ L^{-1}$)
This work	Rice straw	Dilute Sulphuric Acid Treatment with Shear Stress	44	–
[41]	Wheat Bran	Dilute Sulphuric Acid Treatment	53.1	21.3
[42]	Corn Fiber	(a) Dilute Sulphuric Acid Treatment	29.8	4.3
		(b) Dilute Sulphuric Acid + Enzyme Hydrolysis	54.3	22.4
[43]	Wheat Straw	(a) Dilute Sulphuric Acid	25.4	17.8
		(b) Dilute Sulphuric Acid + Enzyme	41.9	19.1
		(c) Dilute Sulphuric Acid + Enzyme (Simultaneous Saccharification and Fermentation)	25.6	5.2
[44]	Marine Macroalga + Mannintol	Milling	26.3	4.7

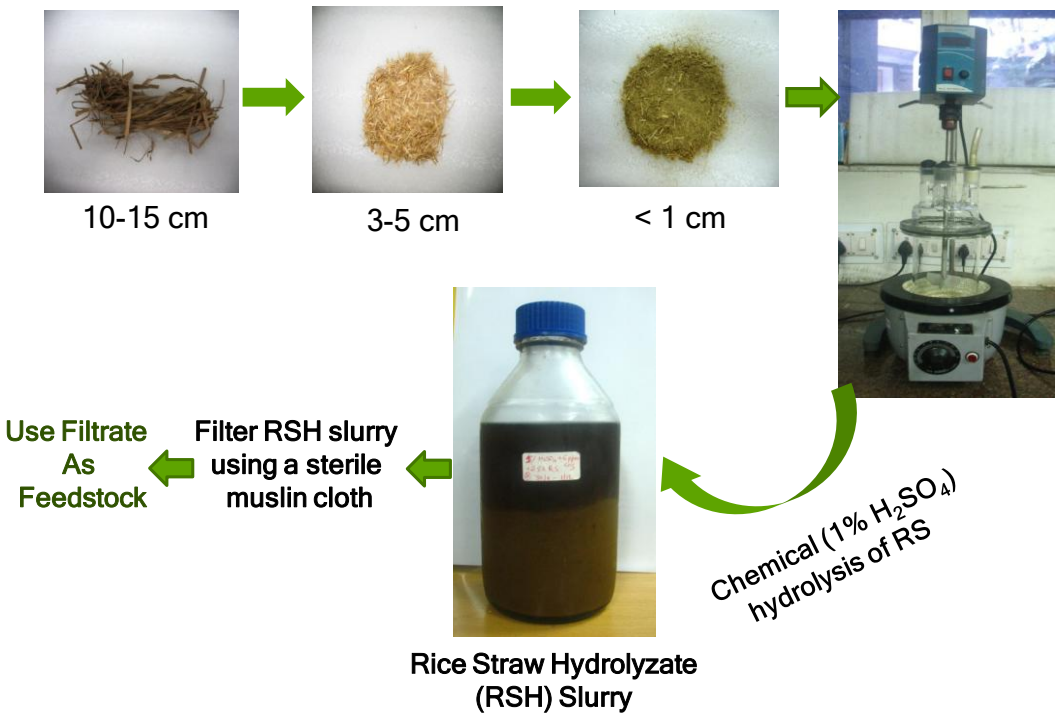

Figure 5.11: Schematic of optimized process for rice straw pretreatment

Table 5.5: Production of ABE from various alternate feedstock

Reference	Microorganism	Substrate	Yield			Total ABE yield
			Acetone	Butanol	Ethanol	
Ranjan et.al. (This Work)	*C. acetobutylicum* NCIM 2337	Rice straw	0.12	0.1	0.02	0.24
[41]	*C. beijerinkii* ATCC 55025	Wheat Bran	0.10	0.41	0.03	0.54
[42]	*C. beijerinckii* BA101	Detoxified SACFH	0.11	0.27	0.008	0.39
		ETCFH	–	0.26	–	0.35
		Glucose	0.156	0.244	0.016	0.39
		Xylose	0.104	0.26	0.02	0.39
[42]	*C. beijerinckii* P260	(a) WSH (Dilute Sulphuric Acid)	0.1	0.25	0.02	0.37
		(b) WSH (Dilute Sulphuric Acid + Enzyme)	0.1	0.2	0.02	0.32
		(c) WSH (Dilute Sulphuric Acid + Enzyme with SSF)	0.13	0.26	0.03	0.42
		(d) WSH (Dilute Sulphuric Acid + Enzyme + Gas Stripping)	–	–	–	0.41
[44]	*C. acetobutylicum* ATCC 824	Marine Macroalga + Mannitol	0.03	0.12	0.013	0.16
[45]	*C. beijerinckii* BA101	Starch	0.13	0.26	0.02	0.41
		Packing peanuts	0.097	0.27	0.005	0.37
		Model Agriculture waste	0.14	0.3	0.002	0.44
		Actual Agriculture waste	0.09	0.19	0.003	0.28

Chemical hydrolysis via acid and alkali treatment results in release of soluble sugar. Table 5.4 and 5.5 compares the various pretreatment methodology adapted by many researcher for hydrolysis of various types of alternative substrate. This indicates that optimum pretreatment of rice straw in this study is at par with other existing methodologies in terms of sugar release. However, the solvent yield achieved from the optimized rice straw hydrolyzate is very less as compared to previous studies. Fig. 5.11 depicts a schematic flow chart of methodology selected as final optimized process. Probable reason for low solvent yield in fermentation of rice straw

hydrolyzate obtained in optimum pre–treatment could be given as follows: Rice straw itself acts as incomplete nutrient source for the microbial metabolism. Rice straw lacks vitamins, minerals and other nutrients important of the *clostridial* metabolic cycle. These nutrients are essentially required for efficient function of enzymes involved in acidogenesis and solventogenesis. In this Chapter, we have optimized the pretreatment methodology for achieving maximum sugar release. In our next Chapter, we shall address the issue of nutritional enhancement of the hydrolyzate, which could increase the solvent yield and selectivity during fermentation of the hydrolyzate.

5.4. Conclusion

Among different methods of rice straw pretreatment (hydrolysis) prior to fermentation, steam exploded (or autoclaved), acid hydrolyzed RSH has liberated the highest amount of total sugar (nearly 44%). *Clostridium acetobutylicum* MTCC 481 was capable of utilizing hydrolyzates obtained from all kinds of pre–treatment of RS (physical, chemical, enzymatic, and combined), and the best ABE productivity and yield was achieved in fermentation of hydrolyzate obtained from steam exploded and acid hydrolyzed RS.

Abbreviations

ABE	Acetone Butanol Ethanol
CMM	Cooked Meat Medium
CTCC	Microbial Type Culture Collection
IMTECH	Institute of Microbial Technology
NCIM	National Collection of Industrial Micro-organisms
RCA	Reinforced Clostridial Agar
RCM	Reinforced Clostridial Medium
RS	Rice Straw
RSH	Rice Straw Hydrolysate

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CHAPTER 6

DEVELOPMENT OF SEMI-DEFINED RICE STRAW BASED MEDIUM FOR BUTANOL PRODUCTION AND ITS KINETIC STUDY

6.1 Introduction

In Chapter 2 we presented a comprehensive review and analysis of literature on economics of butanol manufacture on commercial scale [1]. Out of different cost components of a commercial biobutanol process, the most significant component of total production cost, that contributes to per unit manufacturing cost of butanol, was the cost of substrate. Conventional substrates for biobutanol are molasses and corn. However, as these substrates also have other outlets that fetch higher cost, employment of these substrates is not feasible for economic production of biobutanol, with per unit production price lesser (or at least at par) with petroleum based fuels. Obviously, reduction in the cost of substrate by employing cheap substrates such as agro- and forest residues [2] will give a big boost to the economy of biobutanol. Significant research has been dedicated in last two decades for exploration of variety of alternate cheap renewable feedstocks for ABE fermentation such as bagasse, wheat straw, wheat bran, corn fibre and other agriculture waste. We have presented a

comprehensive review of literature in this area in Chapter 2. As stated earlier, the aim of this thesis is to develop a rice straw based fermentation process for biobutanol.

However, there are inherent difficulties in direct utilization of an agro- residue like rice straw for fermentation, as lignocellulose (comprised of cellulose, hemicellulose and lignin) is the main constituent of these biomasses [3,4]. Natural lignocellulosic biomass is resistant to hydrolysis because of the crystallinity of cellulose, protection of cellulose by lignin, cellulose sheathing by hemicelluloses and the limited accessible surface area of the biomass matrix. Pre-treatment of biomass via physical, chemical, enzymatic or microbial route is critical to collapse the close knit structure of lignocellulosic biomass, thus providing hydrolysis agents with a more direct access to the feedstock [5]. The general process for alcoholic biofuel production using lignocellulosic biomass as feedstock involves pre-treatment and hydrolysis of feedstock followed by fermentation [6-8]. Various modes of pre-treatment have been attempted for hydrolysing lignocellulosic biomass for release of fermentable sugars, such as acid hydrolysis, alkaline hydrolysis, steam treatment or autoclaving, microwave treatment and enzymatic hydrolysis etc. [9-15], and in Chapter 5, we have presented our studies on optimization of pre-treatment of rice straw. In this chapter, we extend the theme of process development of rice straw based ABE fermentation with the next stage of medium development.

Rice straw hydrolysate resulting from optimized pre-treatment (as presented in chapter 5) is nutritionally deprived, and hence, does not act as complete fermentation medium. For an efficient ABE fermentation, the rice straw hydrolyzate needs to be supplemented with additional components or nutritional factors comprising of vitamins, minerals and salts (collectively called as fermentation medium) that support the growth and metabolism of *clostridial* cultures, which eventually manifests in enhanced solvent production [16,17]. These vitamins, minerals and other nutrients act as inducers and / or co-

factors for enzymes that participate in the metabolism leading to solvent production. The literature reports several studies on growth media (i.e. supplementary components in the fermentation medium that support growth) for *clostridial* cultures [18-20]. These growth media comprise of numerous components such as amino acids, trace metals, vitamins, growth factors, carbon sources, etc. Some of these components could be critical for cell growth or productivity, while others may be toxic at certain levels, and many may be involved in complex interactions within the cell. Li et al. [21] has reported that the adequate addition of yeast extract in fermentation medium could promote phase shift (acidogenesis to solventogenesis) by increasing gene transcription to 16-fold, and indirectly enhance butanol synthesis through accelerating the accumulation of histidine and aspartic acid families. Similarly, Haapalainen et al. [22] has reported the importance of potassium and chloride ion for the activation of the enzyme thiolase, which play a key role in *clostridial* metabolism for the production of butanol. Dehydrogenases (butanol dehydrogenase, ethanol dehydrogenase, alcohol dehydrogenases etc.) are the important enzymes of *clostridial* metabolic pathway leading to formation of ethanol, butanol and reversible oxidation of ethanol to acetaldehyde with the concomitant reduction of NAD. The major requisites for the normal functioning of these enzymes includes Zn^{2+}, Fe and other vitamins and minerals, which can be fulfilled by addition of yeast extract, PABA, Zn salts, iron salts etc. to the fermentation medium [23]. In order to find the best composition of these components in the medium for fermentation aimed at maximization of production of butanol, an optimization study is essential.

In our approach, we first studied the effect of selected nutritional factors reported in the literature on the production of butanol by *C. acetobutylicum* MTCC 481 strain using the classical ‘one-variable-at-a-time’ method. The significance of the effect of these nutritional factors on butanol production was then analyzed using a statistical Taguchi experimental design [24,25]. Finally, the solvent production in the optimized fermentation medium was

tested first on shake flask scale, and later in a 2 L stirred tank bioreactor operated in batch mode without pH control.

6.2 Materials and Methods

6.2.1 Micro-organism, Culture Revival and Maintenance

Lyophilized cells of *Clostridium acetobutylicum* MTCC 481 were procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology (Chandigarh, India). The cells were revived anaerobically inside an anaerobic culture bag system (Himedia) in RCA (Reinforced Clostridial Agar) and RCM (Reinforced Clostridial Medium: Broth) culture media at 37^{o}C. The inoculums were prepared in RCM containing following components (with concentration mentioned in g L^{-1}): glucose, 5.0; yeast extract, 3.0; starch, 1.0; beef extract, 10.0; peptone, 10.0; sodium chloride, 5.0; sodium acetate, 3.0; Agar, 0.5; cysteine hydrochloride, 0.5. The pH of medium was 6.8 ± 0.2. 100 mL of media was autoclaved at 121^{o}C, 15 lb pressure and inoculated in a custom fabricated 250 mL screw capped Erlenmeyer flask. In addition, CMM (Cooked Meat Medium) was also used for the maintenance of *clostridia*. Anaerobic condition in broth culture was maintained by adding 0.05% of cysteine hydrochloride, and regular sparging of nitrogen to fermentation broth. All chemicals were of analytical grade procured either from Merck (Germany), Sigma Aldrich (Germany) or Himedia (India). The revived cells were maintained on RCM agar plates and slants at 4^{o}C, and were used as a stock. The cells were sub-cultured every month.

6.2.2 Preparation of rice straw hydrolysate (RSH)

After initial comminution process (washing, drying, cutting, grinding) of rice straw, an aqueous mixture of 5% w/v RS was prepared. This mixture was hydrolysed using 1% H_2SO_4 and was autoclaved for 15 min at 15 lb steam pressure. This RSH was further stirred

Table 6.1: Solvent production from RSH supplemented with single variable (nutrient) at time

S. No.	Components	Acetone g L^{-1}	Butanol g L^{-1}	Ethanol g L^{-1}
1	Magnesium nitrate hexahydrate	0.3		
2	Ferric nitrate	-	-	-
3	Ammonium nitrate	-	-	-
4	Yeast extract	3.61	0.158	-
5	p-aminobenzoic acid (PABA)	3.71	0.167	0.02
6	Biotin	3.62	0.026	-
7	PABA +Biotin	4.01	0.043	-
8	Calcium chloride	-	-	-
9	Potassium chloride	-	-	-
10	Sodium chloride	3.93	-	0.01
11	Magnesium sulfate	3.69	0.182	0.04
12	Sodium acetate	3.58	0.055	-

at 60^{o}C and 200 rpm resulting in smooth slurry. This was then filtered using sterile muslin cloth and the supernatant was used as fermentation broth. This was further supplemented with nutrients as per the methodology of optimization as mentioned later in the text. All chemicals used as supplements ($MgNO_3 \cdot 6H_2O$, $FeNO_3$, NH_4NO_3, Yeast extract, PABA (p-aminobenzoic acid), Biotin, $CaCl_2 \cdot 2H_2O$, KCl, NaCl, $MgSO_4 \cdot 7H_2O$ and CH_3COONa) were of analytical grade and were procured from either Merck or Himedia.

6.2.3 Fermentation conditions

The initial screening of the media constituents was done using conventional method of one-variable-at-a-time approach. *C. acetobutylicum* MTCC 481 strain was cultured in acidified RSH medium. 12 sets of fermentation experiments supplemented with following single additives (in g/L) were carried out: $MgNO_3 \cdot 6H_2O$, 3.0; $FeNO_3$, 3.0; NH_4NO_3, 3.0; Yeast extract, 3.0; PABA, 0.02; Biotin, 0.01; PABA and Biotin, 0.02 and 0.01; $CaCl_2 \cdot 2H_2O$, 0.02; KCl, 0.5; NaCl, 0.01; $MgSO_4 \cdot 7H_2O$, 0.2 and CH_3COONa, 3.0. These media components and their concentrations were selected on the basis of previous literature [18-21, 26].

Table 6.2: Taguchi design matrix and corresponding butanol production by *Clostridium acetobutylicum* (MTCC 481) in shake flask

Experimental Run No.	Parameters (Factor)				Butanol ($g\ L^{-1}$)	S/N Ratio (dB)
	Yeast Extract ($g\ L^{-1}$)	PABA ($mg\ L^{-1}$)	Sodium acetate ($g\ L^{-1}$)	$MgSO_4$ ($g\ L^{-1}$)		
1	1(3)	1(2)	1(3)	1(0.2)	2.14	6.13
2	1(3)	1(2)	2(5)	2(0.5)	2.38	7.15
3	1(3)	2(4)	1(3)	2(0.5)	5.5	14.61
4	1(3)	2(4)	2(5)	1(0.2)	3.28	10.02
5	2(5)	1(2)	1(3)	2(0.5)	0.99	-0.62
6	2(5)	1(2)	2(5)	1(0.2)	1.5	3.27
7	2(5)	2(4)	1(3)	1(0.2)	1.45	2.41
8	2(5)	2(4)	2(5)	2(0.5)	1.44	2.18

Table 6.1 depicts the solvent (acetone, butanol and ethanol) production in above 12 sets of experiments. Out of these 12 components, 4 components, viz. yeast extract, PABA, $MgSO4{\cdot}7H_2O$ and CH_3COONa showed noteworthy potential for butanol production and were short-listed for further Taguchi experimental design, the details of which are described in next section. The optimization studies were carried out in custom fabricated 250 mL anaerobic Erlenmeyer flasks (picture supplied as supplementary material) with bottom port for sample withdrawal and nitrogen sparging in order to minimize oxygen contamination and maintain strict anaerobic conditions. Each flask contained fermentation medium with desired composition (i.e. mixture of 100 mL of acidified RSH and nutritional factors), and was inoculated with 2% of 18 h old, *C. acetobutylicum* MTCC 481 culture. All flasks were sparged with nitrogen at the start and after every 24 h of fermentation to maintain anaerobic conditions. These flasks were kept in an incubator shaker (Make: Scigenics Biotech, Model: Orbitek) operating at 200 rpm and 37^{o}C. The samples of fermentation broth were withdrawn after every 2 days up to a period of 10 days. The initial pH of each set of acidified rice straw hydrolysate based medium was 1.0 ± 0.2.

6.2.4 Taguchi's Orthogonal Array

A well-known experimental design technique, namely L8 orthogonal array design [27, 28] was employed to study the effect of four parameters, viz. yeast extract, PABA, sodium acetate, and $MgSO_4$ on the butanol production. Essentially, the design consisted of a total of 8 experiments with the four parameters, each having two levels, i.e. L8 (2^4). Table 6.2 presents the combinations of experimental conditions adopted as per the design, along with butanol concentration as the observed response in each run. All experiments in this study were performed in custom fabricated 250 mL Erlenmeyer flasks with combination of parameters according to the experimental design (Table 6.2). To study the bio-butanol production in the batch shake flasks, 100 mL of optimized RSH medium was inoculated with seed culture and agitated in an incubator shaker (Make: Scigenics Biotech, Model: Orbitek) for 10 days at 37^{o}C and 200 rpm. Samples were taken periodically for the analyses of the residual sugar and ABE (acetone, butanol and ethanol) solvent production in the fermentation medium. All batch shake flask experiments were conducted in triplicate. The optimal conditions with respect to the experimental factors or parameters (which are essentially the nutritional factors) tested have been determined on the basis of average of signal to noise ratio (S/N) for the factor or parameter at each factor level (Table 6.3). Butanol production by *Clostridium acetobutylicum* MTCC 481 was considered as a desired variable, and a higher concentration was preferred. In each experimental run, the response was recorded as the butanol production and corresponding signal-to-noise (S/N) ratio was calculated using Equation 1 with an overall objective of estimating the effects of various parameters on solvent production, where a large S/N ratio is preferred [29, 30].

Table 6.3: Value of average S/N ratio through Taguchi analysis of factors affecting butanol production by *C. acetobutylicum*

Level	Factors			
	Yeast extract g L^{-1}	**PABA mg L^{-1}**	**Sodium acetate g L^{-1}**	**$MgSO_4$ g L^{-1}**
1	9.472	2.4869	4.1367	5.4554
2	0.3142	7.3025	5.6527	4.3340
Delta*	9.1611	4.8157	1.5161	1.1213
Rank	**1**	**2**	**3**	**4**

$$\frac{S}{n} = -10 \times \log\left(\frac{{}^{1}/_{Y^2}}{n}\right) \quad (1)$$

where Y is the response and n is the number of experimental runs. The statistical significance of each factor was determined using ANOVA (Analysis of Variance). Finally, the optimum conditions for butanol production by *C. acetobutylicum* MTCC 481 were again determined using ANOVA performed using MINITAB® Release 15.1, PA, USA (Trial version).

6.2.5 Validation of results

ABE fermentation was performed to validate the results obtained in Taguchi model. Thereafter, the developed RSH based fermentation media was tested in a 2 L microprocessor controlled bioreactor (Zenith, India) with 1 L of developed RSH medium and 2% v/v of clostridial inoculum. The temperature was controlled at 37°C, 99.98% pure nitrogen was sparged at a rate of 0.1 vvm (vol gas per vol liq per min). The initial pH of the media and the agitation rate were set at 1.0 ± 0.2 and 200 rpm, respectively. The samples of fermentation broth were withdrawn after every 48 h up to a period of 10 days for analysis. The experiments in both shake flask and bioreactor were carried out in duplicate.

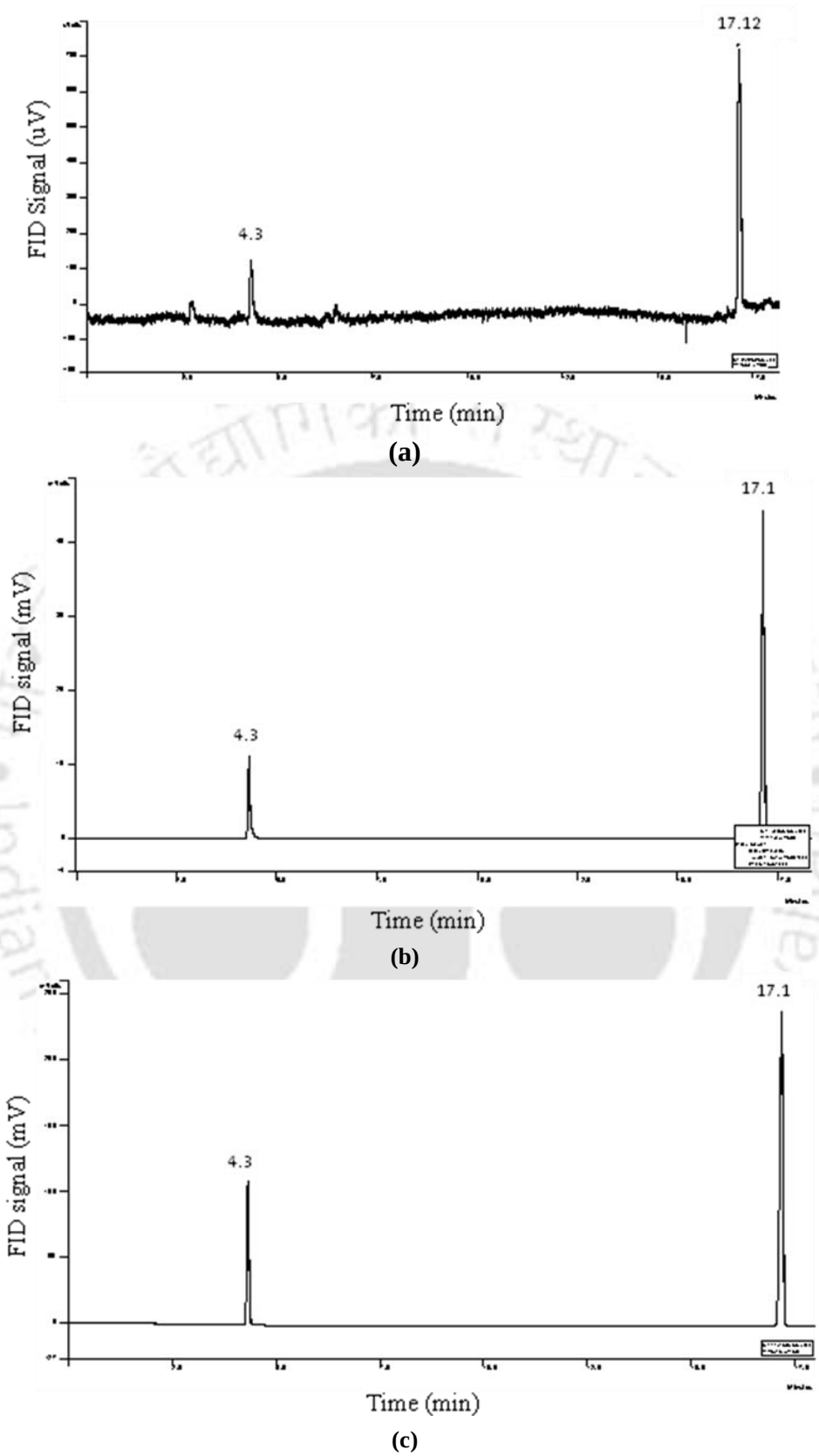

Figure 6.1: Gas chromatograms of fermentation broth for reactor scale experiments (employing optimized rice straw hydrolyzate based medium) at various stages of fermentation: (a) 2 days, (b) 4 days, (c) 10 days.

6.2.6 Analysis

The optical density of the cells in the broth as measured using UV-Vis spectrophotometer (Thermo Fischer) with absorbance at 600 nm after appropriate dilution in water. Glucose was analysed using Glucose (GO) assay kit procured from Sigma Aldrich, USA (GAGO20–1KT). Total sugar analysis was done by Anthrone test as directed by Hedge and Hofreiter [31]. Quantification of reducing sugar has been done by using DNS (Dinitrosalicylic Acid) method proposed by Miller [32]. Samples were filtered with 0.2 µm filter and diluted appropriately for the qualitative and quantitative determination of the solvents and sugars.

Solvent production in the fermentation broth was monitored on a gas chromatograph (Varian) using a CP Wax 52CB (250 mm × 0.25 mm × 0.39 mm) capillary column and a Flame Ionization Detector. The injector and detector temperatures were 230 and 250^{o}C, respectively. The oven temperature was programmed from 45 to 100^{o}C with an increment of 3^{o}C/min, and after 100^{o}C, an increment of 5^{o}C/min up to 200^{o}C. Gas chromatograph was used to quantify acetone, butanol and ethanol during the fermentation of rice straw hydrolysate. This was achieved by plotting standard calibration curves (peak area vs. concentration) using GC grade acetone, butanol and ethanol procured from Sigma Aldrich. R^2 values for standard curve of acetone, butanol and ethanol was observed to be 0.98, 0.99 and 0.98, respectively. Samples were injected at regular intervals. Retention time for acetone was noted to be 4.3 min, while for butanol and ethanol 17.11 min, 7.23, respectively. Representative chromatograms of the fermentation broth after 2, 4 and 10 days of fermentation are depicted in Fig. 6.1.

6.3. Results and Discussion

Preliminary conventional type screening experiments helped in determining the influence of supplemental nutritional factors to RSH on solvent production. However, in the

present study, we have emphasized on butanol production as the response of all experiments. Table 6.1 shows the results obtained after implementing one-variable-at-a-time approach. It could be inferred from Table 6.1 that among the 12 supplemental nutritional factors, $MgNO_3 \cdot 6H_2O$, $FeNO_3$, NH_4NO_3, Biotin, $CaCl_2 \cdot 2H_2O$, KCl, NaCl, and PABA and Biotin had an insignificant impact on butanol production, while PABA, yeast extract, $MgSO_4 \cdot 7H_2O$, and sodium acetate had a significant impact on the products yield. The results obtained with supplemental $MgNO_3 \cdot 6H_2O$, NH_4NO_3, Biotin, NaCl, and (PABA + Biotin) showed production of acetone and ethanol, while no solvent (ABE) production was observed when RSH was supplemented with $FeNO_3$, $CaCl_2 \cdot 2H_2O$ and KCl. These results are in agreement with the work reported by Li et al. [21]. According to Li et al. [21], yeast extract contains abundant of vitamins, minerals, and amino acids, which are necessary for cell growth and synthesis of various enzymes. Rice straw hydrolysate is a deficient medium for nitrogen, vitamins and minerals and other trace elements, which act as cofactor for many enzymes. Yeast extract and PABA being a rich source of nitrogen as well as vitamins and minerals resulted in enhanced *clostridial* growth and metabolism under batch conditions [18, 19]. Reportedly, PABA acts as coenzyme in the metabolism and utilization of protein. PABA converts to folate in presence of enzyme synthetase .deficiency of pab genes in *C. acetobutylicum* can limit the bacterial growth. Hence, supplementation of external PABA to the medium supports folate synthesis, and thus, helping in growth and reproduction of clostridial cells. Sodium acetate, being a source of acetate, which is also a product during acidogenesis phase probably acted as an inducer of solventogenesis. $MgSO_4$ (also known as Epsom salt) is known for its effect on improvement of uptake of other nutrients, which results in enhancement of growth. Somda et al. [20], Gawande et al. [33], Birch et al. [34], Pasternak et al. [35] have also found Mg^{2+} to be essential for enzyme production and consequently alcohol release. Mori et al. [36] showed that magnesium acts as activator of some enzymes,

especially transferases and decarboxylases, which play an important role in biochemistry of alcohol production. Thus, the results of our preliminary or one-variable-at-a-time experiments were in good agreement with earlier study. Thus, the four factors, viz. yeast extract, PABA, $MgSO_4$ and sodium acetate were selected as main or significant factors, and were allowed to undergo further statistical optimization through Taguchi Design of Experiments (DOE) method.

6.3.1 Taguchi analysis and ANOVA

Table 6.2 depicts the final total concentration of alcohols formed with media comprising of different compositions of significant factors, i.e. yeast extract, PABA, $MgSO_4$ and sodium acetate. The results given in Table 6.2 indicate that the production of solvents is a major function of media composition. Among all 8 experimental runs, the highest total solvents (8.04 g L^{-1}) and butanol (5.5 g L^{-1}) concentration was attained in run 3. The percentage effect of each factor on butanol and total solvent production is given in Table 6.3. Out of 4 factors examined, yeast extract and PABA had major effect on butanol production, as indicated by their high delta S/N value of 9.16 and 4.82 db, respectively. In Taguchi statistical design, S/N ratio is an important parameter for identifying optimal conditions for the process. A high S/N ratio indicates higher significance of the factor. Based on this logic, the order of effect of various factors on total alcohol production was determined as yeast extract > PABA > CH_3COONa > $MgSO_4 \cdot 7H_2O$. The percent individual contribution of these four factors to the main effect mean (or delta S/N) was in order: 55.14% (yeast extract) > 28.99% (PABA) > 9.13% (sodium acetate) > 6.75% (magnesium sulphate), indicating yeast extract and PABA to be the factors of greater (relative) significance, and sodium acetate and magnesium sulphate as relatively insignificant factors with lower percent contribution for butanol production (< 10%). Fig. 6.2 displays the main effect of means of process variables, viz., Yeast extract, PABA, $MgSO_4$.$7H_2O$ and sodium acetate. The total solvent production

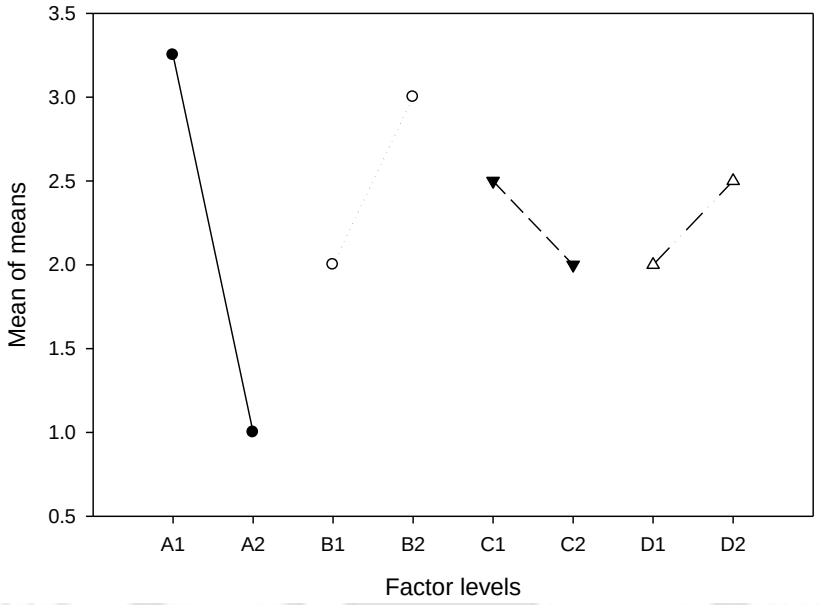

Figure 6.2: Main effects plot of mean for the process variables. A1 & A2: Two levels of yeast extract, B1 & B2: Two levels of PABA, C1 & C2: Two levels of $MgSO_4$, D1 & D2: Two levels of sodium acetate.

Table 6.4: Analysis of variance (ANOVA) of butanol production

Source	**DF***	**SS#**	**MS**	***F* ratio (*F*)**	***p* value (*p*)**	**Confidence level (%)**	**Percent contribution**
Yeast extract	1	5.9004	5.9004	98.33	$p < 0.005$	96.09	55.14
PABA	1	2.036	2.036	14.45	$p < 0.005$	78.32	28.985
Sodium acetate	1	0.2076	0.2076	3.6	$p > 0.005$	58.32	9.125
$MgSO_4$	1	0.3541	0.3541	1.89	$p > 0.005$	68.96	6.749
Residual error	3	1.1118	0.2779				
Model	7	9.6099	2.12				100

was higher at level 1 for factors yeast extract and $MgSO_4$, while factors PABA and sodium acetate produced more solvents at level 2. The comparatively insignificant effect of sodium acetate and $MgSO_4$ on production of butanol can be explained in terms of understanding *clostridial* metabolism, where cells themselves produce acetate by utilizing carbon source from the medium, and hence, additional supplementation of acetate resulted in no net gain in terms of butanol production [37]. Similarly, as stated earlier, Mg^{2+} from $MgSO_4$ is required

for proper functioning of enzymes that are key components of xylose and glucose metabolism [38].

ANOVA was done to quantify the variation in product formation caused due to each factor, and also to determine as whether the lower or higher value of a factor is essential for preferred result, i.e. higher butanol production. Table 6.4 depicts the results of ANOVA for all factors. The ANOVA results corroborate the results based on S/N ratio in that the significant factors for butanol production (which had more than 10% contribution to the main effect with p value < 0.005, F value > 5, and higher confidence level) were yeast extract and PABA, while sodium acetate and $MgSO_4$ were insignificant factors, as indicated by $p > 0.005$ and F < 5 [27, 39]. The ANOVA of butanol production (Table 6.4) had a model SS (Sum of squares), MS (Mean of squares), and F value of 9.61, 2.12, and 29.56, respectively. The model obtained from ANOVA had R^2 (multiple regression coefficient) value of 0.88, which is indicative of the robustness of the model in that it can explain ~ 88% variation in the response. Thus, optimized RSH based fermentation medium for butanol production consisted of stress assisted acid treated RS supplemented with 3.0 g L^{-1} yeast extract and 4.0 mg L^{-1} PABA.

6.3.2 Prediction of butanol and total solvents production with optimized medium

To predict the butanol production (denoted by Y_{opt}) for the optimized fermentation medium mentioned above the following formula was used:

$$Y_{opt} = \bar{T} + \left|A_1 - \bar{T}\right| + \left|A_2 - \bar{T}\right| + \left|B_1 - \bar{T}\right| + \left|B_2 - \bar{T}\right| \quad (2)$$

Here, $\bar{T}$ denotes the average butanol production in all trial results, while A_1, A_2 are the average butanol productions at two levels of yeast extract, while B_1 and B_2 are average butanol productions for the two levels of PABA [29, 40]. Using above formula, the predicted values of butanol and total solvent productions for the optimized fermentation medium were 5.5 g L^{-1} and 8.04 g L^{-1}, respectively.

6.3.3 Validation and comparison of results

Confirmatory experiments to assess butanol and total solvent production with optimized fermentation medium were carried out in duplicate, at both shake flask level as well as in a 2 L bioreactor (Make: Zenith, India). Both solvent and butanol concentration were substantially similar to the predicted optimal values (mentioned in previous section). Moreover, these results were quite comparable to the values previously attained in the experiment trial 3, for which the yeast extract and PABA concentrations were 3 g L^{-1} and 4 mg L^{-1}, respectively (Table 6.3). Shake flask confirmatory experiments with optimized nutritional conditions at 37^{o}C, pH 1.0 and 200 rpm for 10 days resulted in production of 7.4 g L^{-1} of total solvents, and 4.67 g L^{-1} of butanol. The time history of solvent production and sugar utilization by *C. acetobutylicum* MTCC 481 at shake flask scale and reactor scale is depicted in Figs. 6.3 and 6.4, respectively. It was observed that *clostridia* were able to utilize most of the sugar present in RSH based fermentation medium. Nearly 91.4% glucose, 90% reducing sugar, and 83% total sugar were utilized during 10 days of fermentation. These values of high sugar utilization with concurrent production of solvents corroborate the suitability of optimized RSH based fermentation medium for *C. acetobutylicum.* With scaling up of this process in a 2 L bioreactor (or fermentor), results were more encouraging. Nearly 6.0 g L^{-1} of butanol, and 8.7 g L^{-1} of total solvent were produced with almost complete utilization of all sugar in only 8 days. These values are in good agreement with the predicted results by Taguchi method. The reduction in fermentation time, elevated solvent production, and higher substrate utilization profile in the bioreactor could be attributed to control of physical parameters with greater precision, in addition to maintenance of ideal anaerobic conditions in the microprocessor based control system in the bioreactor, as compared to the

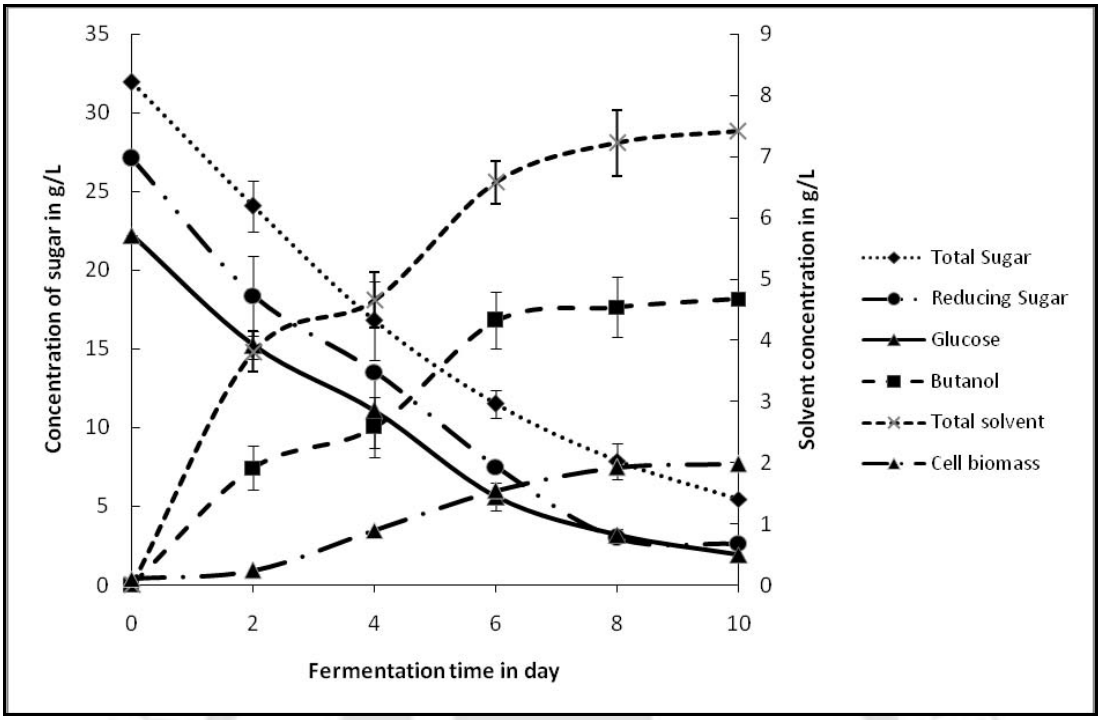

Figure 6.3: Time history of solvent production and sugar utilization in the shake flask experiment with optimized fermentation medium.

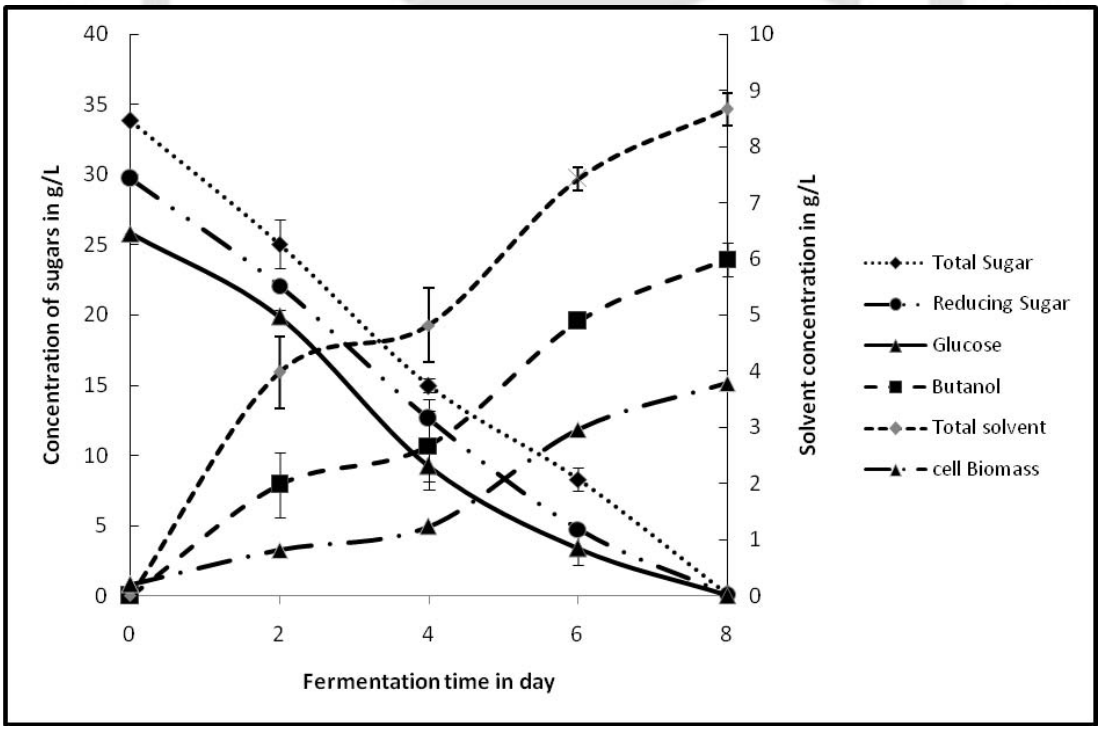

Figure 6.4: Time history of solvent production and sugar utilization in 2 L bioreactor experiment with optimized fermentation medium.

Table 6.5: Comparative evaluation of butanol production with rice straw and other alternate substrates

Reference	Microorganism	Substrate	Butanol titre ($g\ L^{-1}$)
This study	*C. acetobutylicum* MTCC 481	Rice straw	6.0
Virunanon et al. [45]	*C. butyricum, S. cerevisiae*	Cassava pulp	2.51
He and Chen [46]	*C. acetobutylicum* ATCC 824	Corn stover + glucose/xylose	9.64
Guo et al. [47]	*C. beijerinckii* IB4	Corn fiber	6.8
Qureshi et al. [41]	*C. beijerinckii* BA101	Corn fiber	5.8
Efremenko et al. [48]	Immobilized *C. acetobutylicum* B1787	Arthrospira platensis (algae)	0.43
Wang et al. [49]	*C. beijerinckii* BA 101	Distillers Dried Grains with Solubles (DDGS)	3.62
Qureshi et al. [42]	*C. beijerinckii* P260	Wheat Straw (under different processes*)	Process I – 6.05 Process II – 8.09 Process III – 7.4 Process IV – 5.7

* Process I – dilute acid hydrolysed wheat straw; Process II – Acid + enzyme hydrolysis of wheat straw; Process III – acid + enzyme treated wheat straw followed simultaneous saccharification and fermentation; Process IV – acid + enzyme treated wheat straw with solvent removal by gas stripping.

shake flask experiments, where the control of process parameters and anaerobic conditions could have been less precise.

Table 6.5 presents the comparison of results of the present study with those reported in earlier studies using cheap alternate substrates. It can be perceived from the results presented in Table 6.5 that rice straw is indeed a potential substrate giving results that are at par with other agro-residues like wheat straw and corn fiber. As compared to other substrates like cassava pulp, algal biomass and DDGS, the butanol yield is much higher with rice straw.

An interesting result is that under similar conditions as used in this work (i.e. acid hydrolysis of substrate prior to fermentation), the butanol production from wheat and rice straw are almost similar, as evident from work of Qureshi et al. [41, 42]. However, if acid treatment of substrate is also supplemented with a suitable enzyme that helps release of more sugar due to breakage of β-glycosidic linkages of cellulose, greater solvent productivity is seen as evident from results of Qureshi et al. [41, 42].

6.3.4 Kinetic analysis of solvent production, biomass growth, and utilization of sugars under optimized nutrient conditions

The time history of butanol production, *clostridial* biomass growth, and utilization of sugar using optimized fermentation medium in shake flask experiments and also experiments using microprocessor controlled bioreactor (Make: Zenith) have been illustrated in Figs. 6.3 and 6.4. It can be seen from Figs. 6.3 and 6.4 as compared to the shake flask experiments, the fermentation in scaled-up bioreactor resulted in reduced lag phase with an exponential phase of up to 36 h, followed by an extended stationary phase of 96 h, during which production of solvents occurred. This observation is consistent with our study on the *clostridial* biomass growth and butanol production (chapter 5). Solvent production profiles in shake flask and bioreactor experiments showed that the solvent production (including butanol) occurred till 192 h of fermentation cycle. The concentration profiles of sugars (total sugar, reducing sugar and glucose) in the medium for both shake flask and bioreactor scale experiments showed that they get readily utilized by *clostridial* cells. Shake flask experiment resulted in utilization of 91% of total sugar, 95% of reducing sugar and 96% of glucose at the end of fermentation cycle, while reactor scale optimized run resulted in complete utilization of all sugar displaying the enhanced fermentation efficiency at reactor scale.

Further, to estimate the bio-kinetic constants involved in the process, models based on Monod kinetics reported in literature [43, 44] were fitted to the experimental data of butanol production, *clostridial* biomass growth, and utilization of sugar. Table 6.6 presents the kinetic models applied in this study along with the calculated kinetic parameters by fitting the experimental data using Matlab 7.12. These models are essentially unstructured logistic models originally proposed by Mercier et al. [43] for describing the kinetics of biomass growth, substrate consumption, and product accumulation. These models have also been applied earlier by Davery and Pakshirajan [30] and Rodrigues et al. [44] to explain the sphorolipid and biosurfactant production kinetics, in their respective studies. The estimated kinetic parameters values obtained from these models for both shake-flask and bioreactor scale experiments are listed in Table 6.6. Fig. 6.5 shows trends of experimental results (i.e. butanol Production, biomass growth and sugar utilization) along with the trends predicted by the models. The regression coefficients (R^2) for the models fitted to the experimental data are $\geq$ 0.9, which is indicative that the model prediction is in good agreement with the experimental data. Further, goodness of the model fitting is also determined in terms of lower values of the root-mean-square deviation (RMSD) or root-mean-square error (RMSE), and these values have been listed in Table 6.6. The values of P_{max}, i.e. maximum product concentration predicted by the model for both shake flask (4.752 g L^{-1}) and bioreactor (5.03 g L^{-1}) matches well with the experimentally observed values of 4.67 and 6 g L^{-1}, respectively, mentioned in previous section. For both shake flask and bioreactor scale experiments, the specific growth rate of *clostridial* cultures has been quite low ($\mu \approx 0.03\ h^{-1}$), which indicates that optimized RSH is a suitable fermentation medium, where the cell utilizes most of its energy for product formation rather than cellular growth.

Table 6.6: Models applied for the estimation of biokinetic constants and kinetic parameters estimated by fitting the models to experimental data obtained for Shake flask and reactor scale study.

Component	Rate equations	Flask scale process*			Reactor scale process*		
		Kinetic Parameter	Regression Coefficient (R^2)	RMSE (Root Mean Square Error)	Kinetic Parameter	Regression Coefficient (R^2)	RMSE
Butanol	$P = \frac{P_0 P_{max} e^{p_t t}}{P_{max} - P_0 + P_0 e^{p_t t}}$	P_0 = 0.4692 P_{max} = 4.752 P_t = 0.0282	0.9616	0.469	P_0= 0.5286 P_{max} = 5.03 P_t = 0.0289	0.9005	0.749
Biomass	$X = \frac{X_0 X_{max} e^{\mu t}}{X_{max} - X_0 + X_0 e^{\mu t}}$	X_0= 0.06284 X_{max}= 2.01 μ = 0.03278	0.9977	0.040	X_0=0.07351 X_{max}=3.996 μ = 0.0351	0.9733	0.687
Total Sugar	$S_{M0} - S_M = \frac{1}{Y_{P/M}}(P_0 - P_{max}) + \frac{1}{Y_{X/M}}(X - X_0)$	S_0 = 31.08 $Y_{P/M}$ =0.5234 $Y_{X/M}$ =0.1676	0.9915	1.212	S_0 =32.43 $Y_{P/M}$=0.59 $Y_{X/M}$=0.2041	0.9588	3.127

* under optimized conditions

(a) (b)

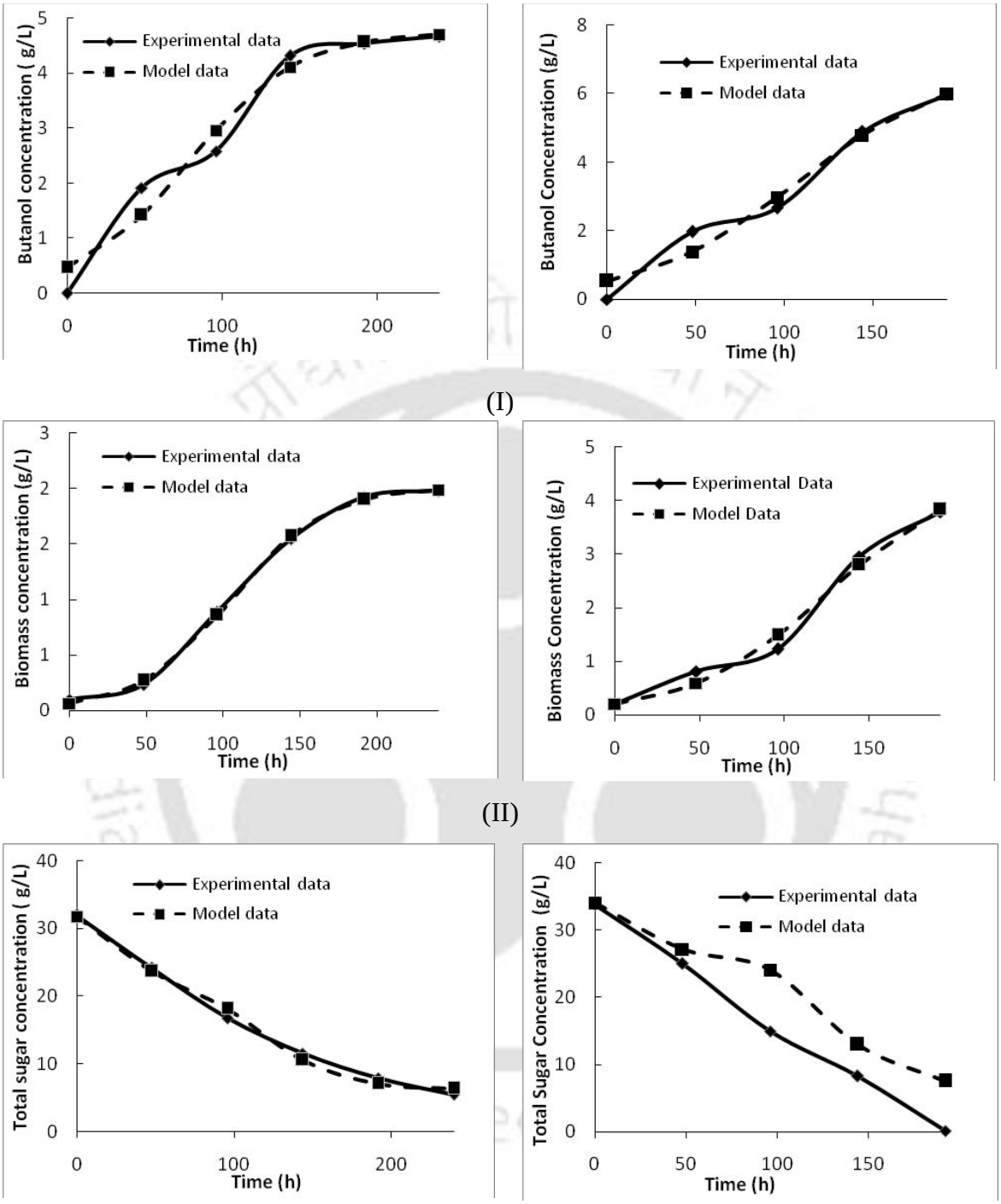

Figure 6.5: Verification of experimental data through comparative study with model data. (a) Flask scale optimized process; (b) Reactor scale optimized process. [(I) Comparative study of experimental and Model data for *Clostridial* butanol production during ABE fermentation; (II) Comparative study of experimental and Model data for *Clostridial* biomass growth during ABE fermentation; (III)Comparative study of experimental and Model data for total sugar utilization in terms of total solvents and biomass].

From the kinetic parameter values presented in the Table 6.6, an interesting observation could be made that the values of P_t and μ are practically same for both shake flask and bioreactor experiments. These parameters are characteristics of the kinetics of the fermentation process. Practically similar values of μ and P_t for shake flask and bioreactor scale experiments using optimized fermentation medium indicate that the effect of scale of operation on the fermentation kinetics and product profile becomes insignificant when fermentation medium is under optimized conditions.

6.4 Conclusion

The results of this study reveal that nutritionally optimized RSH medium (with added yeast extract and PABA) could be a potential alternative feedstock for biobutanol production. Furthermore, our results also reveal that production of ABE solvents through fermentation of pretreated RSH by *C. acetobutylicum* MTCC 481 is a strong function of composition of the media. The optimization of fermentation medium in terms of supplemental nutritional factors by Taguchi design has identified key factors responsible for enhanced production of solvents with emphasis on butanol, which is a potential alternate liquid transportation fuel. The results of this study could give vital inputs for design of an economic and efficient RSH based fermentation process.

Abbreviations and Notations

ABE	Acetone Butanol Ethanol
ANOVA	Analysis of Variance
CMM	Cooked Meat Medium
DDGS	Distillers Dried Grains with Solubles
DNS	Dinitrosalicylic Acid
DOE	Design of Experiments
μ	Specific growth rate (h^{-1})
MS	Mean of squares
MTCC	Microbial Type Culture Collection
NAD	Nicotinamide Adenine Dinucleotide
NCIM	National Collection of Industrial Micro-organisms
P	Product Concentration (g dm^{-3})
P_0	Initial product concentration (g dm^{-3})
P_{max}	Maximum product concentration (g dm^{-3})
P_t	Kinetic constant
PABA	p-aminobenzoic acid
RCA	Reinforced Clostridial Agar
RCM	Reinforced Clostridial Medium
RMSD	Root Mean Square Deviation
RMSE	Root Mean Square Error

RS	Rice Straw
RSH	Rice Straw Hydrolysate
SS	Sum of squares
X	Biomass concentration (g dm^{-3})
X_m	Maximum biomass concentration (g dm^{-3})
X_0	Biomass concentration (g dm^{-3})
$Y_{P/S}$	Product yield on the utilized substrate
$Y_{X/S}$	Biomass yield on the utilized substrate

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CHAPTER 7

PROCESS OPTIMIZATION FOR BUTANOL PRODUCTION FROM DEVELOPED RICE STRAW HYDROLYSATE

7.1. Introduction

In the previous two chapters, we presented results of our study on pre–treatment of rice straw and development of the rice straw based fermentation medium. In this chapter, we take ahead the theme of process development for rice straw based butanol synthesis through ABE fermentation with optimization of the process parameters for maximum butanol production. The metabolic pathway of the micro–organism (which essentially comprises of a series of enzyme–catalyzed reactions, determining the yield and selectivity of a fermentation process) is a major function of physical (temperature, pH, agitation, humidity) and physiological (inoculum size and age) parameters.

Optimization of the fermentation process can be conducted either by changing one factor at a time, or by varying several factors at the same time, and looking for interactions using statistical analysis. The statistical design of experiments is an organized approach that

gives more reliable information per experiment than unplanned approaches. Statistical data analysis permits the identification of the experimental parameters and also visualization of the interactions between them, which could influence the productivity or the quality of the product [1].

This present study is aimed at optimizing the rice straw based ABE fermentation process in terms the physical and physiological parameters using a statistical approach. The optimization is carried out in two phases, viz. the first phase comprising of preliminary experiments to assess the relative influence of different parameters, and the second phase of Taguchi design of experiments. The results of first phase were used to decide the experimental factors and their levels in the Taguchi experimental design. The solvent production under the optimized fermentation process has been tested, initially on shake flask scale, followed by a 2 and 5 L stirred tank bioreactor operated in batch mode. The kinetics of the fermentation process under optimized condition has been analyzed using an unstructured model. After complete process optimization, we have also attempted to assess the role played by precipitate produced in the fermentation medium. This chapter is presented in two sections, viz. Section A describing the preliminary set of experiments, while Section B gives the accounts of actual statistical experiments based on Taguchi DOE.

7.2. Materials and Methods

As stated earlier, the optimization study of the ABE fermentation process has been carried out in two steps. However, some general experimental and analytical techniques were common for both sections, which have been described below.

7.2.1 Micro–organism, Culture Revival and Maintenance

The micro–organism and the procedure followed for the culture revival and maintenance was

same as that during the study of optimization of pre–treatment described in Chapter 5.

7.2.2 Preparation of Rice Straw Hydrolysate

The initial procedure for preparation of rice straw hydrolysate was same as described in Chapter 6. This hydrolyzate was further supplemented with two nutritional factors, viz. yeast extract (3.0 g L^{-1}) and PABA (2 mg L^{-1}). Initially, pH of broth was set at 3.0, 5.0 and 7.0 using NaOH pellets, in accordance to Taguchi experimental design and the broth was sterilized at 15 lb pressure for 15 min. After autoclaving, broth was allowed to cool and the final pH was observed as 4.0, 6.0 and 8.0 ± 0.5, respectively. All chemicals were of analytical grade and were procured from either Merck or Himedia and used without further treatment/purification.

7.2.3 General Conditions of Fermentation

As stated earlier, the optimization of fermentation process was carried out in two steps, viz. the preliminary experiments and the statistical experiments based on Taguchi Design of Experiments (DOE). We describe in this section general conditions of fermentation employed in these experiments: *C. acetobutylicum* MTCC 481 strain was cultured in pre–optimized RSH based medium. The optimization studies were carried out in custom fabricated 250 mL anaerobic Erlenmeyer flasks with bottom port for sample withdrawal and nitrogen sparging in order to minimize oxygen contamination, and strict anaerobic conditions were maintained. Each flask contained 100 ml of optimized RSH based fermentation medium, which was inoculated with *C. acetobutylicum* MTCC 481.

7.2.4 Analysis

The optical density of the cells in the broth was measured using UV–Vis spectrophotometer (Thermo Fischer) with absorbance at 600 nm after appropriate dilution in water. Glucose was analysed using Glucose (GO) assay kit procured from Sigma Aldrich, USA (GAGO20–1KT). Quantification of total sugar was done by Anthrone test as directed by Hedge and Hofreiter [2], and reducing sugar by dinitrosalicylic acid (DNS) method proposed by Miller [3]. Samples were filtered with 0.2 µm filter and diluted appropriately for the qualitative and quantitative determination of the solvents and sugars.

Solvent production in the fermentation broth was determined using a gas chromatograph (Varian) using a CP Wax 52CB (250 mm × 0.25 mm × 0.39 mm) capillary column and a Flame Ionization Detector. The method was developed by keeping injector and detector temperatures at 230 and 250^{o}C, respectively. Column temperature was programmed at 45–100^{o}C with an increment of 3^{o}C per min. between the temperature intervals of 100–200 ^{0}C, increments was 5^{o}C per min; split ratio was set at 70 and carrier gas (nitrogen) flow rate was maintained at 2 ml/min.

SECTION A: PRELIMINARY EXPERIMENTS FOR SCREENING AND SELECTION OF EXPERIMENTAL PARAMETERS AND THEIR LEVELS

7.3 Materials and Methods

Initial screening of levels of each parameter was done using conventional methodology of studying effect of one variable at a time. Besides the parameter considered, all the other physical and cultural conditions were kept constant for that set of preliminary experiment. All flasks were sparged with nitrogen at the start, and after every 24 h of fermentation to maintain anaerobic conditions. The samples of fermentation broth were withdrawn at constant intervals up to a period of 10 days. Each experiment was conducted in duplicates to assess the reproducibility of the results.

7.3.1 Effect of temperature

The experiments were carried out in four custom fabricated 250 mL anaerobic Erlenmeyer flasks. Each flask contained 100 mL of optimized RSH based fermentation medium, which was inoculated with 2% v/v of 18 h old *C. acetobutylicum* MTCC 481 culture at an initial pH of 1.0 ± 0.2. Four flasks were incubated in an incubator shaker (Make: Scigenics Biotech, Model: Orbitek) at 100 rpm under 4 temperatures, viz. 30, 37, 42 and 45^{o}C.

7.3.2 Effect of agitation

These experiments were also performed in 4 sets with working volume of 100 ml hydrolyzate in each flask. Fermentation was carried out in the shaker incubator at different agitation rates of 100, 150, 200, 250 rpm, while all the other physical and cultural conditions were maintained constant (pH: 1.0 ± 0.2, Temperature: 37^{o}C, Inoculum size: 2% v/v, Inoculum age: 2%).

7.3.3 Effect of pH

This study was conducted in set of 5 flasks, each flask containing 100 mL of optimized RSH based fermentation medium, which was further inoculated with 2% v/v of 18 h old *C. acetobutylicum* MTCC 481 culture. 4 flasks were incubated in an incubator shaker (Make: Scigenics Biotech, Model: Orbitek) at 100 rpm under five different pH conditions of 1.0, 4.0, 6.0, 8.0 and 10.0 ± 0.5, respectively using 1 N H_2SO_4 and NaOH pellets. pH of fermentation broth and clostridial growth was monitored at definite intervals of fermentation cycle to detect the switch over of acidogenesis phase to solventogenesis.

7.3.4 Effect of inoculum size

The preliminary experiments for study of effect of inoculum size were performed in set of 4 flasks with a working volume of 100 ml of developed rice straw hydrolyzate. These flasks were inoculated with different inoculums size of 2, 5, 10 and 15% v/v. All the other physical and cultural parameters were kept constant for all the set of flasks (pH: 1.0 ± 0.2, temperature: 37^{o}C, agitation rate: 100 rpm, inoculum age: 18 h).

7.3.5 Effect of inoculum age

The preliminary experiments for assessing effect of inoculum age were performed in set of 4 flasks with a working volume of 100 ml of developed rice straw hydrolyzate. These flasks were inoculated with inoculums of different age viz., 18, 24, 36, 48 h. Other physical and cultural parameters were: pH = 1.0 ± 0.2, Temperature = 37^{o}C, Agitation rate = 100 rpm, Inoculum size = 2%.

7.4 Results and Discussion

7.4.1 Study of Effect on Temperature on Solvent Production

Bacteria of the genus *Clostridium* are gram–positive spore–forming bacillary anaerobes, very diverse in both their physiology and genetics. They are mesophiles, which

Table 7.1 Clostridial fermentation with different substrates at varied temperature ranges

Micro–organism	Temperature (^oC)	Substrate	Butanol (g L^{-1})	Reference
C. acetobutylicum MTCC 481	37	RSH	5.11	This Paper
C. acetobutylicum CIC 8008	35	Corn Straw Hydrolyzate	6.5	[4]
C. saccharoperbutyl–acetonicum N1–4(ATCC 13564)	30	Glucose	8.69	[5]
C. acetobutylicum DSM 792	37	Lactose	1.0	[6]
C. acetobutylicum DSM 792	37	Cheese whey	1.5	[6]
C. beijerinckii NRRL B592	34	Glucose	9.1	[7]
C. saccharoperbutyl–acetonicum DSM 2152	31.5	Molasses	4.5	[8]
C. acetobutylicum ATCC 4259	30	Whey	2.2	[9]
C. acetobutylicum ATCC 4259	37	Whey	7.8	[9]

grow in a diverse range of temperature between 30 to 60^{o}C [10]. For example, the optimal growth temperature for clostridia varies from 34–37°C for *C. beijerinckii* to 75–78°C for *C. thermohydrosulfuricum* [11]. Thus, depending upon the species, optimal conditions for growth and metabolism of clostridial strain vary. Table 7.1 depicts various clostridial cultures producing butanol at varied temperature range. Present work is an attempt to study the optimal temperature conditions for the *C. acetobutylicum* MTCC 481 strain for enhanced butanol production using a pre–optimized RSH based medium. Optimized RSH based medium has been observed to release 38.68 g L^{-1} of total sugar, 32.95 g L^{-1} of reducing sugar and 27 g L^{-1} of glucose after 24 h of pre–treatment process. Time history of release of these sugars is depicted in Fig. 7.1.

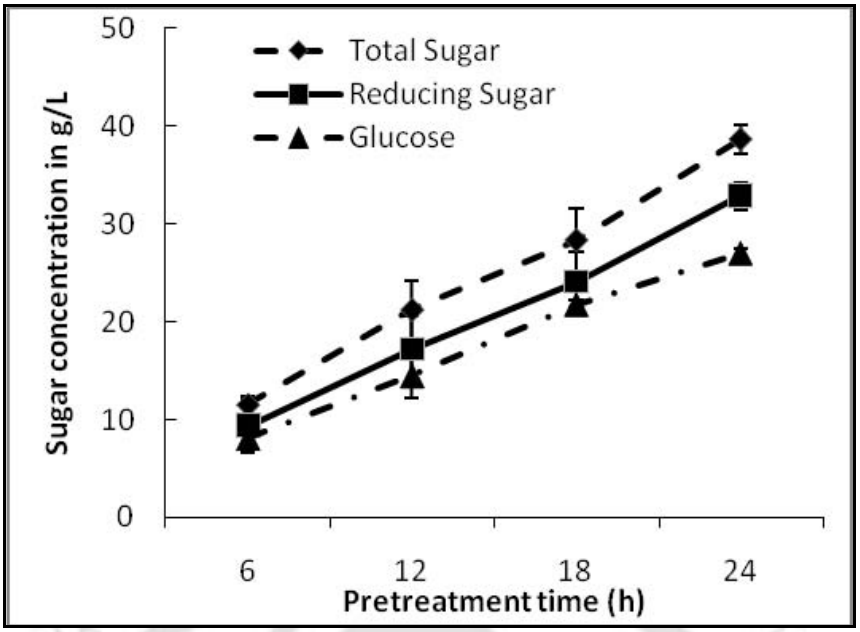


Figure 7.1: Time history of sugar release during pretreatment

Fermentation of optimized RSH at varied temperature of 30, 37, 42, and 45°C was performed keeping all the other physical (fermentation time, temperature, pH and agitation) and cultural conditions (inoculum size and age) constant. Time history of clostridial fermentation performed at 30, 37, 42 and 45°C is shown in Figs. 7.2–7.5. At 30°C, *C. acetobutylicum* shows reduced solvent production of 2.74 g L^{-1} with only 0.44 g L^{-1} of butanol utilizing 60.5% of total sugar, 63.3% of reducing sugar and 61.23% of glucose. Major solvent contribution was observed from acetone (2.2 g L^{-1}), resulting in poor butanol selectivity of nearly 0.13. Overall solvent and butanol yield at 30°C was very small, viz. 0.07, and 0.01, respectively. When the same experiment was performed at elevated temperature of 37°C, the results were very encouraging. It resulted in enhanced production of solvents with 8.35 g L^{-1} of total solvent and 5.11 g L^{-1} of butanol utilizing 72.26% of total sugar, 70.5 g L^{-1} of reducing sugar and 70% of glucose. Unlike 30°C, at 37°C butanol contributed maximum to the total solvent concentration, this resulted in high butanol selectivity of 0.53 and high butanol yield of 0.13.

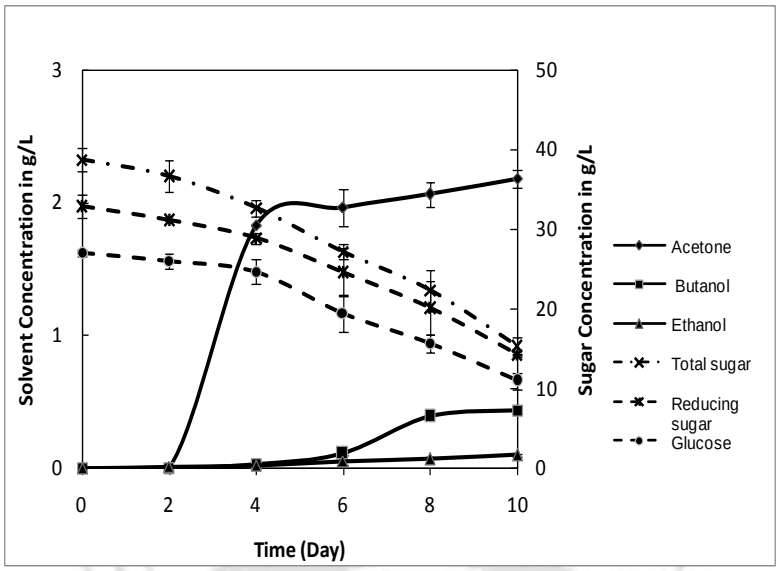


Figure 7.2: Sugar utilization and solvent production during fermentation at 30°C

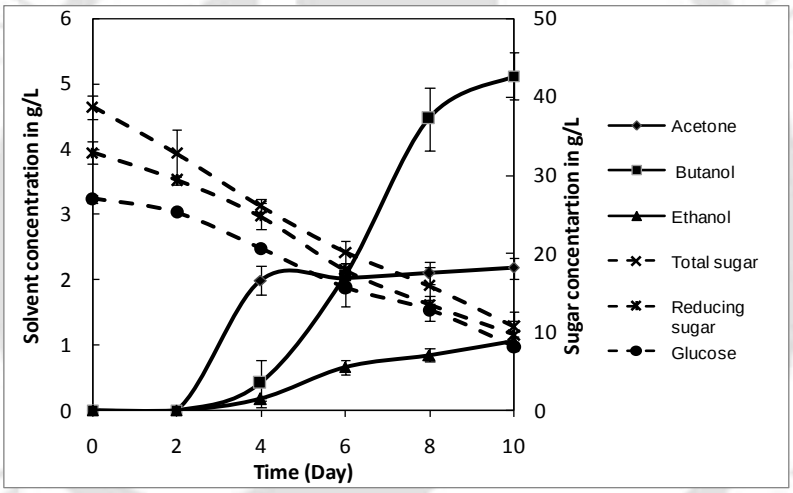


Figure 7.3: Sugar utilization and solventproduction during fermentation at 37°C

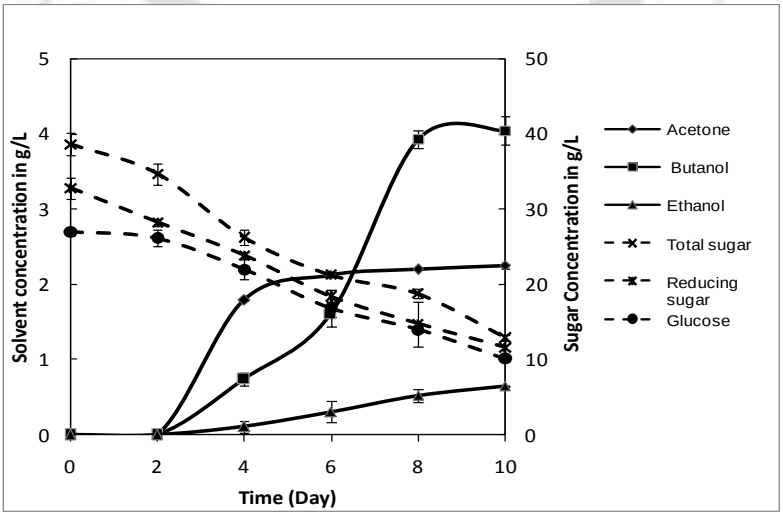


Figure 7.4: Sugar utilization and solventproduction during fermentation at 42°C

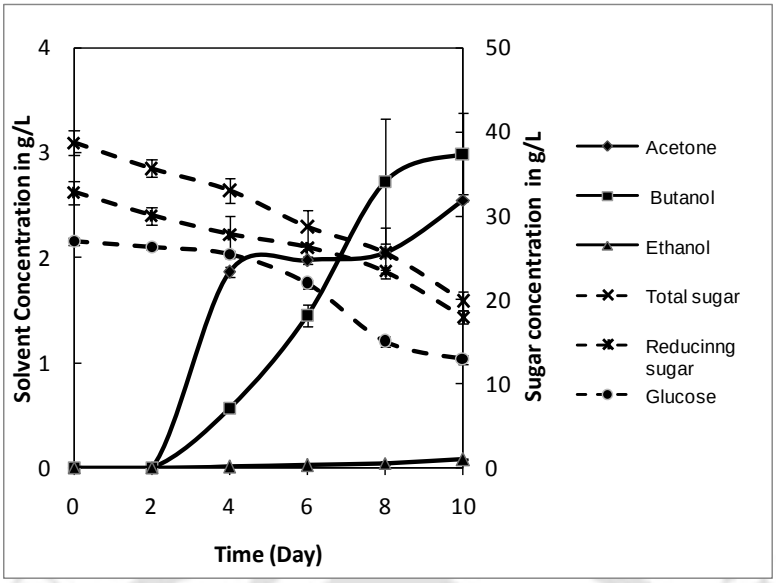


Figure 7.5: Sugar utilization and solventproduction during fermentation at 45°C

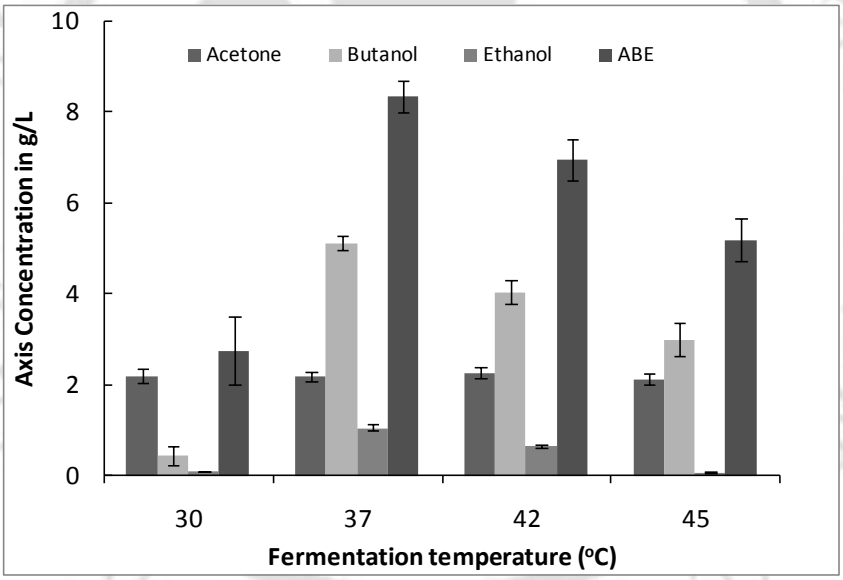


Figure 7.6: Comparative assesment of solvent production at varied temperature.

Table 7.2. Summary of solvent yield and selectivity at varied temperature

Fermentation Temperature	Yield		Butanol selectivity	*k* (g/L-day)	R^2	RMSE
	Butanol	Total solvent (ABE)				
30°C	0.01 ± 0.003	0.07 ± 0.005	0.13	0.064	0.85	2.5
37°C	0.13 ± 0.05	0.22 ± 0.07	0.53	0.092	0.93	1.9
42°C	0.1 ± 0.01	0.18 ± 0.06	0.50	0.078	0.91	2.0
45°C	0.07 ± 0.002	0.13 ± 0.06	0.47	0.055	0.80	2.7

Similar experiments further at 42 and 45°C resulted in reduced butanol production, viz. 4.0 g L^{-1} and 2.9 g L^{-1}, respectively. Total solvent concentration observed at 42 and 45°C was 6.95 and 5.19 g L^{-1}, respectively with a nearly similar butanol selectivity of 0.5 and 0.47 respectively. Butanol and total solvent yield at 42°C was slightly higher than 45°C. Fig. 7.6 illustrates the comparative assessment of solvent production at varied temperature, while Table 7.2 summarizes the total solvent and butanol yield achieved by performing fermentation at varied temperature.

Overall, it was observed that for the production of butanol using *C. acetobutylicum* MTCC 481 among the studied temperatures, 37°C was most suitable; in terms of elevated butanol yield and selectivity, with maximum utilization of sugar. These results are very much in concurrence with the work performed by Berezina et al. [11]. They worked with different strains of *C. acetobutylicum* and examined their solvent and butanol production at 30 and 37°C. They observed that *C. acetobutylicum* VKPM B–4786, *C. acetobutylicum* 6, and *C. acetobutylicum* 7 grown at 30°C in 6% flour medium gave the highest acetone yield (6.9, 6.1, and 7.0 g/L, respectively), while strain *C. acetobutylicum* ATCC 824 produced only 0.3 g/L acetone under the same conditions. While at 37°C, *C. acetobutylicum* VKPM B–4786, *C. acetobutylicum* 6, and *C. acetobutylicum* 7 synthesized 7.2, 8.3, and 6.9 g/L of butanol, respectively. Thus, it was observed that for each clostridial strain, a definite optimal temperature is required, which contributes to enhanced production of target product.

Temperature plays a critical role in solvent production as it regulates the activity of enzymes participating actively in cellular metabolism [12–14]. For *C. acetobutylicum* MTCC 481, 37°C temperature was observed to be the most ambient which supports the activity of enzyme involved in acidogenesis and solventogenesis. Temperature lower and higher than 37°C resulted in reduced solvent yield. In addition to this, according to Berezina et al. [11], it is advantageous from the economic stand–point to conduct fermentation at 37°C, as this

allows the energy consumption connected with product separation to be decreased.

7.4.1.1 Kinetic Analysis of Effect of Temperature

Both kinetics and thermodynamics of equilibrium microbial reactions (which are essentially a set of enzymatic reactions) are affected by temperature [15]. Fermentation reactors can be operated at psychrophilic (5 – 25°C), mesophilic (25 – 40°C), thermophilic (40 – 65°C), extreme thermophilic (65 – 80°C) or hyperthermophilic (> 80°C) temperatures [16]. Enzymes work optimally at a specific temperature. Increases in temperature will double the enzymatic activity until the optimal temperature is reached, above which the enzymatic activity may rapidly decrease [16]. Kinetic and thermodynamic data provides key insight into the actual mechanism by which temperature exerts its effect on bioconversion of substrate (glucose) to solvents. The substrate consumption data for 27.01 g/L initial substrate concentration was used for calculating rate constants at four fermentation temperatures and activation energy of the fermentation process was calculated from it. The glucose utilization data fitted well in first order kinetic equation with values of R^2 ranging from 0.85 to 0.95. The values of first order rate constant (k) at 30, 37, 42 and 45^{o}C temperatures were calculated to be 0.064, 0.093, 0.078 and 0.056 (g L^{-1} day^{-1}) respectively (Fig. 7.7). The k values clearly depict that for 27.01 g/L initial glucose concentration, the rate of glucose bioconversion is highest at 37^{o}C and the lowest at 45^{o}C. The value of rate constant follows a parabolic path. The value of rate constant increases from 30 to 37^{o}C to reach a peak, and decreases thereafter, in a parabolic profile. Fig. 7.8 depicts the comparative assessment of experimental data and model data during fermentation at varied temperature. The rate constant being the highest at 37^{o}C shows that the maximum probability of conversion of reactants/substrate to product occurs at the optimum temperature of 37^{o}C, and is less for temperatures lower (30^{o}C) and higher than 37^{o}C (i.e. 42 and 45^{o}C).

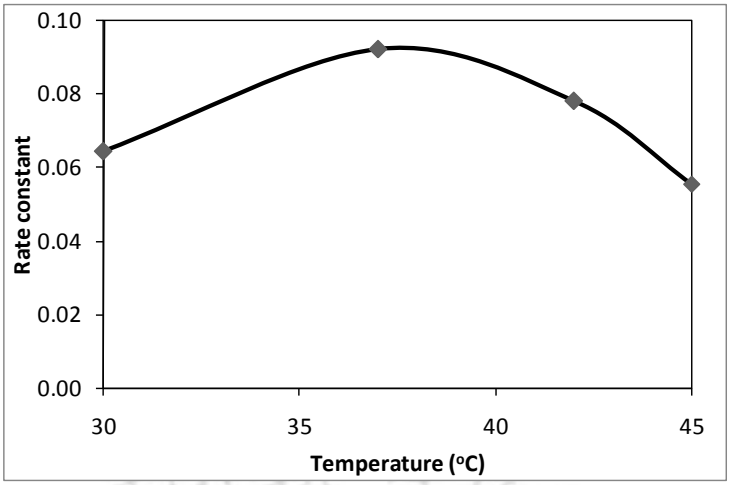


Figure 7.7: Kinetic study of butanol fermentation at varied temperature

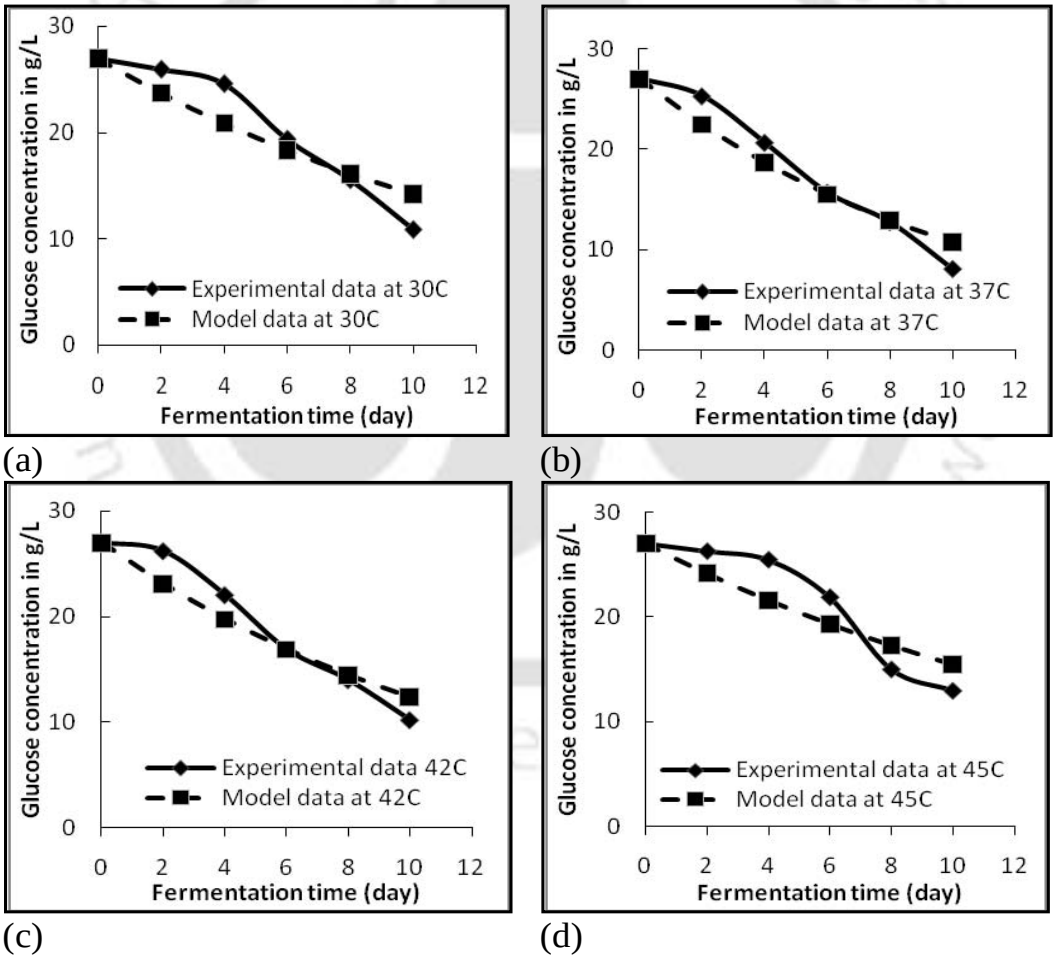


Figure 7.8: Comparative assessment of experimental data with model data for glucose utilization during ABE fermentation at varied temperature. (a) Temperature = 30°C, (b) Temperature = 37°C, (c) Temperature = 42°C, (d) Temperature = 45°C.

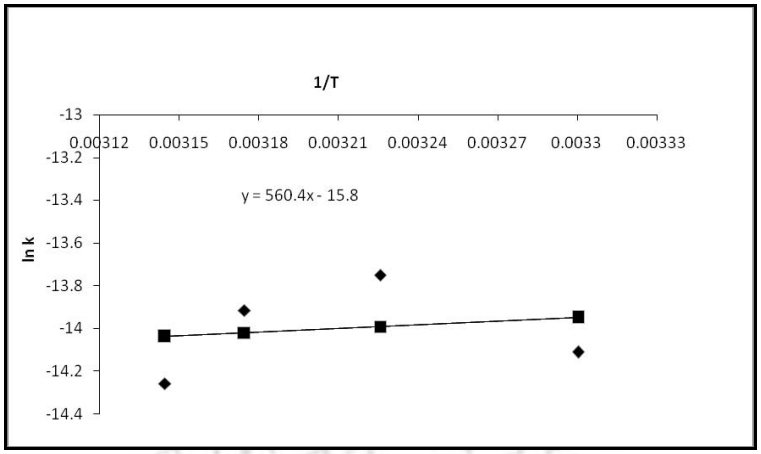


Figure 7.9: ln *k* vs 1/*T* (K^{-1}) curve for calculating activation energy (E_a) of glucose bioconversion to solvents

Thus, a higher *k* value at 37°C gave a higher total product yield. This is because of higher enzyme activity at 37°C, which enables greater substrate utilization and consequently, higher solvent production. Activation energy (E_a) for bioconversion of glucose (substrate) was found to be 19.475 kcal/mol using Arrhenius equation (Fig. 7.9). Bajpai and Margaritis [17] have studied the effect of temperature on ethanol production and have observed similar results. Ethanol yields were relatively constant over the temperature range 25–35°C, and but dropped sharply beyond 35°C. Other kinetic parameters, specific growth rate, specific ethanol production rate, and specific total sugar uptake rate were maximum at 35°C. This observation suggests that at an optimum temperature (37°C for present study), the increase in metabolic activity due to an increase in temperature is outweighed by the denaturation of the enzyme system within the cell. At temperatures above 37°C, the effect of denaturation will become more and more pronounced over the increase in metabolic activities. Figures 7.2–7.5 shows the effect of temperature on total sugar utilization. The rate of total sugar utilization increased with temperatures up to 37°C, but was found to decrease as the temperature increased beyond 37°C. The residual total sugar concentration were only between 8 and 10 g/L over the

temperature range 30–37°C**.** Above a temperature of 37^{o}C**,** the residual total sugar concentration increased sharply. King and Hossain [18] and Lee et al. [19] have also reported similar trend of results. In the typical Arrhenius plot of changes in rate processes with temperature, rates hook downward at either end of the 'optimal' temperature range for the enzyme or organism studied [20]. Study of La Para et al. [21] at genetic scale indicated that elevated temperature failed to improve substrate utilization rates, demonstrating reduced metabolic potential.

7.4.2 Effect of agitation on solvent production

Agitation of the fermentation broth is known to have a vital effect on the kinetics and yield of fermentation process. Agitation essentially determines the level of convection in the system and also determines the heat and mass transport characteristics of the system. Therefore, optimization of agitation provided in the fermentation broth is one of the important parameters to be considered, while optimization of the process. In aerobic fermentations, agitation increases the interfacial area between the gas and liquid, and also improves the aeration and dissolved oxygen content of the medium. However, in anaerobic fermentations, the fermentation broth has to be absolutely oxygen–free, and hence, the role of agitation is mainly for maintaining the uniform and homogeneous solid liquid suspension, and to ensure good transport of nutrients and metabolites across cell membrane [22]. In addition to assistance in enhancing mass transfer between the different phases present in the culture, agitation also helps maintain homogeneous chemical and physical conditions in the culture. Agitation creates shear forces, which affect micro–organisms in several ways, causing morphological changes, variation in their growth and product formation and also damaging the cell structure [23]. Very slow agitation rates may lead to the improper mixing of nutrient and microbial biomass in the fermentation broth resulting in settling of cells at the

base of fermentation vessel. This results in poor product yield. However, vigorous agitation can generate intense shear forces which could damage the cell structure, which could finally lead to cell lysis and poor product formation [22]. The effects of agitation on the growth, production of solvents and sugar utilization by *Clostridium acetobutylicum* MTCC 481 has been explored in this work. Earlier studies have reported that the rate of agitation plays an important role in controlling the metabolism of *C. acetobutylicum*. This study is performed to determine the optimal range of agitation rate by the clostridial cells.

In our preliminary experiments of process optimization, we have studied the effect of degree of agitation on the production rates of the solvents by varying agitation speed in the range 100 to 250 rpm. Fermentation was carried out at varied agitation rates of 110, 150, 200 and 250 rpm, keeping all the other physical and cultural parameters constant (temperature: 37^{o}C, pH: 1.0, inoculum size 5%, inoculum age: 18 h). Time history of biomass growth, sugar utilization (total sugar, reducing sugar and glucose), and solvent production (acetone, butanol, and ethanol) during the fermentation cycle are presented in Figs. 7.10–7.13 for different agitation rates employed. Agitation of fermentation broth at 100 rpm resulted in production of 5.5 g L^{-1} of total solvents with only 2.3 g L^{-1} of butanol. The residual total sugar remaining unutilized in fermentation broth at the end fermentation cycle was observed to be 14.5 g L^{-1}, resulting in total solvent yield of 0.27 and butanol yield of 0.11, whereas butanol selectivity was observed to 0.34. Agitating fermentation broth at 150 rpm, resulted in comparatively increased solvent yield of 0.35 with an elevated butanol selectivity of 0.5. During this process nearly 88% of glucose, 80% of reducing and total sugars were utilized. An interesting result of experiments at different agitation rates is that the solvent yield showed only marginal to negligible variation with agitation rates.

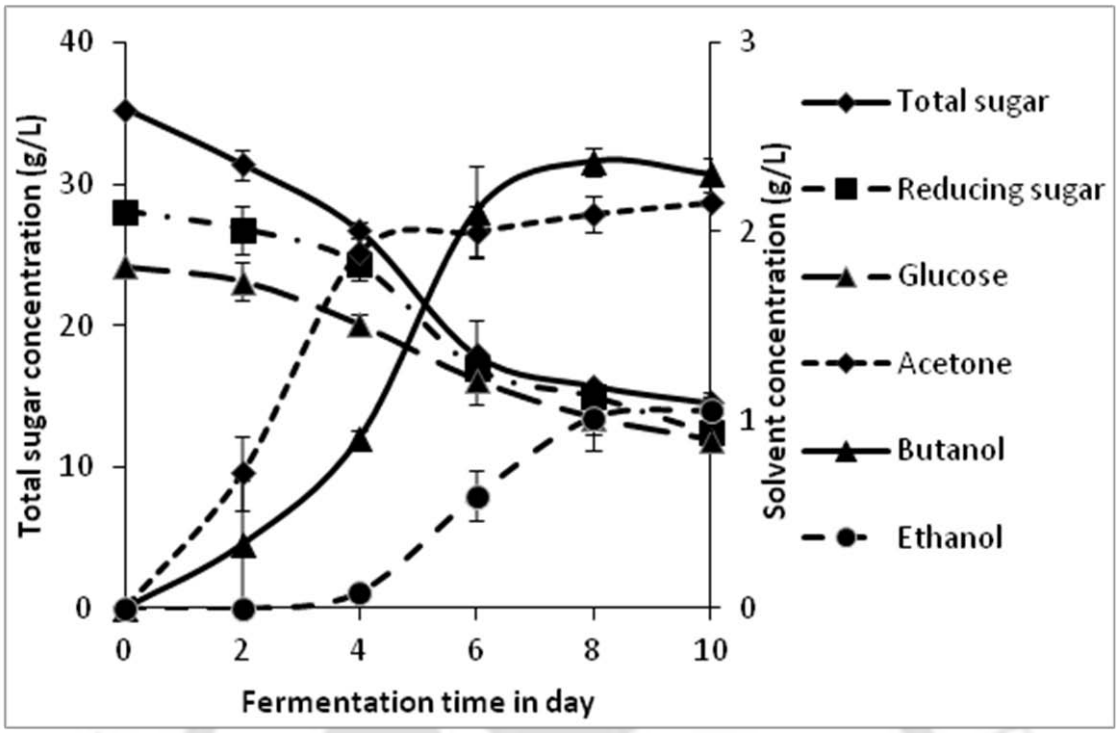


Figure 7.10: Characteristic trends of fermentation at 100 rpm
(Time history of formation of products, viz. acetone, butanol and ethanol, with time history of consumption of substrates, viz. total sugar, reducing sugar and glucose).

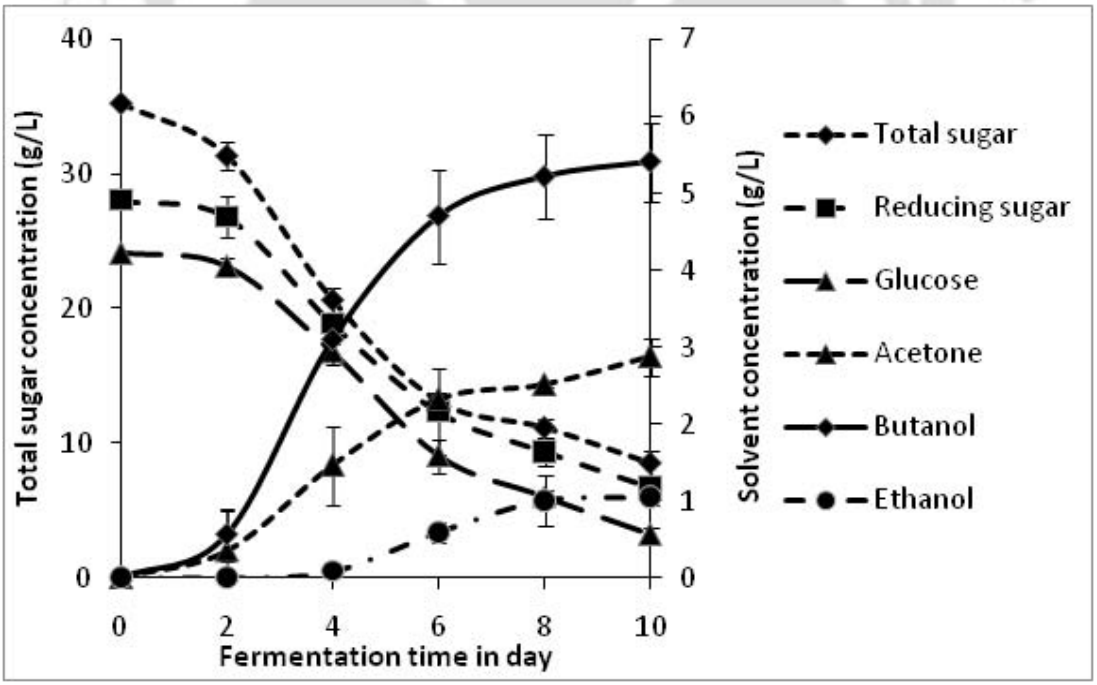


Figure 7.11: Characteristic trends of fermentation at 150 rpm
(Time history of formation of products, viz. acetone, butanol and ethanol, with time history of consumption of substrates, viz. total sugar, reducing sugar and glucose).

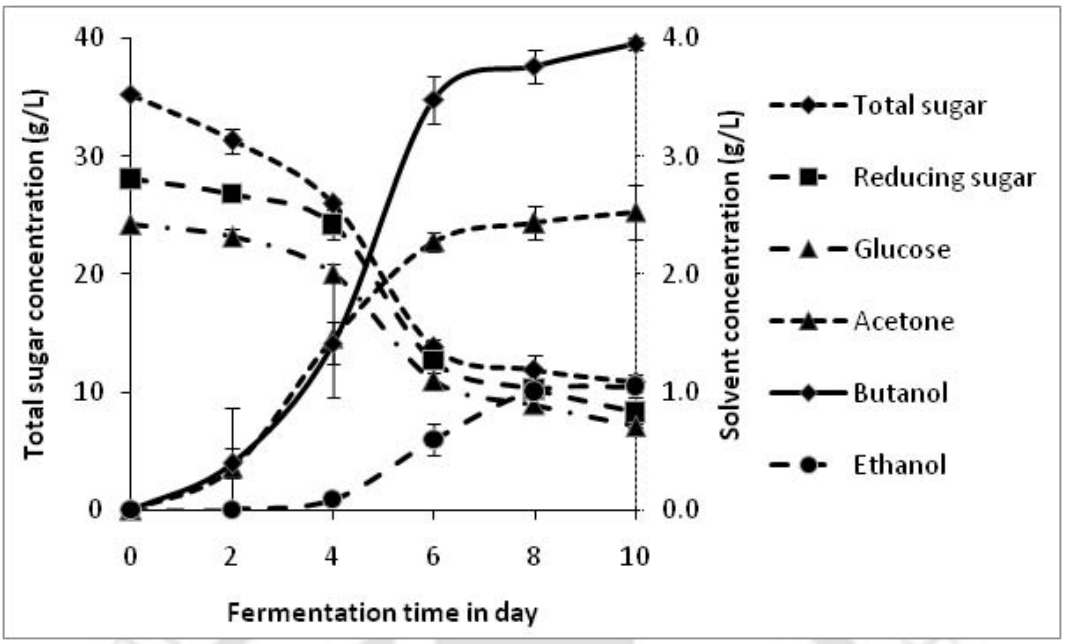


Figure 7.12: Characteristic trends of fermentation at 200 rpm
(Time history of formation of products, viz. acetone, butanol and ethanol, with time history of consumption of substrates, viz. total sugar, reducing sugar and glucose).

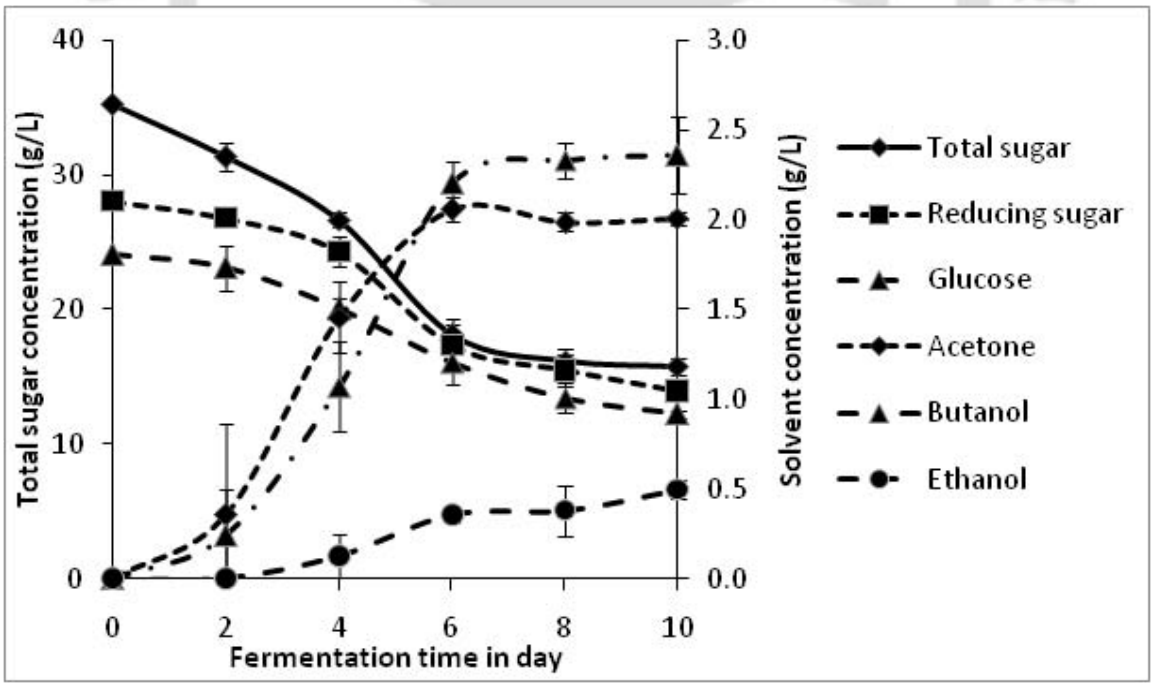


Figure 7.13: Characteristic trends of fermentation at 250 rpm
(Time history of formation of products, viz. acetone, butanol and ethanol, with time history of consumption of substrates, viz. total sugar, reducing sugar and glucose).

Table 7.3: Effect of agitation on solvent yield and butanol selectivity during fermentation by *C. acetobutylicum*

Agitation (rpm)	Yield (g/g)		Butanol selectivity (mol/mol)
	Butanol	Total solvent (ABE)	
100	0.11±0.03	0.27±0.04	0.34
150	0.21±0.07	0.35±0.08	0.5
200	0.16±0.04	0.31±0.05	0.45
250	0.12±0.07	0.31±0.08	0.41

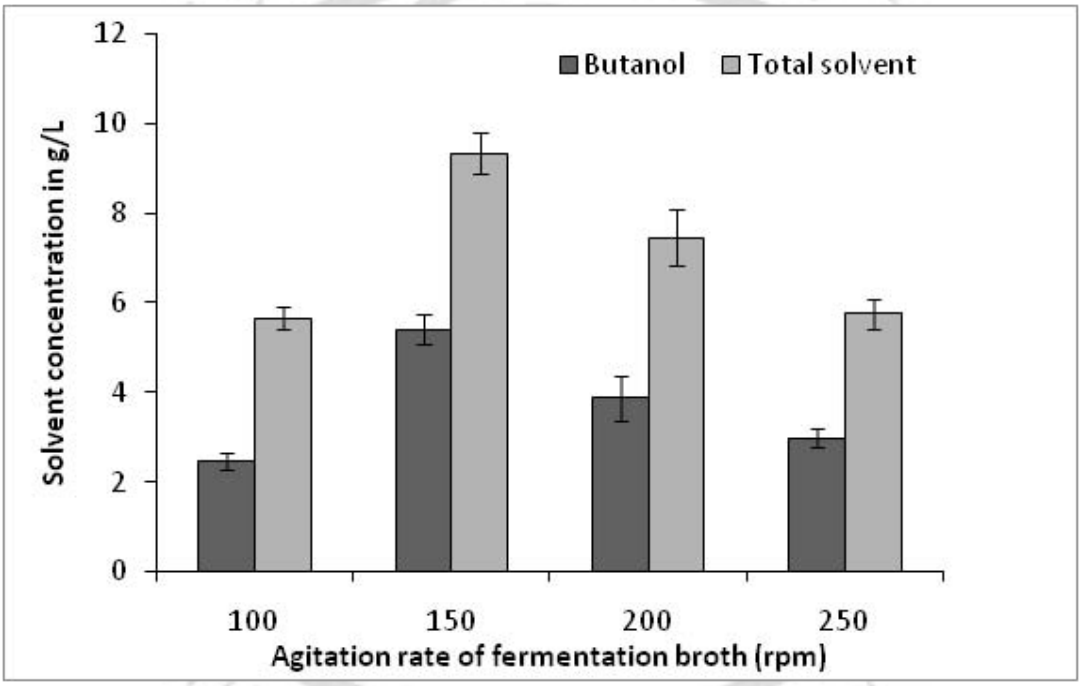


Figure 7.14: Comparative study of solvent production during clostridial fermentation performed at varied agitation rate.

More specifically, the total solvent yield (in g/g) was 0.27, 0.35, 0.31, 0.31 at agitation rates of 100, 150, 200 and 250 rpm, respectively. Although the total solvent yield was indifferent to the agitation rate in the fermentation broth, some variation in the yield and selectivity of individual solvents was observed under varying agitation rate (especially the yield and selectivity of desired product, butanol).

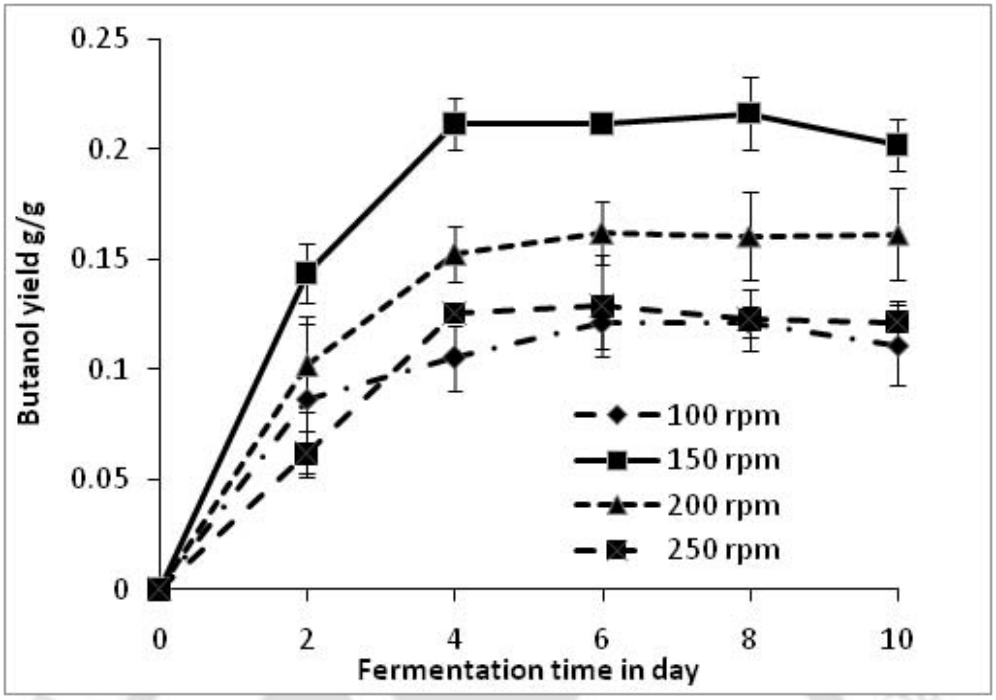


Figure 7.15: Time history of butanol yield in experiments conducted at different agitation rates

The highest yield and selectivity towards butanol was obtained for fermentation conducted at agitation rate of 150 and 200 rpm. A comparison of total solvent production (in g/L) along with butanol production (in g/L) is shown in Fig. 7.14, while the yield (in g/g) and selectivity for butanol is illustrated in Table 7.3. Fig. 7.15 depicts the time history of butanol yield achieved during 10 days of fermentation cycle. Ethanol production in experiments at all four values of agitation rpm was observed to be very less: in the range of 0.4 to 1.0 g L^{-1}. On the basis of these preliminary experiments, on a whole, it could be concluded that the agitation rate in the range of 150 to 250 rpm is optimal for the solvent production using *C acetobutylicum* MTCC 481 strain. Based on these experiments, we have selected 3 levels of agitation rate, viz. 150, 200 and 250 rpm, for further statistical optimization of complete fermentation process, as described in the subsequent sections.

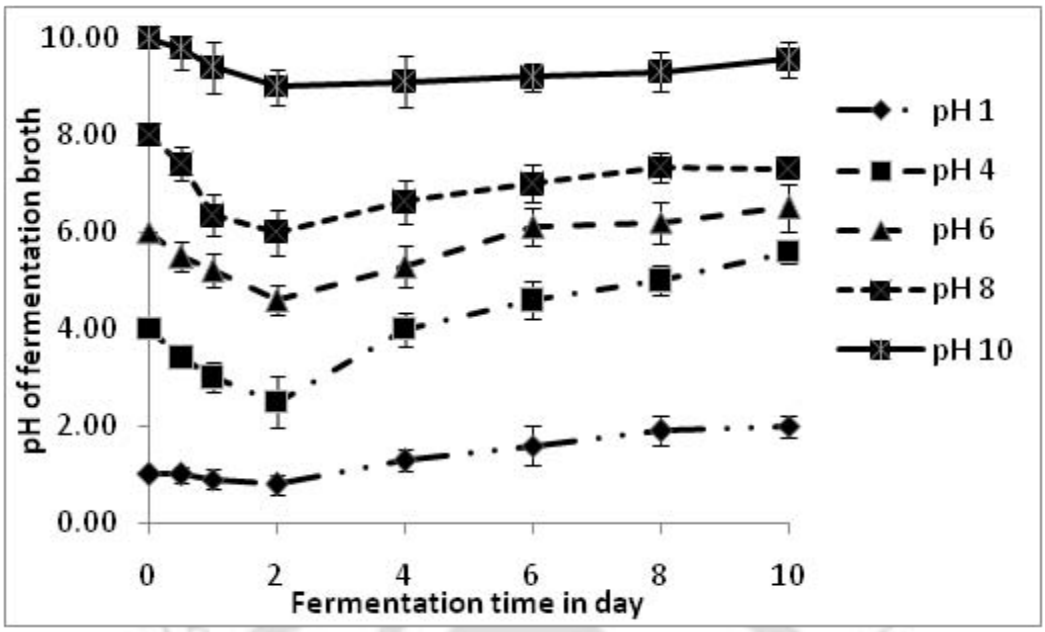


Figure 7.16: Time history of pH trend followed by *Clostridium acetobutylicum* at varied external pH of 1.0, 4.0, 6.0, 8.0 and 10.0 ± 0.5

7.4.3. Study of effect of pH on solvent production

The results obtained after fermentation of developed rice straw based medium at varied pH revealed that cells *C. acetobutylicum* were able to grow under the diverse range of external pH of fermentation broth. However, the ability of clostridial culture to produce solvents differed at varied pH. Highly acidic and alkaline pH of fermentation broth resulted in reduced biomass growth, poor utilization of sugar, and low production of solvents. Figs. 7.16 and 7.17 depict the time history and growth curve of pH observed for *C. acetobutylicum* MTCC 481 cultures for initial pH of 1.0, 4.0, 6.0, 8.0 and 10.0 ± 0.5 of the fermentation broth. *Clostridial* microbial culture efficiently utilizes the developed RS based medium, with a smaller lag phase of 10–12 h for the fermentation carried out at pH 4.0, 6.0 and 8.0. For fermentation carried out at extreme pH, a comparatively longer lag phase was observed (18–20 h). After lag phase, an exponential growth phase of 18–24 h was observed in experiments conducted at all 5 values of initial pH.

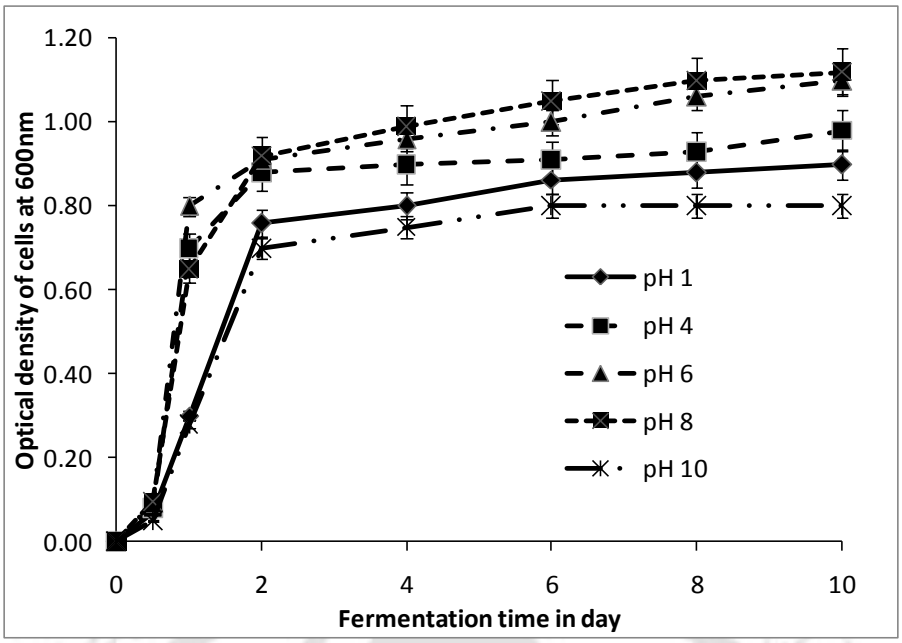


Figure 7.17: Time history of clostridial growth cycle observed at varied pH of 1.0, 4.0, 6.0, 8.0 and 10.0 ± 0.5

An extended stationary phase, which lasted for 8–10 days of fermentation cycle, was observed for all initial pH. Lag phase indicated the time taken by Clostridial culture to adapt its new environmental condition, while exponential phase denotes the stage during which clostridia rapidly divide and increase their cell density/counts. As stated earlier in Chapter 2 (section 2.2), during this period, clostridial cells enter acidogenesis phase and produce acids like, acetic acid, butyric acid etc., utilizing the carbon components present in the fermentation medium. Acids produced during acidogenesis get readily converted in to solvents like acetone, butanol and ethanol. This process is referred to as "solventogenesis". Acidogenesis results in decreasing the pH of fermentation broth and solventogenesis results in increasing of pH of fermentation broth. This trend of initial decrease and later increase of external pH is often referred as, "Butterfly shift". This switchover from acidogenesis to solventogenesis is a pH sensitive process, and the time history of this process at varied external pH is depicted in Fig. 7.16. It can be readily inferred from Fig. 7.17 that highly acidic and alkaline pH of

fermentation broth resulted in reduced growth and poor acid to solvent switchover.

Time history of solvent production at different initial pH of fermentation broth in a 10–day fermentation cycle is depicted in Figs. 7.18 to 7.22. The fermentation broth consisting of developed rice straw based medium contained 35.2 g L^{-1} of total sugar, 28.24 g L^{-1} of reducing sugar with a glucose content of 24 g L^{-1}. Clostridial fermentation of soluble sugar at an initial pH 1.0 ± 0.5 resulted in utilization of 44% of total sugar and nearly 50% of glucose, with the production of 2.53 g L^{-1} of acetone, 2.26 g L^{-1} of butanol and 1.05 g L^{-1} of ethanol, at the end of 10–day fermentation cycle. The overall solvent yield was 0.4 with a low butanol yield of 0.16 g/g and butanol selectivity of 0.31 mol/mol. The same fermentation experiment conducted at an initial pH of 4.0 ± 0.5 resulted in enhanced solvent production with 2.09 g L^{-1} of acetone, 4.82 g L^{-1} of butanol and 0.06 g L^{-1} of ethanol, utilizing ~ 69% of total sugar and 76.3% of glucose.

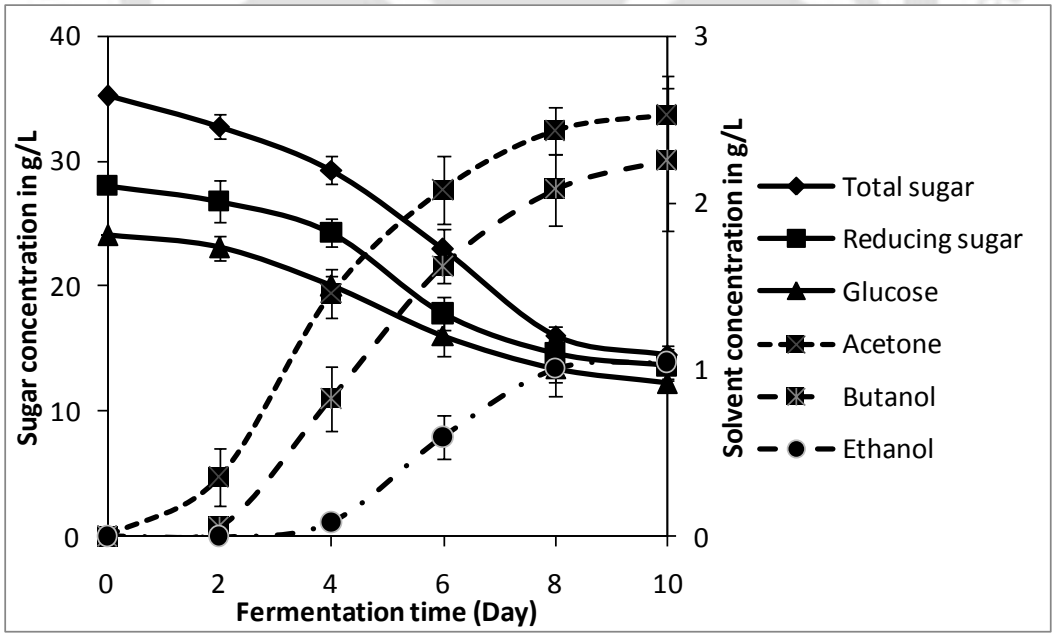


Figure 7.18: Time history of solvent production and sugar utilization at pH 1.0 ± 0.5

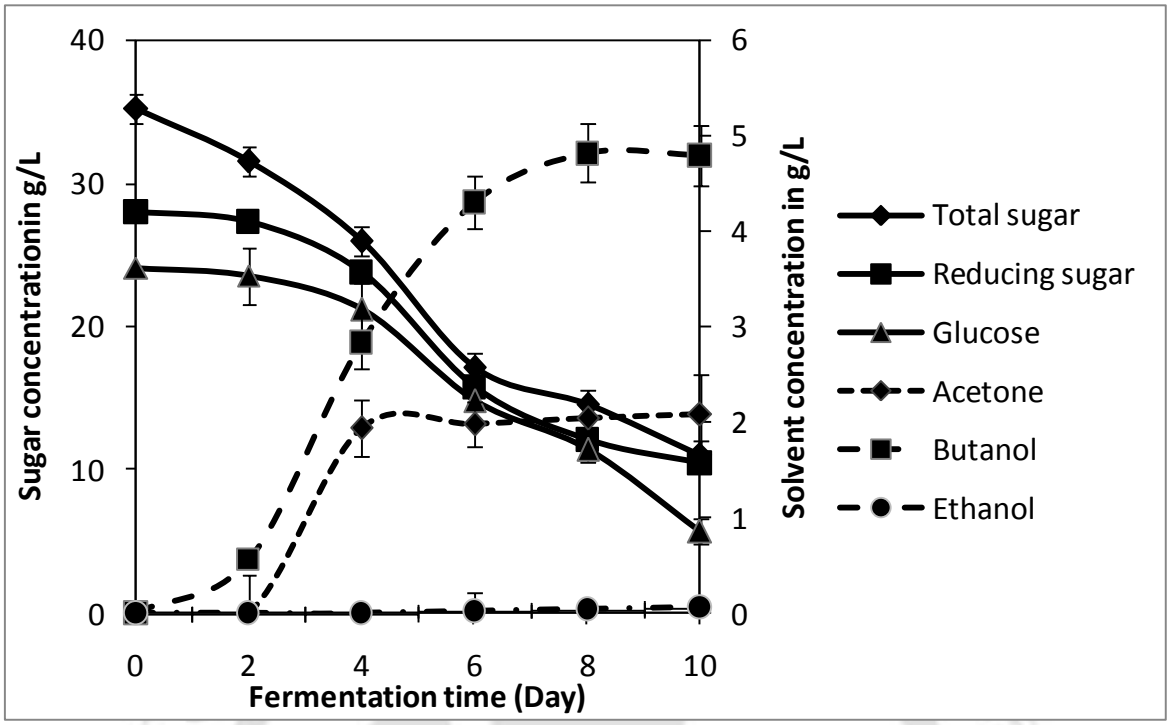


Figure 7.19: Time history of solvent production and sugar utilization at pH 4.0 ± 0.5

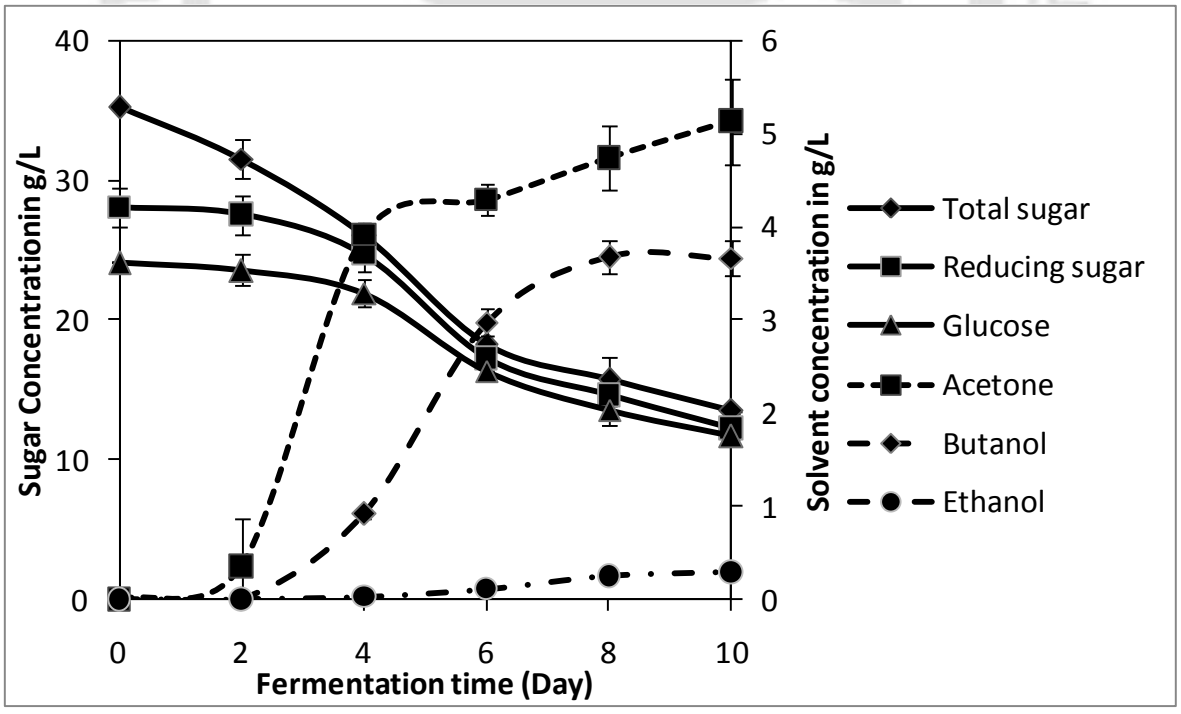


Figure 7.20: Time history of solvent production and sugar utilization at pH 6.0 ± 0.5

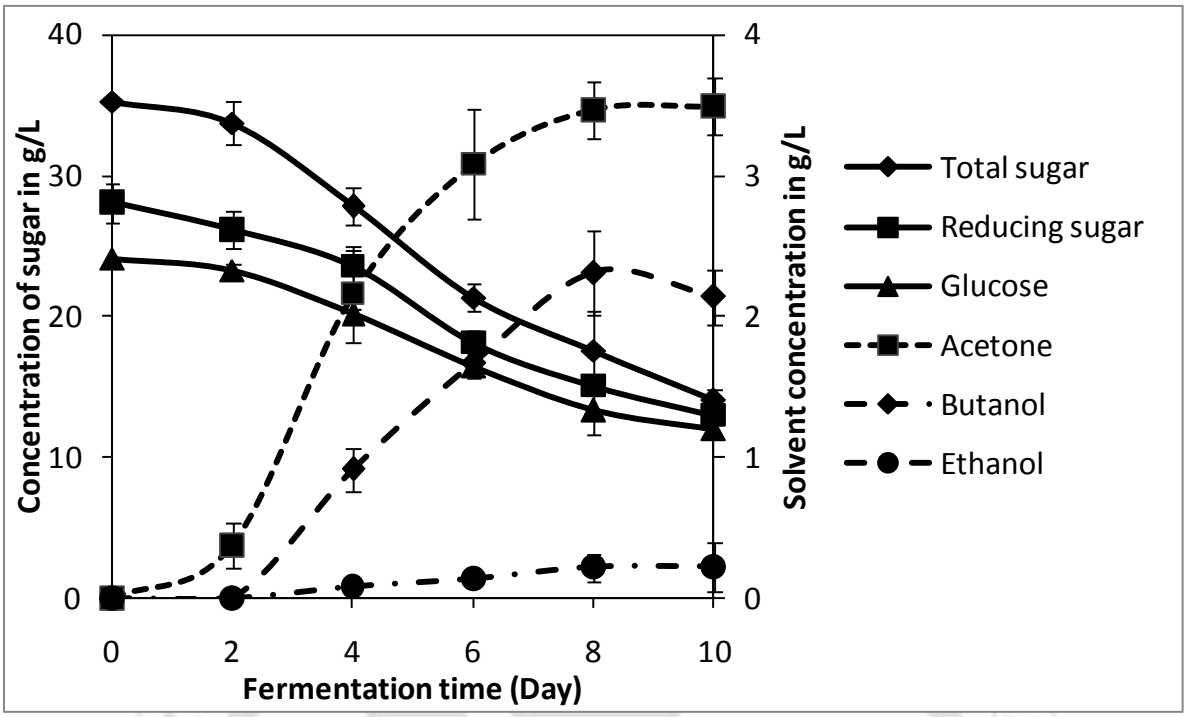


Figure 7.21: Time history of solvent production and sugar utilization at pH 8.0 ± 0.5.

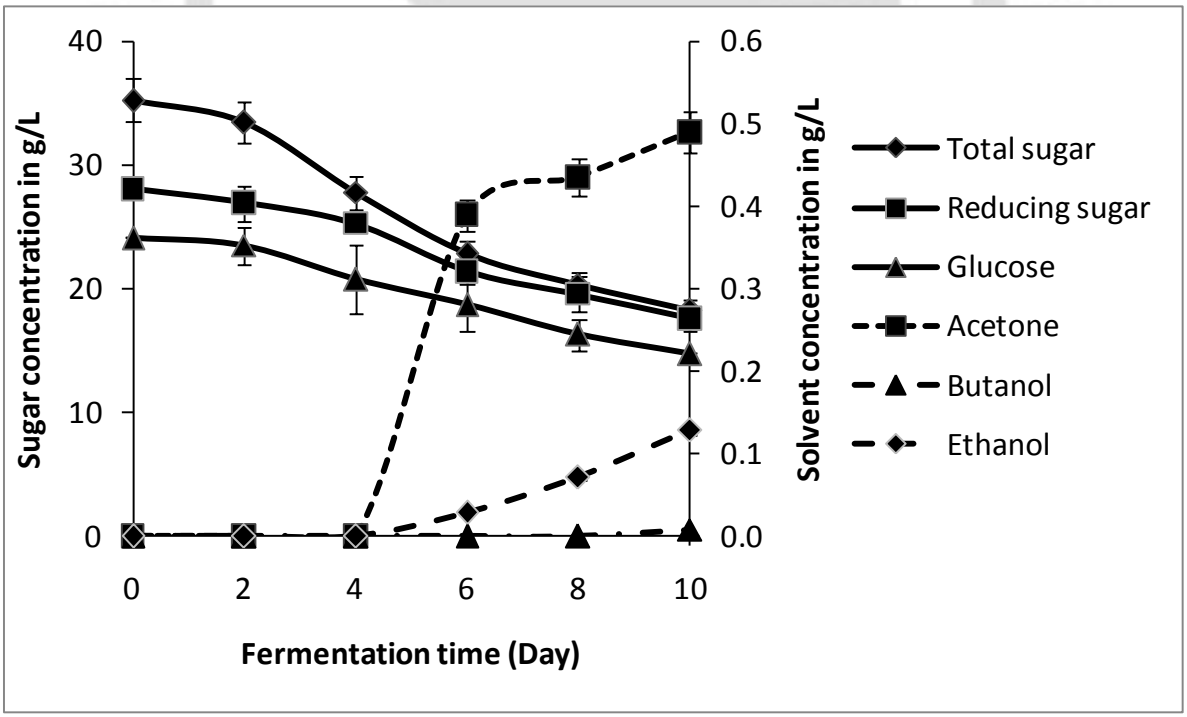


Figure 7.22: Time history of solvent production and sugar utilization at pH 10.0 ± 0.5

The solvent yield at initial pH 4 was found to increase to 0.63, with an enhanced butanol yield of 0.43 and butanol selectivity of 0.66 mol/mol. Similar experiments performed at initial pH 6.0 ± 0.5 resulted in comparable solvent yield of 0.67, with shifting of selectivity of products towards acetone. 5.14 g L^{-1} of acetone, 3.67 g L^{-1} of butanol and 0.3 g L^{-1} of ethanol was observed at the end of fermentation cycle, with a reduced butanol yield of 0.27 and butanol selectivity of 0.34 mol/mol.

Table 7.4: Comparative study of solvent yield, butanol yield and butanol selectivity achieved after carrying out the fermentation at varied of pH 1.0, 4.0, 6.0, 8.0 and 10.0.

Initial pH of Fermentation Broth (± 0.5)	Yield in terms of Total sugar(g/g)		Butanol selectivity (mol/mol)
	Butanol	Total solvent (ABE)	
1	0.12 ± 0.012	0.37 ± 0.04	0.2652
4	0.38 ± 0.018	0.58 ± 0.05	0.6349
6	0.27 ± 0.013	0.67 ± 0.04	0.3425
8	0.17 ± 0.01	0.43 ± 0.04	0.3271
10	0.01 ± 0.00	0.03 ± 0.01	0.0821

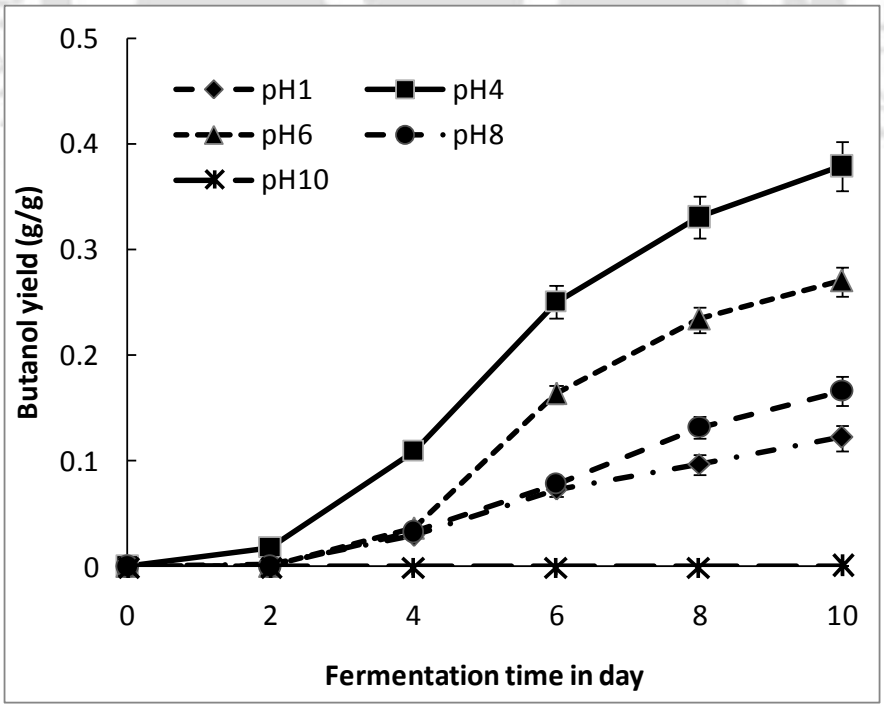


Figure 7.23: Time history of butanol yield at varied pH, achieved during 10–day of fermentation cycle

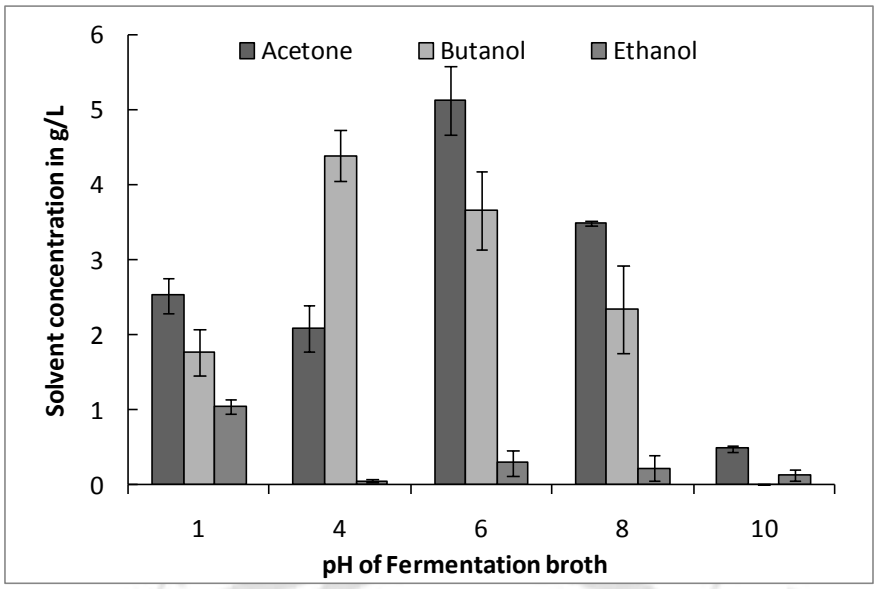


Figure 7.24: Comparative study of solvent production by *C acetobutylicum* MTCC 481 under varied external pH of fermentation broth.

Maintaining the external initial pH of fermentation broth in slightly alkaline range, resulted in poor solvent yield of 0.4, with the declined production of 3.5 g L^{-1} of acetone, 2.14 g L^{-1} of butanol and 0.22 g L^{-1} of ethanol. Overall, a reduced butanol yield of 0.15 and butanol selectivity of 0.3 was achieved at pH 8.0 ± 0.5. For fermentation carried out at highly alkaline initial pH of 10.0 ± 0.5, nearly no butanol production was observed with reduced production of acetone and ethanol viz., 0.49 and 0.13 g L^{-1}, respectively. This resulted in a solvent yield of 0.03, with a butanol selectivity of 0.08 mol/mol. A summarised comparative chart of solvent yield, butanol yield and butanol selectivity for the fermentation experiments at different initial pH of 1.0, 4.0, 6.0, 8.0 and 10.0 is given in Table 7.4. Time history of butanol yield at different pH, achieved during 10–day fermentation cycle is depicted in Fig. 7.23, where yield of butanol is calculated in terms of total sugar utilized. Fig. 7.24 illustrates the time history of solvent production under different initial pH of fermentation broth. Huang et al. [25] has reported that the transition from acidogenesis to solventogenesis is dependent on external pH (pH of fermentation broth).

Table 7.5 Clostridial fermentation with different substrates at varied optimal pH

Reference	Organism	Substrate	Optimum pH	Mode of fermentation	Butanol yield/ concentration
This paper	*C. acetobutylicum* MTCC 481	Rice straw	4.0 ±0.5	Batch	0.38
[24]	*C. acetobutylicum* DSM 1731.	Glucose	4.3–5.9	Continuous	150 mM
[25]	*C. acetobutylicum* ATCC 824	Glucose	4.5	Continuous	150 mM
[26]	*C. acetobutylicum* DSM 1731.	Synthetic	4.3	Continuous	
[27]	*C. saccharo–butylicum* DSM 13864	gelatinised sago starch	4.5	Batch	10.1 g/L
[28]	*C. acetobutylicum*	Sugarcane juice	5.3	Batch	0.26%
[29]	*C. beijerinckii* P260.	Wheat straw	6.5	Batch	20 gL^{-1}
[30]	*C. acetobutylicum* ATCC 824	Glucose	3.7	Batch	18.8 g L^{-1}

The results achieved by Huang et al are in concurrence with our experiments, where an external pH of 4.0 ± 0.5 resulted in increased production of solvents [25]. The clostridial cells can maintain a relatively high intracellular pH under acidic growth conditions by redirecting metabolism from organic acid synthesis to solvent synthesis. Both acidic and neutral fermentation products have been shown to interfere with membrane related functions such as ATP (a measure of energy) generation, which are responsible for generation of the trans–membrane pH gradient [31,32].The enhanced ability of solvent–producing cells to generate a trans–membrane pH gradient is presumably due to greater capacity of clostridial cells to convert acids into solvents [25]. Bahl et al. [26] reported that clostridial fermentation performed at neutral pH, mainly resulted in production of acids and gaseous products; however, performing the fermentation at reduced pH of 4.3 resulted in elevated production of solvents. Gottwald and Gottsehalk [24] stated that *C.* acetobutylicum DSM 1731 is unable to keep a constant pH inside the cells, which allowed production of weak organic acids (acetate, butyrates) in a first phase, and solvents in the second phase. Since the undissociated forms of

these acids are able to freely permeate through the cytoplasmic membrane, they will accumulate in the cell interior at large values and decrease the internal pH [33]. At external pH of 5.9 and 4.3, the cells kept a constant pH gradient of 0.9 to 1.3 [24]. Similarly, Liew et al. [27] reported that batch fermentation of gelatinised sago starch at pH 4.5 resulted in increased butanol production of 10.11 g L^{-1} during the stationary phase of metabolic cycle. Table 7.5 illustrates the previous attempts of many researchers with different Clostridial strains at varied optimal pH ranges, with various substrates. Highly acidic pH resulted in production acidic components but the switch over to solventogenesis got restricted, while highly alkaline conditions did not allow commencement of acidogenesis successfully, which results in nearly no solventogenesis and finally failure to produce solvents. Thus, among the range of external initial pH studied in this work, values 4.0, 6.0 and 8.0 were found to be more conducive for solvent production. Among these set of values of pH, pH = 4.0 gave best results in terms of butanol production for *C. acetobutylicum* MTCC 481 strain.

7.4.4 Effect of Inoculum Size on Clostridial Fermentation

In addition to physical parameters of pH, temperature and agitation rate, the physiological or cultural parameters also influence the outcome of a fermentation process. Size of the inoculum added to fermentation broth is an important physiological parameter. The influence of inoculum size is essentially manifested in terms of duration of the lag phase [34]. The lag time (or lag phase) is extended when the cells are severely stressed by starvation or other means [34]. This results in very low cell densities. The minimal inoculum size is the critical volume of inoculum required to initiate culture growth, due to the diffusive loss of cell materials into the medium. The subsequent culture growth cycle is dependent on the inoculum size, which is determined by the volume of medium and size of the culture vessel [35-36].

Table 7.6: Effect of inoculum size on solvent yield and butanol selectivity

Inoculum Size %(v/v)	Yield (g/g)		Butanol selectivity (mol/mol)
	Butanol	Total solvent (ABE)	
2	0.23 ± 0.011	0.36 ± 0.2	0.58
5	0.29 ± 0.013	0.44 ± 0.23	0.59
10	0.28 ± 0.011	0.42 ± 0.21	0.59
15	0.26 ± 0.012	0.39 ± 0.23	0.59

In our preliminary experiments in process optimization, we have tried to identify the relative influence of this parameter. In the preliminary experiments, we screened four inoculum sizes, viz. 2, 5, 10 and 15% v/v broth for butanol production. For experiments performed by inoculating the developed RSH with 2% v/v clostridial culture, nearly 63% of total sugar with 72% of glucose was utilized, with production of 2.23 g L^{-1} of acetone, butanol 4.55 g L^{-1}, and 0.27 g L^{-1} of ethanol. The same experiment conducted with 5% v/v inoculum size resulted in increased sugar utilization (70% of total sugar and 75 % of glucose) with elevated solvent production of 2.4 g L^{-1} of acetone, 7.0 g L^{-1} of butanol and 1.08 g $L^{-1}$of ethanol. Similar experiment conducted with even higher inoculum size of 10 and 15% v/v resulted in utilization of 72–74% of glucose with production of 8.75 and 8.05 g L^{-1} of total solvents (acetone + butanol + ethanol). Figs. 7.25 to 7.28 illustrate the time history of sugar utilization and solvent production in fermentations carried out with 2, 5, 10 and 15% v/v inoculum size. Table 7.6 compares the butanol and solvent yield, and butanol selectivity achieved during fermentation with varied inoculum sizes. Butanol yield achieved with 5, 10 and 15% v/v inoculum sizes was observed to be 0.28, 0.29, 0.26 g g^{-1} with a similar butanol selectivity of 0.59 mol mol^{-1}.

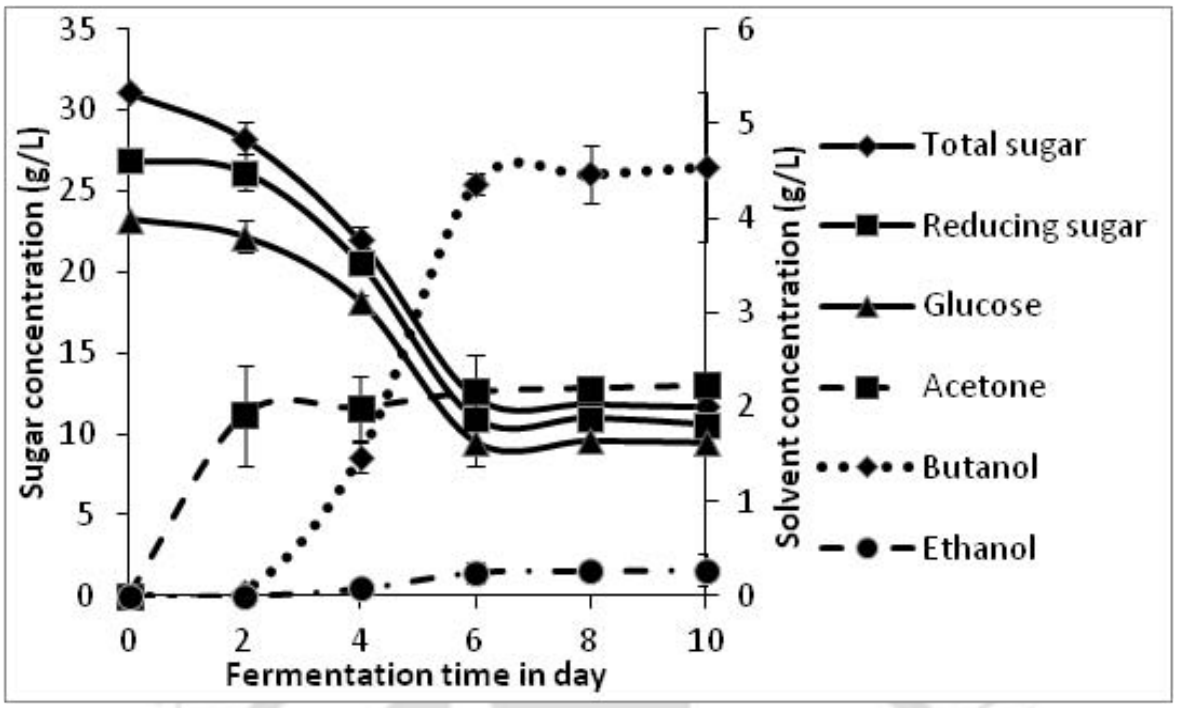


Figure 7.25. Time history of sugar utilization and solvent production for fermentation with 2% v/v inoculum

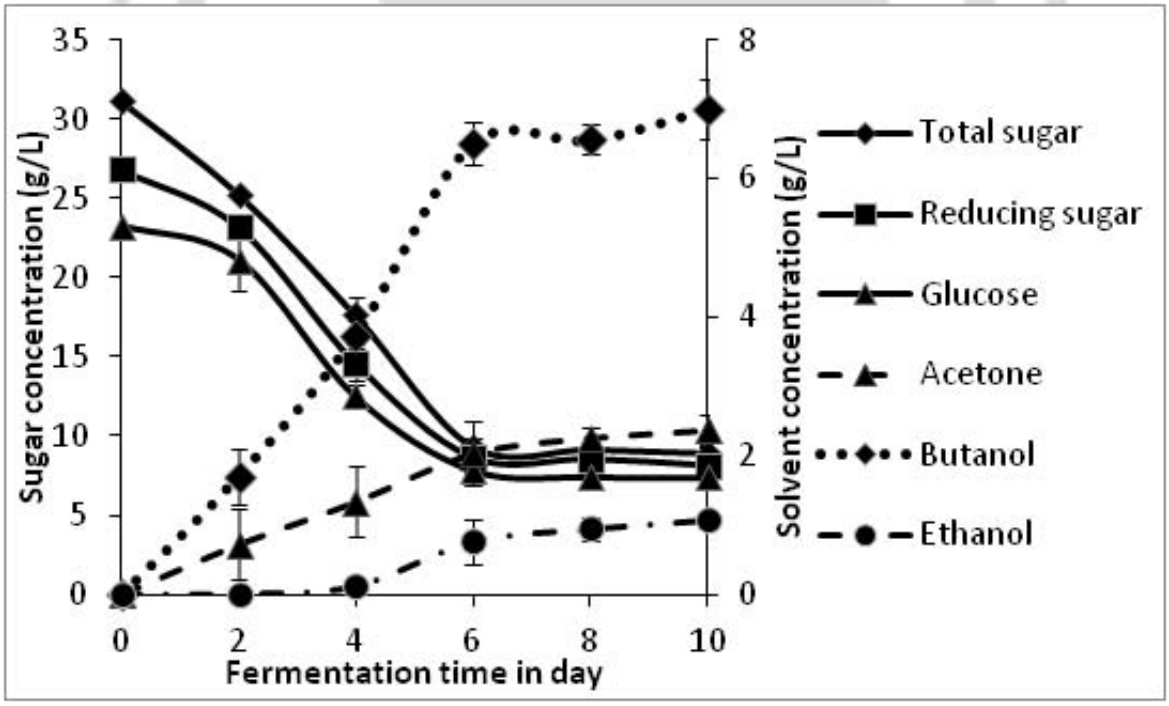


Figure 7.26. Time history of sugar utilization and solvent production for fermentation with 5% v/v inoculum

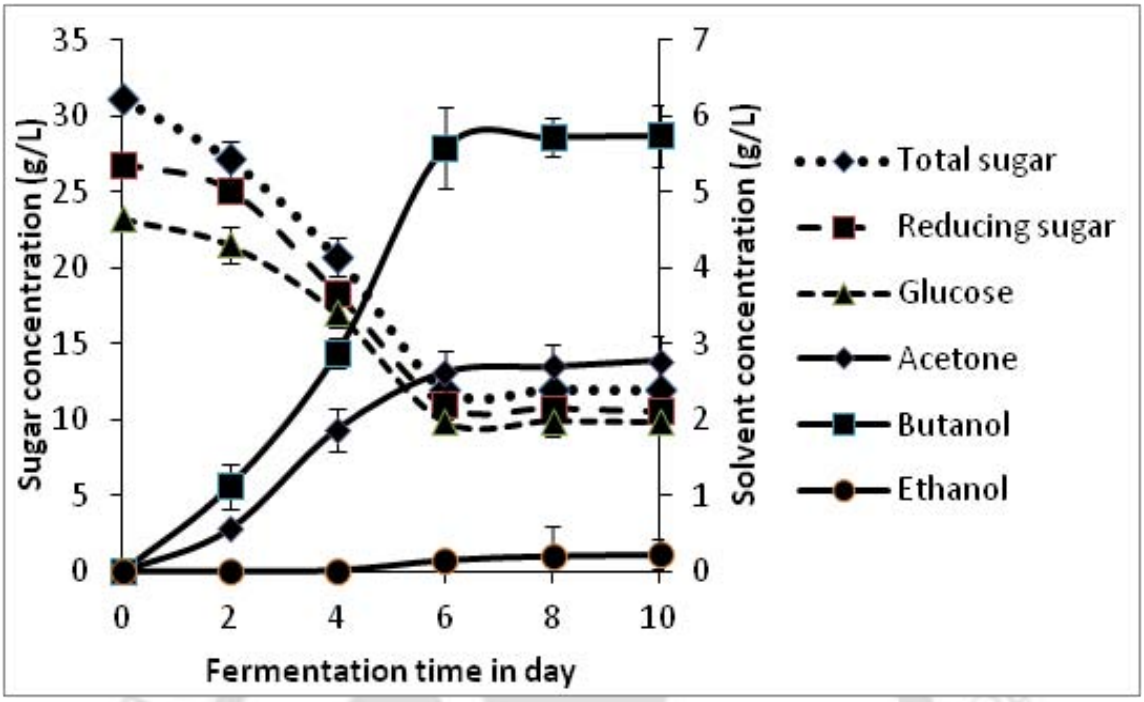


Figure 7.27: Time history of sugar utilization and solvent production for fermentation with 10% v/v inoculum.

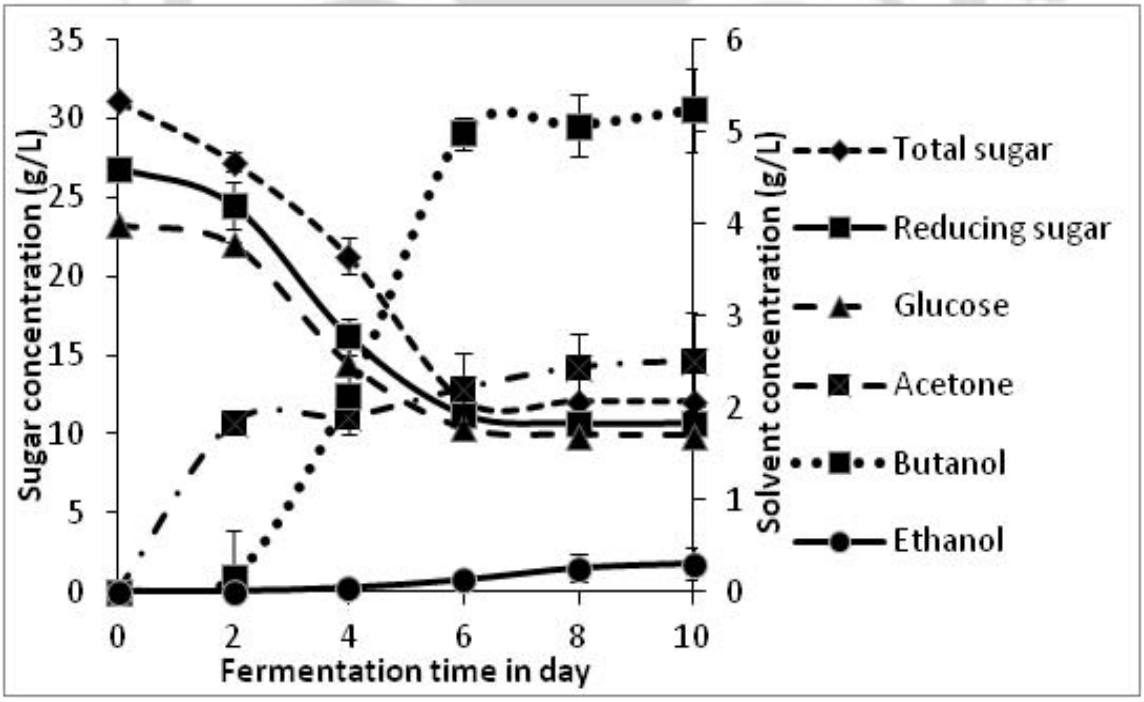


Figure 7.28: Time history of sugar utilization and solvent production for fermentation with 15% v/v inoculum

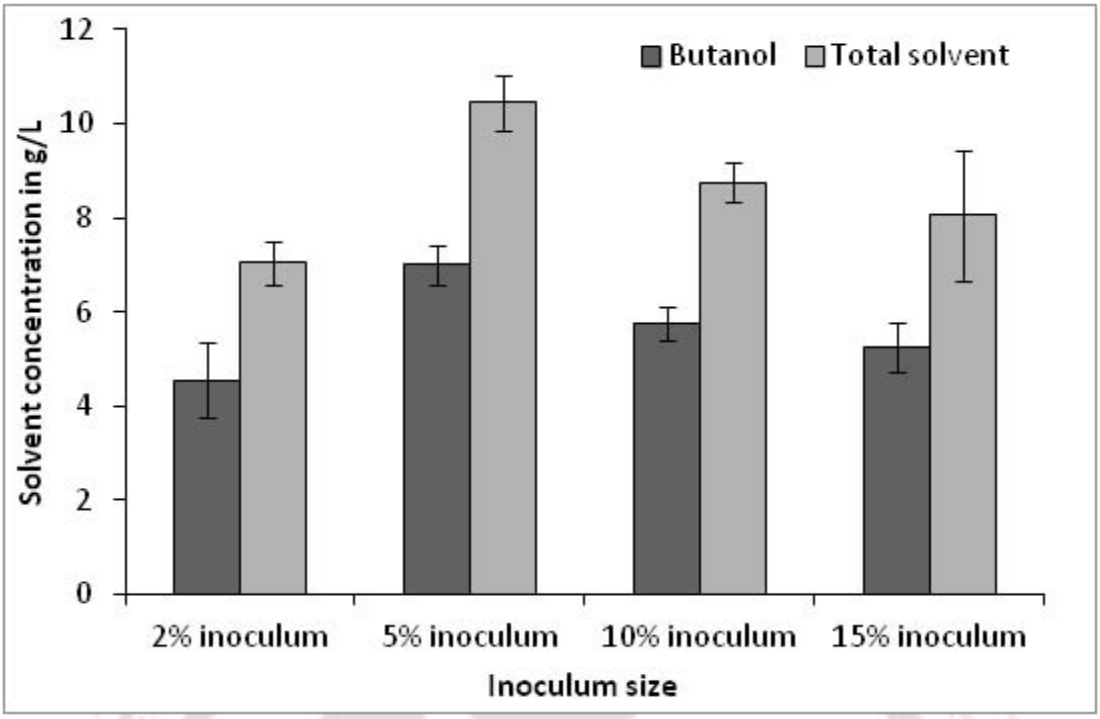


Figure 7.29: Butanol and total solvent (ABE) concentration achieved with different inoculum size

Table 7.6 gives a comparative evaluation of butanol yield and selectivity achieved in preliminary experiments carried out with different inoculum sizes, while the total solvents and butanol production in these experiments is shown in Fig. 7.29. The time histories of butanol yields in these experiments are compared in Fig. 7.30. From results shown in Figs. 7.29 and 7.30 and Table 7.6, it could be perceived that a low inoculum size of 2% v/v was relatively inefficient for solvent production, while an inoculum size ranging from 5 – 15% v/v resulted in enhanced solvent production. An interesting observation can also be made that for an inoculum size ≤ 5% v/v, the yields were nearly the same, irrespective of the increased in cell density due to higher inoculum size. This result indicated that an inoculum size of 5% was most optimum for clostridial fermentation of developed rice straw for the combination of physical parameters (pH, temperature and agitation) used in these preliminary experiments. However, in the overall process optimization, variation in the physical parameters mentioned above could influence the optimum inoculum size, and the optimum value of 5% v/v, as seen

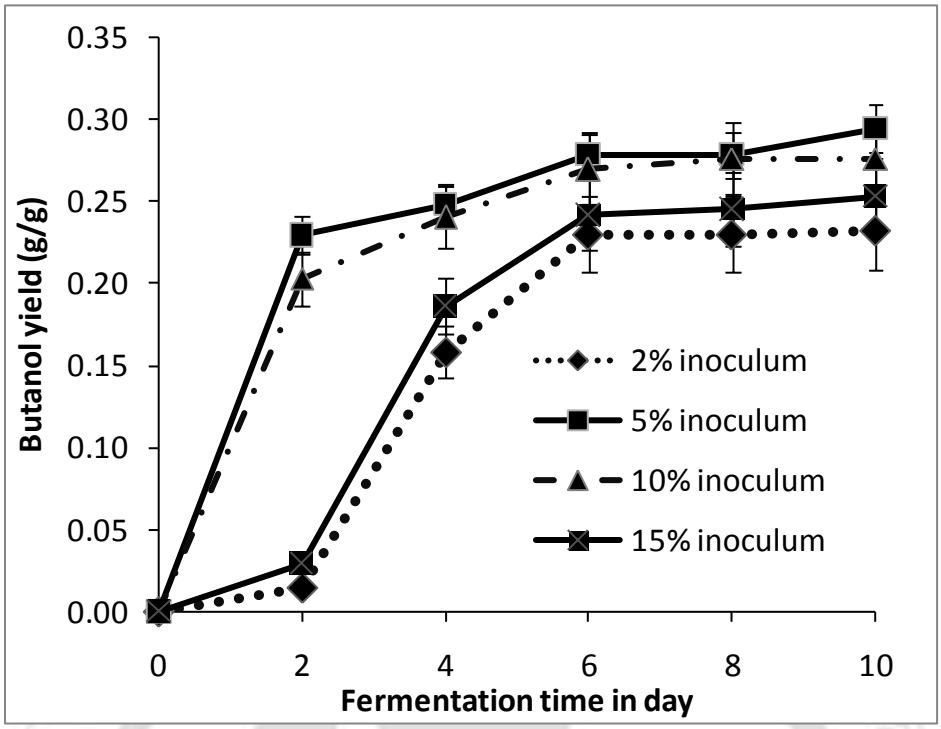


Figure 7.30: Comparative study of butanol yield with different inoculum sizes

in the preliminary experiments may not hold for other combinations of physical parameters. With this consideration, we have selected 3 levels of inoculum sizes, viz. 5, 10 and 15% v/v for further statistical optimization of the process.

7.4.5 Effect of Inoculum age on clostridial fermentation

Next to the inoculum size, the age of the inoculum is an important physiological factor influencing fermentation process. In the preliminary experiments prior to statistical optimization of the fermentation process, the potential of clostridial culture to produce solvent with respect to time duration was investigated by using an inoculum grown for 18, 24, 36 and 48 h. The respective optical density observed for these set of inoculum age was 0.75, 0.85, 0.9, 1.0. Figs. 7.31 to 7.34 illustrate these trends of solvent production and sugar utilization. From our previous studies, as described in Chapter 4, in a typical clostridial

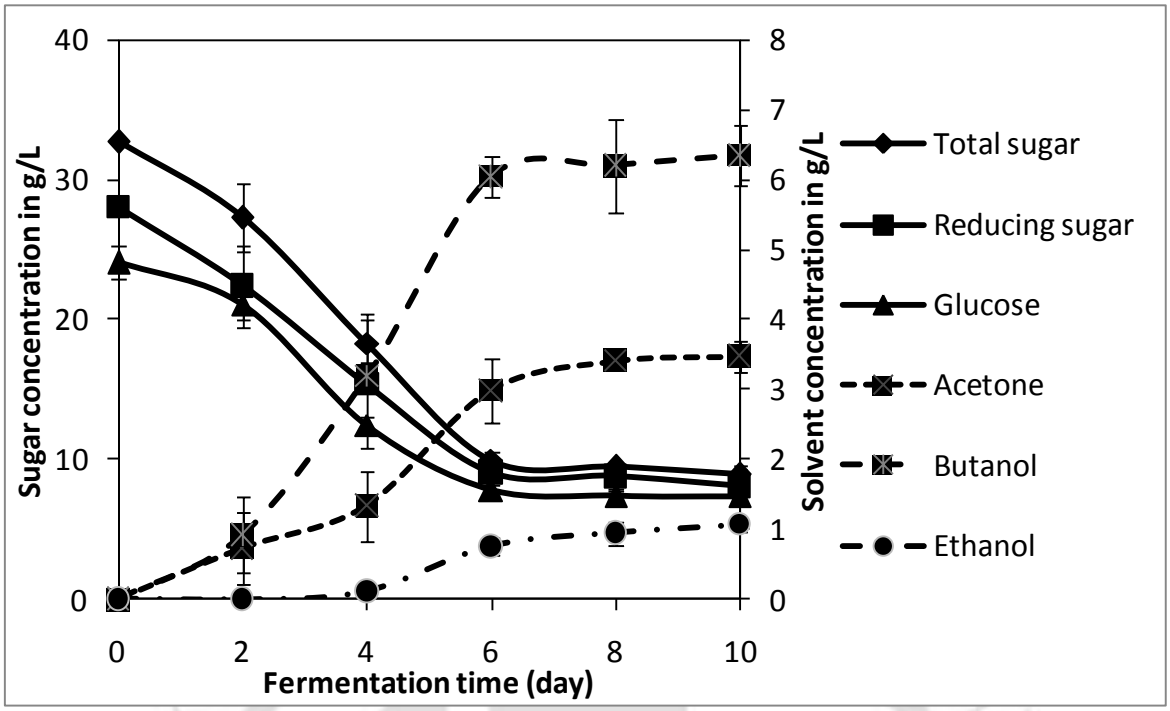


Figure 7.31: Time history of sugar utilization and solvent production with fermentation with 18 h old inoculum

growth curve, 18 h old Clostridial culture are observed in the initial exponential phase, while a 24 old culture lies towards the end of exponential growth. Thus, in both the case cells remains in the rapidly dividing and growing stage, while a 36 and 48 h old inoculum is reported to present in the very initial and mid stationary phase of clostridial growth cycle [37]. Thus, 36 and 48 h old inoculum are comparatively metabolically less inactive. For experiment performed with 18 h old inoculum, formation of nearly 3.48 g L^{-1} of acetone, 6.35 g L^{-1} of butanol and 1 g L^{-1} of ethanol was observed. While performing the same experiments with 24 h old inoculum, slightly elevated solvent concentrations of 3.86 g L^{-1} of acetone, 5.83 g L^{-1} of butanol and 0.23 g L^{-1} of ethanol were observed. Similar experiments with 36 and 48 h old inoculum resulted in relatively reduced production of solvent, with 3.07 and 2.7 g L^{-1} of butanol.

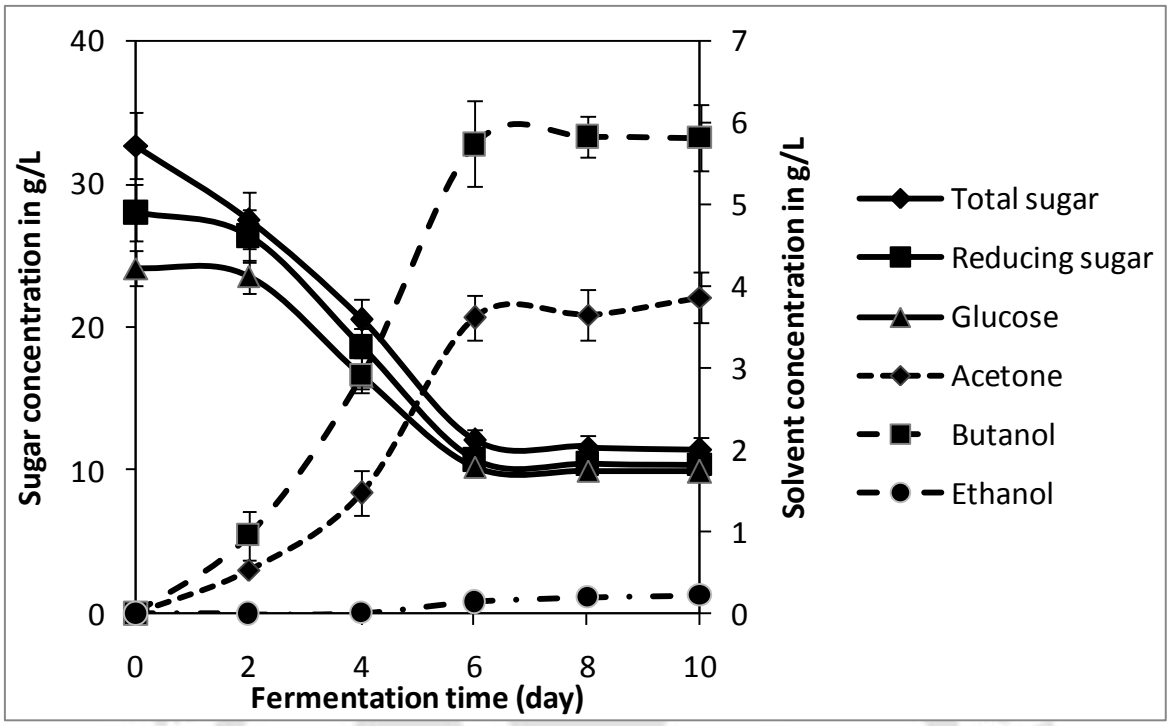


Figure 7.32: Time history of sugar utilization and solvent production with fermentation with 24 h old inoculum

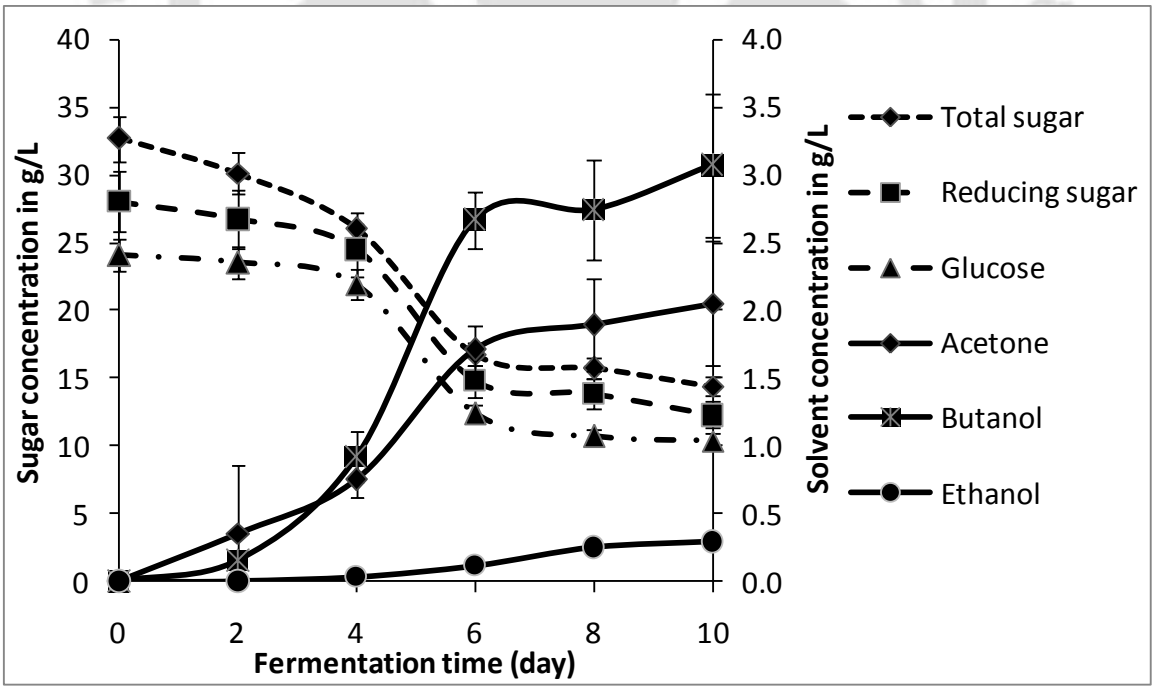


Figure 7.33: Time history of sugar utilization and solvent production with fermentation with 36 h old inoculum

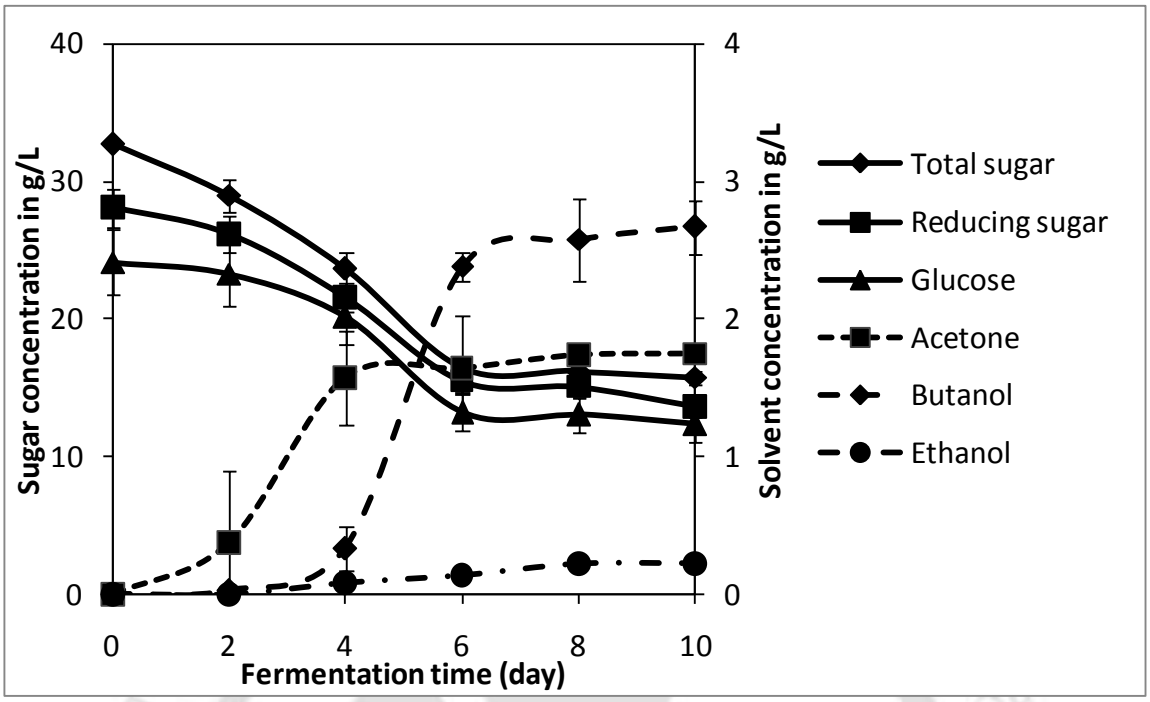


Figure 7.34: Time history of sugar utilization and solvent production with fermentation with 48 h old inoculum.

Table 7.7: Comparative study of solvent yield and butanol selectivity at varied inoculums age

Inoculum Age (h)	Yield (g/g)		Butanol selectivity (mol/mol)
	Butanol	Total solvent (ABE)	
18 (OD = 0.75)	0.27 ± 0.013	0.46 ± 0.023	0.51
24 (OD = 0.85)	0.27 ± 0.012	0.47 ± 0.022	0.52
36 (OD = 0.9)	0.17 ± 0.010	0.3 ± 0.017	0.51
48 (OD = 0.95)	0.16 ± 0.011	0.3 ± 0.019	0.51

Table 7.7 gives a compares the yield and selectivity of butanol obtained in preliminary fermentation experiments carried out with inoculums of different age (or at different stages of clostridial growth cycle). Fermentation of developed RSH with inoculums of different age resulted in butanol yield of 0.27 g/g for 18 and 24 h old inoculum, 0.17 g/g for 36 h old inoculum and 0.16 g/g for 48 h old inoculum, with an uniform butanol selectivity of 0.51 mol/mol. Fig. 7.35 represents the time history of butanol yield, while Fig.7.36 displays the

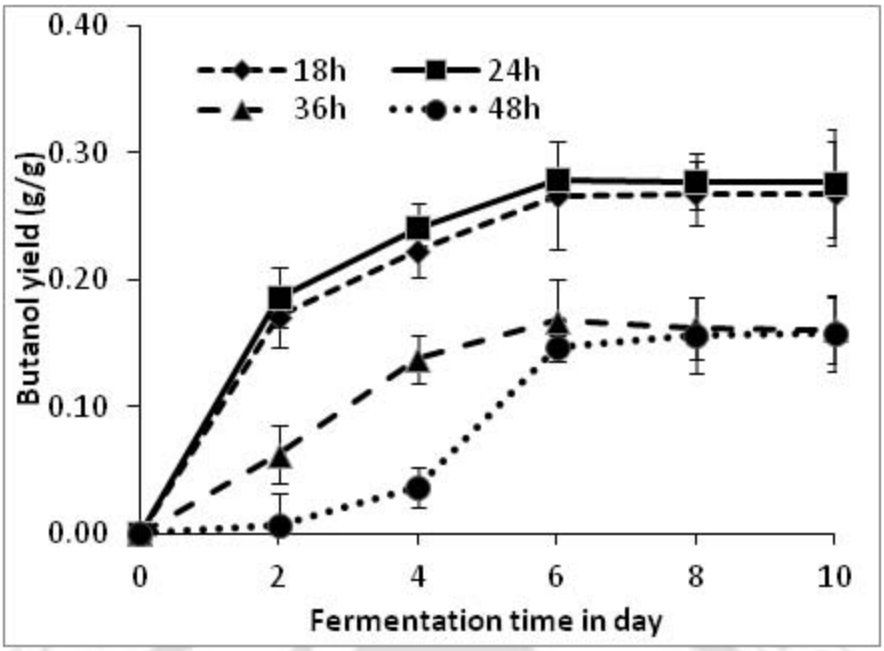


Fig 7.35: Comparative study of time history of butanol yield achieved during fermentation performed with varied inoculum age

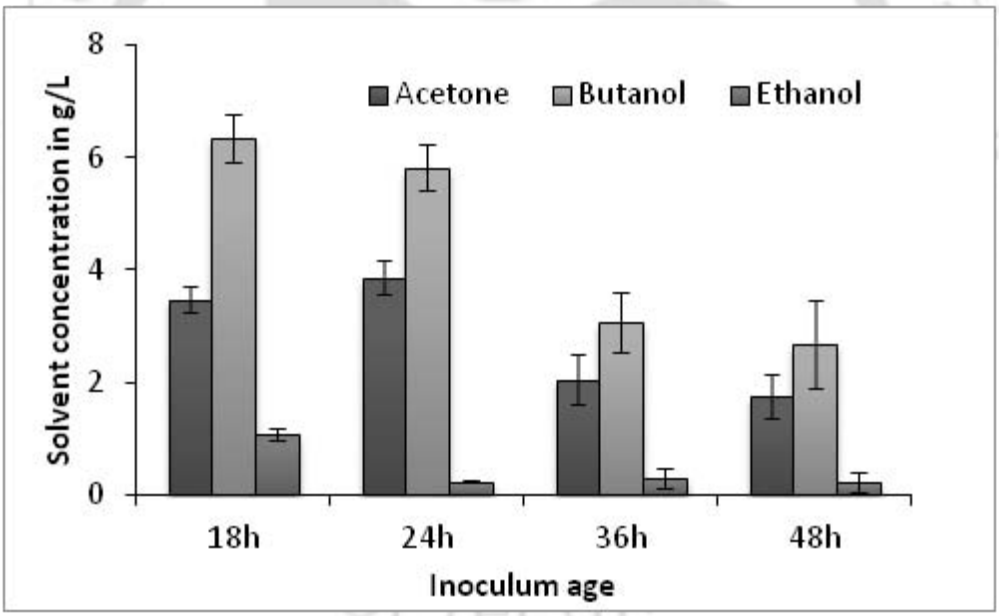


Figure 7.36: Comparison of production of three solvents in preliminary fermentation experiments with different inoculum age

time history of butanol solvent production in fermentations with different inoculum age. Nearly 70% of total sugar and 74% of glucose was utilized during all fermentation experiments. Thus, on the basis of these preliminary experiments, the 3 levels of inoculum age screened for further statistical optimization were 18, 24 and 48 h.

SECTION B: STATISTICAL EXPERIMENTS FOR OPTIMIZATION USING TAGUCHI DOE METHOD AND ANOVA

7.5 Materials and Methods

7.5.1 Fermentation Conditions for Process Optimization Using Statistical Approach

The results of the preliminary experiments yielded the levels of the parameters (or factors) for the design of statistical experiments using Taguchi L18 array. The combinations of experimental parameters and their levels are given in Table 7.8 given on next page. The basic methodology followed for fermentation experiments and their analysis with combinations of parameters as described in Table 7.8 is already explained in section 7.2 and 7.3. Total 18 set of experiments were done as described in Table 7.8. Moreover, each experiment was conducted in triplicates to assess the reproducibility of the results.

7.5.2 Taguchi's Orthogonal Experimental Array

A well–known experimental design technique, namely L18 OA design was employed to study the effect of five parameters, viz. temperature, pH, inoculum size, inoculum age, and agitation on the butanol production by *C. acetobutylicum* MTCC481 [38–40]. Essentially, the design consisted of a total of 18 experiments with five parameters—one parameter having two levels and the other four having three levels, i.e., L18 ($2^1 \times 3^4$). Levels of five parameters (as decided on the basis of preliminary experiments described in preceding section) under study were as follows: (1) Temperature (2 levels) – 37 and 42^{o}C, (2) pH (3 levels) – 6.0, 8.0 and 4.0, (3) Inoculum size (3 levels) – 5, 10 and 15%, (4) Inoculum age (3 levels) – 18, 24, and 36 h old, and (5) Agitation speed (3 levels) – 150, 200 and 250 rpm, respectively.

Table 7.8: Taguchi design matrix and corresponding butanol production by *Clostridium acetobutylicum* (MTCC 481) in shake flask.

Experiment Run No.	Parameters (Factors)										Butanol (g L^{-1})	S/N (db)
	Temperature		pH		Inoculum size		Inoculum age		Agitation			
	Coded level	Actual value (^{0}C)	Coded level	Actual value (±0.5)	Coded level	Actual value (%)	Coded level	Actual value (h)	Coded level	Actual value (rpm)		
1	1	37	1	6	1	5	1	18	1	150	5.34	14.51
2	1	37	1	6	2	10	2	24	2	200	4.67	13.35
3	1	37	1	6	3	15	3	36	3	250	4.12	12.26
4	1	37	2	8	1	5	1	18	2	200	4.42	12.88
5	1	37	2	8	2	10	2	24	3	250	3.30	10.37
6	1	37	2	8	3	15	3	36	1	150	3.3	10.30
7	1	37	3	4	1	5	2	24	1	150	11.93	21.34
8	1	37	3	4	2	10	3	36	2	200	6.89	16.71
9	1	37	3	4	3	15	1	18	3	250	7.60	17.52
10	2	42	1	6	1	5	3	36	3	250	2.74	8.65
11	2	42	1	6	2	10	1	18	1	150	6.42	16.11
12	2	42	1	6	3	15	2	24	2	200	2.77	8.23
13	2	42	2	8	1	5	2	24	3	250	2.46	7.74
14	2	42	2	8	2	10	3	36	1	150	2.89	9.22
15	2	42	2	8	3	15	1	18	2	200	3.46	10.75
16	2	42	3	4	1	5	3	36	2	200	4.75	13.52
17	2	42	3	4	2	10	1	18	3	250	5.49	14.74
18	2	42	3	4	3	15	2	24	1	150	6.04	15.57

Table 7.8 presents the experimental combinations adopted as per the experimental design. All experiments were performed in custom fabricated 250 mL Erlenmeyer flasks. In each flask, 100 ml of the optimized RSH medium was inoculated with seed culture and agitated in an incubator shaker (Make: Scigenics Biotech, Model: Orbitek) for 8 days. Pre–optimized RSH medium consisted of stress assisted acid treated RS supplemented with 3.0 g L^{-1} yeast extract and 4.0 mg L^{-1} PABA [41]. To vary the age of seed culture/inoculum, clostridia were grown at different times (18, 24 and 36 h) in three flasks. Each of these flasks contained 50 ml of the seed culture medium (RCM), and was incubated at 37°C and 150 rpm in a rotating orbital incubator shaker.

Samples were taken periodically for the analyses of the residual sugar and solvent (acetone, butanol and ethanol) production in the fermentation medium. The optimal conditions with respect to the experimental factors or parameters have been determined on the basis of average of signal to noise ratio (S/N) for the factor or parameter at each level (Table 7.9). Butanol production by *Clostridium acetobutylicum* MTCC 481 was considered as a desired variable, and a higher concentration was preferred. In each experimental run, the response was recorded as the butanol production and corresponding signal–to–noise (S/N) ratio was calculated using Equation 1 with an overall objective of estimating the effects of various parameters on solvent production, where a large S/N ratio is preferred [42].

$$\frac{S}{n} = -10 \times \log\left(\frac{1/Y^2}{n}\right) \quad (1)$$

where Y is the response and n is the number of experimental runs. The statistical significance of each factor was determined using ANOVA. Further, the optimum conditions for butanol production by *C. acetobutylicum* MTCC 481 were again determined using ANOVA performed using MINITAB® Release 15.1, PA, USA (Trial version).

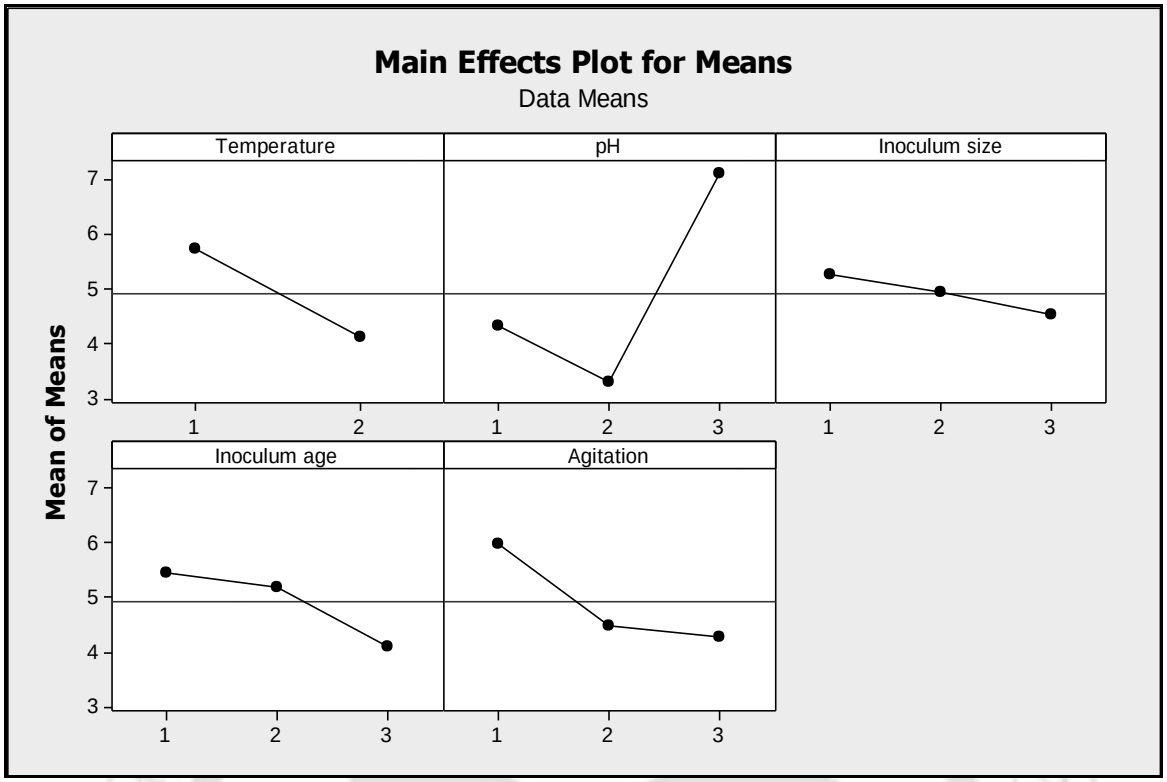


Figure 7.37: Main effects plot of mean for the process variables

(The lines connecting the points are only for convenience of reader and they do not necessarily show trends)

7.5.3 Validation of Results

Further validation of ABE fermentation process with optimized process parameters, as revealed from preceding study with Taguchi DOE, was done using 250 mL custom fabricated Erlenmeyer flasks. The duration of fermentation was 8 days. The samples of fermentation broth were withdrawn after every 48 h for the analysis of biomass, solvents and sugar. This experimental protocol (using developed RSH based fermentation media) was tested in a 2 L microprocessor controlled bioreactor (Zenith, India) with 1 L of developed RSH medium and 5% v/v of Clostridial inoculum. The temperature was controlled at 37^{o}C, 99.98% pure nitrogen was sparged at a rate of 0.1 vvm. The initial pH of the media and the agitation rate were set at 4.0 ± 0.5 and 150 rpm, respectively. The fermentation cycle lasted

for 6 days and samples were withdrawn after every 24 h. Later, the same experimental protocol was scaled up in 5 L bioreactor with 3 L of optimized fermentation medium under the optimized process conditions. Fermentation cycle in a 5 L bioreactor lasted for 5 days, and samples of fermentation broth were withdrawn every 24 h.

7.5.4. Fermentation with Precipitate Removal

Optimized rice straw achieved after statistical optimization was observed to contain some precipitate in the fermentation broth while maintain the broth pH at 4.0± 0.5. In order to check the effect of this precipitate during fermentation, it was removed by centrifuging (Make: Hermle, Germany) the broth at 6000 rpm for 10 min. Centrifuged broth was allowed to undergo fermentation under optimum condition of 37^{o}C, 4.0 ± 0.5 pH, 5% v/v inoculums, 18 h old inoculums at 150 rpm for a 10 day cycle. Samples were withdrawn at an interval of 2 days and experiment was conducted in duplicate.

***Analysis of precipitate*:** Precipitate obtained after centrifuging the fermentation broth was allowed to air dry in a hot air oven (JSGW, India) at 60^{o}C for 24 h. Dried sample was then calcined in a silica crucible at 500^{o}C for 5 h using a muffle furnace (Labtech, Korea). Qualitative characterization of precipitation was done by using XRD (Bruker D–8, Germany) technique.

Table 7.9: Value of Average S/N ratio through Taguchi analysis of factors affecting butanol production by *C. acetobutylicum*

Level	Factors				
	Temperature	**pH**	**Inoculum Size**	**Inoculum Age**	**Agitation**
1	14.36	12.19	13.10	14.42	14.51
2	11.61	10.21	13.42	12.76	12.57
3		16.57	12.44	11.78	11.88
Delta*	2.75	6.36	0.98	2.64	2.63
Rank	**2**	**1**	**5**	**3**	**4**

Table 7.10: Analysis of variance (ANOVA) of Butanol production

Source	DF*	SS#	MS	*F* ratio (F)	*P* value (P)	Confidence level (%)	Percent contribution
Temperature	1	11.75	11.75	F > 5	p < 0.05	95	18
pH	2	46.56	46.56	F > 5	p < 0.05	99.47	41.44
Inoculum Size	2	1.572	1.572	F < 5	p > 0.05	37.67	6.38
Inoculum Age	2	6.049	6.049	F < 5	p > 0.05	10.20	17.19
Agitation	2	10.32	10.32	F < 5	p > 0.05	87.37	17.12
Residual error	8	15.78	15.78				
Model	17	92.02					100

* Degrees of Freedom, # Sum of Squares

7.6 Results and Discussion

7.6.1 Taguchi analysis and ANOVA

In the statistical optimization experiments, we have emphasized on optimization of process parameters for butanol production as the response of all experiments, rather than total solvent concentration, as butanol is the desired target product. Table 7.8 depicts the butanol concentration produced from developed RSH based media comprising of different combinations of processing conditions and cultural parameters, i.e. temperature, pH, inoculum size, inoculum age, and agitation. The results given in Table 7.8 indicate that the butanol production in ABE fermentation process is a major function of the process parameters (or physical conditions) and cultural environment. Among all 18 experimental runs, the highest total solvents (15.56 g L^{-1}) and butanol (11.93 g L^{-1}) concentration was attained in run 7. It also displayed the highest signal to noise ratio. The percentage effect of each factor on butanol and total solvent production is given in Table 7.9. Out of 5 factors examined, pH, temperature, inoculum age and agitation had major effect on butanol production, as indicated by their high delta S/N value of 6.36, 2.75, 2.64 and 2.63 db, respectively. Based on this result, the order of significance of effect of various factors on

butanol production was determined as pH > Temperature > Inoculum age > Agitation > Inoculum size. The percent individual contribution of these four factors to the main effect mean (or delta S/N) was in order (Table 7.10): 41.44% (pH) > 18% (Temperature) > 17.19% (Inoculum age) > 17.12% (Agitation) > 6.38% (Inoculum size), indicating pH to be the factor of the highest (relative) significance, and temperature, inoculum age and agitation rate of moderate significance and inoculum size as relatively least significant factor, with the lowest percent contribution for butanol production (< 10%). Fig. 7.37 displays the main effect of means of process variables, viz. pH, temperature, inoculum age, agitation rate and inoculum size. The total solvent production was higher at level 1 for factors temperature, inoculum size, inoculum age and agitation, while for factor pH, level 3 produced more solvents. The critical volume of inoculum required to initiate culture growth, due to the diffusive loss of cell materials into the medium is referred as inoculum size. The subsequent culture growth cycle is dependent on the inoculum size, which is determined by the volume of medium and size of the culture vessel.

Reducing the inoculum size results in reduction of number of cells or cell concentration. If the number of cells inoculated is below a critical value, inoculum will be without cells throughout the culture and may result in longer lag phase. On the contrary, if the cell concentration is above a certain critical value (> 5% in this study), it will result in a reduced lag phase with highly active cells, irrespective of its higher concentration [43]. This explains the comparatively insignificant effect of inoculum size on production of butanol above the critical value of 5%, as mentioned above. pH is one of the most important controlling factors of anaerobic fermentation process. It was observed from Table 7.8 that the acidic pH resulted in enhanced solvent production in comparison to the neutral pH. In ABE clostridial fermentation under batch conditions, exponentially growing cells produce organic acids, which lower the pH of the medium (acidogenesis). As the culture enters the stationary

phase, the metabolism of the organism changes, converting pre–formed organic acids into organic solvents (solventogenesis). It is suggested that the switch over to solvent production is an adaptive response of the cell to the low medium pH, which is a consequence of acid production or acidogenesis [44]. Solvent production usually occurs only after the external pH falls below 5.0. The acetic and butyric acids produced during the fermentation are weak organic acids; they would be expected in their protonated form to be freely permeable through the cell membrane. Consequently, at sufficiently high concentration these acids would tend to equilibrate intra–cellular pH and external pH of the fermentation broth. Thus, the switch from acid to solvent production may not be the result of the low external pH directly, but a result of a drop in internal pH of the cells [10]. This indicates the critical role of pH in solvent production. Thus, a lower external pH helps the clostridial cells to achieve solventogenesis easily, and at a faster pace. It is evident from these results that the pH 4 ± 0.5 is the optimum pH needed to produce maximum butanol and other solvents. Cellular growth and metabolism is a strong function of the temperature of the cell environment. Temperature above and below the tolerance limit of the organism can prevent enzyme catalysed reactions that are important for cellular metabolism. Clostridia are mesophiles; they grow optimally between 20 and 45°C. Above and below this limit, *C. acetobutylicum* loses its ability to produce acids as well as solvents efficiently [45]. The optimum temperature of 37^{o}C obtained by us is within the above limits of optimum temperature. Solid–liquid mass transfer is an important aspect of all fermentation processes, which are usually carried out in agitated vessels. Rate of agitation is, thus, a critical parameter that has to be optimized. Under–agitated systems may lead to incomplete fermentation due to inadequate interaction of substrate and cells, while over–agitated systems may result in damage of cells due to high shear stress, which adversely affects the yield of fermentation [46]. In concurrence of these arguments, we have obtained a moderate value of 150 RPM as the optimum for ABE

fermentation.

ANOVA of the experimental results was done to quantify the variation in product formation caused due to each factor, and also to determine as whether the lower or higher value of a factor is essential for preferred result, i.e. higher butanol production. Table 7.10 depicts the results of ANOVA for all factors. The ANOVA results corroborate the results based on S/N ratio in that the significant factors for butanol production (which had more than 10% contribution to the main effect with p value < 0.005, F value > 5, and higher confidence level) were pH, temperature and agitation rate, while inoculum age and inoculum size were insignificant factors, as indicated by p > 0.005 and F < 5 [39, 47]. The ANOVA of butanol production (Table 7.10) had a model SS and DF value of 92.02, and 17.00, respectively. The model obtained from ANOVA had R^2 (multiple regression coefficient) value of 0.94, which is indicative of the robustness of the model in that it can explain ~ 94% variation in the response. Thus, optimized fermentation process for butanol production using optimized RSH includes fermentation at 37°C, 4 ± 0.5 pH and 150 rpm. This indicates that the process parameters affect the solvent production more significantly than the cultural conditions. This result is very important from scale–up point of view of the process in that it indicates that variation in solvent production due to cultural conditions could be compensated by adjusting the process parameters (such as rpm, temperature and pH), which are easy to control and manipulate.

7.6.2 Prediction of butanol and total solvents production with optimized medium

To predict the butanol production (denoted by Y_{opt}) for the optimized fermentation conditions mentioned above, the following formula was used:

$$Y_{opt} = \bar{T} + |A_1 - \bar{T}| + |A_2 - \bar{T}| + |B_1 - \bar{T}| + |B_2 - \bar{T}| + |B_3 - \bar{T}| \quad (2)$$

Here, $\bar{T}$ denotes the average butanol production in all trial results, while A_1, A_2 are the

average butanol productions at two levels of temperature, while B_1, B_2 and B_3 are average butanol productions for the three levels of pH [42, 49]. Using above formula, the predicted values of butanol and total solvent productions for the optimized process parameters were 11.89 and 19.15 g L^{-1}, respectively.

7.6.3 Validation and Comparison of Results

Confirmatory experiments to assess butanol and total solvent production under optimized process conditions (pH, agitation and temperature as mentioned in previous section) were carried out in duplicate, at both shake flask level as well as reactor level, using two reactors, viz. a 2 L bioreactor (Make: Zenith, India) and 5 L bioreactor (Make: Sartorius B–lite, India). Shake flask confirmatory experiments resulted in production of 12.05 g L^{-1} of total solvents, and 9.28 g L^{-1} of butanol.

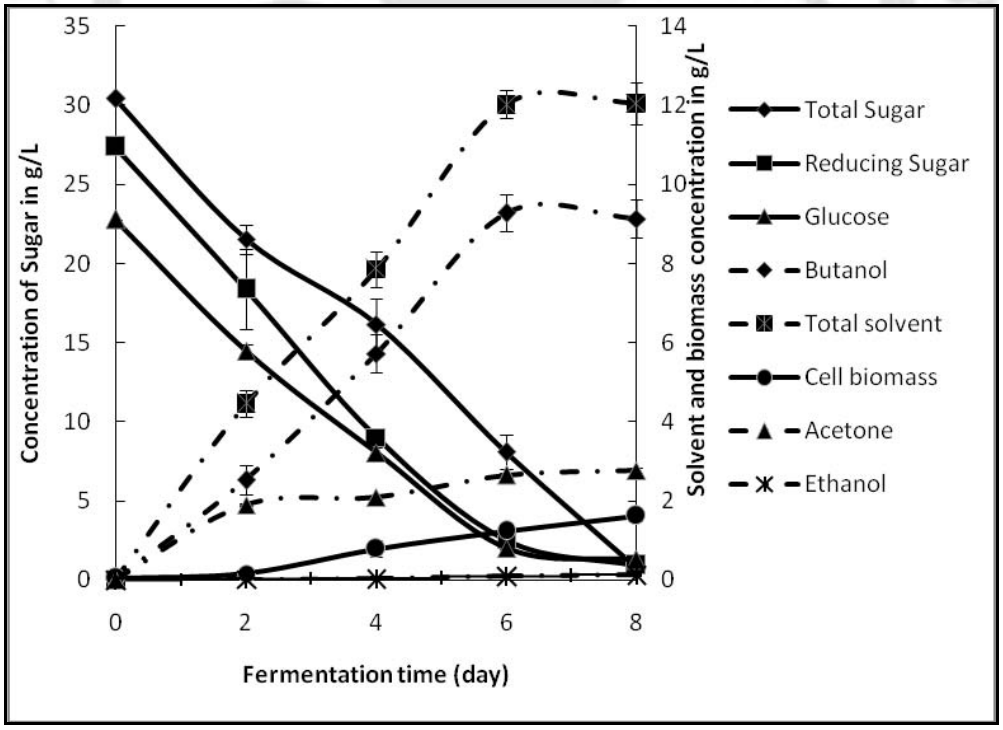


Figure 7.38: Time course of solvent production and sugar utilization in the shake flask experiment with optimized fermentation medium.

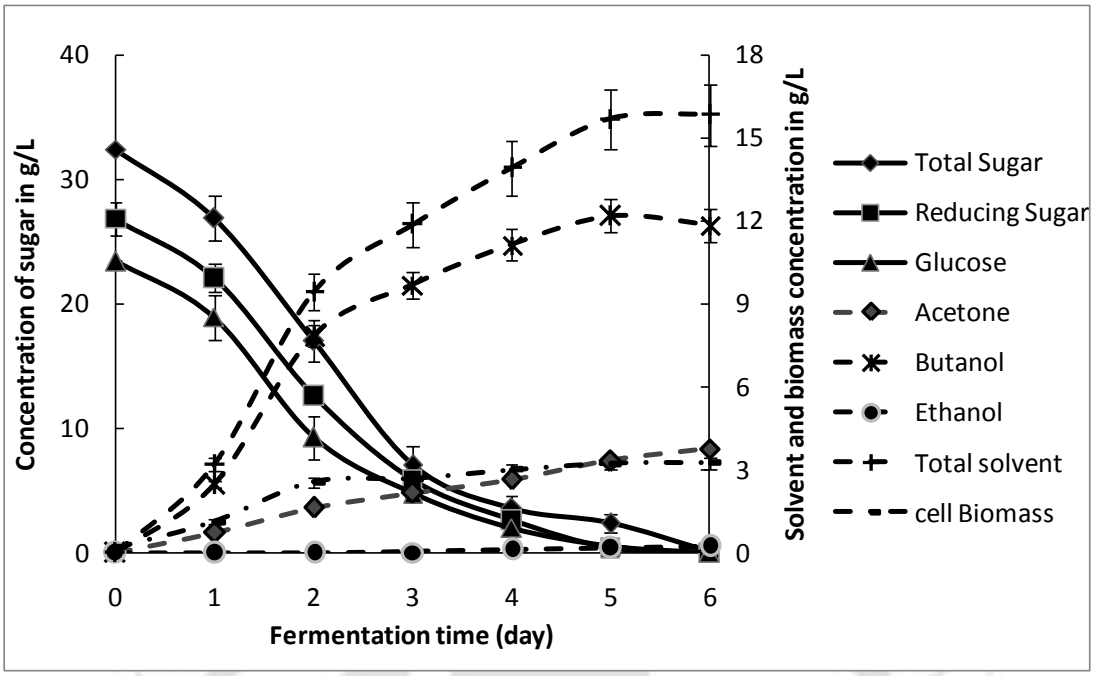


Figure 7.39: Time course of solvent production and sugar utilization in 2 L bioreactor experiment with optimized fermentation medium.

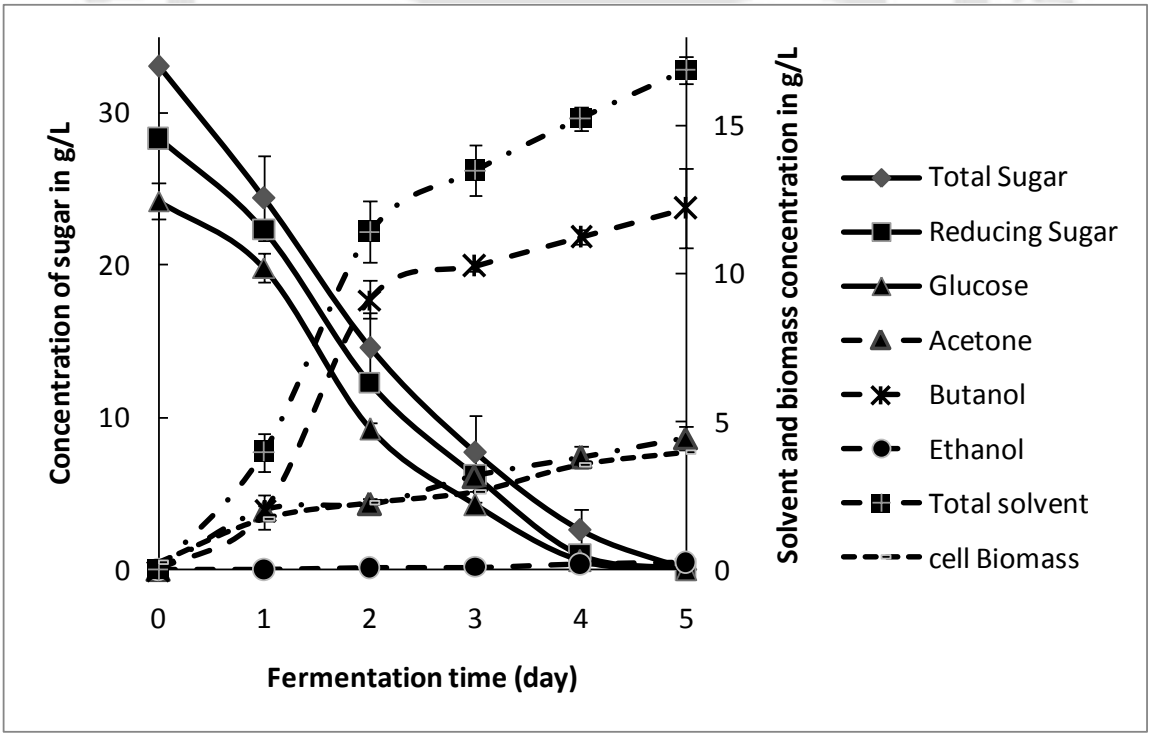


Figure 7.40: Time course of solvent production and sugar utilization in 5 L bioreactor experiment with optimized fermentation medium.

Table 7.11: Summarization of butanol and total solvent yield, and butanol selectivity

Process Optimized at Different Scale	Yield of total sugar (g/g)		Selectivity for Butanol (mole / mole)
	Butanol	Total solvent (ABE)	
Shake flask	0.31 ± 0.016	0.39 ± 0.02	0.7
2 L Bioreactor	0.38 ± 0.02	0.49 ± 0.024	0.7
5 L Bioreactor	0.38 ± 0.021	0.51 ± 0.025	0.7

The time course of solvent production and sugar utilization by *C. acetobutylicum* MTCC 481 at shake flask scale and reactor scale is depicted in Figs. 7.38, 7.39 and 7.40, respectively. It was observed that clostridia were able to utilize most of the sugar present in RSH based fermentation medium. Nearly 94.44% glucose, 96.59% reducing sugar, and 97.47% total sugar were utilized during 8 days of fermentation. These results of high sugar utilization with concurrent production of solvents corroborate that the values of optimum process parameters as obtained by Taguchi ODE are indeed optimum values for *C. acetobutylicum* MTCC 481. With scaling up of this process in a 2 and 5 L bioreactor, results were more encouraging. Nearly 12.17 g L^{-1} of butanol, and 15.8 g L^{-1} of total solvent were produced in only 6 days in a 2 L fermentor, while nearly 12.2 g L^{-1} of butanol, and 16.9 g L^{-1} of total solvent were produced in only 5 days in a 5 L fermentor with almost complete utilization sugar. The variations of clostridial biomass concentration with fermentation time and solvent concentration is shown in the surface plot in Fig. 7.41A, while Fig. 7.41B shows the variations of butanol concentration with glucose concentration and fermentation time. These values are in good agreement with the predicted results by Taguchi method. Moreover, these results were quite comparable to the values previously attained in the experiment trial 7, for which the fermentation was carried out at pH 4.0 ± 0.5, 37^{o}C, agitated at 150 rpm with 5% of 24 h old inoculums (Table 7.8). Table 7.11 further summarizes yield and selectivity for the

target product, i.e. butanol. We would like to specifically mention that yield of a product (butanol and total solvent) is calculated on the basis of initial concentration of total sugar analyzed in the fermentation medium, while butanol selectivity is determined on the basis of moles of butanol produced in the process against total moles of the solvent. Yield of butanol and total solvents at reactor scale was higher than flask scale, while selectivity for butanol, at all the scale of operation was found to be nearly similar (~ 0.7). The reduction in fermentation time, elevated solvent production, and higher substrate utilization profile in the bioreactor is attributed to precise control of physical parameters resulting in generation of ideal anaerobic conditions, in the microprocessor based bioreactor control systems. On the other hand, in shake flask experiments, the control of process parameters and anaerobic conditions could have been less precise, due manual operations.

Table 7.12 gives a comparative evaluation of our results with earlier optimization studies of ABE fermentation published in literature. We would like to specifically point out that a direct quantitative comparison of our results with these studies is not possible because the values of optimized parameters as well as the butanol production achieved under optimized parameter is highly system specific, as it depends on the substrate used, microbial strain employed and mode of operation of fermentation. However, this Table 7.12 can be used as a reference for qualitative assessment of the feasibility of rice straw as substrate for butanol production. It could be perceived from this Table 7.12 that the butanol production observed with rice straw (12.7 g L^{-1}) in our study at optimized condition is at par with those reported by other authors with substrates such as sweet potato (11.36 g L^{-1}), Jerusalem artichoke (13.6 g L^{-1})and maize stalk juice (11.5 g L^{-1}). The cost of rice straw, however, is far lower than these substrates. Thus, rice straw based fermentation at optimized conditions is a potential process for highly economic production of biobutanol.

Table 7.12: Table of comparison of the results of present study with earlier literature

Reference	Micro–organism	Substrate	Optimization Approach	Mode of Fermentation	Butanol ($g\ L^{-1}$)	Parameters
Ranjan et al. (This paper)	*C. acetobutylicum* MTCC 481	Rice straw	Statistical	Batch	12.7	Physical [Temperature: 37°C, pH = 4.0 ± 0.5, Agitation = 150 rpm] and cultural [Inoculum age = 18 h, Inoculum size = 5%]
[45]	*C. acetobutylicum.* NCIMB 13357	Date fruit	Conventional	Batch	4.4	Physical [Temperature = 35°C and pH = 7] and Nutritional
[49]	*C. acetobutylicum* IFP 904	Jerusalem artichoke	Conventional	Batch	13.6	Physical [pH = 5.5] and Nutritional
[50]	*C. acetobutylicum* IFP 904	Corn cobs	Conventional	Batch	10.5	Cultural and Nutritional
[51]	*C. acetobutyficum* P262	Sweet potato	Statistical	Batch	11.36	Nutritional
[52]	*C. acetobutyficum* MEMS–7	Molasses	Conventional	Batch	13.2	Nutritional
[53]	*C. beijerinckii* NCIMB 8052	Rice Bran	Conventional	Batch	12.24	Pretreatment
[54]	*C. acetobutylicum*	Glucose	Statistical	Continuous	7–8 g/L/h	Nutritional
[55]	*C. beijerinckii* ATCC 55025	Wheat bran	Conventional	Batch	8.8	Nutritional
[56]	*C. pasteurianum* DSM525	Glycerol	Statistical	Batch	6.0	Nutritional
[57]	*C. beijerinckii* NCIMB 8052	Maize stalk juice	Statistical	Batch	11.5	Physical [pH = 6.7, agitation = 48 rpm and Nutritional
[58]	*C. acetobutylicum* ATCC 824	Corn stover	Conventional	Batch	8.3	Pretreatment
[59]	*C. beijerinckii* ATCC 10132	Synthetic medium (Glucose/ Sucrose/ Fructose/ Molasses/Malt extract/ Glycerol)	Conventional	Batch	20.0	Physical [pH = 6.5, Temperature = 37°C] and Nutritional
[60]	*C. acetobutylicum* CICC 8008	Corn straw	Statistical	Batch	6.57	Nutritional
[61]	*C. butylicum* TISTR 1032 *and Bacillus subtilis* WD 161	Cassava starch	Statistical	Batch with Coculture	6.12	Nutritional
[62]	*Clostridium sp.*	Synthetic medium (Glucose)	Statistical	Batch	12.4	Nutritional

7.6.4 Kinetic analysis of solvent production, biomass growth, and utilization of sugars

It can be seen from Figs. 7.38, 7.39 and 7.40 that as compared to the shake flask experiments, the fermentation in bioreactor resulted in reduced lag phase, with an exponential phase of up to 36 h, followed by an extended stationary phase of 96 h, during which production of solvents occurred. This observation is consistent with results reported in our earlier paper on the clostridial biomass growth and butanol production [63]. Solvent production profiles in shake flask and bioreactor experiments showed that the solvent production (including butanol) occurred until 192 h of fermentation cycle. The concentration profiles of sugars (total sugar, reducing sugar, and glucose) in the medium for both shake flask and bioreactor scale experiments showed that they are readily utilized by clostridial cells.

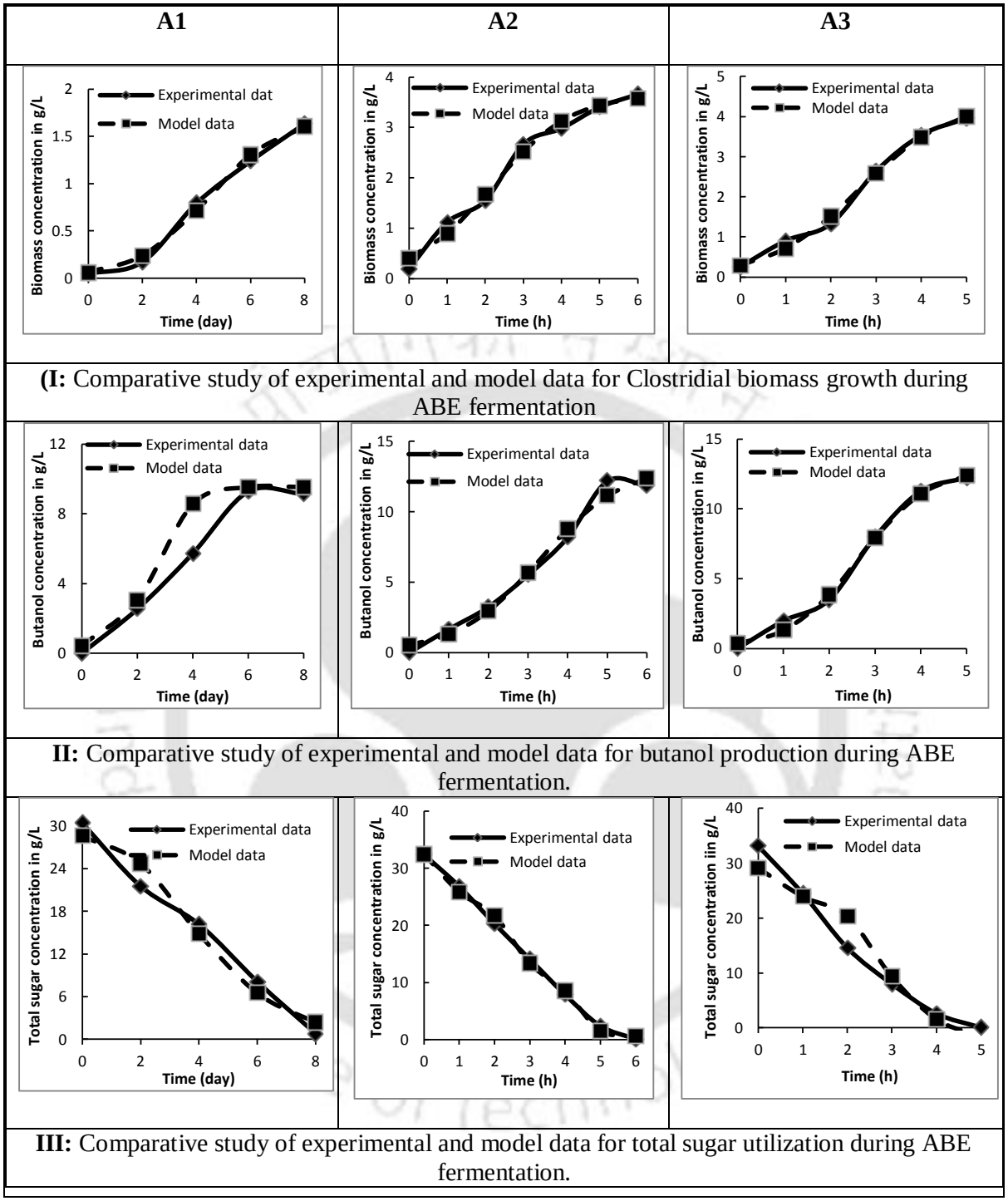
Further, to estimate the bio–kinetic constants involved in the process, models based on Monod kinetics reported in literature were fitted to the experimental data of butanol production, clostridial biomass growth, and utilization of sugar [62, 64]. Table 7.13 presents the kinetic models applied in this study along with the calculated kinetic parameters by fitting the experimental data using Matlab 7.12. These models are essentially unstructured logistic models originally proposed by Mercier et al. [64] for describing the kinetics of biomass growth, substrate consumption, and product accumulation. The estimated kinetic parameters values obtained from these models for both shake–flask and bioreactor scale (2 and 5 L) experiments are listed in Table 7.13. Fig. 7.42 summarizes the comparative study of experimental and model data. The regression coefficients (R^2) for the models fitted to the experimental data are ≥ 0.93, which is indicative that the model prediction is in good agreement with the experimental data. Further, goodness of the model fitting is also determined in terms of lower values of the root–mean–square deviation (RMSD) or root–mean–square error (RMSE), and these values have been listed in Table 7.13. The values of

P_{max}, i.e. maximum product concentration predicted by the model at shake flask scale is 9.59 g L^{-1} which is in good agreement with experimental results attained being 9.27 g L^{-1}. Similarly model P_{max} values were reportedly 13.33 and 12.92 g L^{-1} for 2 and 5 L reactor respectively, which was again in good sync with the experimental data, being 12.17 and 12.22 g L^{-1} for 2 and 5 L bioreactor systems respectively, mentioned in previous section.

For both shake flask and bioreactor scale experiments, the specific growth rate of clostridial cultures has been moderate ($\mu \approx 0.7$ to 0.99 h^{-1}), which is yet another corroboration that the process parameters obtained from Taguchi experiments are indeed optimum for the fermentation process for butanol production, where the cells utilize most of their energy for product formation rather than cellular growth. The kinetic parameter values listed in Table 7.11 show an interesting trend in that the values of P_t and μ are higher for bioreactors than shake flasks. These parameters are characteristics of the kinetics of the fermentation process taking place at that particular level. Elevated values of μ and P_t for bioreactor scale experiments using optimized process parameters indicate that at these conditions the scale of operation of the process has a conducive effect on the product formation, in that the extent of product formation increases.

7.7 Study of effect of removal of precipitate from optimized fermentation medium

In this section, we have attempted to study the effect of removal of precipitate produced during the maintenance of pH of RSH based fermentation broth during final process optimization experiments. During pre–treatment, biomass was subjected to severe conditions such as a combination of high temperature and reaction with chemicals such as (i) dilute sulfuric acid, (ii) dilute alkali, (iii) agitation, (iv) hot water, and (v) high pressure.



(I: Comparative study of experimental and model data for Clostridial biomass growth during ABE fermentation

II: Comparative study of experimental and model data for butanol production during ABE fermentation.

III: Comparative study of experimental and model data for total sugar utilization during ABE fermentation.

Figure 7.41: Verification of experimental data through comparative study with model data (A.1) Flask scale optimized process; (A.2) 2 L Reactor scale optimized process; (A.3) 5 L Reactor scale optimized process

Table 7.13: Kinetic parameters (estimated by fitting model rate equations to experimental data) for fermentation under optimized conditions at levels of shake flask, 2 L bioreactor and 5 L bioreactor.

Component and Rate Equation	Flask Scale Experiments			2 L Reactor Scale Experiments			5 L Reactor Scale Experiments		
	Kinetic Parameters	**R^2**	**RMSE**	**Kinetic Parameters**	**R^2**	**RMSE**	**Kinetic Parameters**	**R^2**	**RMSE**
Butanol $X = \frac{X_0 X_{max} e^{\mu t}}{X_{max} - X_0 + X_0 e^{\mu t}}$	P_0 = 0.43 P_{max} = 9.6 P_t = 0.9	0.99	0.65	P_0 = 0.53 P_{max} = 13.33 P_t = 0.96	0.98	0.2	P_0 = 0.38 P_{max} = 12.92 P_t = 1.32	0.99	0.51
Biomass $P = \frac{P_0 P_{max} e^{p_t t}}{P_{max} - P_0 + P_0 e^{p_t t}}$	X_0 = 0.06 X_{max} = 1.7 μ = 0.75	0.99	0.09	X_0 = 0.41 X_{max} = 3.67 μ = 0.96	0.98	0.76	X_0 = 0.29 X_{max} = 4.39 μ = 1	0.99	0.16
Total Sugars $S_{M0} - S_M = \frac{1}{Y_{P/M}}(P_0 - P_{max}) + \frac{1}{Y_{X/M}}(X - X_0)$	S_0 = 28.04 $Y_{P/S}$ = 1 $Y_{X/S}$ = 0.09	0.96	3.2	S_0 = 30.74 $Y_{P/S}$ = 0.82 $Y_{X/S}$ = 0.2	0.99	1.14	S_0 = 28.55 $Y_{P/S}$ = 1.01 $Y_{X/S}$ = 0.2	0.93	3.7

It has been previously reported (Qureshi et al. [65]) that such severe pretreatment conditions may lead to production of certain fermentation inhibitors such as salts, phenolic acids (r–coumaric and ferulic), and aldehydes etc. It has also been reported that certain salts act as potent fermentation inhibitors. Salt type depends upon the biomass in use (Soni et al. [66]). In this section we have removed the precipitate via centrifugation and the hydrolyzate was allowed to undergo fermentation under completely optimized conditions (pH = 4.0 ± 0.5; temperature = 37^{o}C, agitation = 150 rpm, inoculums size = 5%, inoculums age = 18 h). Table 7.14 presents a comparative study of fermentation carried out with and without precipitate removal. An interesting result observed was that removal of salt precipitate resulted in reduced solvent production. Removal of precipitate resulted in production of only 2.8 g L^{-1} of acetone, 5.2 g L^{-1} of butanol and 0.12 g L^{-1} of ethanol, while as reported in previous section fermentation carried out with precipitate resulted in production of 2.8 g L^{-1} of acetone, 9.2 g L^{-1} of butanol and 0.14 g L^{-1} of ethanol. Fig. 7.43 illustrates the time history of solvent production and sugar utilization for flask–scale fermentation performed with removal of precipitate The phase purity of the calcined residue was analyeed by X–ray diffraction (XRD). Fig. 7.44 shows the XRD patterns of the calcined residue.

The pattern of the solid residue matches well with the standard patterns of iron oxide (hematite, JCPDS file No. 72–0469). Some of the major peak pattern of the solid reside can be readily indexed to Fe_2O_3 with rhomb–centered lattice structure, where the diffraction peaks at 2θ values of 33.118, 35.612, 65.971 can be ascribed to the reflection of (104), (110), (220) and (125), respectively. There is no detectable CuO phase present in the sample. There are some significant XRD peak that could not be indexed. Thus, XRD pattern suggest the presence of iron salt as major compound in the sample. The detection of iron salt in the precipitate supports our finding. Until the pH of fermentation broth was acidic, salt was in ferrous state, and hence, soluble in the broth.

Table7.14: Study of effect of removal precipitate from fermentation broth on solvent production

Experiment with Optimized Process	Yield in terms of total sugar (g/g)		Butanol selectivity in terms of total solvents
	Butanol	Total solvent (ABE)	
After removal of precipitate	0.19±0.020	0.29±0.02	0.62
Without precipitate removal	0.31±0.016	0.4±0.02	0.70

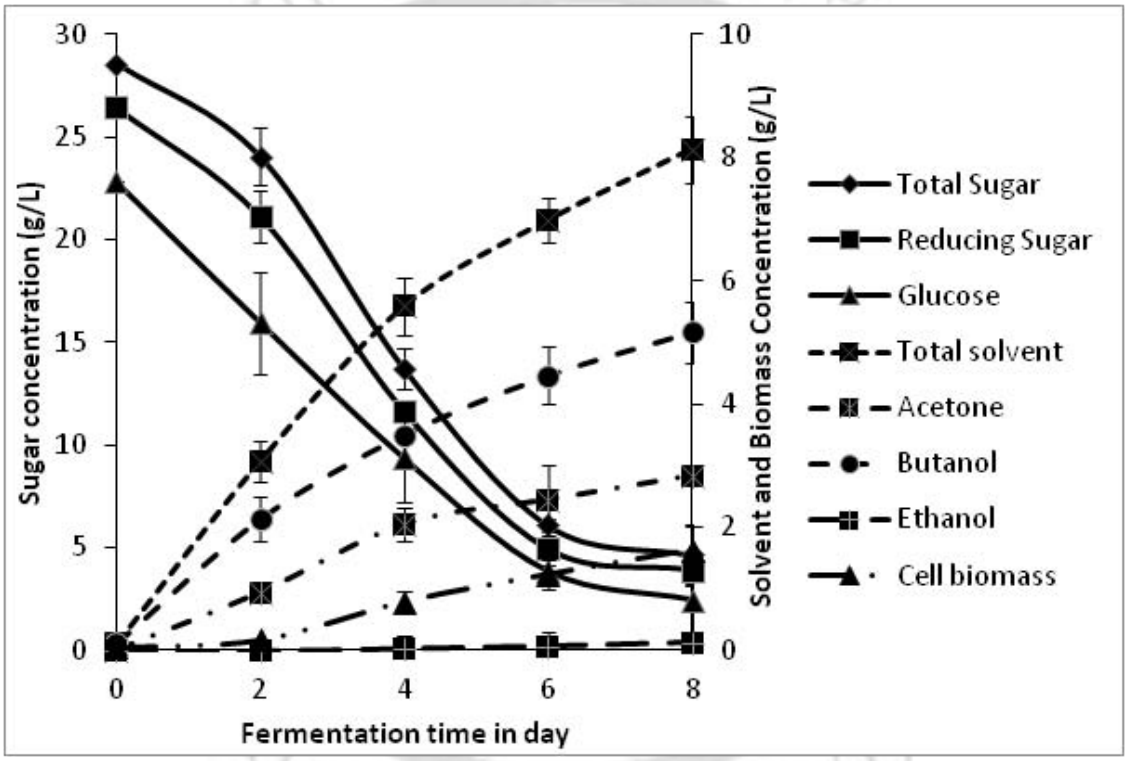


Figure 7.42: Time history of sugar utilization, solvent and biomasss production when fermentation was carried out with precipitate removal

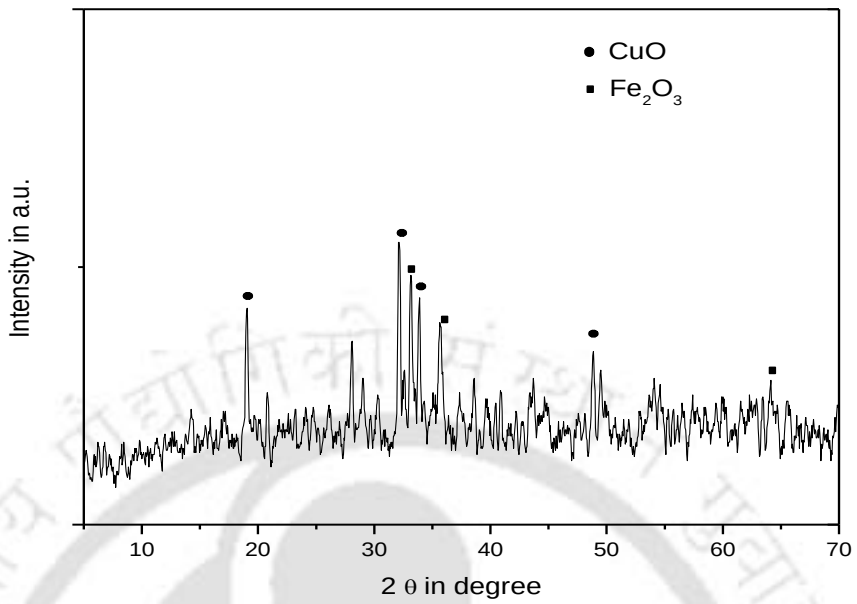


Figure 7.43: XRD diffractogram of the calcined precipitate

However, the addition alkali resulted in rise of pH changing ferrous to ferric state resulting in the formation of precipitates. Dehydrogenases are the key enzymes of clostridial metabolic cycle. One of the family of alcohol dehydrogenases, are iron containing ones. They occur in bacteria and fungi and are oxygen–sensitive. Members of the iron–containing alcohol dehydrogenase family includes *Clostridium* acetobutylicum's NADPH and NADH dependent butanol dehydrogenases (genes adh1, bdhA and bdhB) enzymes, which play key role in solvent production (Wiesenborn et al. [67]; Demain [68]). During initial exponential phase, when clostridia undergoes acidogenesis (pH), these dehydrogenase utilize the Fe^{+2} ions as their cofactors resulting in higher enzyme activity, leading to enhanced solvent production. Because of these reasons the removal of precipitate from fermentation broth resulted in reduced production of solvent.

7.8 Conclusion

In this Chapter, we have presented our studies in optimization of the rice straw based ABE fermentation process for biobutanol production. This study has been carried out in two phases, viz. preliminary phase in which we determined the influence of different physical and cultural parameters on the fermentation process, and in the second phase we carried out experiments based on Taguchi L18 array method for optimization of the process.

The optimum conditions of the rice straw based ABE fermentation process with the microbial strain of *Clostridium acetobutylicum* MTCC 481 are: temperature = 37^{o}C, pH = 4.0 ± 0.5, inoculum size = 5% v/v, inoculum age = 18 h, agitation rate = 150 rpm. Experiments done at higher scale of 2 and 5 L bioreactor corroborate these results. The analysis of experimental results has given an interesting result that the physical parameters of pH, temperature, and agitation rate are of more significance for butanol production than cultural parameters such as inoculums size and age. This result has high significance from the viewpoint of scale–up of the process. Another interesting result of this study is that at optimum process conditions, the scale of the experiments has conducive effect on butanol production in that the extent of production increases with scale of operation. The extent of butanol production at optimized conditions is at par with the production with other alternate substrates reported in literature. However, relatively much lower cost of rice straw than these alternate substrates make the rice straw based fermentation process economically more sound and viable. The process of ABE fermentation with rice straw as substrate can well be extended into a biorefinery. The main products of the process would be butanol, ethanol and acetone. The solid residue remaining from the fermentation can be utilized as animal fodder after separation, washing and drying and nutritional enhancement. The CO2 during fermentation is extremely pure and can be utilized by soft drinks industry.

Abbreviations and Notations

ABE	Acetone Butanol Ethanol
ANOVA	Analysis of Variance
CMM	Cooked Meat Medium
DNS	Dinitrosalicylic Acid
DOE	Design of Experiments
Ea	Activation Energy
μ	Specific growth rate (h^{-1})
K	Kinetic rate constant
MS	Mean of squares
MTCC	Microbial Type Culture Collection
NCIM	National Collection of Industrial Micro-organisms
P	Product Concentration (g dm^{-3})
P_0	Initial product concentration (g dm^{-3})
P_{max}	Maximum product concentration (g dm^{-3})
P_t	Kinetic constant
PABA	p-aminobenzoic acid
RCA	Reinforced Clostridial Agar
RCM	Reinforced Clostridial Medium
RMSD	Root Mean Square Deviation
RMSE	Root Mean Square Error

RS	Rice Straw
RSH	Rice Straw Hydrolysate
SS	Sum of squares
X	Biomass concentration (g dm^{-3})
X_m	Maximum biomass concentration (g dm^{-3})
X_0	Biomass concentration (g dm^{-3})
$Y_{P/S}$	Product yield on the utilized substrate
$Y_{X/S}$	Biomass yield on the utilized substrate

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CHAPTER 7

CHAPTER 8

OVERVIEW AND SCOPE FOR FURTHER RESEARCH

8.1 Overview

Fast depletion of fossil fuels leading to large hike in the prices of crude oil, coupled with concerns of global warming due to green house gas emission have made quest for an alternate, renewable liquid transport fuel mandatory. Among the several alternate fuels that have emerged in the past two decades, biobutonal offers a special merit over other fuels such as biodiesel, bioethenol & biomethanol due to its very similar properties as gasoline. The economic analysis of biobutanol manufacture on commercial scale has shown that cost of feedstock (or the recurring cost of raw materials) forms the major component contributing to per unit manufacturing cost of biobutanol. Use of a cheap, alternate substrate for the ABE fermentation process can boost the economy of biobutanol manufacture. It is in this spirit that current thesis work was undertaken, and an attempt was made to develop a lab-scale process for rice-straw based ABE fermentation, and optimize it in terms of various physical and

physiological facets. The previous chapters have given detailed accounts of our research. These results when put together give an interesting story of step-by-step evolvement of rice-straw based fermentation process. We summarize the major findings and conclusions of different chapters of this thesis (which essentially addressed different facets of the fermentation process) below:

In Chapter 2, we presented a comprehensive review of literature in the area of ABE fermentation that addressed seven important aspects of this process such as basic biochemistry and metabolic pathway, microbial cultures, substrates, solvent recovery, fermentation design and economics.

In Chapter 3, we presented an overview of the literature addressing use of rice-straw (which is an abundantly available biomass in India throughout year) for synthesis of liquid and gaseous biofuels. In addition, we also presented a brief account on structure of rice-straw and its physical and chemical properties.

In Chapter 4, we presented results of study on screening and selection of microbial culture for the process among those available in microbial were MTCC-481, NCIM-2337, NCIM-2877, NCIM-2878. The media used was reinforced clostridial media supported with glucose (2% w/v). The best results with consistency were obtained with MTCC-481 strain, and hence, it was chosen for further process development. To further understand the physiology of ABE fermentation, experiments were performed with MTCC 481 strain in RCM supplemented with 2%, 4%, 6%, and 8% w/v glucose and 2.5%, 4%, 5% and 6% xylose. An interesting

result was obtained with these results in that the total production showed a maxima with substrate concentration (6% for glucose and 5% for xylose). We attributed the reduction in total solvent production with higher substrate concentration to inhibition induced by substrate. Concurrently with screening of microbial cultures, we also screened different alternate substrates such as fruit waste, rice-straw, rice-husk and bagasse for ABE fermentation. All substrates were acid hydrolyzed (0.5% w/v sulfuric acid) prior to fermentation. The highest total solvent yield along with the highest selectivity for butanol was obtained for rice straw hydrolyzate, and thus, rice straw was confirmed as the biomass feedstock for further experiments.

Chapter 5 describes the studies in the next stage in process development, i.e. optimization of the pretreatment of rice-straw for release of sugars prior to fermentation, which influences the total yield of solvent. Being energy intensive, this stage is also a cost intensive component of the process. In order to optimize this stage, we attempted different individual techniques as well as their combination such as physical treatment of washing/cutting/grinding, steam explosion, agitation, chemical treatment of acid/alkali hydrolysis and enzymatic treatment. Another parameter of this process to be optimized was concentration of rice-straw in aqueous solution. Four values of this parameter, viz. 1, 3, 5 and 7% were attempted. The major result of this study was that physical treatment was necessary, but it needed to be assisted by suitable chemical/enzymatic treatment for effective sugar release. Among all treatment techniques attempted, the best results were obtained with physical treatment (autoclaving at 15 psi; pressure at 121^{o}C for 15 min) followed by 1% w/v acid treatment at 5% w/v rice-straw concentration.

In Chapter 6, we presented the results on study on development of rice straw based medium (with nutritional enhancement) for butanol production. Initially, using method of one variable at a time, we assessed extract of 12 nutritional factors on solvent production. Four factors among these were selected on the basis of butanol production. A Taguchi L_8 experimental array was designed using these factors, and butanol production in each of these was assessed, and two significant factors (i.e., PABA and Yeast extract) were indentified on the basis of signal to noise ratio. ANOVA based on the results of Taguchi experimental design matrix also gave the similar result.

The validation of the optimum medium composition predicted by the Taguchi experimental design was done initially at shake flask level scale followed by reactor scale. The kinetic data of these experiments was analyzed using unstructured models. At this stage, under optimized condition of media, we obtained an interesting result that the kinetic parameters of the model were essentially same at both shake flask as well as reactor scale experiments.

Chapter 7 addresses the issue of optimization of the process in terms physical and physiological parameters. The physical parameters were pH, agitation rate and temperature, while physiological factors were size and age of the inoculum added to fermentation reactor. Preliminary experiments were done to assess the relative influence of these parameters. This was followed by a statistical optimization of the process based on L18 Taguchi experimental design. The values (or levels) of the above-mentioned parameters used in Taguchi experimental design were decided on the basis of preliminary experiments. The medium used for these experiments was optimized medium (obtained in the study described in Chapter 6). Results of Taguchi experimental design revealed following optimum conditions for maximum production of butanol by Clostridial strains: temperature = 37^oC, pH = 4 ± 0.5,

Table 8.1: Improvements in process performance at various stages of optimization (Yardsticks: Solvent yield, Butanol yield and Butanol selectivity)

Sl. no	Particulars	Solvent yield	Butanol yield	Butanol selectivity	Increase in butanol yield (n fold – denoted as ×)
1.	Un-optimized process	0.087	0.009	0.083	-
2.	Optimized RS pretreatment	0.14	0.06	0.34	6.6×
3.	RS based developed medium	0.25	0.15	0.57	17×
4.	Complete Process optimized	0.4	0.31	0.7	34.4×
5.	Removal of salts	0.29	0.19	0.32	21.1×

inoculums size = 5% v/v, inoculum age = 18 h, agitation rate = 150 rpm. Among all parameters, pH, temperature, and agitation were the most significant parameters influencing solvent production. The optimized physical and physiological parameters were further verified at shake-flask and bioreactor scale (2 and 5 L bioreactor). Experiments using 2 L and 5 L bioreactor under optimized process conditions showed nearly complete sugar utilization of soluble sugar with production of 15.849 g/L of total solvents (including 12.84 g/L of butanol) in 2 L bioreactor, and 16.91 g/L of total solvents (including 12.22 g/L of butanol) in a 5 L bioreactor, respectively. This again was an interesting result in that the solvent production increased with scale of operation under optimized condition of physical and physiological parameters. Finally, we have also attempted to study the effect of removal of precipitate produced during maintenance of pH of developed medium under optimized physical conditions. Removal of precipitate resulted in reduced solvent production. This was justified by XRD analysis of precipitate, which reported dominant presence of Fe^{2+}. Fe^{2+} being and important cofactor of key enzymes involved in Clostridial fermentation as mentioned in Chapter 2 was a desired nutrient for enhanced solvent production, and

therefore, removal of Fe^{2+} precipitate resulted in poor solvent yield.

Table 8.1 presents a comprehensive overview of process development study presented in this thesis using the total solvent production and butanol production as yardsticks. The improvement in these two yardsticks clearly indicates the step-by-step optimization of process, and the betterment of process obtained therewith. If this process is scaled up in its present state, where the total butanol yield in 5 liter bioreactor is 0.38 g/g total sugar, with total sugar yield in this being 55% w/w of rice straw, the potential of bio butanol production is about 25 million ton per 100 million ton of rice straw.

8.2 Scope for Further Research

This thesis has attempted to explore feasibility of rice straw based ABE fermentation process for production of butanol on lab scale. The results of study that addressed different facets of the fermentation process have clearly established the potential of this process, and also its promise for scale up, which could further lead to commercialization. Improvement of the butanol yield and selectivity by different means can further boast the promise of this process for commercialization. We give below some suggestions for further research to take ahead the theme. We believe that the results of the present study form important input and guideline for further research.

- The mode of fermentation used in the present experiment study was batch mode. The yield can be improved using fed batch and continuous mode of operation. These modes of fermentation are more suitable for a large-scale process than the batch mode, which is more susceptive towards solvent inhibition.
- Used of immobilized culture can give better control over cell density in the system, and also reduces solvent inhabitation and cell recycle.

- In situ solvent removal and recovery can also help reduce the solvent inhabitation and improve yields. Several methods have been reported in literature (see Chapter 2) for efficient removal of solvent such as liquid extraction, gas stripping, membrane based separation etc; and these can be attached along with fed batch and continuous mode of fermentation.
- Used of better genetically engineer/ microbial strains that have better selectivity towards butanol as well as higher tolerance to solvent removal can further enhance the economics of this process.
- The hydrolysis of rice straw prior to fermentation is an energy intensive process this could be eliminated by use of co-culture of clostridia with saccharolytic fungi such as *T. reesei*. Simultaneous Saccharification and Fermentation (SSF) with co-cultures of Clostridia and *T. reesei* could be another process alternative, which is worth further investigation.

CHAPTER 8

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RESEARCH OUTPUT OF THE THESIS

JOURNAL PAPERS

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