Biobutanol from lignocellulosic biomass by a novel *Clostridium sporogenes* BE01

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DECLARATION

I hereby declare that the work presented in this thesis entitled "**Biobutanol from lignocellulosic biomass by a novel *Clostridium sporogenes* BE01**" is a bonafide record of research carried out by Ms Lalitha Devi Gottumukkala (Reg # 4179), under my supervision, at the Centre for Biofuels, Biotechnology Division of CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum, India. I also declare that this work or no part of this work has been submitted for the award of any degree, diploma, associateship or any other title or recognition. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the doctoral committee of the candidate has been incorporated in the thesis.

Rajeev Kumar Sukumaran

Thiruvananthapuram

24 July 2014

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Biobutanol from lignocellulosic biomass by a novel *Clostridium sporogenes* BE01**" is original work done by me under the supervision of Dr Rajeev Kumar Sukumaran, at the Centre for Biofuels, Biotechnology Division of CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum, India. I also declare that this work did not form part of any dissertation submitted for the award of any degree, diploma, associateship, or any other title or recognition from any University/Institution

Lalitha Devi Gottumukkala

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CONTENTS

Chapter 1

Introduction and Review of literature

1. Introduction

1.1. Butanol: An alternative fuel

Next generation biofuels from renewable sources have gained interest among research investigators, industrialists, and government due to major concerns on volatility of oil prices, climate change and depletion of oil reserves (Dellomonaco et al, 2010). Recently, among the liquid transportation biofuels, biobutanol has drawn significant attention from researchers worldwide, due to its superior fuel properties than ethanol. Advantages of butanol over ethanol are its high energy content, better blending with gasoline, less hydroscopic nature, lower volatility, direct use in convention engines, low corrosiveness etc. Fuel properties of butanol are given below in table 1.1. Butanol is chemically synthesized by Oxo synthesis, Reppe synthesis and crotonaldehyde dehydrogenation (Lee et al, 2008).

Table 1.1: Fuel properties of Butanol

	Methanol	Ethanol	Butanol	Gasoline
Energy density (MJ/L)	16	19.6	29.2	32
Air-fuel ratio	6.5	9	11.2	14.6
Heat of vaporization (MJ/Kg)	1.2	0.92	0.43	0.36
Research octane number	136	129	96	91-99
Motor octane number	104	102	78	81-89

Source, Lee et al, 2008

1.2. Microbial production of butanol

1.2.1. History of ABE fermentation

Microbial production of butanol has a long history and is referred as ABE fermentation, due to the mixture of acetone, butanol and ethanol produced in the ratio 3:6:1 during fermentation. The first report on production of butanol was in 1861 by Louis Pasteur. It was only in 1905 that Schardinger reported acetone production during fermentation. In 1911, Fernbach isolated a culture which was able to ferment potatoes, but not maize starch to produce butanol. In 1914, Weizmann isolated a culture capable of producing high quantities of butanol from various starchy substrates and this culture was later identified as *Clostridium acetobutylicum* (Jones & Woods, 1986)

Microbial production of acetone from potatoes and maize as the substrate was largely commercialized for rubber manufacture. During the First World War, acetone production expanded further for the manufacture of munitions. After the end of First World War, ABE fermentation was completely ceased due to the inefficient supply of substrates. Later, molasses came into picture as an efficient substrate and ABE fermentation was given top priority once again during Second World War. By 1960s ABE fermentation was virtually ceased in US and many other countries, as there was acute competition between fermentation and chemical routes due to growth in petrochemical industry and increased cost of the molasses, due to its demand for various processes (Jones & Woods, 1986). The ABE fermentation process in South Africa and Russia was continued till 1980 to 1990s. It was reported that Russian fermentation industry is focusing on conversion of biomass to butanol (Lee et al, 2008).

1.2.2. Clostridia in ABE fermentation

Clostridia are known for ABE fermentation for years and are classified into many species. Solventogenic Clostridia are re-classified into four species, *Clostridium acetobutylicum, C. beijerinckii, C. saccharobutylicum, C. saccharoperbutylacetonicum* (Johnson et al, 1997, Jones & Keis et al, 1995, Keis et al, 1995, Keis et al, 2001). *C. aurentibutylicum, C. pasteurianum* and *C. tetanomorphum* were also reported for butanol formation as major product (Gottwald et al, 1984, Jones & Woods, 1986, Biebl, 2001). Among all these bacteria identified till date for butanol production, *C. acetobutylicum* was well studied for its production and metabolism at genetic level.

1.2.3. Metabolism

Butanol fermentation is biphasic and occurs in two distinctive phases, acidogenic phase and solventogenic phase (Johnson, 1931). The first acidogenic phase occurs during exponential growth of the cells. Cells divide rapidly and produce acids. In the second phase, cell growth slows down and involves production of solvents by assimilating acids (Durre, 2005).

Elucidation of biochemical and transcriptional regulation of the acidogenic/solventogenic pathways deduced only fraction of metabolic network. The functionality of the pathway mostly depends on the byproducts and redox equivalents obtained from core metabolic pathway, ex: acetyl coenzyme A, ATP, NADP, NADPH etc. Transcriptome analyses have revealed that the complex network of solvent formation is interlinked with sporulation (Janssen et al, 2010, Jones et al, 2008). Combined usage of metabolomics, isotope racers and quantitative flux modeling directly mapped the metabolic events associated with acidogenic-solventogenic transition. Reduction in pyruvate carboxylase, amino acid biosynthesis, flux in reductive branch of TCA cycle

was revealed in association with NADPH and acetyl CoA drop during solventogenesis (Amadour-Noguez et al, 2011). Metabolic pathway with enzymes and factors involved for acidogenesis and solventogenesis in *C. acetobutylicum* is represented in detail in Fig 1.1.

Fig 1.1: Metabolic pathway for ABE fermentation in *Clostridium acetobutylicum*

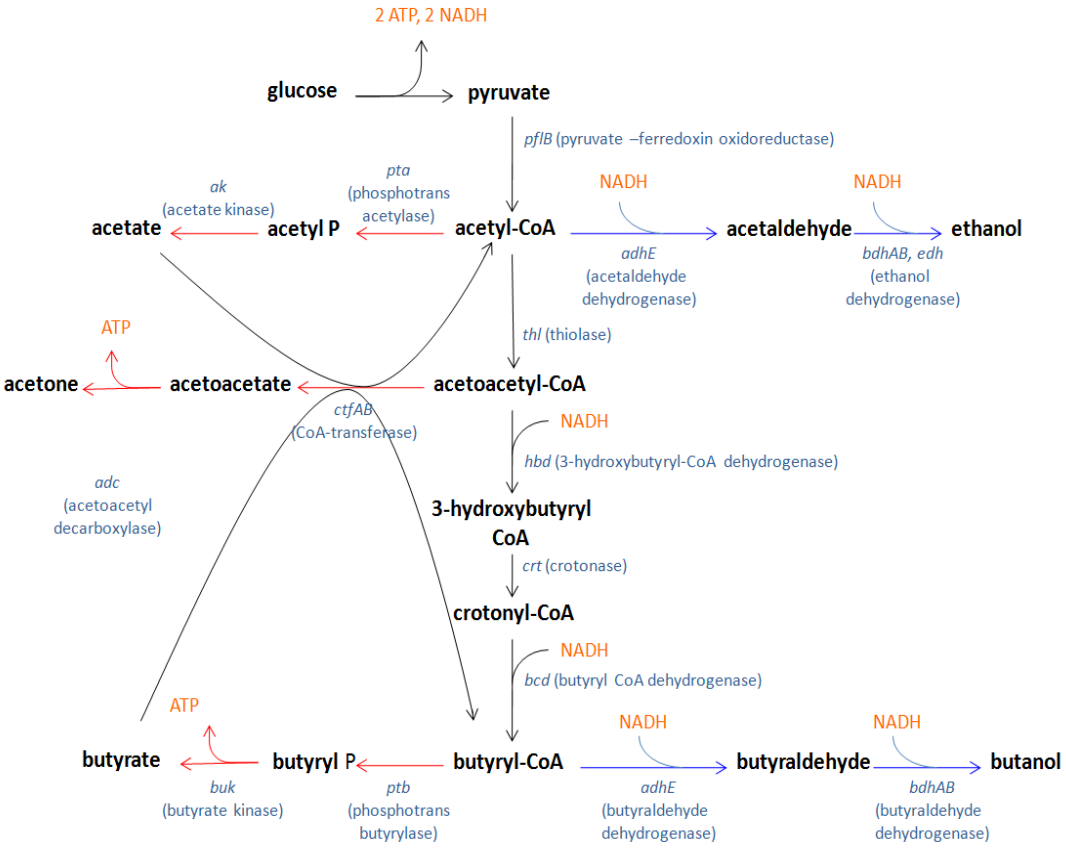


Figure 1: Metabolic pathways in *C. acetobutylicum*. Reactions during acidogenic phase and solventogenic phase are represented in red and blue arrows respectively. Pale blue letters describe the genes (in italics) and enzymes involved in the reactions

1.3. Butanol fermentation and downstream processing: Bottle necks and strategies for improvement

ABE fermentation is a well established bioprocess, but for industrial production of butanol, there are limitations and draw backs with the microbial processes. Cost of the

substrate, unproductive sporulation, ineffective carbohydrate utilization and reutilization of end products, solvent toxicity to the organism and recovery of butanol are the major challenges in biobutanol production. Inability of the Clostridial cultures to attain high cell density and large quantities of acetone formation are additional limitations in ABE fermentation.

Classical fed-batch and continuous cultivation do not seem to be economically feasible, because of solvent toxicity and the biphasic nature of acetone–butanol fermentation. To overcome this problem, fed-batch culture has been coupled with an *in-situ* recovery process (Ezeji et al, 2004 a,b), and multistage continuous fermentation has been conducted (Godin & Engasser, 1990). Two-stage fermentation is effective to increase the production of acids in acidogenic phase and to control the pH and improve solvent yields in solventogenic phase (Mustschlechener et al, 2000)

For selective improvement of butanol production in ABE fermentation, strategies like metabolic engineering of *Clostridia* for enhanced butanol production and reduced acetone formation, directed evolution for solvent tolerance, media engineering, advanced fermentation techniques, high cell density fermentation and in-situ solvent recovery methods were tried and reported by several researchers (Table 1.2).

Table 1.2: ABE Fermentation using simple sugars

	Substrate	Organism	Fermentation type	Butanol yield	References
Simple sugar	Maltodextrin/ Glucose	*C. beijerinckii* BA101	Batch	19 g/L	Formanek et al, 1997
	Glucose	*C. acetobutylicum* ATCC824	Batch	13.86 g/L	Monot et al, 1982

Glucose	*C. acetobutylicum*	Two stage continuous culture under phosphate limitation	175 Mm	Bahl et al, 1982
Glucose	*C. acetobutylicum* and *C. beijerinckii*	batch with CaCO3 supplementation	14.78 g/L	Richmond et al, 2011
Glucose	*C. beijerinckii* NRRL B592	Two stage continuous culture in semi synthetic medium	9.1 g/L	Mustschlecheher et al, 2000
Glucose	*C. acetobutylicum*	Controlled pH	12.3 g/L	Yang et al, 2013
Glucose	*C. acetobutylicum*	Cryo gel beads immobilization	14.47 g/L	Tripathi et al, 2010
Glucose	*C. acetobutylicum*	Batch fermentation, pervaporation	32.8 g/L Total solvents	Evans & Wang, 1988
Glucose	*C. acetobutylicum*	Fed batch fermentation, pervaporation	165 g/L Total solvents	Evans & Wang, 1988
Glucose	*C. beijerinckii* BA101	Continuous fermentation, gas stripping	460 g/L Total solvents	Ezeji et al, 2004a

Since acetone is not qualified as fuel, due to its corrosiveness to engine parts made of rubber or plastic, trials were ongoing to reduce the production or even completely suppress it by cell or bioreactor engineering. Reducing the acetone production was considered as an important objective to eliminate the undesirable conversion of substrate to acetone and increase the butanol yield per unit mass of substrate utilized. Attempts to reduce acetone by metabolic engineering were made by down regulating ctfB gene and adc gene by antisense RNA (asRNA) and mobile group II

intron respectively, but were not successful, as it also resulted in decreased butanol production (Lee et al, 2012). A natural non-acetone forming butanol producer will be of great importance for an efficient conversion of substrate to butanol. It also has great significance in downstream processing, as butanol and ethanol mixture without acetone contamination can directly go as transportation fuel.

1.3.1 Recovery methods

Gas stripping, perstraction, pervaporation and liquid-liquid extraction are the common product recovery methods reported for the recovery of low concentrations of butanol from fermentation medium and increasing the solvent yields (Zheng et al, 2009) Fig 1.2.

1.3.1.1 Liquid-liquid extraction

In liquid –liquid extraction, a water insoluble organic extractant is mixed with the fermentation broth. Since butanol is more soluble in the extractant, it gets concentrated in the organic phase. Solvents thus concentrated in the organic phase can be recovered by back extraction into another solvent or by distillation (Maddox, 1989). Oleyl alcohol which is relatively non toxic is generally used as the extractant (Evans &Wang, 1988, Karcher *et al*, 2005). Disadvantages of liquid –liquid extraction include toxicity of the extractant, formation of emulsions, loss of extractant and formation of rag layer by microbial accumulation at the liquid- liquid interface.

1.3.1.2 Perstraction

Perstraction is similar to liquid –liquid extraction in that it recovers butanol and other solvents into an extractant but a direct contact of the extractant and the fermentation broth is prevented by introduction of a contact membrane that separates the phases but allows

diffusion of ABE (Traxler *et al*, 1985, Grobben *et al*, 1993). While Perstraction can address toxicity of the extractant and also provide some selectivity in recovery of solvents by selection of membranes, there could be serious fowling issues and the process is relatively costly to operate.

1.3.1.3 Pervaporation

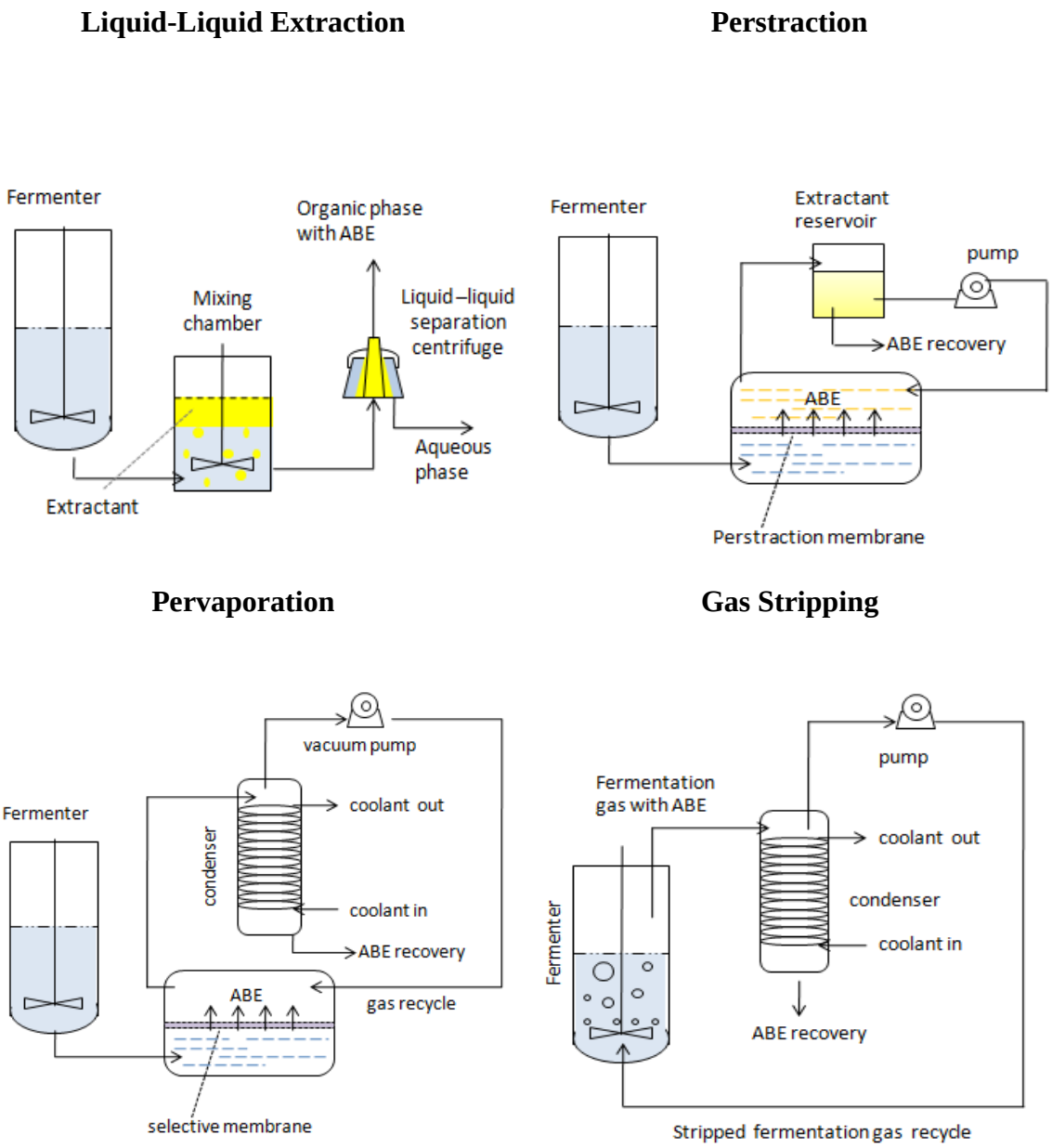
Pervaporation allows selective removal and recovery of volatiles from fermentation broth. Here the fermentation broth containing solvents is passed over a selective membrane in a Pervaporation module where the other side of the membrane is a gaseous phase (either vacuum or an inert sweep gas) when the volatiles are extracted into the gaseous phase from where it can be condensed and recovered (Shao & Huang 2007). Use of Pervaporation techniques in fermentation, in particular ABE fermentation was reviewed in Vane, 2005, Ezeiji *et al*, 2006 and Qureshi & Ezeiji, 2008. Membrane fouling and loss of fermentation intermediates are considered as the major drawbacks of Pervaporation (Ezeiji *et al*, 2010). In contrast to the membrane based techniques for product separation.

1.3.1.4 Gas stripping

Gas stripping is a simple and cost effective technique that can be integrated with ABE fermentation. Here, oxygen free N_2 or the fermentation gases comprising of CO_2 and H_2 is continually sparged into the reactor and the effluent gases are channeled through a condenser where the volatized solvents are recovered by cooling it (Ezeiji *et al*, 2003). Gas stripping has now become one of the most promising strategies for in situ removal and recovery of ABE due to its simplicity in operation, no toxicity or removal of nutrients and intermediates from the fermentation broth. Integration of gas stripping with

ABE fermentation have resulted in highly improved productivities and yield (Ezeiji *et al*, 2004b, 2007, Qureshi & Blaschek, 1999).

Fig 1.2: Various techniques used in ABE fermentation for *in situ* product removal



1.4. Low cost substrates: Focus on lignocellulosic biomass

Clostridium species can utilize and ferment broad range of carbohydrates which occur in dairy and wood wastes (Jones & Woods, 1986). Several substrates like cheese whey, starchy substrates, glycerol and lignocellulosic biomass were tried for butanol fermentation (Table1. 3).

Lignocellulose is abundant in nature and is mainly composed of cellulose, hemicellulose and lignin and the percentage of these three components mainly depend on the source of biomass, whether it is from hardwood, softwood or grasses. In most of the of the agricultural residues, the lignocellulosic biomass is made up of 10-25% lignin, 20-30% hemicellulose and 40-50% cellulose. Cellulose is highly crystalline in nature and is major component of cell wall with repeated units of hexoses. Hemicellulose is amorphous and is polymer of mainly pentoses and few hexoses. Lignin contains aromatic alcohols and forms protective layer on cellulose and hemicellulose (Anwar *et al*, 2014). The complex network of lignocelluloses is represented in Fig 1.3.

Fig 3: Lignocellulose structure

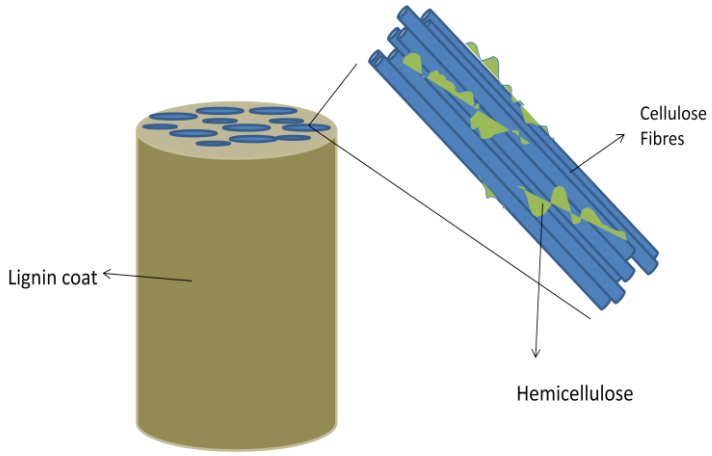


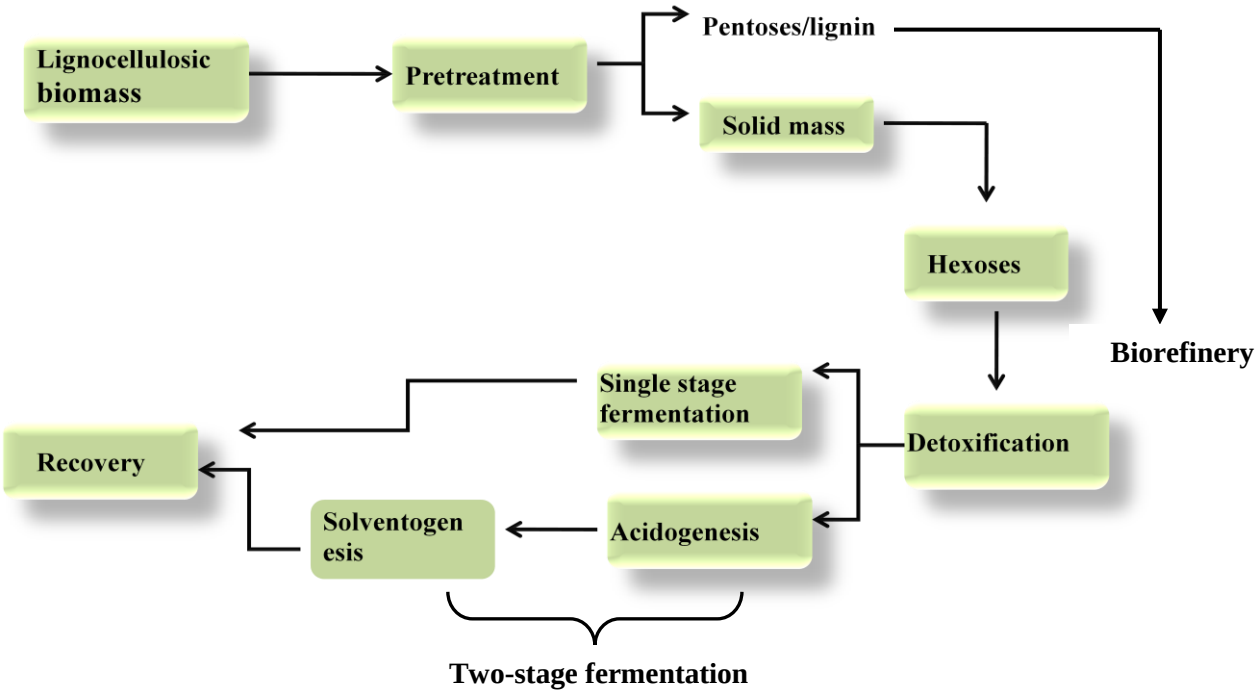
Table 1.3: ABE Fermentation employing complex substrates

	Substrate	Organism	Fermentation type	Butanol yield	References
Lignocellulose and starch	Corn fiber	*C. beijerinckii*	Batch	6.5 g/L	Qureshi et al, 2008
	Wheat straw	*C.beijerinckii* P260	Batch	12 g/L	Qureshi et al, 2007
	Rice straw (Acid hydrolysate)	*C.acetobutylicum* MTCC 481	Batch	12 g/L	Ranjan et al, 2012
	Barley straw	*C.beijerinckii* P260	Batch with gas stripping	47.2 g/L (Total solvents)	Qureshi et al, 2010a
	Corn stover	*C.beijerinckii* P260	Batch with gas stripping	50.14 g/L (Total solvents)	Qureshi et al, 2010b
	Cassava starch	*C.saccharoper-butylacetonicum* N1-4	Batch	16.9 g/L	Thang et al, 2010
	Corn starch	*C.saccharoper-butylacetonicum* N1-4	Batch	16.2 g/L	Thang et al, 2010
	Sago starch	*C.saccharoper-butylacetonicum* N1-4	Batch	15.5 g/L	Thang et al, 2010
Others					
	Cheese whey	*C. acetobutylicum*	Continuous	4.93 g/L	Raganati et al, 2013
	low grade glycerol + Glucose	*C. acetobutylicum* 4259	Continuous	7.6 g/L	Andrade et al, 2003

The complex structure of lignocellulosic biomass keeps cellulose inaccessible to cellulose degrading enzymes. Though chemical digestion breaks down cellulose to

sugars, it has the drawback of high inhibitors generation. For enzymes to act efficiently on lignocellulosic biomass; several pretreatment strategies are reported and choice of pretreatment should be based on the biomass type. Rice straw is an abundant lignocellulosic raw material and can be considered as an attractive renewable source for biofuel production. It is widely studied for bioethanol production (Binod et al, 2010), but for butanol production, use of rice straw as substrate is being considered very recently and there are not many detailed reports. The general process for conversion of lignocellulosic biomass to butanol is given in Fig 1.4.

Fig 1.4: Schematic showing butanol production from lignocellulosic biomass by *Clostridium* species



The complexity of lignocellulosic biomass, inhibitors formed during pretreatment and hydrolysis are severe draw backs in conversion of agriculture wastes to butanol. Developing a feasible technology for conversion of lignocellulosic biomass to butanol needs fine tuning of every single step involved in the process.

1.5. Conclusion

Clostridium species known till date produce acetone, butanol and ethanol (ABE) in the ratio 3:6:1. Among the solvents formed during fermentation, acetone does not qualify as fuel and separation of butanol from acetone is energy consuming process. As research at the genetic level to down regulate butanol production is under progress, a natural non acetone forming butanol producer, capable of utilizing various substrates will be of great importance. An efficient strain capable of growing in lignocellulose biomass hydrolysate and produce butanol without any supplementation of vitamins and minerals will be an added advantage for the process. It was therefore decided to focus on identifying the novel strain which was isolated for its capability to produce butanol without forming acetone and to further improve the process for butanol production from lignocellulosic biomass.

Chapter 2

Materials and Methods

2.1. Preparation of Rice Straw hydrolysate

2.1.1. Dilute Acid pretreatment of Rice straw

Rice straw obtained locally was knife milled to a powder with maximum particle size of ~4mm and pretreated with 4% (w/w) H_2SO_4 at a solid loading of 15% (w/w). Pretreatment was performed at the temperature 121 °C for 60 min. The pretreated biomass was neutralized by 10N NaOH to bring the pH to ~5.00. Solids were separated by wet sieving and were air dried at room temperature to remove excess moisture. The final moisture content of the biomass was determined using a moisture analysis balance and the pretreated straw was used for enzymatic hydrolysis.

2.1.2. Enzymatic hydrolysis of rice straw

Enzymatic hydrolysis was performed with commercial cellulase (Zytex India Limited, Mumbai) at 10% (w/w) solids loading. Cellulase activity of the enzyme was measured using filter paper assay according to IUPAC (Ghose, 1987). Cellulase was used at a concentration of 30 FPUs/g (dry substrate) for saccharification of the pretreated rice straw. Hydrolysis was done at pH 4.8 at 50 °C and 200 rpm for 48 h in 0.05M Citrate buffer. The hexose stream obtained from hydrolyzed rice straw was analyzed for sugars and the presence of acidic inhibitors like acetic acid and formic acid using HPLC.

2.2. Micro organism and culture conditions

C. sporogenes BE01 was isolated from contaminated cooked meat medium at the Biotechnology division of CSIR-NIIST. The culture was maintained as a spore suspension at 4 °C. It was cultivated in Tryptone/Glucose/Yeast extract (TGY) medium

to generate the pre-inoculum and inoculum for butanol fermentation. TGY medium contained in g/l -Tryptone-5.0, Yeast extract-5.0, K_2HPO_4 -1.0 and Glucose-1.0. Cysteine HCl-0.5 g/l was added as chemical reducing agent and resazurin, 0.01% (w/v) was added as redox indicator in the medium (Fukushima *et al*, 2002). To prepare the pre-inoculum, spores were heat shocked at 75 °C for 2 min and kept in ice to bring it to room temperature. Heat shocked spores (10% v/v) were inoculated into TGY medium and incubated at 37 °C for 12 h in an anaerobic chamber (Bactron I, Shell labs, USA) purged with nitrogen gas. Actively growing cells from pre-inoculum were inoculated into fresh TGY medium to generate inoculum for fermentation. Growth of the organism was measured as absorbance at 600 nm using a UV-Vis spectrophotometer.

2.3. Butanol production

Semi-defined P2 medium containing glucose, trace elements, vitamins and reducing agent [In g/L: Glucose- 50.0, Ammonium acetate -1.5, $MgSO_4.7H_2O$ - 0.2, KH_2PO_4 - 0.5, K_2HPO_4 - 0.5 NaCl - 0.01, $MnSO_4.H_2O$ -0.01, $FeSO_4.7H_2O$ - 0.01, Yeast extract -1.5, Para amino benzoic acid (PABA) - 0.001, Biotin - 0.00001, Thiamin - 0.001] (Qureshi and Blaschek, 1999) was used for butanol production. Calcium carbonate was added as buffering agent and the medium pH was adjusted to 6.8. Cysteine HCl- 0.5 g/l was added as chemical reducing agent. The fermentation medium was sterilized by autoclaving (121 °C for 15 min) and cooled down to 37 °C under continuous purging of nitrogen gas. Actively growing 12 h old culture of *C. sporogenes* BE01 at 10% v/v was used as inoculum for butanol production, unless age and percentage of the inoculum is specified otherwise.

Fermentation with rice straw hydrolysate was done with or without supplementation of modified and optimized concentrations of P2 minerals [In g/L

$(NH_4)_2SO_4$ -1.5, $MgSO_4.7H_2O$ - 6.0, KH_2PO_4 - 0.5, NaCl-0.01, $MnSO_4.H_2O$ - 0.01, $FeSO_4.7H_2O$ - 0.01] (Hartmanis *et al*, 1986; Qureshi and Blaschek, 1999) Yeast extract (1.5 g/l) was added as the source of organic nitrogen, vitamins and other essential nutrients. Calcium carbonate was added as buffering agent in the hydrolysate and the medium pH was adjusted to 6.7.

2.4. Analytical methods

2.4.1. Sugars and organic acids analysis

Sugars were analyzed and quantified with Rezex ® RPM monosaccharide analysis column (Phenomenex, USA) by Shimadzu prominence UFLC with RI detector. 85 °C oven temperature was maintained and de-ionized water at a flow rate of 8 ml/min was used as mobile phase. Rezex® ROA organic acid analysis column (Phenomenex) and PDA detector was used for separation and detection of acidic inhibitors and volatile fatty acids present in hydrolysate before and after fermentation. Oven temperature 50 °C was used and mobile phase used for separation was 0.05M H_2SO_4 at the flow rate 0.6 ml/min.

2.4.2. Solvents analysis

Butanol and ethanol produced during fermentation were analyzed by gas chromatograph (Chemito GC 8610). Poropak Q ® column was used for separation by maintaining the oven temperature as a gradient with rise in temperature from 50 °C to 200 °C at the rate of 8 °C/min. and were detected by flame ionization detector (FID). Injector and detector temperature were 150 °C and 250 °C respectively.

Chapter 3

Identification and characterization of *Clostridium sp* producing biobutanol

3.1. Introduction

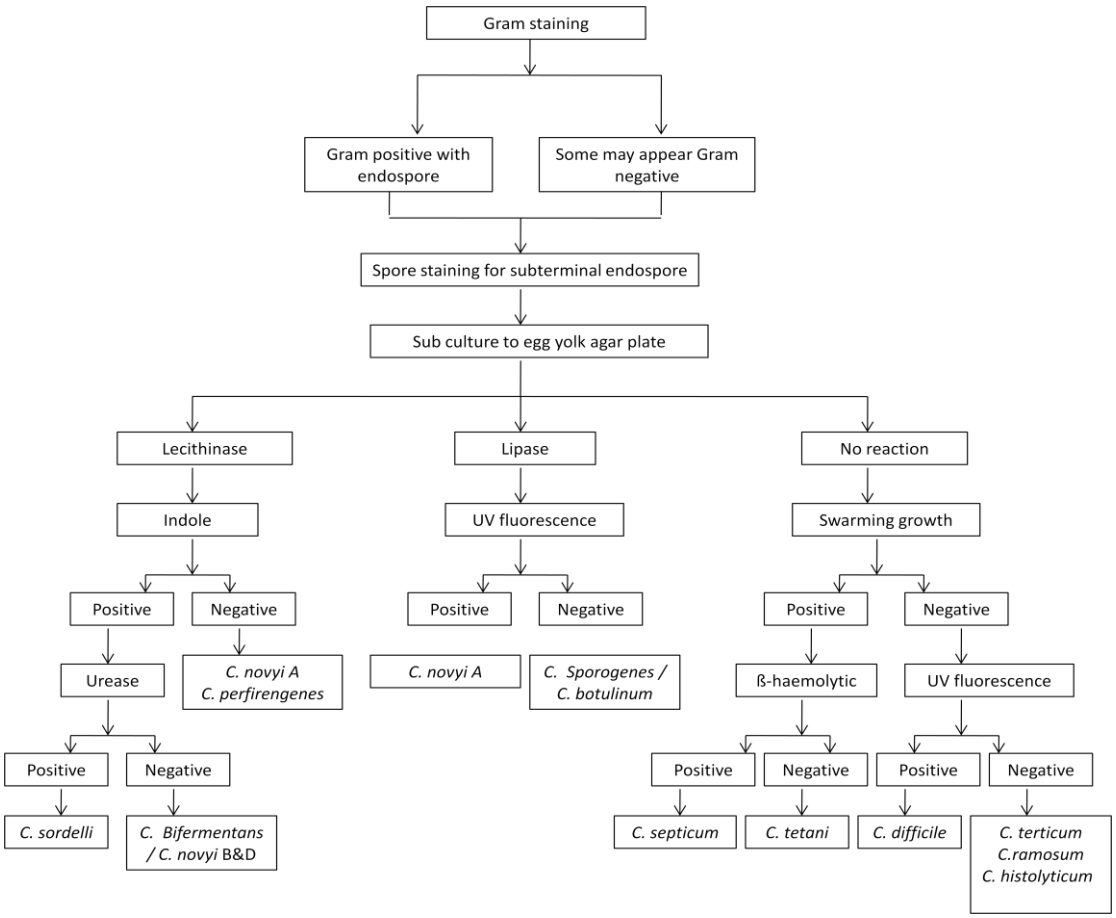
More than 100 species are assigned to the genera *Clostridium* after it was first proposed by Prazmowski in 1880. To be included in *Clostridium* genera, any bacteria should meet the following criteria. 1. It must be obligate anaerobe; 2. It should be able to form endospores; 3. Dissimilatory sulfate reduction should be possible; 3. Motile- they are normally with peritrichous flagella; 4. Straight or curved rods with Gram positive type cell wall (Hippe et al, 1992). Though the main distinguishing factor of *Clostridium* genera is its sub-terminal endospore, the trigger associated with spore formation is still unknown. Unlike many *Bacillus* species, spore formation is not due to nutrients limitation. There are certain *Clostridium* species which are slightly aero tolerant, but in those conditions, cells divide without forming spores. This specifies that for spore formation complete anaerobic conditions are required and this is the feature that distinguishes *Clostridium* form other spore forming *Bacillus* (Smith, 1977).

Substrate spectrum of *Clostridium* species is very wide and covers a wide range of natural organic compounds. They degrade organic compounds to acids, alcohols, carbon dioxide and hydrogen. The smell associated with *Clostridium* species is generally due to butyric acid and in some cases hydrogen sulfide also contributes to the characteristic foul smell. Based on their ability to degrade natural organic compounds, they are divided in to 4 classes 1. Saccharolytic *Clostridia,* which are again sub classified into amylolytic, cellulolytic, pectinolytic and chitinolytic. These *Clostridia* produces the required enzymes responsible for biodegradation of natural polymers; 2. Proteolytic –

Clostridia belonging to this category are able to degrade proteins and utilize amino acids to form branched chain fatty acids; 3. Proteolytic and Saccharolytic clostridia, which can degrade both proteins and sugars, and they are normally pathogenic in nature except *C. oceanicum*. 4. Specialists- *Clostridium* belonging to this category are neither proteolytic nor saccharolytic. They survive on special substrates like purines, uric acid, alcohols and acids (Hippe et al, 1992).

Egg yolk reactions are of great importance for the identification of *Clostridium* species (Barrow & Feltham, 1993). (Fig 3.1)

Fig 3.1: Chart to differentiate lecithinase and lipase producing *Clostridium* species based on Egg yolk agar plate technique.



Modified from Bacteriology – Identification, UK Standards for Microbiology Investigations

Clostridium species also exhibit characteristics like granulose formation and capsule formation during their vegetative phase and clostridial phase. Granulose is a polyglucan

reserve material and is associated with cigar shaped clostridial stage, presporulation cells, capsule formation and solvent production (Reysenbach et al, 1986).

Gram-positive anaerobic bacteria capable of forming endospores were originally assigned to *Clostridium* genera. This made the genera very diverse by including very distantly related organisms to the type species of *Clostridium* (Gupta et al, 2009). Whereas, few species belonging to *Clostridium* genera are very closely related and can be distinguished through very specific molecular markers or biochemical tests. One such example is more than 99% similarity between *C. botulinum* and *C.sporogenes*. *C. sporogenes* is highly similar to *C. botulinum* except for its inability to produce lethal toxins and is considered as non neuro toxinogenic counter part of *C. botulinum* (Sebaihia et al, 2007). There is almost same kind of similarity with *C. butanolgenum* and *C. beijerinkii*. High genome diversity, difference in physiological and genetic traits within the same species of *C. acetobutylicum* became apparent over decades of studies (Keis et al, 2007). This suggests that identifying a bacteria falling in *Clostridium* genera needs detail and in depth studies. In this chapter, a detailed morphological, biochemical and phylogenetic studies were conducted to identify a butanol producing novel *Clostridium sp*.

3.2. Materials and Methods

3.2.1. Staining methods

Gram staining was done for routine bacterial identification and endospore staining was used to determine endospores. Gram staining, spore staining and capsule

staining were performed using staining kits from Himedia India following the standard protocols..

3.2.2. Biochemical characterization

Production of extracellular enzymes like lipase, protease, amylase, endoglucanase, amylase, xylanase and lecithinase were determined by plate assay method. Lipase and protease plate assay was done on tributyrin agar plates and skim milk agar plates respectively. Amylase plate assay was done by flooding Lugol's iodine on soluble strach agar plate (Singh et al, 2012). Congo red plate assay method was used for endoglucanase and xylanase activity (Pointing, 1999). Halo zones around the colonies in plate assays indicate the positive reactions. Specific biochemical tests like ONPG (β-galactosidase), lysine utilization, ornithine utilization, urease, phenylalanine deamination, nitrate reduction, H_2S production, citrate utilization, Voges Proskauer's test, methyl red test, indole test and malonoate utilization were performed. Phenol red was used as indicator for sugar fermentation tests. Phenol red turns to yellow color with acids formation during sugar fermentation.

3.2.3. Molecular Identification of the isolate

Molecular identification was carried out by sequencing and BLAST analysis of amplified regions of 16S rRNA gene.

3.2.3.1. Genomic DNA isolation and PCR amplification of rRNA

Genomic DNA from the bacterial isolate was performed using GeneJet Genomic DNA isolation kit as per the manufacturer's protocol (Fermentas, USA). A portion of the 16S rRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using the universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and

1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane et al, 1985). PCR reactions contained 0.5 units of *Taq* DNA polymerase, 1x *Taq* buffer, 200 µM of each dNTPs, 2.0 µM $MgSO_4$ (All from Fermentas, USA), 0.2 µg genomic DNA, and 0.5 µM forward and reverse primers. Reaction conditions for PCR amplification were an initial 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. An Eppendorf ® gradient PCR system was used for the amplification. PCR products were separated by electrophoresis on a 1 % agarose gel and products were visualized in long range UV trans-illumination for documentation. Nucleotide sequences of the PCR amplicons were determined by dye terminator sequencing following the manufacturers' protocols.

3.2.3.2. Phylogenetic analysis

Identity of the sequence assembly was established by BLAST analysis (Altschul et al. 1990). Later a homology search was performed and based on the results and a Phylogenetic tree was constructed using the neighbor joining method implemented in MEGA 4 software applying neighbor-joining (NJ) method, which is a simplified version of minimum evolution (ME) method (Tamura et al, 2007).

3.2.4. Growth and butanol production

Three different media- Cooked meat medium, reinforced Clostridial broth and reinforced Clostridial agar (Himedia, India) was used for culture revival and growth studies. Butanol fermentation was studied in P2 glucose synthetic medium mentioned in section 2.3.

3.3. Results and discussion

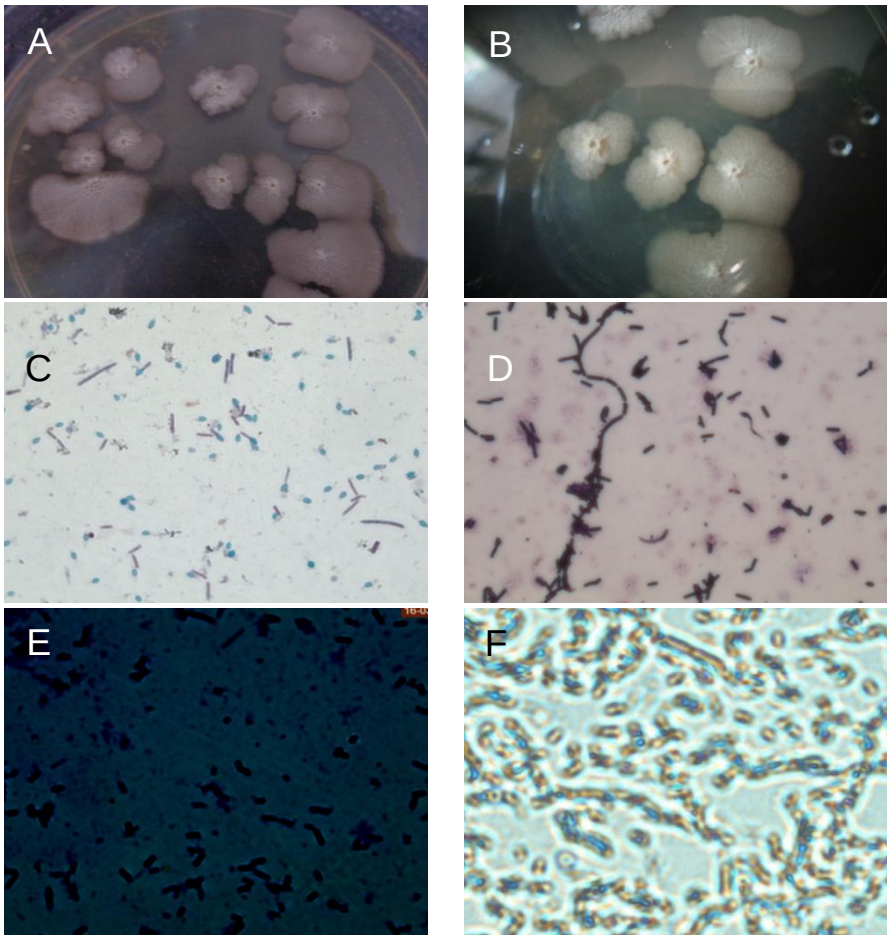
3.3.1. Morphological characteristics

Surface colony morphology of the pure culture grown anaerobically on a reinforced clostridial agar plate was large and flat with wide spreading, irregular, coarse rhizoid margin and raised centre (Medusa head colony). They are 2-6 mm in diameter with wooly periphery composed of entangled filaments (Fig 3.2a & b).

Culture was able to grow luxuriously in reinforced clostridial broth and cooked meat medium under strict anaerobic conditions at 37 °C. In cooked meat medium, there was blackening of meat particles, a characteristic feature of proteolytic clostridia. In both the media, culture started sporulation after 72 h. Gram staining and spore staining indicated that the bacteria is Gram-positive with sub terminal endospore. Rod shaped and cigar shaped vegetative cells were observed with Gram staining, and spore staining revealed that spores are sub terminal and oval in shape (Fig 3.2c).

Sheathed cells in the form of chains were noticed by Gram staining of bacterial film settled at the bottom in reinforced clostridial broth. Cells in these long chains were not in physical contact with one another, rather they were held together by a sheath (Fig 3.2d). In clostridia, these sheaths maintain linear integrity of the chains and that gives the resistance to culture under test. A variant of *C. sporogenes* was reported for its ability to form sheathed cells and this sheath was assumed to be made of polysaccharides (Betz, 1970). Positive result for capsule staining can also be correlated with the ability of the culture to form exo-polysaccharide (Fig 3.2e)

Fig 3.2: Morphological features of bacterial isolate



A &B- colony morphology on reinforced clostridial agar plate, C- spore staining; D- sheathed cells, E- capsule staining, F- granulose staining;

Iodine vapor staining method gave positive result for granulose formation (Fig 3.2f). In most of the *Clostridium* species, granules are made up of polyglucan and key enzymes involved in the synthesis are ADP glucose pyrophosphorylase and glycogen synthase. These granules were found in clostridial stage of cells or at the end of exponential stage for the isolate. Long rods, representatives of vegetative phase cells did not show the presence of granules. It was reported that granules degrade during sporulation and hence they can be counted as source of energy for spore formation in Clostridia (Robson et al, 1974, Long et al, 1983). Microscopic observation of stained

cells and colony characteristics indicated that bacteria could belong to *Clostridium* species.

3.3.2. Biochemical characteristics

Clostridium species are very diverse in biochemical characteristics. They are reported well for their proteolytic, lipolytic and saccharolytic activity.

3.3.2.1. Lipase

Plate assays performed for lipase were positive with a clear halo zone in tributyrin (tributyryl glycerol) agar plate (Fig 3.3a). A zone of tributyrin hydrolysis is indicative of either lipase or esterase activity. It was further confirmed by *p*-Nitro phenyl palmitate spectrophotometric assay for lipase activity. Though short chain esters can be used, their hydrolysis generally is indicative of only esterase activity but not lipase, hence *p*-Nitro phenyl palmitate should be used for lipase assay. Lipases (triacylglycerol acyl hydrolase; EC 3.1.1.3) are versatile biocatalysts and can act at the oil/water interface, unlike other enzymes that act in aqueous phase. They hydrolyze triacyl glycerols to release free fatty acids and glycerol (Gupta et al, 2003)

Among *Clostridium* species, *C. sporogenes* and *C. botulinum* are reported for lipase activity. Lipase assay is generally used to distinguish *C. sporogenes* and *C. botulinum* from other *Clostridium* species (Barrow & Feltham, 1993). *C. sporogenes and C. botulinum* are very closely related and possess almost similar biochemical characteristics except for minor differences.

3.3.2.2. Protease

Skim milk agar plate assay was performed for protease activity. Clearance around the colonies indicated that culture is proteolytic (Fig 3.3b). Proteases are group of

enzymes that hydrolyze peptide bonds of proteins and break them into polypeptide or free amino acids. Both *C. botulinum* belonging to class 1 and *C. sporogenes* are proteolytic in nature. It was reported that nutritional and biochemical requirements of proteolytic *C. botulinum* and *C. sporogenes* are indistinguishable. Extracellular protease production by *C. sporogenes* was reported during end of active growth phase or stationary phase under energy-limiting conditions (Allison & Macfarlane, 1990). *Clostridium* species like *C. botulinum* and *C. tetani* belonging to proteolytic class are pathogenic, where as *C. sporogenes* is considered as non-toxic variant of *C. botulinum* (Sebaihia et al, 2007).

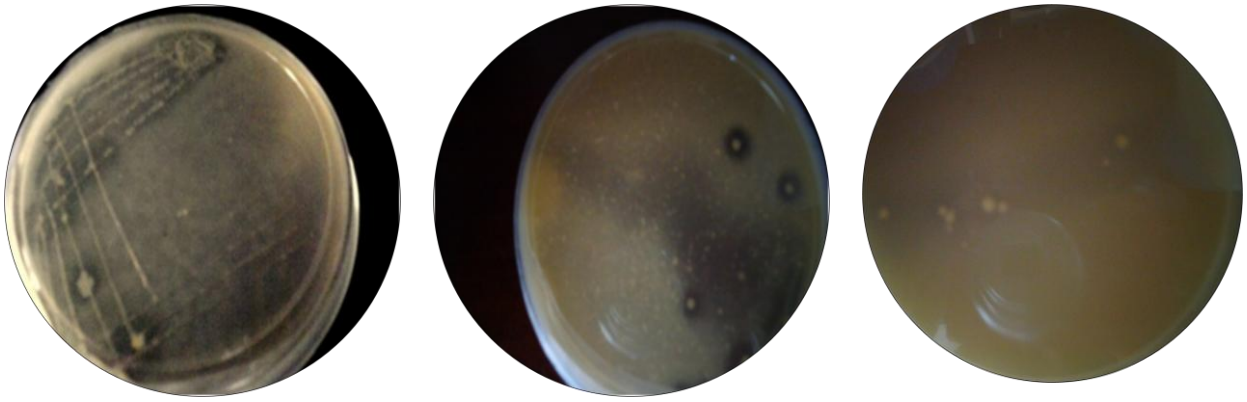
3.3.2.3. Lecithinase

Lecithinase (Phospholipase C) is an enzyme that splits the phospholipid lecithin. Phospholipids are charged molecules and are soluble in water, but the diglyceride formed during splitting consists of two long hydrocarbon chains which is not soluble in water. Lecithinase acting on egg yolk emulsion causes turbidity and on egg yolk agar it forms precipitate (Kushner, 1957). Precipitation around the streak of bacteria indicates lecithinase production (Harbour, 1954). Culture streaked on egg yolk agar did not form any precipitate around the colonies and hence was confirmed as lecithinase negative (Fig 3.3c). Lecithinase plate reactions in connection with other studies can be considered for distinguishing *Clostridium* species. Precipitate and zone formation varies with different species of *Clostridium*.

From the lipase test and flow chart analysis, it was indicative that the isolate could be either the *C. botulinum* or *C. sporogenes*. Egg yolk plate reaction for lecithinase helps in distinguishing *C. botulinum* from *C. sporogenes* (Barrow & Feltham, 1993). *C. botulinum* gives a positive reaction for lecithinase with zone of luster and precipitation

extending beyond the colonies. *C. sporogenes* is generally lecithinase negative, even if the precipitation forms with few strains, it deposits under the colony and rarely spreads with extended incubation time (Mc Clung & Toabe, 1947). From the lipase and lecithinase assay, it was speculated that culture could be *Clostridium sporogenes*.

Fig 3.3: Biochemical characterization of isolate BE01



A: Lipase B: Protease C: Lecithinase

3.3.2.4. Amylase

Starch is a glucose polymer containing the linear polymer-amylose and the branched polymer-amylopectin and needs a combined action of alpha-amylase, beta-amylase, pullulanae and glucoamylase for complete hydrolysis. Amylase breaks down starch to dextrins and maltose and its activity can be identified by soluble starch agar plate assay. Culture grown on soluble starch agar plate, which is capable of degrading starch forms a zone of clearance around the colonies, when the culture plate is flooded with Lugol's iodine (Singh et al, 2012).

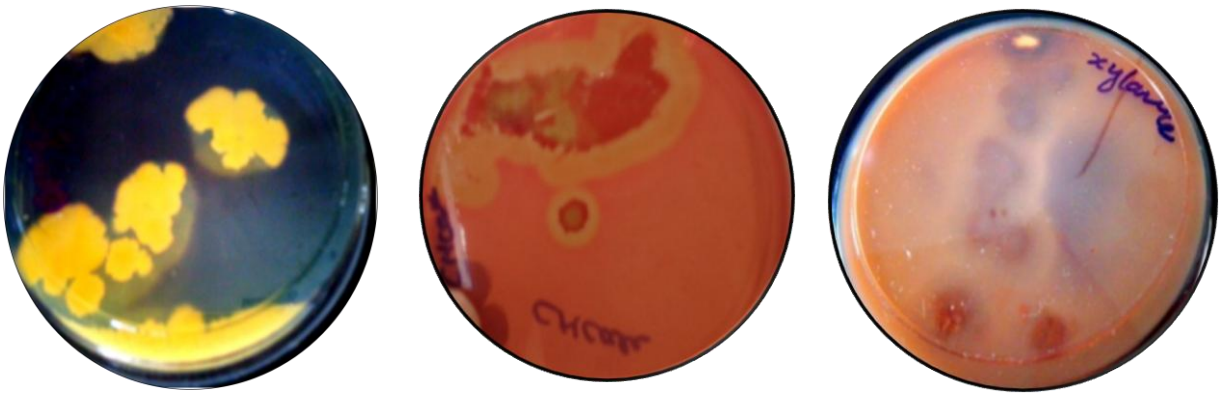
Soluble starch agar plate assay was positive for the isolate indicating amylase activity (Fig 3.4a). *C. botulinum* produces only beta-amylase and catalyses the removal of maltose molecules from non-reducing ends of the starch polymers. But, due to the

absence of other secretary starch degrading enzymes, *C. botulinum* cannot hydrolyze starch completely (Sebaihia, 2007). Both beta-amylase and alpha-amylase genes and their catalytic domains were reported in *C. sporogenes*, but their secretion and amylase activity is not studied yet (Sudarsanam, 2008, Fulton, 2008).

3.3.2.5. Endoglucanase and xylanase

Congo red assay for endoglucanase activity on carboxymethyl cellulose plate and xylanase activity on Birchwood xylan plate yielded positive results (Fig 3.4b & c). Endoglucanase catalyze the hydrolysis of cellulose by breaking down internal ß-1, 4 linkages and acting synergistically with exoglucanses and ß-glucosidases. Xylanases degrade xylan by acting on 1, 4-beta-D-xylosidic linkages. Though endoglucanase and xylanase activity in *C. sporogenes* is not reported till date, they are reported in few *Clostridium* species like *C. thermocellum*, *C. cellulolyticum*, *C. cellulolovorans* etc (Kosugi et al, 2001, Jean-Charles et al, 2007, Morag et al, 1990). As mentioned previously, *C. sporogenes* is considered as non-toxic surrogate strain of *C. botulinum* and the latter was reported to harbor sequences of glycoside hydrolase family, glycosyl transferase family, carbohydrate esterase family and carbohydrate-binding module family in its genome, though detailed studies on enzymatic activities have not been performed.

Fig 3.4: Biochemical characterization of isolate BE01



A: Amylase B: Endoglucanase C: Xylanase

3.3.2.6. Sugar utilization spectrum and biochemical characterization

Isolated *Clostridium* strain could utilize and ferment glucose, fructose, sucrose and maltose. In xylose, mannose and cellobiose, weak fermentation was observed. Arabinose, lactose, cellulose and starch did not give any color change with phenol red in the first 48 h (Fig 3.5). The color change is mainly based on the acids formed due to fermentation of sugars and reduction in medium pH. Phenol red is a pH indicator that turns yellow under acidic conditions helps in understanding the formation of acids during fermentation. Sugar utilization in *Clostridia* varies from species to species. *C. butyricum* was reported to ferment most of the sugars except mannose and *C. sporogenes* was known for just glucose fermentation. *C. botulinum*, which is closely related to *C. sporogenes*, can utilize glucose, maltose and trehalose (Barrow & Feltham, 1993). *C. sporogenes* basically a proteolytic organism was reported to utilize sucrose and maltose in presence of L-proline and no turbidity without proline supplementation. Even with glucose as carbon source, increased growth was reported with proline supplementation (Lovitt et al, 1987).

Fig 3.5: Sugar Utilization Spectrum for isolate BE01

Biochemical tests for the isolate gave positive result for lysine utilization, ornithine utilization, H_2S production, Voges Proskaeur's test, methyl red test and the bacteria was negative for ONPG, urease, phenylalanine deamination, nitrate reduction, citrate utilization, indole test and malonate utilization (Fig 3.6a & b). Few *Clostridium* species are known for Stickland reaction in amino acid metabolism by using pairs of amino acids as electron donors and acceptors. Ornithine is converted to proline and proline is further reduced by proline reductase. Proline reductase was shown to be associated with *C. sporogenes* membrane and takes part in vectorial proton translocation. Lysine mutase, a B12 dependent amino mutase is involved in lysine utilization (Ljungdahl et al, 1989). Hydrogen sulphide production is reported for few sulfate reducing *Clostridium* species like *C. perferingens, C. sporogenes, C. botulinum*, *C. pasteurianum* etc. In *C. botulinum*, group I and II produce H_2S where as group III and IV are negative for H_2S production. Strains belonging to I and II are very different from III and IV (Oquma et al, 1986). It was reported that many of the *Clostridium* species follow inducible dissimilatory type sulfate reduction pathway (Harrison, 1984). Acetoin production is reported in just few species of *Clostridium* and acids production (positive for methyl red test) is common with all *Clostridium* species.

Fig 3.6: Biochemical tests using HiIMViC Test Kit

A

B

A-Control, B-Strip inoculated with C.sporogenes BE01

Based on the morphological characteristics and biochemical tests, the isolate was found to be *C. sporogenes*. Primarily lipase assay and other biochemical characteristics revealed that the isolate could be either *C. botulinum* or *C. spororgenes*. Negative reaction for lecithinase assay confirmed it as *C. sporogenes*, as *C. botulinum* is a lecithinase positive. Since *C. sporogenes* is not a well studied strain for its metabolism and difficult to confirm by just biochemical characterization, molecular characterization and phylogenetic analysis was also performed to confirm the identity of the organism.

3.3.3. Molecular Identification & Phylogenetic analysis

PCR amplification of 16S rDNA yielded a 910 bp nucleotide sequence which on BLST analysis was found to be 100 % similar to *C. sporogenes* with maximum identity to strains CL3 and CL2 16S ribosomal RNA genes (Table 3.1)

Isolate BE01>

CTGGACTTGAGCAGTCGAGCGATGAAGCTTCCTTCGGGAAGTGGATTAGCGGCGGACGGGTGAGTAACACGTGGGTAAC
CTGCCTCAAAGTGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATAACATAAGAGAATCGCATGATTTTCTTATC
AAAGATTTATTGCTTTGAGATGGACCCGCGGCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGT
AGCCGACCTGAGAGGGTGATCGGCCACATTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGGGTGATGAAGGTCTTCGGATTGTAAAGCCCTGTTTTCTG
GGACGATAATGACGGTACCAGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGC
GTTGTCCGGATTTACTGGGCGTAAAGGGTGCGTAGGCGGATGTTTAAGTGGGATGTGAAATCCCCGGGCTTAACCTGGG
GGCTGCATTCCAAACTGGATATCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGAT
TAGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGCTGAGGCACGAAAGCGTGGGTAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGGTGTAGGGGGTATCAACTCCCCCTGTGCCGCAGTTA
ACACAATAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGC
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC

Table 3.1. Sequences with significant similarity to
16S rDNA partial gene sequence of isolate BE01 identified by BLAST analysis

Description	Max score	Total score	Query cover	E value	% Identity	Accession
C. sporogenes strain CL3 16S ribosomal RNA gene, partial sequence	1663	1663	0.98	0	100%	JF836014.1
C. sporogenes strain CL2 16S ribosomal RNA gene, partial sequence	1663	1663	0.98	0	100%	JF836013.1
C. sporogenes strain CL1 16S ribosomal RNA gene, partial sequence	1663	1663	0.98	0	100%	JF836012.1
C. sporogenes strain CG3 16S ribosomal RNA gene, partial sequence	1663	1663	0.98	0	100%	JF836010.1
C. sporogenes strain CG1 16S ribosomal RNA gene, partial sequence	1663	1663	98%	0	100%	JF836008.1

Phylogenetic analysis of the isolated strain which was designated as *C. sporogenes* BE01 based on the biochemical characterization and BLAST analysis revealed that it is closely related to *C. sporogenes* CL2 strain and *C. sporogenes* DSM 795. Phylogenetic analysis represented the closely related species in a very obvious manner. Sulfate reducing species *C. sulfidigens* and *C. thiosulfatireducens* shared the same clade. Different strains of *C. sporogenes* and *C. botulinum* were listed in the same

clade and *C. acetobutylicum* and *C. tyrobutyricum* were seen completely out-branched in the phylogenetic tree. Other butyric acid and butanol producing saccharolytic and moderately saccharolytic strains-*C. butyricum, C .saccharoperbutylacetonicum* and *C. cellulovorans* clustered together in the same clade. As obvious from the phylogenetic tree *C. sporogenes* is a diverse species and few strains are far related from the type strains (Fig 3.7).

Phylogenetic tree construction of *C. sporogenes* BE01 with various strains of *C. sporogenes* revealed that it is very closely related to reported hydrogen producers of the species (Fig 3.8). *C. sporogenes* CL1, *C. sporogenes* CL2, *C. sporogenes* CL3, *C. sporogenes* CG1 and *C. sporogenes* CG3 are reported for hydrogen production under respective NCBI accession numbers. *C. botulinum* A strain Hall, which is considered as very closely related species to *C. sporogenes* is distinguishably out-branched (Sebaihia et al, 2007).

Fig 3.7: Phylogenetic tree of *Clostridium sporogenes* BE01 isolate with various *Clostridium* species

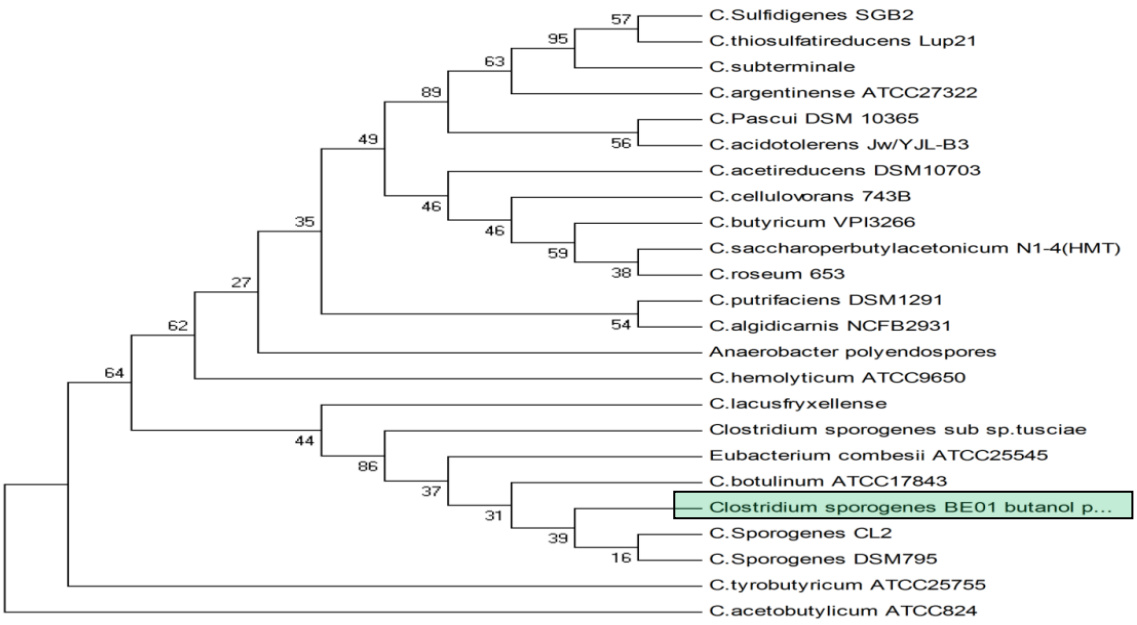
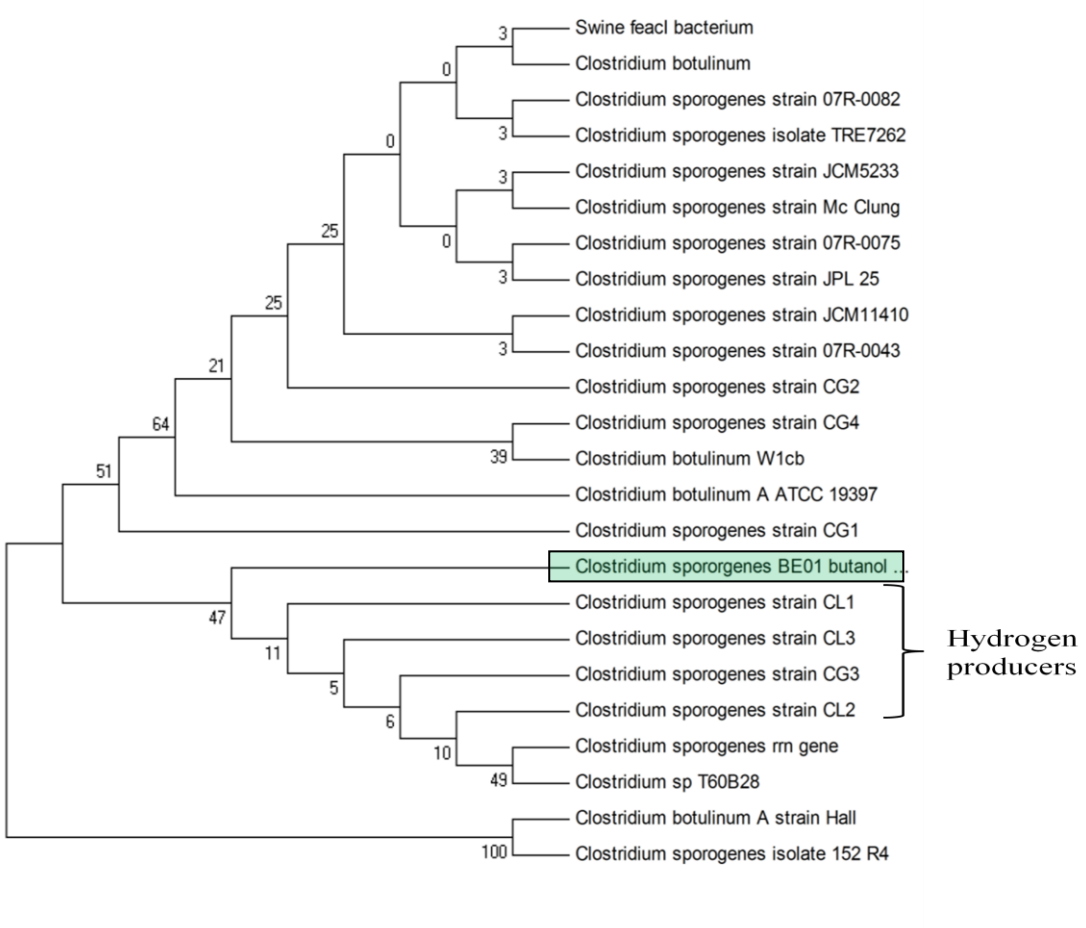


Fig 3.8: Phylogenetic tree of isolated *C. sporogenes* BE01 with various reported *C. sporogenes* strains



3.3.4. Growth and butanol production

C. sporogenes BE01 was able to grow in both reinforced Clostridial broth (RCB) and cooked meat medium (CMM). Growth was more profound in CMM, though there was lag phase observed for 24h. In RCB, there was no lag phase observed, but growth reached stationary phase at 24h (Fig 3.9). Culture grown in RCB medium was used as inoculum to check for butanol production in P2 glucose synthetic medium. *C. sporogenes* BE01 was able to produce ethanol, butanol, acetic acid and butyric acid in P2 medium. Acetone production was not observed with P2 medium (Fig 3.10). This could

be attributed to probable absence of acetoacetate decarboxylase enzyme in *C. sporogenes*, a surrogate strain of *C. botulinum* (Sebaihia et al, 2007).

Fig 3.9: *Clostridium sporogenes* BE01 growth in cooked meat medium (CMM) and reinforced clostridial broth (RCB).

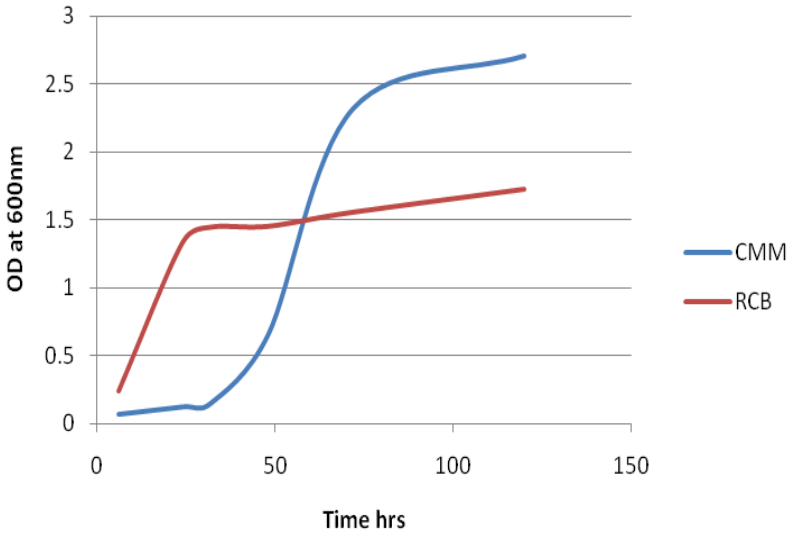
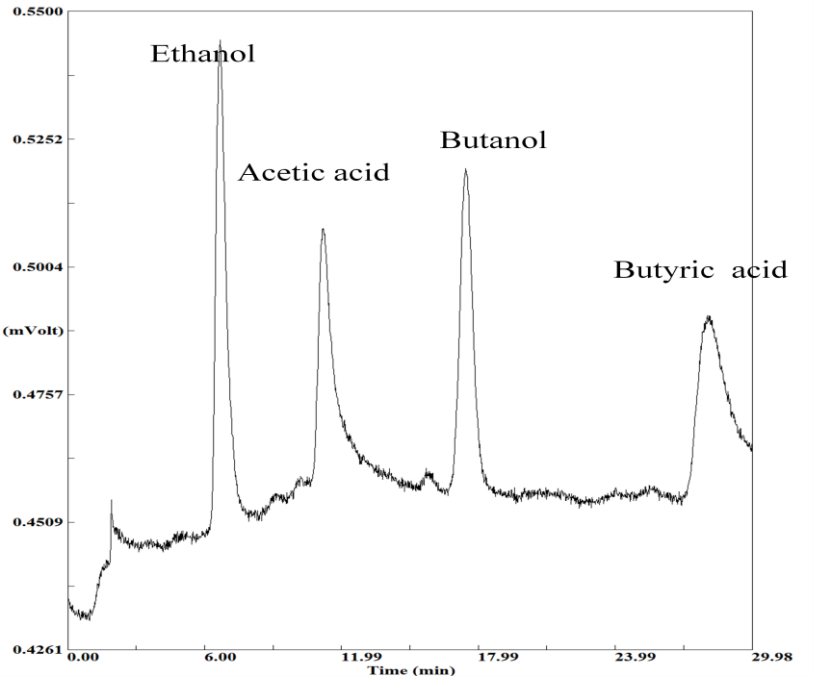


Fig 3.10: Solvents and organic acids production by *C. sporogenes* BE01 in P2 medium



3.4. Conclusion

C. sporogenes BE01 isolated from contaminated cooked meat medium is a Gram-positive, spore forming, obligate anaerobe with medusa head shaped colonies on reinforced clostridial agar plate. It is a proteolytic, saccharolytic, lipolytic strain and is lecithinase negative. Fermentation was efficient with glucose, fructose, sucrose and maltose and with xylose, mannose and cellobiose, week fermentation was observed. Phylogenetic analyses revealed that it is closely related to the reported hydrogen producing *C. sporogenes* species. It produced butanol, ethanol, acetic acid and butyric acid in P2 glucose medium without forming acetone.

Chapter 4

Growth characteristics of *Clostridium sporogenes* BE01 and its butanol production in glucose medium

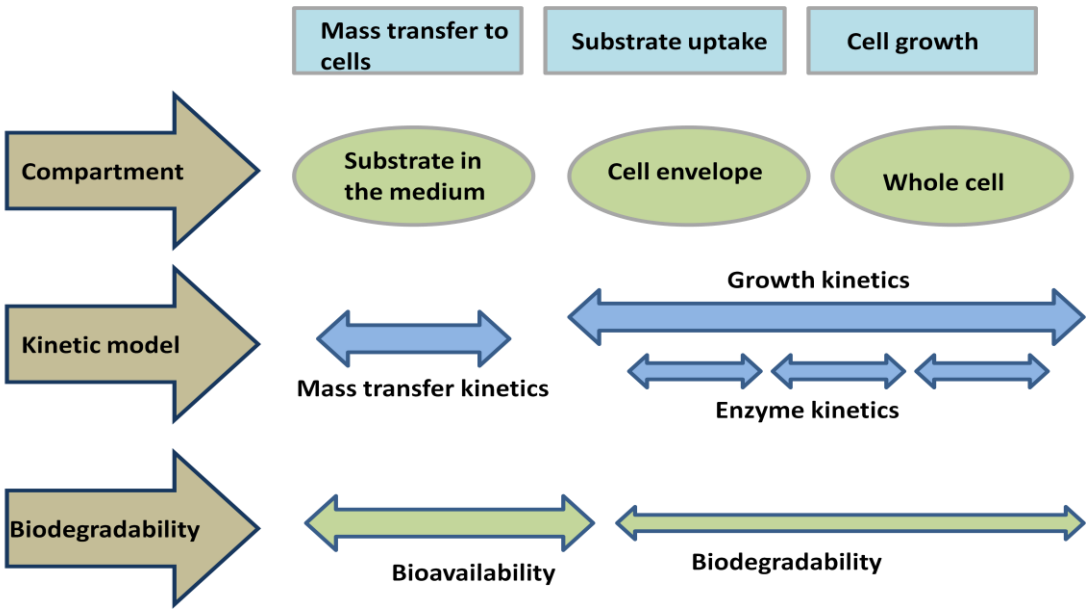
4.1. Introduction

Growth characteristics and physiology of bacteria varies significantly with the culture conditions. *Clostridia*, which belong to the group of anaerobic bacteria, require very stringent conditions for their growth and metabolism. Presence of oxygen strongly inhibits the growth of anaerobic bacteria. Other than carbon and nitrogen sources present in the medium, there are many factors that influence the growth of *Clostridia*.

C. sporogenes is a proteolytic organism and its amino acid and vitamin requirements for the growth were well studied. Amino acid complete medium (MACC) and low phosphate basal medium (LPBM) for the growth of *C. sporogenes* NCIB 8053 have been reported previously. LPBM and MACC media contains L-proline and glucose in the concentration 20 and 10 mmol/L respectively, with all the other trace elements, minerals, amino acids and vitamins (Lovitt et al, 1987a).

Growth kinetics is a relationship between specific growth rate and concentration of the substrate (Kovarova-Kovar & Egli, 1998). There are numerous kinetic models for growth rate estimation, out of which Monod model is more popular for both simple and complex growth kinetic studies. Monod had proposed a relatively simple empirical model considering the growth-limiting substrate (Monod, 1949). To understand the effect of substrate concentration on the growth of the organism, several factors like mass transfer, substrate transport, substrate utilization, biodegradability should be considered (Fig 4.1).

Fig 4.1: Flow chart showing kinetic process which affect microbial growth, specific with respect to compartment, kinetic model and biodegradability



(Adapted from Kovarova-Kovar & Egli (1998))

Substrate and product inhibition kinetics of the growth of *C. sporogenes* BE01 provides key insights into the improvement of butanol production. Semi defined glucose based P2 medium was reported extensively for butanol production by *Clostridium* species (Qureshi et al, 2000). ABE production and sugar utilization by *Clostridia* is normally restricted to 20 g/L and 60 g/L respectively, indicating both substrate and product inhibition (Ezeji et al, 2004). Other than solvents, butyric acid is also reported as growth and fermentation inhibitor (Zhu & Yang, 2004).

In the current chapter, growth kinetics, substrate inhibition and product inhibition kinetics were studied in detail in semi-defined glucose containing P2 medium. The medium composition was further fine tuned by statistical methods for improving butanol fermentation.

4.2. Materials and Methods

4.2.1. Growth kinetics

The growth rate of the cells in the batch system was estimated by taking absorbance at regular intervals. Specific growth rate is defined by the following equation.

$$\mu = \frac{1}{x}\frac{dX}{dt} = \frac{d \ln X}{dt} \quad (1)$$

Where, X is the cell concentration in absorbance unit at 600 nm (OD). Specific growth rate (μ) was determined at the log phase of the growth (Junag & Tsai, 2006).

Monod growth kinetics relates specific growth rate to substrate concentration and can be defined as

$$\mu = \mu_m \left[\frac{S}{K_S + S}\right] \quad (2)$$

Where, μ_m is maximum specific growth rate and K_s is saturation constant. S is substrate concentration. Whenever S >> K_s, then μ= μ_m and the cells grow at their maximum rate for a given medium and environmental conditions (Hill & Robinson, 1974).

4.2.2. Growth inhibition kinetics

Specific growth rate was calculated by sampling the cells at regular time intervals (Naher et al, 2008). Varying concentrations of inhibitors in glucose containing P2 production medium were used for inhibition kinetic studies. Linearized Haldane growth inhibitory equation was used for representing growth inhibition kinetics and determination of growth inhibition constant (Kumar et al, 2005)

Specific growth rate $$\mu = \frac{(\log_{10} Z - \log_{10} Z_0)2.303}{t - t_0} \quad (3)$$

Haldane equation $$\mu = \frac{\mu_{max} S}{K_S + S + \left(\frac{S^2}{K_i}\right)} \quad (4)$$

At concentrations where, S >> K_S, Haldane equation reduces to the below equation

$$\mu = \frac{\mu_{max} S}{S + \left(\frac{S^2}{K_i}\right)} \quad (5)$$

Linearised Haldane equation $$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \left(\frac{1}{K_i . \mu_{max}}\right) S \quad (6)$$

Linear graph was plotted with 1/μ vs initial inhibitor concentrations (S). μ_{max} was obtained from the slope of the curve and inhibition constant K_i was derived from intercept on X-axis.

4.2.3. Optimization for butanol production

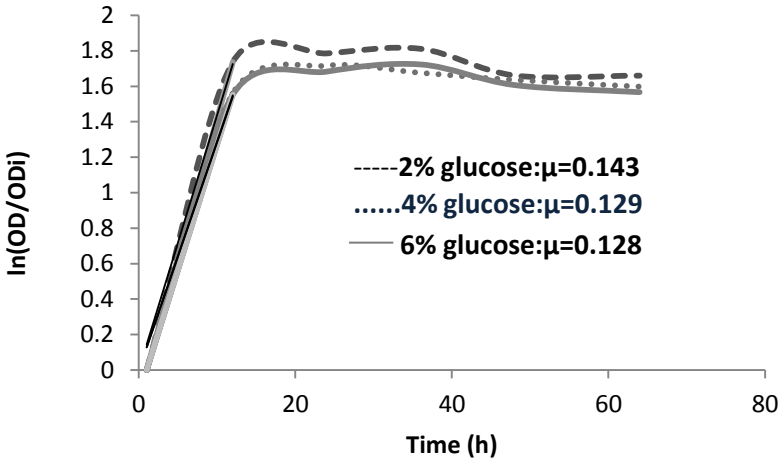
A Plackett and Burman (1946) design was employed to determine the effect of individual parameters on butanol fermentation by *C. sporogenes* BE01. Glucose based P2 medium components and pH were screened in a design with seven variables in a total of 20 experiment runs. The parameters tested were glucose, yeast extract, ammonium sulfate, calcium carbonate, biotin, inoculum size and pH and these were tested at two levels. The variables identified as significant by Plackett and Burman design was optimized using response surface central composite design (Box & Wilson, 1951). The effects of selected variables were studied at five different levels.

4.3. Results and Discussion

4.3.1. Growth kinetics

C. sporogenes BE01 could grow with glucose as the carbon source in semi defined P2 medium, without amino acid supplementation, though it is mainly a proteolytic organism and grows well under glucose limited conditions in presence of amino acids (Lovitt et al, 1987a). Specific growth rate (μ) was high with 2.0 % glucose concentration and decreased with 4.0 % and 6.0 % glucose concentration (Fig 4.2).

Fig: 4.2: Growth kinetics of *C. sporogenes* BE 01 at different glucose concentrations

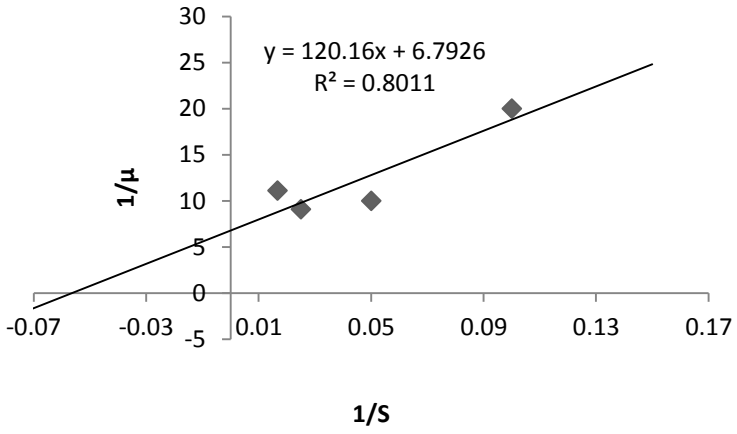


4.3.2. Monod Kinetics

Maximum specific growth rate (μ_m) and saturation constant (K_s) of *C. sporogenes* BE01 was determined by applying Monod equation. Monod equation is purely empirical and K_s describe overall affinity of the organism for its growth-limiting substrate. There are reports where K_s was related with K_M considering the enzymes involved or a single enzyme involved primarily in substrate uptake by the organism (Snoep et al, 2009). In semi defined glucose P2 medium, *C. sporogenes* BE01 gave maximum specific growth rate (μ_m) 0.147 h^{-1} and saturation constant or growth affinity constant (K_s) was 17.65 g/L (Fig 4.3). With research progressing on mathematical modeling to describe conversion of

nutrient to biomass and products, it became evident that bacterial adaptations effect growth affinity constants, which is leading to uncertainty (Ferenci, 1999).

Fig: 4.3: Monod kinetics of *C. sporogenes* BE01 in P2 glucose medium

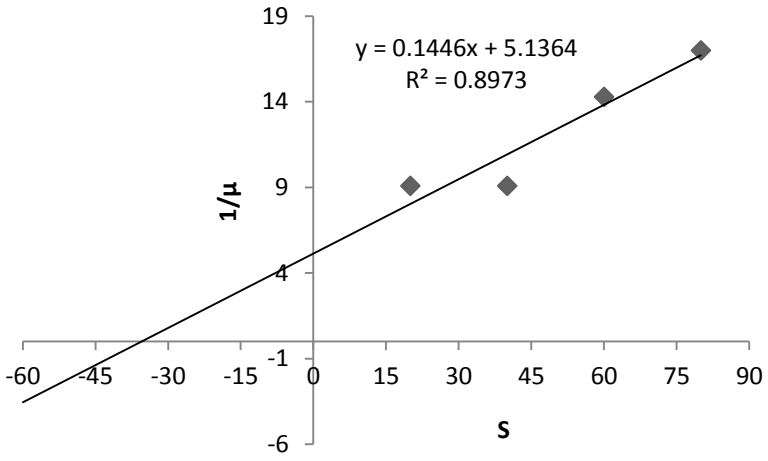


Maximum specific growth rate of *C. sporogenes* cultivated in pressure tubes in glucose-limited medium in batch culture was 0.38 h^{-1} (Lovitt et al, 1987a). Lovitt et al (1987b) also reported the importance of proline in the medium for the efficient growth of *C. sporogenes* NCIB 8053.

4.3.3. Substrate inhibition

Preliminary experiments revealed that glucose was inhibitory even at moderate concentrations. Haldane equation was applied to determine the inhibition constant (K_i) of the substrate. Inhibition constant for glucose as substrate was 36.55 g/L (Fig 4.4). *C. sporogenes* MD1 showed maximum specific growth rate 0.3 h^{-1} in Trypticase, Yeast extract medium, but when 35 mM glucose was supplemented, it declined to 0.2 h^{-1} (Montville et al, 1985). This supports our observation that there is an inhibitory effect of glucose at moderate concentrations.

Fig: 4.4: Substrate inhibition of *C.sporogenes* BE01 in P2 glucose medium

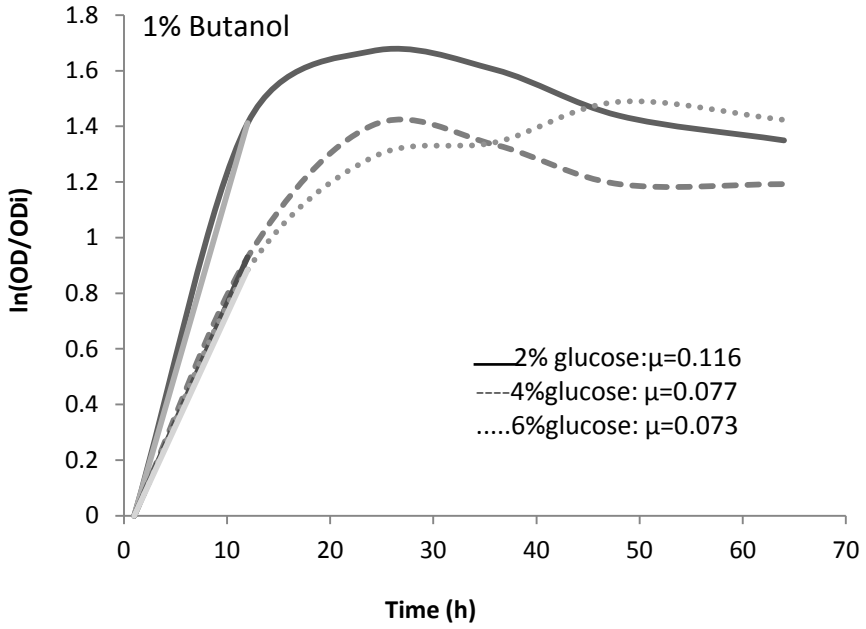


Microbial growth can be inhibited in several different ways by various end products formed during fermentation. In ABE fermentation, inhibitory effect of end products, especially butanol and butyric acid was reported, but it varies from species to species and even between strains of the same species.

4.3.4. Inhibitory effect of end products

As glucose in higher concentrations negatively impacted growth rate of *C. sporogenes* BE01, the effect of glucose concentration on butanol tolerance was tested with 1.0% (v/v) butanol. The growth of *C. sporogenes* BE01 decreased as such with 1% (v/v) butanol in all the glucose concentrations tested and glucose concentration had shown marked effect on the percentage growth reduction in the presence of butanol. Percentage growth reduction in 2.0%, 4.0 % and 6.0% glucose concentrations in presence of 1.0% (v/v) Butanol was 18.8, 40.3 and 42.9 % respectively (Fig 4.5).

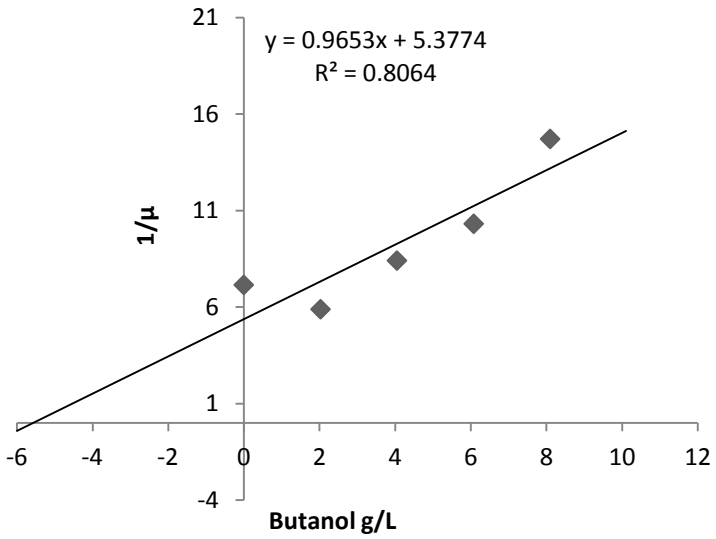
Fig: 4.5: Butanol tolerance at different glucose concentrations



C. acetobutylicum ATCC824 showed growth rate of 0.156 h^{-1} in corn starch soluble medium and its butanol tolerant mutant, SA-1 showed faster growth rate 0.197 h^{-1} under unchallenged conditions. At butanol concentration 15 g/L, parent strain showed negative growth rate and SA-1 showed 50% inhibition of specific growth rate at 15.5 g/L. Negative growth rate is due to cell lysis (Lin & Blaschek, 1983).

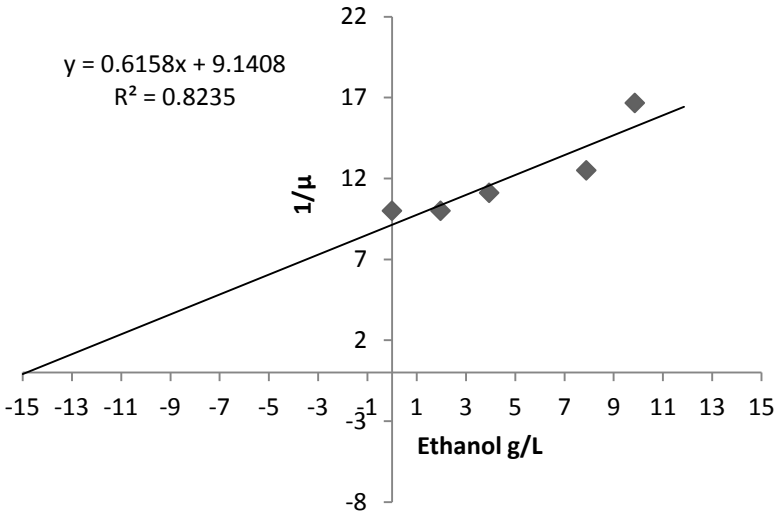
Growth inhibition kinetics by applying Haldane equation revealed that the inhibition constant (K_i) for *C. sporogenes* BE01 was 5.57 g/L with butanol (Fig 4.6). Inhibitory constant is where inhibition on cell growth starts and cannot be related to complete inhibition. Complete inhibition was at 15 g/L butanol, with specific growth rate reduced to 0.03 and at 20 g/L butanol growth rate was negative indicating cell lysis.

Fig: 4.6: Growth inhibition kinetics of *C. sporogenes* BE01 by butanol



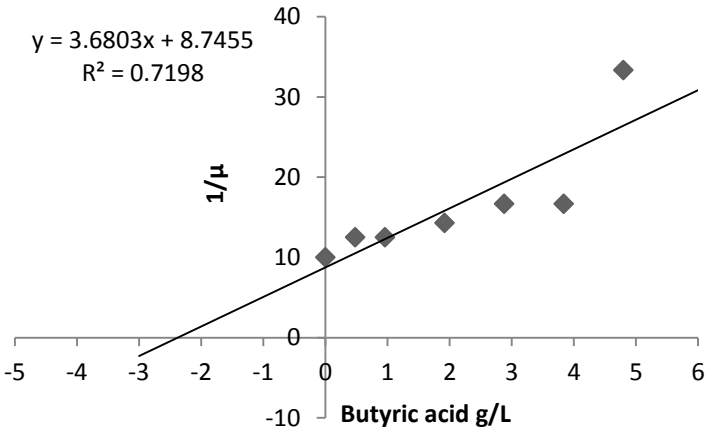
Butanol inhibition of *C. acetobutylicum* was reported for its differential regulation by glucose and xylose. For cultures growing in glucose, butanol inhibited the complete cell growth at 14 g/L and in xylose complete inhibition was reported at 8 g/L of butanol (Ounine et al, 1985). *Clostridium saccharoperbutylacetonicum* ATCC 27022 was inhibited completely by butanol at 30 g/L and butyric acid at 8.7 g/L (Soni et al, 1987). Inhibitory effect of ethanol was not well reported and in certain reports it was considered not inhibitory (Yang & Tsao, 1994). In case of *C. sporogenes* BE01, ethanol was not found as inhibitory as butanol, and it showed inhibition at higher concentrations only. Inhibition constant (K_i) for ethanol was 14.8 g/L (Fig 4.7).

Fig: 4.7: Growth inhibition kinetics of *C. sporogenes* BE01 by ethanol



Butyric acid in association with intracellular pH change was reported inhibitory. The influence of the pH was correlated with inhibitory effect of un-dissociated butyric acid. Cellular growth of *Clostridium acetobutylicum* was inhibited at a butyric acid concentration as low as 0.5 g/L, and solvents formation started at 1.5 g/L (Monot et al, 1984). Growth of *C. sporogenes* BE01 was also inhibited by butyric acid and inhibition constant (K_i) was 2.38 g/L and growth rate was reduced to half at 4.5 g/L (Fig 4.8)

Fig: 4.8: Growth inhibition kinetics of *C. sporogenes* BE01 with butyric acid



4.3.5. Optimization of parameters for improving butanol production by *C. sporogenes* BE01

Single parameter optimization was done to evaluate three important parameters l-inoculum source, calcium carbonate and inorganic nitrogen source. Culture was grown for 24 h in cooked meat medium (CMM) and glucose containing Tryptone, yeast extract (TGY) medium. Butanol fermentation was tested with glucose and xylose as carbon source. Though solvents production was observed in both glucose and xylose containing P2 medium, production was not satisfactory (Fig 4.9). Butanol production was further improved by adding calcium carbonate and substituting the inorganic nitrogen source-ammonium acetate with ammonium sulfate. Significant quantities of acetic acid and butyric acid were observed as end products (Fig 4.10 a & b).

Fig 4.9: Butanol production by *C. sporogenes* BE01

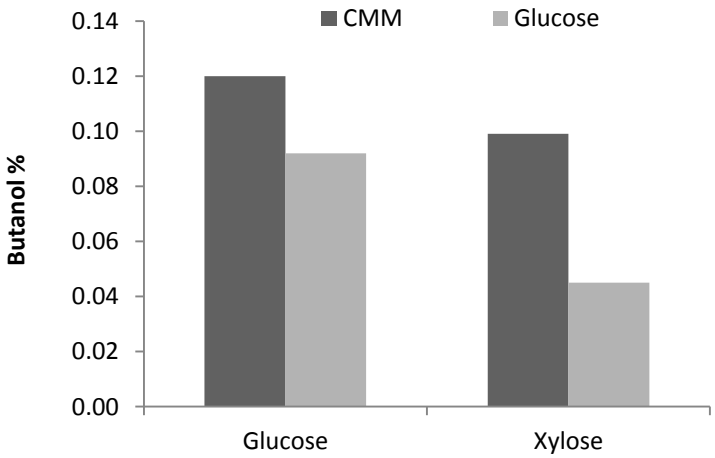
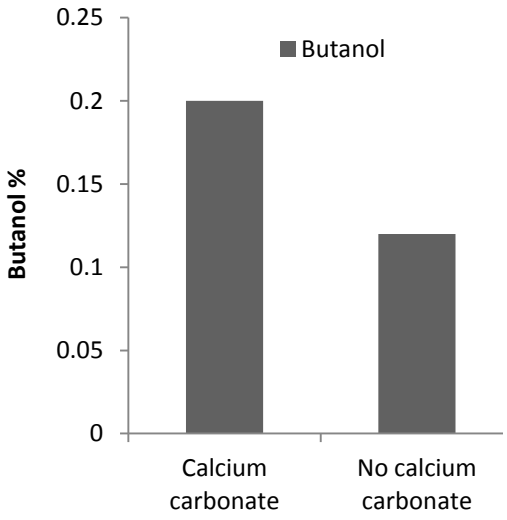
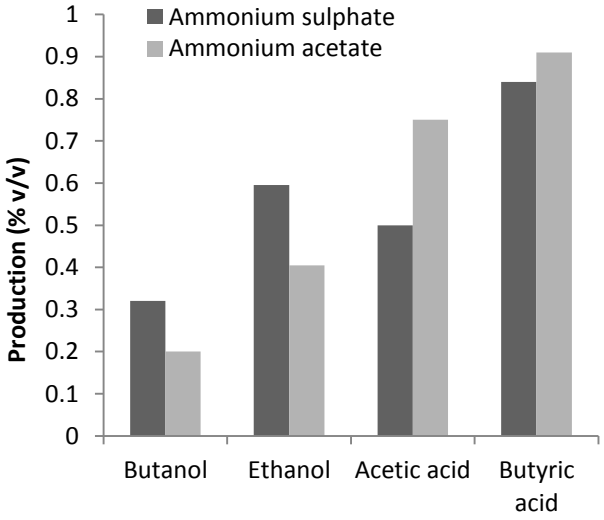


Fig 4.10. Production of solvents and acids by *C. sporogenes* BE 01





A: CaCO3 addition B: Production of solvents and acids

4.3.6. Screening of parameters by Plackett & Burman design matrix

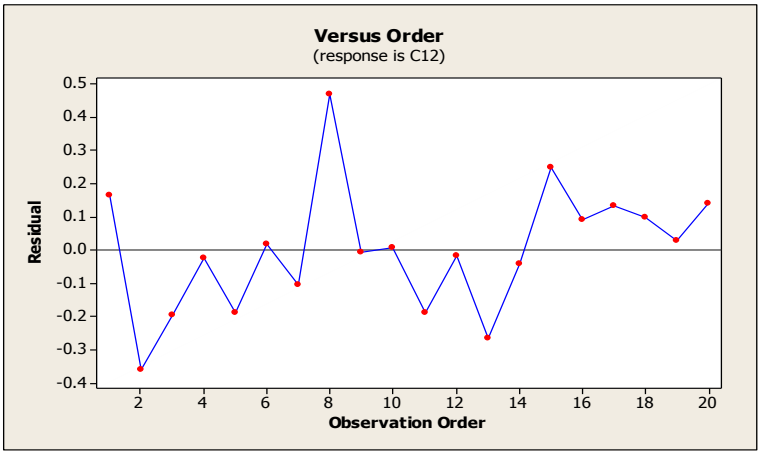
To determine the most significant parameters, seven important variables were screened. These parameters include environmental factors - carbon source, organic nitrogen source, inorganic nitrogen source, inoculums, vitamin and buffering agent. The response, butanol production varied significantly between the runs tested and was in the range of 1.76 g/L to 3.0 g/L (Table 4.1 and Fig 4.11)

Table 4.1: Design matrix for screening of parameters by Plackett & Burman method

Run Order	pH	Glucose (g/L)	Yeast extract (g/L)	Inoculum size (%)	Ammonium sulphate (g/L)	Calcium carbonate (g/L)	Biotin	Butanol g/L
1	6.8	3	3	10	1	3	0.00001	2.37
2	5.8	3	3	10	1	10	0.0001	2.53
3	6.8	3	1.5	6	1	10	0.00001	1.97
4	5.8	5	3	6	1	3	0.00001	2.00
5	6.8	5	3	10	1	3	0.0001	2.18
6	5.8	5	1.5	10	3	10	0.0001	2.62

7	6.8	3	1.5	10	3	3	0.0001	1.76
8	6.8	3	3	6	3	10	0.0001	2.80
9	6.8	5	1.5	10	3	3	0.00001	1.90
10	6.8	3	3	10	3	10	0.00001	2.63
11	5.8	3	3	6	3	3	0.0001	1.64
12	5.8	3	1.5	6	3	3	0.0001	1.56
13	6.8	5	1.5	6	3	10	0.00001	1.86
14	5.8	5	3	6	3	10	0.00001	2.40
15	5.8	5	1.5	10	1	10	0.0001	3.0
16	5.8	3	1.5	10	1	10	0.00001	2.67
17	5.8	5	3	10	3	3	0.00001	2.36
18	6.8	5	1.5	6	1	3	0.0001	1.87
19	6.8	5	3	6	1	10	0.0001	2.62
20	5.8	3	1.5	6	1	3	0.00001	1.80

Fig 4.11: Variation of Butanol production corresponding to changes in parameters



A linear regression model was fitted which was significant with P value 0.002 and the parameters with statistically significant effects were identified by ANOVA. Estimated effects and coefficients revealed that calcium carbonate, pH and inoculum size were significant with P <0.05 among the six variables tested (Table 4.2 & Fig 4.12, 13)

Table 4.2: ANOVA for the regression model

Term	Effect	Coef	SE Coef	T	P
Constant		2.23160	0.05356	41.67	0.000
pH	-0.06180	-0.03090	0.05356	-0.58	0.575
Glucose	0.10760	0.05380	0.05356	1.00	0.335
yeast extract	0.25340	0.12670	0.05356	2.37	0.036
Inoculum size	0.34660	0.17330	0.05356	3.24	0.007
Ammonium sulfate	-0.14700	-0.07350	0.05356	-1.37	0.195
Calcium carbonate	0.56700	0.28350	0.05356	5.29	0.000
Biotin	0.06120	0.03060	0.05356	0.57	0.578

Fig: 4.12: Pareto chart showing the effect of each tested parameter on Butanol production

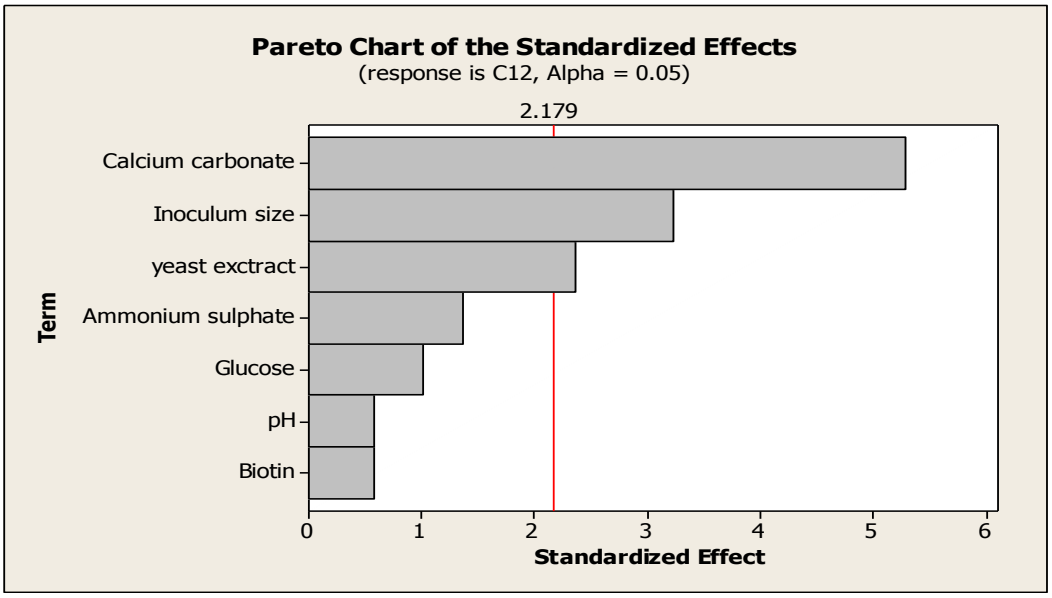
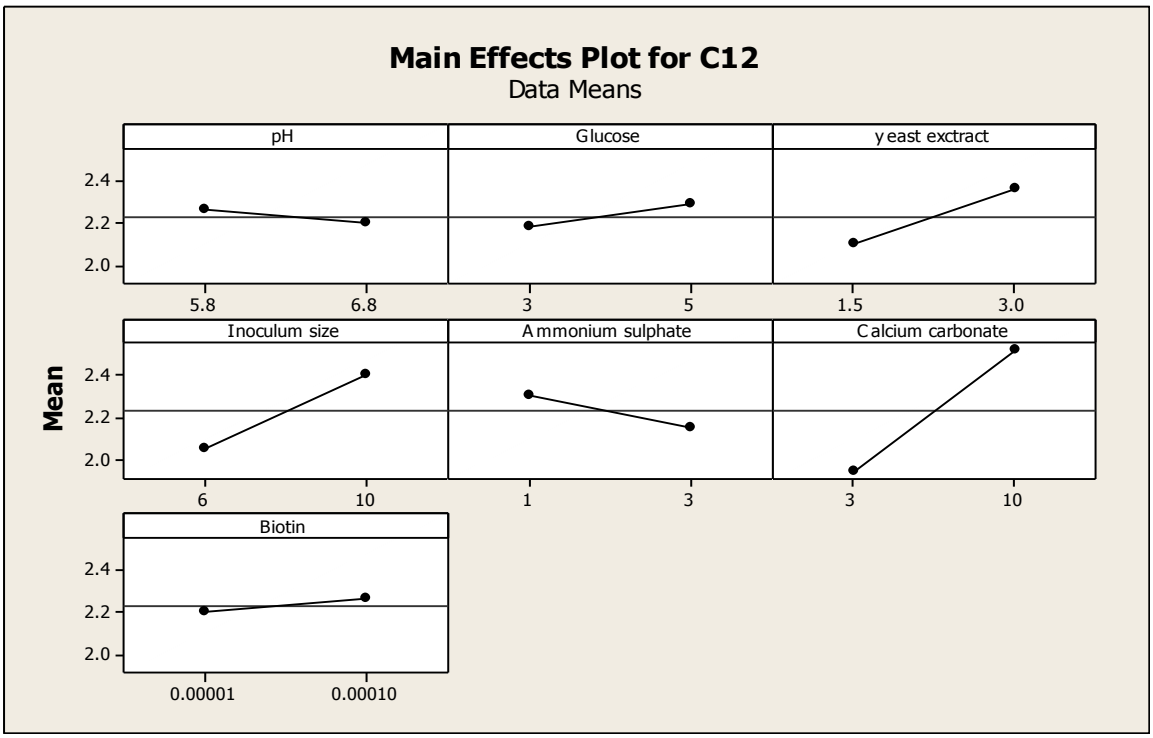


Fig: 4.13: Main Effects Plot for the regression model



From the above results it can be deduced that, with increase in inoculum size, yeast extract and calcium carbonate, there was increase production of butanol.

4.3.7. Central composite experiment design for optimizing butanol production

Based on Plackett & Burman experimental results, though pH was not found significant, considering the correlation between buffering agent and pH, both calcium carbonate and pH were selected in addition to glucose and yeast extract for further optimization of butanol production and to understand the interactions between the parameters. Central composite design (CCD) was used to analyze the variables at five levels. 30 runs were performed for 4 parameters for better and accurate understanding of significant parameters and interactions between them. Table 4.3 shows the CCD design and the responses obtained for butanol production.

Table 4.3: CCD design matrix for optimization Butanol Production

Std Order	Run Order	Pt Type	Blocks	Glucose	pH	Calcium carbonate	yeast extract	Butanol
4	1	1	1	50	6.8	4	1	3.20
11	2	1	1	20	6.8	4	2	3.00
24	3	-1	1	35	6.15	7	2.5	3.68
10	4	1	1	50	5.5	4	2	2.50
19	5	-1	1	35	4.85	7	1.5	1.00
7	6	1	1	20	6.8	10	1	3.50
21	7	-1	1	35	6.15	1	1.5	1.70
5	8	1	1	20	5.5	10	1	1.50
26	9	0	1	35	6.15	7	1.5	1.90
1	10	1	1	20	5.5	4	1	1.80
27	11	0	1	35	6.15	7	1.5	3.32
17	12	-1	1	5	6.15	7	1.5	0.51
2	13	1	1	50	5.5	4	1	2.24
13	14	1	1	20	5.5	10	2	2.00
30	15	0	1	35	6.15	7	1.5	3.33
8	16	1	1	50	6.8	10	1	3.70
3	17	1	1	20	6.8	4	1	2.20
22	18	-1	1	35	6.15	13	1.5	3.80
31	19	0	1	35	6.15	7	1.5	3.32
20	20	-1	1	35	7.45	7	1.5	2.40
15	21	1	1	20	6.8	10	2	3.85
16	22	1	1	50	6.8	10	2	4.50
14	23	1	1	50	5.5	10	2	2.50
23	24	-1	1	35	6.15	7	0.5	2.87
29	25	0	1	35	6.15	7	1.5	3.33
9	26	1	1	20	5.5	4	2	1.92
25	27	0	1	35	6.15	7	1.5	3.25
28	28	0	1	35	6.15	7	1.5	3.32
6	29	1	1	50	5.5	10	1	2.20
12	30	1	1	50	6.8	4	2	2.80
18	31	-1	1	65	6.15	7	1.5	2.20

Estimated regression coefficients for the response butanol and P values are given in Table 4. 4. Among individual parameters tested, pH was significant and among interactions, only pH and calcium carbonate showed significant interaction with a P value <0.05. ANOVA analysis of the regression model suggests that the model was significant with a computed F value of 5.71 and P value 0.001 (Table 4.5). Surface plot of the significant interaction is given in Fig 4.14.

Table 4.4: Estimated Regression coefficients

Term	Coef	SE Coef	T	P
Constant	-23.5577	10.3619	-2.273	0.037
Glucose	0.1637	0.0916	1.787	0.093
pH	7.9303	2.9060	2.729	0.015
Calcium carbonate	-0.8516	0.4579	-1.860	0.081
yeast extract	-1.5576	2.7984	-0.557	0.586
Glucose*Glucose	-0.0016	0.0004	-3.837	0.001
pH*pH	-0.6598	0.2250	-2.933	0.010
CaCO3*CaCO3	0.0018	0.0106	-0.171	0.866
yeast extract*yeast extract	0.4609	0.3802	1.212	0.243
Glucose*pH	-0.0036	0.0130	-0.278	0.785
Glucose*Calcium carbonate	0.0003	0.0028	0.116	0.909
Glucose*yeast extract	-0.0067	0.0169	-0.396	0.697
pH*Calcium carbonate	0.1476	0.0652	2.265	0.038
pH*yeast extract	0.0702	0.3910	0.180	0.860
Calcium carbonate*yeast extract	0.0485	0.0847	0.573	0.575

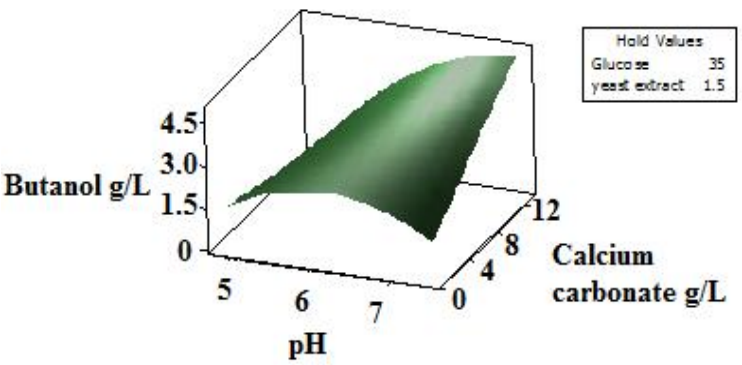
The analysis was done using un-coded units. Estimated Regression Coefficients for Butanol

Table 4.5: Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	20.649	20.649	1.4749	5.71	0.001
Linear	4	12.754	4.211	1.0526	4.07	0.018
Square	4	6.413	6.413	1.6032	6.20	0.003
Interaction	6	1.482	1.482	0.2471	0.96	0.484
Residual Error	16	4.135	4.135	0.2584		
Lack-of-Fit	10	2.422	2.422	0.2422	0.85	0.611
Pure Error	6	1.713	1.713	0.2855		
Total	30	24.784				

R-Sq = 83.32% R-Sq(pred) = 34.31% R-Sq(adj) = 68.72%

Fig: 4.14. Response surface plot showing interaction between CaCO3 and Butanol



Response surface optimization resulted in increased butanol production of 4.5 g/L in modified P2 medium with 50 g/L of glucose, 10 g/L of calcium carbonate, 2 g/L of yeast extract and at pH 6.8. Butanol production by *C. sporogenes* was not reported at this higher level with any kind of substrate. Turton et al (1983a & b) reported that some strains of *C. sporogenes* produce butanol and ethanol, if glucose is provided as carbon source whereas, some strains like *C.sporogenes* MD1 could not utilize glucose efficiently and produced ethanol, acetic acid and butyric acid without producing butanol (Flythe & Rusell, 2006).

4.4. Conclusion

C. sporogenes BE01 growth rate decreased with increase in glucose concentration beyond 20 g/L and substrate inhibition constant was 36.55 g/L. Applying Monod's equation, maximum specific growth rate was found to be 0.145 and saturation constant was 17.65 g/L. Butanol, ethanol and butyric acid also showed inhibitory effect on growth rate of *C. sporogenes* BE01 with inhibition constants 5.57, 14.8 and 2.38 g/L respectively. Single parameter optimization revealed the effect of inoculum source, calcium carbonate, inorganic nitrogen source individually and PB design showed calcium carbonate, inoculum size and yeast extract as significant parameters. Central composite design depicted the interaction between pH and calcium carbonate. Single parameter optimization and multiple parameter optimizations resulted in increase in butanol production in modified P2 medium from 0.97 g/L to 4.5 g/L.

Chapter 5

Biobutanol production from rice straw by a non-acetone producing *Clostridium sporogenes* BE01

5.1. Introduction

Lignocellulosic biomass is a potential renewable source for the cheaper production of biobutanol. Cellulose, hemicellulose and lignin together make the lignocellulosic structure complex for hydrolysis and hence pretreatment is required before hydrolysis for efficient release of sugars that can be fermented by microorganisms (Iranmahboob et al, 2002). The process of biomass pretreatment and its hydrolysis result in the release of several toxic compounds in to the hydrolysate. The variety and concentration of inhibitors generated depends on the pretreatment and hydrolysis methods used for generation of sugars from heterogeneous lignocellulosic polymer (Taherzadeh et al, 2000).

Furan derivatives, phenolics and weak acids are the common inhibitors found in hydrolysate. Furan derivatives like furfurals and hydroxy methyl furfurals are mainly derived by degradation of hexoses and pentoses present in the cellulosic and hemicellulosic portion of lignocellulosic structure and lignin releases phenolic compounds on degradation (Palmquist & Hahn-Hfagerdal, 2000). Acetyl groups present in the hemicellulose degrades into acetic acid, while furan derivatives under high temperatures break down into formic acid. The effect of these toxic compounds varies widely among different strains of bacteria. Weak acids are found highly toxic to cell growth and fermentation. Organic acids, in general are more toxic to bacteria than yeast

(Liu & Blaschek, 2010). Uncoupling and intracellular anion accumulations were the proposed mechanisms to explain the inhibitory effect of weak acids on microorganisms (Russell, 1992).

Rice straw, an agriculture residue is a suitable substrate for biobutanol production (Ranjan et al, 2013). Rice straw is one of the most abundant lignocellulosic biomass available. It has high cellulose and hemicellulose content which can be readily hydrolyzed into hexoses and pentoses (Binod et al, 2010). Hexoses are readily utilized by solventogenic *Clostridium* species, Pentoses can also be utilized, but with the low production rates (Volesky & Szczesny, 1983). Dilute acid pretreatment and enzymatic hydrolysis of rice straw releases acidic inhibitors in to hydrolysate. The sensitivity of the organism to acetic acid depends on the culture conditions (Lawford & Rousseu, 1998). Formic acid at the concentration less than 1mM reduced fermentation efficiency of *C. acetobutylicum* (Wang et al, 2011). Detoxification of hydrolysate is one of the most important steps in conversion of lignocellulosic biomass to biofuels.

C. sporogenes is a non neurotoxigenic counterpart of group 1 *Clostridium botulinum* (Sebaihia et al, 2007) and it produces ethanol and butanol without acetone in the final mixture of solvents, which is an advantage in bioconversion process of biomass to alcoholic fuels (Kannan et al, 2010). The present study evaluated the production of butanol production using an enzymatic hydrolysate of dilute acid pretreated rice straw and optimized the parameters for improving yield of butanol using *C. sporogenes* BE01.

5.2. Materials and Methods

5.2.1. Fermentation

Fermentation was performed with rice straw hydrolysate (prepared as in section 2.1.2) as the medium, in 100 ml screw capped bottles containing 50 ml medium. The medium was supplemented with minerals [in g/L -$(NH_4)_2SO_4$ -1.5, $MgSO_4.7H_2O$-6.0, KH_2PO_4-0.5, NaCl-0.01, $MnSO_4.H_2O$-0.01, $FeSO_4.7H_2O$, 0.01] (Hartmanis *et al*, 1986; Qureshi & Blaschek, 1999) or without. Yeast extract (1.5 g/l) was added as the source of organic nitrogen, vitamins and other essential nutrients. Calcium carbonate (4.5 g/l unless specified different) was added as buffering agent in the hydrolysate and the medium pH was adjusted to 6.7. The fermentation medium was sterilized by autoclaving (121 °C for 15min) and cooled down to 37 °C under continuous purging of nitrogen gas. Actively growing 12 h old culture of *C. sporogenes* BE01 at 10% v/v was used as inoculum for butanol production, unless age and percentage of the inoculum is specified differently.

5.2.2. Effect of inoculum age

C. sporogenes BE01 was used as the inoculum for butanol fermentation in its active growth stage. Initial pH of the fermentation medium was maintained at 6.7. Cells grown in TGY medium were harvested at 6 h, 9 h, 12 h or 24 h of growth and were used as inoculum to study the impact of inoculum age on butanol production. Inoculum concentration (10% v/v) was kept constant and fermentation was continued till 96 h. Samples were collected at regular time intervals and were analyzed for butanol production.

5.2.3. Effect of calcium carbonate

Calcium carbonate was used as the buffering agent to maintain the pH of the medium during fermentation (Richmond et al, 2011). The effect of calcium carbonate on accumulation of butanol in the fermentation medium was studied by adding different concentrations 0, 2, 5 and 10 (g/l) in the fermentation medium. pH was adjusted to 6.7 after adding calcium carbonate into the hydrolysate and the medium was autoclaved at 121 °C for 15 min. Fermentation was performed for 96 h and samples were collected at regular time intervals to analyze butanol production.

5.2.4. Growth inhibition kinetics

In this approach, acidic inhibitors such as acetic acid and formic acid were considered as growth inhibitory compounds. Specific growth rate was calculated by sampling the cells at regular time intervals (Naher et al, 2008). Varying concentrations of acidic inhibitors in glucose containing P2 production medium were used for kinetic studies. Linearized Haldane growth inhibitory equation was used for representing growth inhibition kinetics and determination of growth inhibition constant (Kumar et al, 2005).

Specific growth rate $$\mu = \frac{(\log_{10} Z - \log_{10} Z_0)2.303}{t - t_0} \quad (1)$$

Haldane equation $$\mu = \frac{\mu_{max} S}{K_S + S + \left(\frac{S^2}{K_i}\right)} \quad (2)$$

At concentrations where, S >> K_S, Haldane equation reduces to the below equation

$$\mu = \frac{\mu_{max} S}{S + \left(\frac{S^2}{K_i}\right)} \quad (3)$$

Linearized Haldane equation $\frac{1}{\mu} = \frac{1}{\mu_{max}} + \left(\frac{1}{K_i.\mu_{max}}\right) S$ (4)

Linear graph was plotted with 1/μ vs initial inhibitor concentrations (S). μ_{max} was obtained form the slope of the curve and inhibition constant K_i was derived from intercept on X-axis.

5.2.5. Adsorption experiments for inhibitors removal

To compare four different adsorbent resins for the removal of acidic inhibitors, experiments were performed in batches in 100 ml glass bottles with 50 ml rice straw hydrolysate. The four polymeric adsorbent resins selected for the comparison were XAD 4 (non polar), XAD 7 (Moderately polar), XAD 16 (Polar) and the anion-exchange resin selected was Seralite SRA-400. The XAD adsorbent resins differ in matrix, pore size and surface area, but have the same particle size. Anion-exchange resin, Seralite SRA-400 carries an active functional group on its matrix with ion exchange capacity. Properties of the resins are given in Table 5.2.

Inhibitors such as acetic acid, formic acid, furfurals and hydroxymethyl furfurals (HMF) present in hydrolysate were analyzed prior to detoxification and the detoxification efficiency was determined by analyzing the remaining acids and furfurals present in resin treated samples. Detoxification studies were performed as batch processes. Before using these resins for detoxification studies, they were conditioned as recommended by the respective manufacturer. Resins were added to the hydrolysate at 1% (w/v) and kept at 200 rpm for 8 h at 30 $\pm$ 2°C. Samples were withdrawn at regular intervals to analyze the presence of acidic inhibitors. The affinity of resins towards sugars and minerals were also analyzed by determining the initial and final sugar and mineral concentrations in the hydrolysate.

5.2.6. Adsorption models

Monolayer Langmuir and the empirical Freundlich models were applied to fit the batch adsorption experiments data and to select the better resin favoring the adsorption of acetic acid and formic acid from rice straw hydrolysate.

5.2.6.1. Freundlich adsorption isotherm

According to Freundlich adsorption model, surface of the adsorbent is heterogeneous and allows multilayer adsorption of adsorbate on to adsorbent, which is polymolecular layer adsorption (Freundlich, 1926). The linear from of Freundlich equation is

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \qquad (5)$$

Where q_e = amount adsorbed (mg/g); Ce = equilibrium aqueous concentration (g/L); K_F and $\frac{1}{n}$ are Freundlich constants. The linear plot of log q_e vs log C_e provide the values of slope and intercept, $\frac{1}{n}$ and K_F respectively. The constants $\frac{1}{n}$ and K_F are related to adsorption intensity and adsorption capacity respectively.

5.2.6.2. Langmuir adsorption isotherm

According to Langmuir theory, adsorption is monolayer and no further adsorption takes place, if a site is occupied by solute (Langmuir, 1916). Langmuir equation for monolayer adsorption is expressed as follows.

$$\frac{1}{q_e} = \frac{1}{q_0 . K_L . C_e} + \frac{1}{q_0} \qquad (6)$$

Where C_e = equillibrium concentration (g/L) ; q_e = adsorption capacity (mg/g) and q_0 and K_L are the Langmuir constants. A plot of $1/q_e$ vs $1/C_e$ yields slope and intercept by which the constants can be determined.

The percentage removal of acetic acid and formic acid by the resins from rice straw hydrolysate was calculated by the following equation

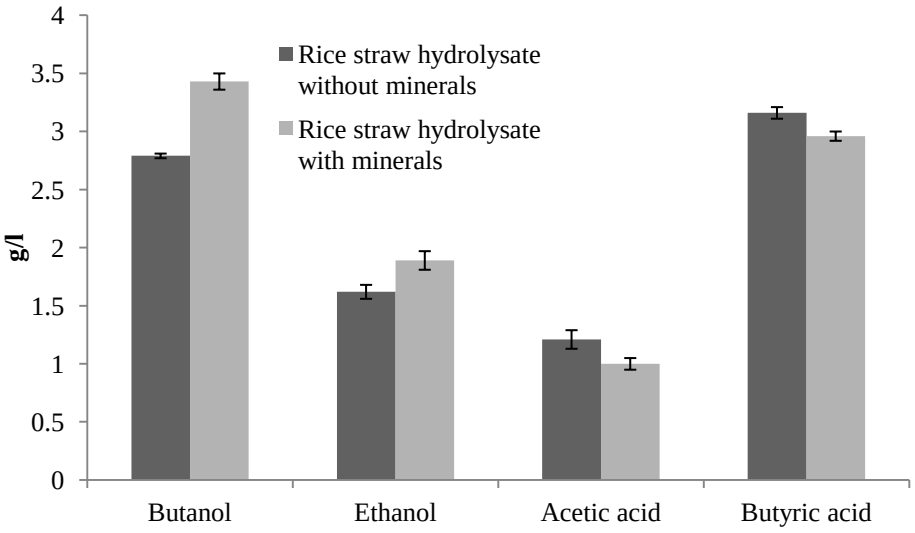
$$\text{Percentage removal} = \frac{C_0 - C_e}{C_0} \times 100 \qquad (7)$$

5.3. Results and discussion

5.3.1. Comparison of Biobutanol production in rice straw hydrolysates with and without mineral supplementation

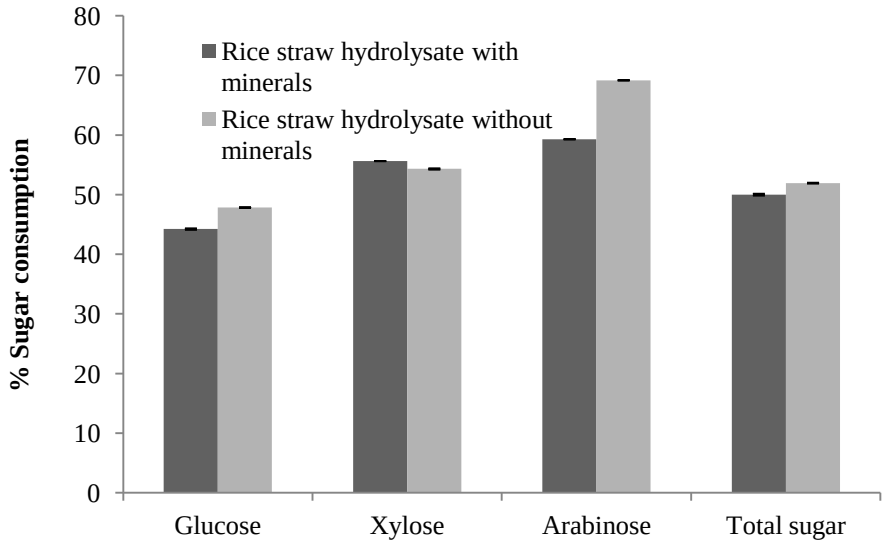
Hydrolysate with 39.02 g/l of glucose, 11.35 g/l xylose and 1.71 g/l arabinose was obtained with dilute acid pretreatment and enzymatic hydrolysis of rice straw. Hemicellulose is removed with dilute acid pretreatment and enhances the enzymatic digestibility of cellulose from residual solids (Mosier et al, 2005). Butanol fermentation using rice straw hydrolysate was tried both with supplementation of minerals or without. With supplementation of minerals in hydrolysate, 2.79 g/l of butanol, 1.62 g/l of ethanol, 1.21 g/l of acetic acid and 3.16 g/l of butyric acid were produced and without mineral supplementation, 3.43 g/l and 1.89 g/l of butanol and ethanol respectively were produced including 1.0 g/l acetic acid and 2.96 g/l butyric acid (Fig 5.1).

Fig 5.1: Butanol fermentation in rice straw hydrolysate by *C. sporogenes* BE01



Both hexoses and pentoses were utilized for butanol production by the organism. Forty four % of glucose, 56 % of xylose and 59 % of arabinose was utilized by the organism from the hydrolysate without mineral supplementation and 48% of glucose, 54 % of xylose and 69% of arabinose was utilized by the organism from the hydrolysate with mineral supplementation (Fig.5.2). Solvent production was higher without mineral supplementation; this could be due to the natural presence of minerals in rice straw like potassium, phosphorous, iron and calcium in their oxidized forms and also small amounts of magnesium, sulfur and sodium (Kadam et al, 2000). Mussatto & Roberto (2005) reported decrease in xylitol production with addition of nutrients to rice straw hydrolysate medium and also mentioned that it could be probably due to imbalance between ionic nutrition. Ammonium sulfate can also have negative effect on the organism and fermentation by releasing sulfate ions and decreasing the pH of the medium (Mussatto & Roberto, 2005).

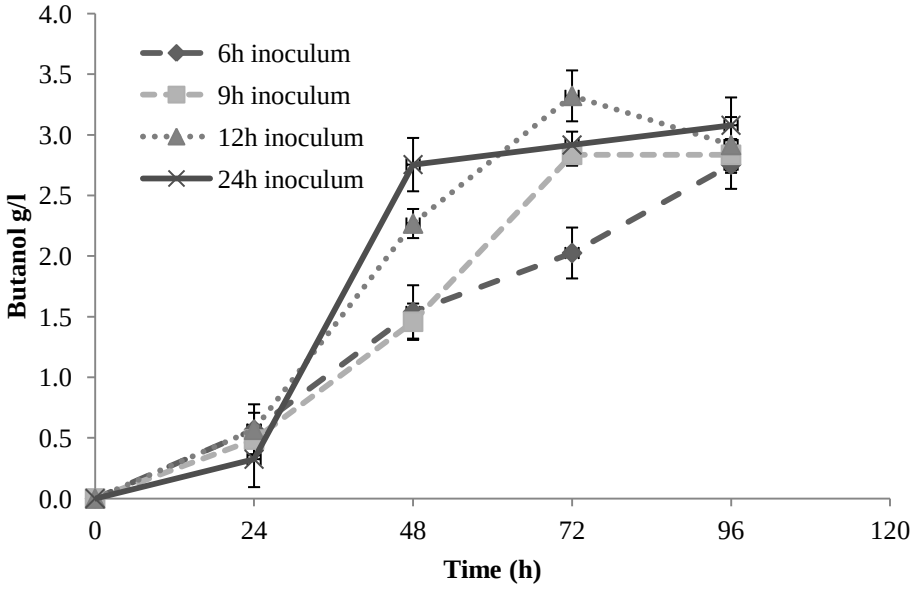
Fig 5.2: Sugars consumption from rice straw hydrolysate by *C. sporogenes* BE01



5.3.2. Effect of inoculum age on butanol fermentation

The results shown in Fig 5.3 indicated that the age of inoculum influences solvent production. The 12 h old inoculum supported higher butanol production (3.32 g/l) when compared to other inocula. There was a decrease in butanol production when 24 h old inoculum was used. Inoculum age and the culture motility control the production of solvents and trigger the activity of enzymes operating in solventogenic phase (Spivey, 1978). Jones *et al* (1982) correlated the morphological changes of *Clostridium acetobutylicum* P262 with its solvent production in molasses medium. It was reported that after 6 h, the chains formed by elongated rods broke and highly motile cells were released and after 14-18 h the motility of the cells decreased. Decrease in motility of the cells results in decrease in solvent production (Spivey, 1978), which explains the higher butanol yield with inoculum grown for 12 h when the cells are actively growing and motile.

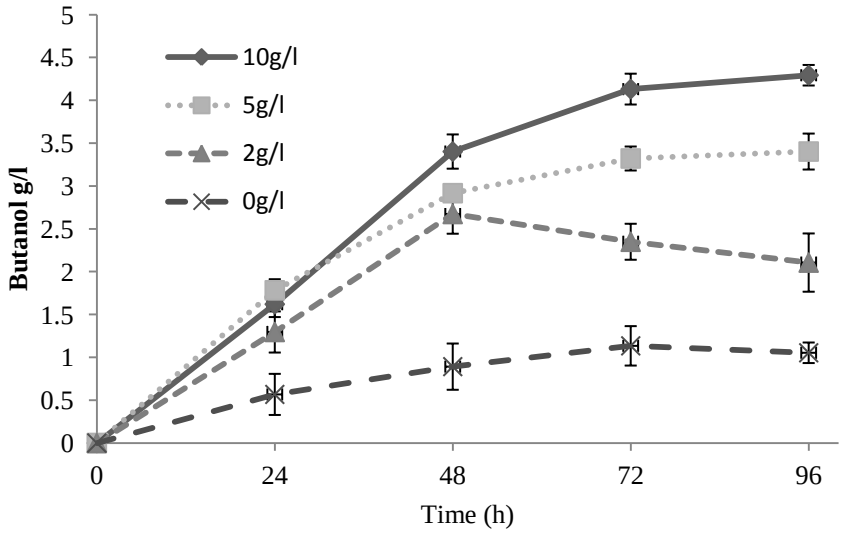
Fig: 5.3: Effect of inoculum age on butanol fermentation in rice straw hydrolysate by *C. sporogenes* BE01



5.3.3. Effect of calcium carbonate on butanol production

Hydrolysate supplemented with 10 g/l calcium carbonate supported the highest production of butanol (4.05 g/l) with a productivity 0.04 $gl^{-1}h^{-1}$. Final butanol concentration with 0, 2, and 5 g/l of calcium carbonate were 3.32 g/l, 2.35 g/l and 1.13 g/l respectively (Fig 5.4). ABE fermentation with poorly buffered medium leads to excessive accumulation of acids. Un-dissociated acetic acid and butyric acid are inhibitory to cell growth, nutrient uptake and butanol production (Ezeji et al, 2005). Richmond et al (2011) reported that calcium carbonate favored growth and butanol production of *Clostridium* species. It also increases the tolerance of organism to butanol and so increases the final butanol accumulation in the fermentation medium.

Fig 5.4: Effect of Calcium carbonate on butanol fermentation in rice straw hydrolysate by
C. sporogenes BE01



5.3.4. Growth inhibition kinetics

Dilute acid pretreatment of lignocellulosic biomass releases some compounds like acetic acid, formic acid, furfurals and hydroxy methyl furfurals which are inhibitory for conversion of sugars to desired products (Wickramasinghe & Grzenia, 2008). Acetic acid and formic acid were the major inhibitors found in enzymatic hydrolysate of dilute acid pretreated rice straw in the concentrations of 3.46 g/L and 7.24 g/L respectively. The specific growth rate of *C. sporogenes BE01* decreased with increase in acidic inhibitors concentration in the medium. Linearized Haldane equation, used to determine the K_i values gave a linear graph with regression coefficients 0.87 and 0.96 for acetic acid and formic acid respectively (Fig 5.5). Growth inhibition constants (K_i) and maximum specific growth rate (μ_{max}) in acetic acid and formic acid are given in Table 5.1. About 3.0 g/L of acetic acid and 2.0 g/L of formic acid individually resulted in 50% decrease of specific growth rate. These results suggest that both the acetic acid and formic acid are toxic to *C. sporogenes BE01*.

Fig 5.5: Kinetics of growth inhibition of *C. sporogenes* BE01 by acetic acid and formic acids

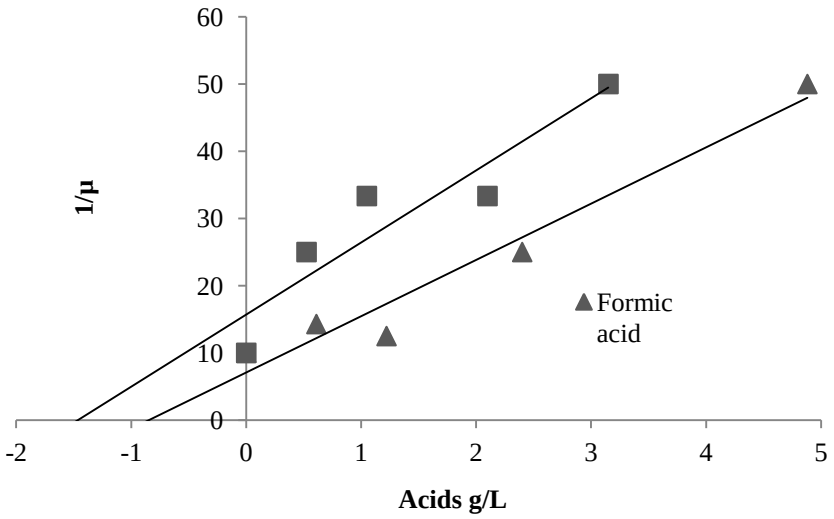


Table 5.1. Kinetic parameters of growth inhibition by *C. sporogenes*

	Acetic acid	Formic acid
μ_{max}	0.06	0.14
K_i	1.60	0.76

Above the threshold concentration, growth rate decreases linearly with increase in inhibitor concentration (Costa & Moreira, 1983). Maximum specific growth rate of *Clostridium acetobutylicum* was reduced to 50% at 4 g/L of acetic acid (Ballongue et al, 1987). On the basis of molar concentration, formic acid was stated as strong inhibitor than acetic acid. Addition of 1mM of formic acid, which was 0.046 g/L to corn mash medium resulted in acid crash and reduced ABE fermentation (Wang et al, 2011). Formic acid is more inhibitory to cell growth than acetic acid due to its smaller molecular size and higher anion toxicity (Larsson et al, 1999).

5.3.5. Solvent production in detoxified rice straw hydrolysate

Adsorption of various inhibitors from different lignocellulosic hydrolysates were

already reported with polymeric resins and anion-exchange resins (Qureshi et al, 2008; Nilvebrant et al, 2001), but the selection of resin for detoxification is different for different kinds of lignocellulosic biomass and the kinds of inhibitors to be removed. Removal of inhibitors is considered necessary to improve the solvent production by the organism. XAD7, XAD4, XAD16 and Seralite SRA-400 were evaluated for removal of inhibitors. Properties of the resins selected are given in Table 5.2.

Table 5.2: Properties of resins selected for de-toxification of inhibitors in biomass hydrolysates

Properties	**XAD 4**	**XAD 7**	**XAD 16**	**Seralite SRA-400**
Matrix and active group	Styrene-divinylbenzene	Acrylic ester	Polystyrene, a polar	Styrene/divenyl benzene with quarternary ammonium functional group
Particle size	20-60 mesh	20-60 mesh	20-60 mesh	20-50 mesh
Surface area	725 m^2/g	~450 m^2/g	800 m^2/g	-
Pore size	~0.98 ml/g pore volume 40 Å mean pore size	1.14 ml/g pore volume	1.82 ml/g pore volume 100 Å mean pore size	-
Ionic form and Ion exchange capacity	-	-	-	Cl^- 3-3.5meq/g

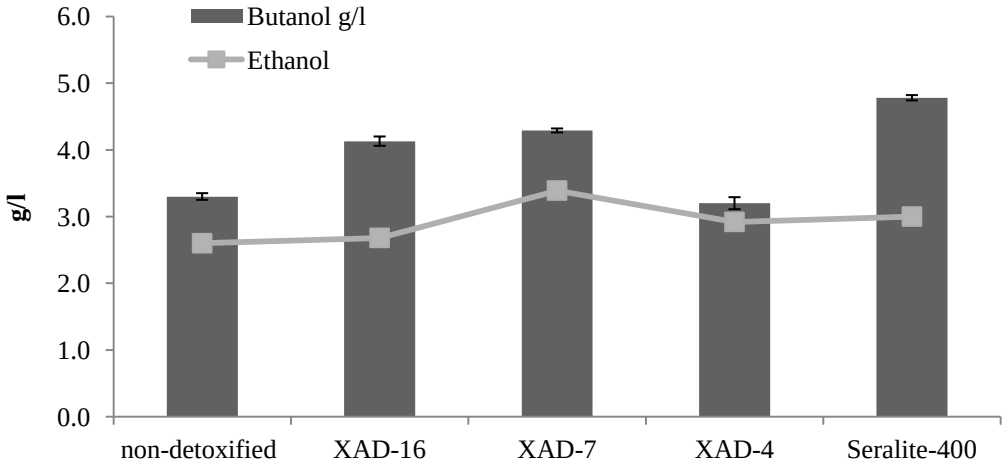
Furfurals were found to be absent in hydrolysate, so removal of acetic acid and formic acid was studied. XAD7, XAD16 and Seralite SRA-400 were found to be efficient in acid removal, where as XAD4 was less effective (Table 5.3).

Table 5.3: Removal of inhibitors in hydrolysates by resins

Resin	Acetic acid(i) (g/l)	Acetic acid(f) (g/l)	% removal	Formic acid(i) (g/l)	Formic acid(f) (g/l)	% removal	% removal of total acids
XAD 4	3.46	1.51	56.36	7.24	4.16	42.54	47.01
XAD 7	3.46	0.94	72.83	7.24	3.21	55.66	61.21
XAD 16	3.46	1.95	43.64	7.24	2.5	65.47	58.41
Seralite SRA-400	3.46	1.06	69.36	7.24	3.28	54.70	59.44

Butanol fermentation was also performed with the detoxified samples. Hydrolysate treated with Seralite SRA-400 supported fermentation most efficiently compared to the other resins, attaining a butanol production of 4.78 g/l. Hydrolysates treated with XAD4 did not support improved production of solvents, but those treated with XAD16 and XAD7 supported higher butanol production reaching 4.13 g/l and 4.29 g/l respectively. XAD4 treated hydrolysates gave similar butanol production (3.20 g/l) when compared to non detoxified hydrolysate, which gave 3.3 g/l. Total solvent yield was also higher in hydrolysate treated with Seralite SRA-400 (7.78 g/l) (Fig 5.6). Enzymatic hydrolysate did not show any presence of furfurals and hydroxymethyl furfurals. Furfurals and hydroxymethyl furfurals which are generally formed by acid hydrolysis can be removed efficiently by XAD4 resin (Qureshi et al, 2008). Anionic exchanger can efficiently remove both anionic and uncharged inhibitors at higher pH 10.0 (Nilvebrant et al, 2001). They can also efficiently remove acids like levulinic acid, acetic acid, formic acid and furfurals (Chandal et al, 2011) which explain the better performance of Seralite SRA400 for detoxifying the hydrolysate.

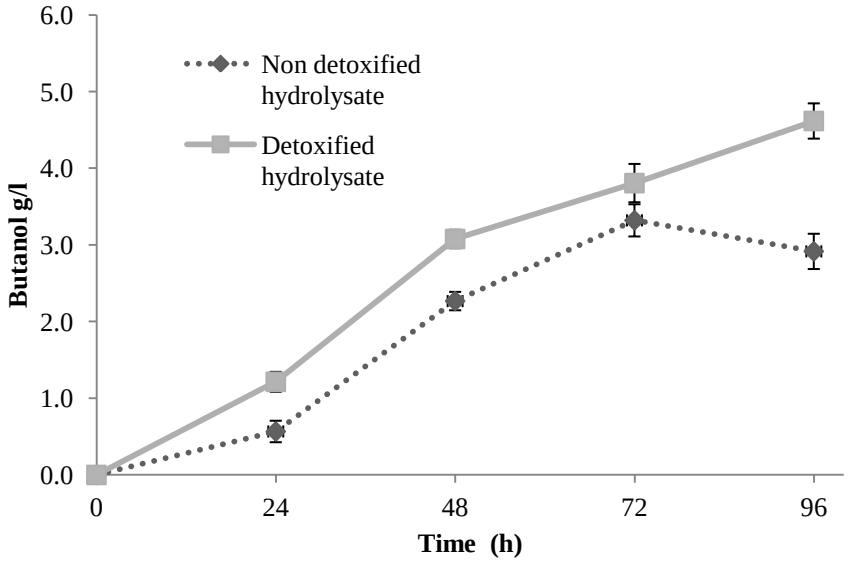
Fig: 5.6: Solvents production in hydrolysate detoxified by different resins



5.3.6. Time course study of butanol production with detoxified and non detoxified hydrolysate

Anionic resin (Seralite SRA400) was found better for detoxification of rice straw hydrolysate and hence used for further detoxification studies. The maximum butanol production (4.62 g/l) was obtained with detoxified hydrolysate at 96h with a productivity of 0.05 $gl^{-1}h^{-1}$. Non detoxified hydrolysate also supported a maximum butanol production of 3.32 g/l at 96 h with a productivity of 0.03 $gl^{-1}h^{-1}$. Butanol production in detoxified hydrolysate increased from 72h to 96h, compared to the non-detoxified hydrolysate in which the butanol production had dropped on extended incubation from 72h to 96h (Fig 5.7).

Fig 5.7: Time course study of butanol production from rice straw hydrolysate detoxifies by Seralite SRA-400



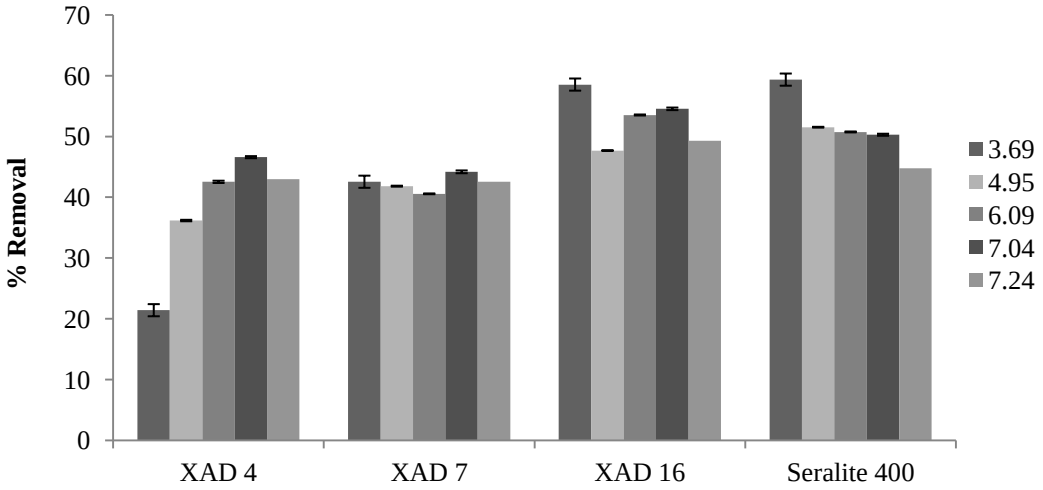
Ranjan et al (2012) had reported a butanol production of 12.7 g/L and a yield of $0.38 gg^{-1}$, which was achieved with acid hydrolyzed rice straw by using *Clostridium acetobutylicum* MTCC 481. In an earlier report, rice straw and sugarcane bagasse hydrolysates were detoxified with ammonium sulfate precipitation and activated charcoal treatment and ABE concentration of 18.1 g/l was obtained (Soni et al, 1982). Qureshi et al (2008) reported that dilute acid treated corn fiber hydrolysate inhibited the cell growth and butanol production due to the presence of furfurals and detoxification with XAD4 resin is needed to improve the fermentation efficiency.

5.3.7. Effect of initial concentration of acidic inhibitors on their removal

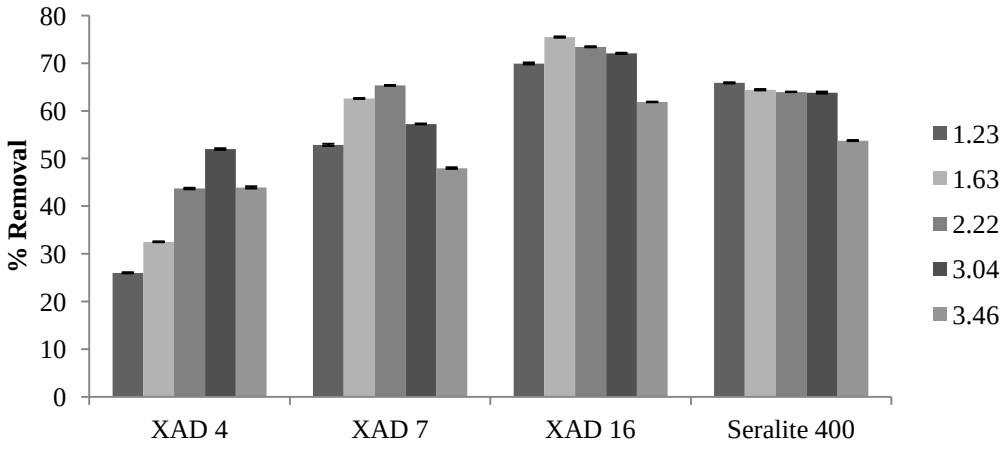
To further improve the detoxification efficiency and butanol production, detailed studies on inhibitors adsorption was done. The hydrolysate diluted to have different initial concentrations of acids was used for adsorption studies. The initial concentrations of acids in the hydrolysate had an impact on their removal by adsorbent resins (XAD 4, XAD 16, XAD 7 and Seralite SRA-400). Adsorption of acetic acid and formic acid on

Seralite SRA-400 decreased with increase in initial acid concentration and XAD resins showed increase in adsorption efficiency with increased acid concentration and then started decreasing after a point (Fig. 5.8a, b).

Fig 5.8: Efficiency of toxin removal by resins



A: Formic Acid Removal



B: Acetic Acid removal

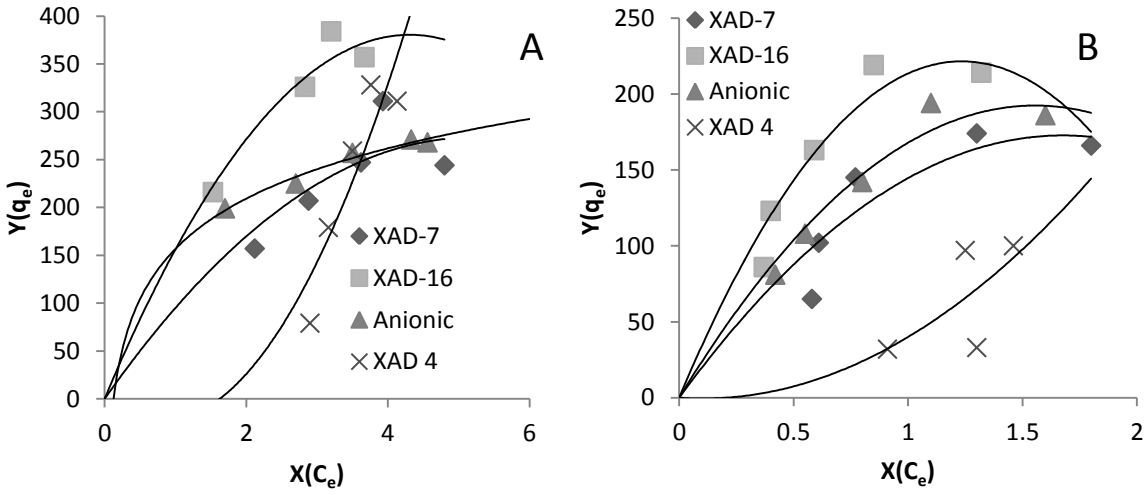
5.3.8. Isotherm modeling

An adsorption model is required to predict the loading of adsorbate on to adsorbent at a particular concentration under ambient conditions. Equilibrium concentration (C_e) and adsorption capacity (q_e) were determined by plotting acid

concentration (g/L) vs time. Rate of adsorption was higher at the beginning and decreased with increase in contact period, this could be due to the availability of large surface area during the initial period of adsorption and also the equilibrium established between the amount of adsorbate adsorbed and remained in the solution.

Equilibrium concentration and time varied with initial concentrations of acids. Adsorption isotherm graphs showed parabolic curves for XAD-7, XAD-16 and Seralite SRA-400 when plotted with $X(C_e)$ vs $Y(q_e)$, except XAD 4 gave unfavorable curve for both acetic acid and formic acid (Fig 5.9a & b). Freundlich adsorption isotherm and Langmuir adsorption isotherms were applied to determine adsorption capacity and intensity of the adsorbent resins.

Fig: 5.9: Adsorption isotherm model for organic acid adsorption by different resins



A- Acetic Acid; B-Formic Acid

5.3.8.1. Freundlich and Langmuir adsorption isotherm

The constants n and K_F were determined by slope and intercepts obtained by the linear plots of log C_e vs log q_e. The equilibrium concentration and the amount of adsorbent adosrbed to the resin evaluate the efficiency of adsorption by any resin. The constants k_F and n obtained represents the adsorption capacity and adsorption intensity

respectively (Garcia-Cuello et al, 2011). Table 5.4a and b shows the n and K_F values of acetic acid and formic acid for XAD 4, XAD 16, XAD 7 and Seralite SRA-400 (anion-exchange resin). .

Table 5.4: Adsorption isotherms of different resins for acetic (A) and Formic (B) acids

A

Adsorbent	Freundlich model			Langmuir model		
	n	K_f	R^2	q_0	K_L	R^2
XAD-4	1.48	1.8	0.82	12	-0.22	0.76
XAD-7	1.52	100.7	0.70	100	0.1	0.83
XAD-16	0.26	155.6	0.74	250	0.2	0.72
SRA-400	3.09	166.7	0.98	250	0.69	0.97

B

Adsorbent	Freundlich model			Langmuir model		
	n	K_f	R^2	q_0	K_L	R^2
XAD-4	0.45	40.1	0.58	23	-0.39	0.55
XAD-7	1.48	130.3	0.66	167	0.17	0.66
XAD-16	1.49	156.3	0.81	200	0.33	0.84
Seralite SRA-400	1.52	207.0	0.90	250	0.5	0.96

XAD 16 gave highest K_F and n values for acetic acid, where as for formic acid, it was Seralite SRA-400 that gave the highest constant values. The difference between the constants obtained with XAD 16 and Seralite SRA-400 for both acetic acid and formic acid adsorption was not found to be significantly different, but the regression coefficient

values showed better fit to Seralite SRA-400. These results suggest that adsorption capacity and adsorption intensity for acidic inhibitors from acid pretreated rice straw hydrolysate was better with Seralite SRA-400.

Langmuir isotherm describes well monolayer adsorption of adsorbent on to adsorbate. The constants q_0 and K_L were obtained from the linear plot of $1/C_e$ vs $1/q_e$. The experimental data was fitted in with Langmuir equation to derive the constant values, which are indicative of surface binding energy and monolayer coverage. Adsorption studies of formic acid and acetic acid on to XAD 4 gave negative constants which indicate that the adsorption was not monolayer and does not fit in Langmuir model. Constants obtained with XAD 7, XAD 16 and Seralite SRA-400 fit in the Langmuir adsorption model, but with regression coefficients were close to and below 0.8 (Table 5.4a & b). For both the acetic acid and formic acid adsorption, the langmuir constants were high for Seralite SRA-400 (0.96 regression coefficient).

Earlier studies showed that when sugarcane bagasse hydrolysate was treated with anion-exchange resin, improved ethanol yield was achieved by removing 84% of acetic acid (Vanzyl et al, 1991). Larsson et al (1999) also reported that anion exchange resin efficiently removed toxic inhibitors from wood hemicellulose hydrolysate. XAD resins were also known to remove both anionic inhibitors and uncharged inhibitors effectively (Nilvebrant et al, 2001).

5.3.8.2. Affinity of resins towards minerals and sugars present in hydrolysate

Minerals (calcium, magnesium, iron and potassium) and sugars in the rice straw hydrolyaste were analyzed before and after the treatment with adsorbent resins. 10-12% of sugars were adsorbed by all the resins, whereas, mineral adsorption varied with resins. XAD-4 adsorbed nearly 50% of calcium, magnesium and iron from the hydrolysate and

less amount of potassium. Affinity towards minerals was different for all the four different resins (Table 5.5) Iron adsorption was comparatively high with all the four resins and potassium was adsorbed less. Calcium and magnesium adsorption varied strongly with the kind of resin used for detoxification.

Table 5.5. Mineral adsorption from hydrolysates by resins

Adsorbents	Calcium	Magnesium	Iron	Potassium	Sugars
XAD4	50.0	50.0	57.8	3.0	10.3
XAD7	25.0	0.0	48.2	3.1	12.1
XAD16	0.0	16.7	48.6	9.4	11.3
Seralite SRA-400	25.0	0.0	43.8	6.5	11.7

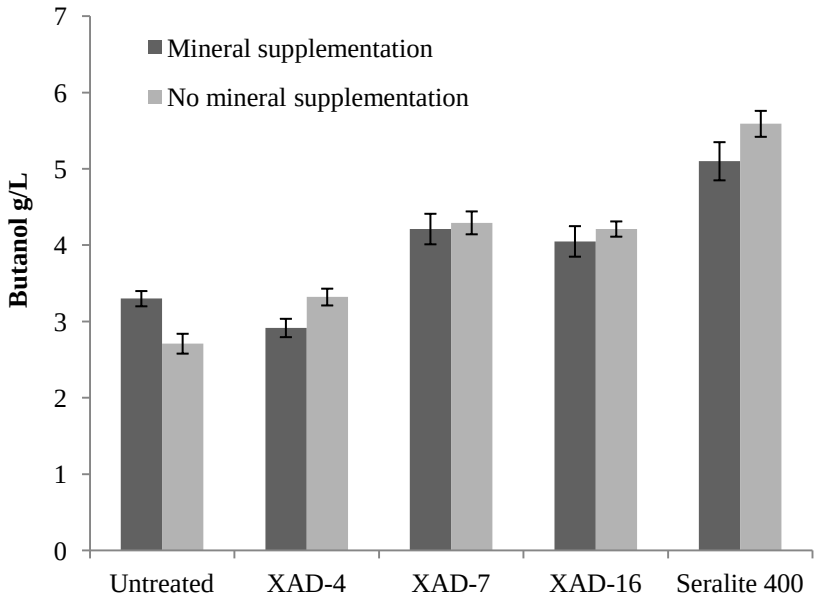
There are reports on metal adsorption by amberlite XAD resins and ion exchange resins and their application for the separation and pre concentration of trace metals (Elci et al, 1992). The criterion to select an adsorbent is high specificity to adsorb inhibitors and little or no specificity for sugars (Weil et al, 2002). Seralite SRA-400 was selected as a suitable candidate for detoxification of rice straw hydrolysate for of its high affinity to acidic inhibitors and less affinity towards sugars and minerals.

5.3.9. Evaluation of mineral supplementation in rice straw hydrolysate treated with resins

Supplementation of minerals to the hydrolysate treated with XAD-4 gave butanol yield similar to untreated hydrolysate, which suggests that adsorption of minerals by XAD-4 during detoxification affected butanol fermentation negatively. Mineral supplementation did not lead to any significant difference in butanol production for

hydrolysate detoxified with XAD-7 and XAD-16. Rice straw hydrolysate treated with Seralite SRA-400 gave 1.7 times increase in butanol yield, while there was slight decrease in hydrolysate supplemented with minerals (Fig 5.10).

Fig 5.10: Evaluation of mineral supplementation in rice straw hydrolysate treated with resins



Reduction of butanol production in untreated rice straw hydrolysate supplemented with minerals was observed in the preliminary experiments and the same was observed here with rice straw hydrolysate detoxified with Seralite SRA-400. Reduced efficiency of hydrolysate detoxified with XAD-4 could be attributed to mineral loss from hydrolysate by adsorption during the process of detoxification. On the contrary, there are reports on XAD-4 as efficient detoxifier, but mostly for the removal of furfurals from hydrolysate, which was later supplemented with minerals and vitamins (Qureshi et al, 2008).

5.4. Conclusion

The ability of the culture to grow and produce butanol in rice straw hydrolysate with an advantage of no acetone in the final fermentation mixture indicates its potential to be used for biobutanol production. Rice straw hydrolysate with yeast extract and calcium carbonate can be used as substrate for butanol fermentation by using *C. sporogenes* BE01. Acetic acid and formic acid were the major acidic inhibitors in dilute acid pretreated rice straw hydrolysate. Presence of acetic acid and formic acid at low concentrations in the medium inhibited the growth of the *C. sporogenes BE01*. Inhibition constants for acetic acid and formic acid were 1.6 g/L and 0.76 g/L respectively. Seralite SRA-400, an anion-exchange resin was efficient in removing acidic inhibitors, with minimal removal of sugars and minerals in rice straw hydrolysate and improving butanol production to 5.59 g/L. XAD-4 was least efficient in removing acidic inhibitors and exhibited high affinity towards minerals present in hydrolysate, therefore had negative effect on butanol fermentation.

Chapter 6

VFA, hydrogen and solvents production: Bio-electrochemical analysis of electron transfer mediators involved

6.1. Introduction

Biobutanol gained significant attention for its properties as liquid transportation fuel, but its production from lignocellulosic biomass is facing challenges both technically and economically (Gracia et al, 2011). VFAs and hydrogen produced during fermentation process can be considered as an added advantage, when an efficient process is in place. Formation of various industrial products from a single substrate in a single run could be considered beneficial for lignocellulosic biorefinery processes. For this, it is essential to understand the redox micro environment of the bioreactors operated with the desired biomass and microbes. Fluctuation in redox environment leads to change in bacterial growth pattern, glucose utilization, products formed etc (Barnes & Ingram, 1956). Redox balance in a bioreactor is one among the many key components that leads to carbon flux and change in metabolic activity of the organism (Rao & Mutharasan, 1987). In order to assess the bio-electric potential of a bioreactor, it is essential to perform the analysis of bio-electrocatalytic efficiency of the microbial catalyst. Electrochemical characterization of microbial bioreactors will help in understanding the redox active species participating in electron transfer reactions (Marsili et al, 2008). The metabolic pathway of glucose to butanol conversion is complex and several enzymes are involved in diverting the pathway towards VFAs (acetic acid and butyric acid) and for assimilation of these VFAs to butanol. It is a highly interlinked chain of redox reactions with many electron transporters involved. Formation of hydrogen and volatile fatty acids is the intrinsic part of biochemical pathway for butanol fermentation by Clostridia

(Geshlagi et al, 2009). Conversion of glucose to acetyl CoA through pyruvate route generates hydrogen and, acetyl CoA is the precursor for VFAs and solvents production (Juanita & Guangyi, 2009; KEGG, 2010). Hydrogen generated by Clostridia is directly related to volatile fatty acids production. Conversion of acetyl CoA to acetate yields hydrogen twice the yield as the conversion to butyrate (Tao et al, 2007). The ratio of acetic acid and butyric acid has tremendous effect on the ratio of solvents formed (Matta-el-Ammouri et al, 1987). Hydrogen produced during the process is an energy rich gaseous fuel and VFAs can be used as precursors for Polyhydroxyalkanoates (Lee et al, 2013). Solvents are the main metabolic products and can be used as biofuels.

This study presents the Bio-electrocatalytic efficiency of *C. sporogenes* BE01, a novel non acetone producing bacteria to convert fermentable sugars generated from hydrolysis of lignocellulosic biomass to solvents, VFAs and hydrogen. It is also focused to understand the possible electron transport mediators and redox reactions involved during the process.

This is the first report on hydrogen production from rice straw hydrolysate by using a pure strain of *C. sporogenes* and also it is the first the report on demonstration of electron transporters mediated redox activity of *Clostridium sp.* during biobutanol fermentation from lignocellulosic biomass.

6.2. Materials and methods

6.2.1. Fermentation

C. sporogenes BE01 was maintained in spore form at 4 ºC. The spores were heat shocked at 80 ºC for 2 min and the temperature was immediately brought down by placing in ice bath. The heat-shocked spores were cultured in TGY medium to develop pre-inoculum. Actively growing cells were inoculated in to fresh TGY medium for inoculum generation. Highly motile, 12h old culture was inoculated in to rice straw hydrolysate. Rice straw hydrolysate was made anaerobic by cooling under nitrogen atmosphere after heat sterilization at 120 ºC for 10min. Bottles with loosened caps were placed inside the anaerobic chamber for 12h before inoculation.

Fermentation was carried out in 100ml bottles having 70ml of rice straw hydrolysate medium. As described in the previous reports, $CaCO_3$ and yeast extract (Himedia, India) were the only supplementation and cystein HCl (Himedia, India) was added as reducing agent. Fermentation was carried out for 96h. Initial pH range of 5.8, 6.2, 6.4 and 6.8 with 10 g/L $CaCO_3$ was chosen to study the effect of initial pH on bioprocess and three different concentrations of $CaCO_3$ were tried to understand the role and effect of $CaCO_3$ on redox micro environment of *C. sporogenes* BE01 inoculated rice straw hydrolysate. pH change of the fermentation medium was monitored every 24h and samples were collected for analysis.

6.2.2. Analytical methods

6.2.2.1. Total gas estimation and hydrogen analysis

Total gas produced by *C. sporogenes* BE01 was analyzed by mounting gradient syringe on every reactor by piercing it through rubber septum. The gas produced

displaced the piston of the syringe and gradient helped to measure the gas produced. Head space volume of the reactor was also taken into consideration.

Percentage hydrogen analysis in the total gas produced was done by gas chromatograph (Nucon, India) equipped with a thermal conductivity detector (TCD). 2.1/8" × 2m SS column with molecular sieve stationary phase of size 60/80 mesh was used for gases separation at the temperature 60 ˚C. Carrier gas used was nitrogen at a flow rate 20 ml/min under 1kg/cm^2 pressure. The detector and injector were operated at 80 and 50^0 C, respectively.

6.2.2.2. Electro chemical analysis

Cyclic Voltammetry (CV) was performed using potentiostat–glavanostat system (Autolab- PGSTAT12, Ecochemie) to understand the redox microenvironment and electron transport mediators involved during the fermentation process. Voltamograms were recorded using platinum wire as the working electrode and a carbon rod as counter electrode against the reference electrode (Ag–AgCl(S)). Potential ramp was applied in the range of +0.5V to -0.5V at the scan rate of 30 mV/s. Redox currents and peaks were recorded for further analysis.

Electro kinetic analysis was performed by plotting Tafle slope using autolab software. Natural log of anodic current (lnI) was plotted against applied range (E/V) for representing the Tafles plot generated by the software. Oxidation slope and reduction slope for every voltamogram was recorded and plotted against time to understand the fluctuations in redox environment with respect to time.

6.3. Results and discussion

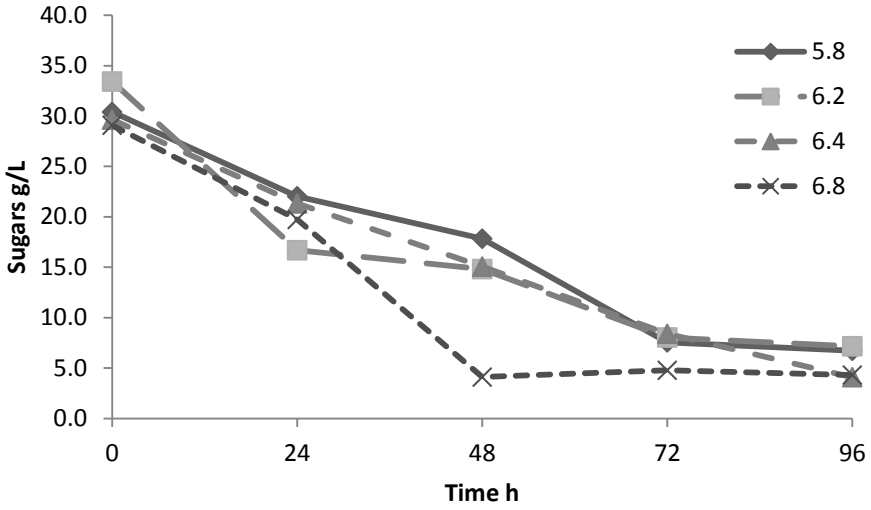
6.3.1. Glucose utilization

Dilute acid pretreatment and enzymatic hydrolysis of rice straw generated 45g/L sugar. Pentoses were found in negligible concentrations in the hydrolysate, as the hemi cellulosic fraction was removed during dilute acid pretreatment. pH was adjusted before autoclaving and glucose estimation was done for every reactor before inoculating *C. sporogenes* BE01. Heat sterilizing the hydrolysate at 121 ºC for 10 min resulted in 15 g/L sugar loss and the final glucose obtained was 30 g/L.

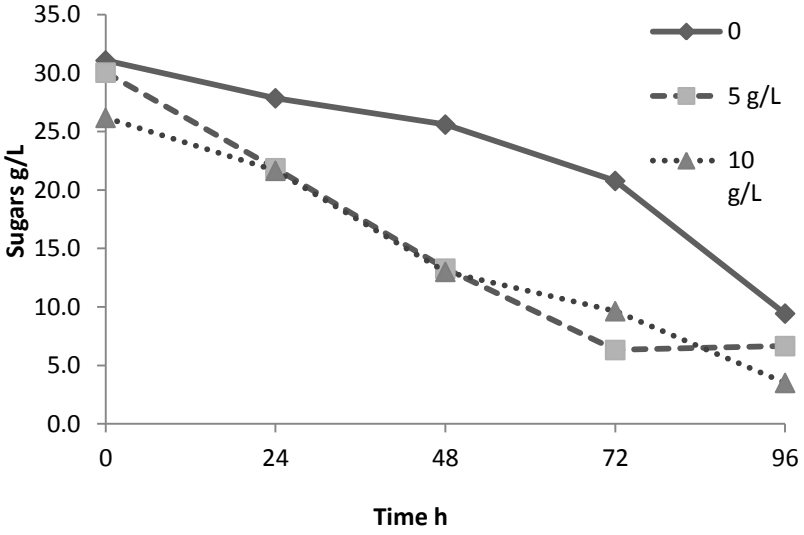
The redox microenvironment of *C. sporogenes* BE01 driven biobutanol fermentation system in enzymatic rice straw hydrolysate was studied at various initial pH and with different $CaCO_3$ concentrations. pH plays a very significant role in microbial catalysis and also in the growth of the bacteria. Especially in butanol fermentation, pH has direct control over the acids formation and their assimilation (Yang et al, 2013). *C. sporogenes* BE01 exhibited change in pattern of glucose utilization with change in micro environment of the bioreactor. The pH of the medium in the range of 5.8 to 6.4 had very less effect on sugar utilization, but the utilization rate enhanced with pH approaching near neutral. This signifies the increased metabolic activity and growth at near neutral pH (Fig 6.1a).

Supplementation of $CaCO_3$ in the medium enhanced glucose utilization and it increased with increasing the concentration of $CaCO_3$ (0, 5 and 10 g/L) (Fig 6.1b).

Fig 6.1: Sugar consumption by *C. sporogenes* BE01 from rice straw hydrolysate



A: At different initial pH supplemented with 10 g/L $CaCO_3$



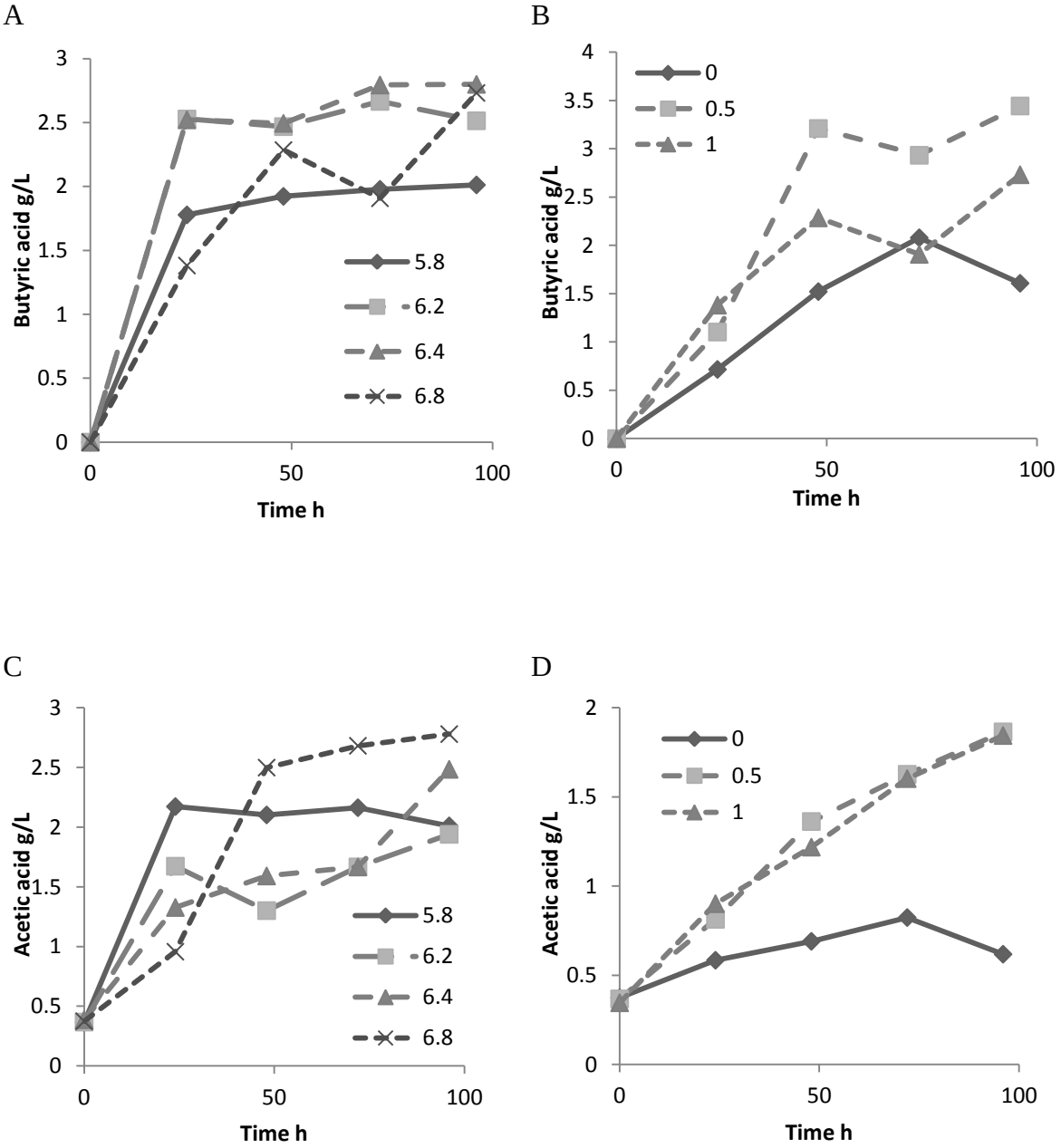
B: With $CaCO_3$ supplementation: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$.

Increased glucose utilization and ABE productivity in the presence of ≥ 4 g/L $CaCO_3$ was reported with *Clostridium beijerinkii* grown in semi defined P2 medium (Han et al, 2013). Calcium ions has various effects at cellular level which could contribute to increased growth, though pH buffering effects might also contribute to stimulatory effects on butanol fermentation (Richmond et al, 2011).

6.3.2. Hydrogen and volatile fatty acids

C. sporogenes is a known producer of butyric acid and was reported for cheese fermentation in combination with *Clostridium butyricum* and *Clostridium beijerinckii* (Le Bourhis et al, 2007, Montville et al, 1985). In *Clostridium tyrobutyricum* butyrate at 15 g/L showed inhibitory effect on acetate formation (Ying & Shang-Tian, 2004). Similarly here, during butanol fermentation, butyric acid production was relatively higher than acetic acid production in all the conditions tested (Fig 6.2a, b, c, d). When overall VFAs production was considered, near neutral pH (6.4 and 6.8) were found to be favorable with 5.2 g/L and 5.5 g/L VFA respectively. The reactor with $CaCO_3$ concentration of 5g/L performed considerably well for conversion of sugars to acids (Fig 6.2b & d). *Clostridium propionicum*, an organic acid producer showed highest growth and organic acid production at pH 7.0 (Stinson & Naftulin, 1991). Acid forming enzymes are highly pH dependent. The activity of the enzymes would be inhibited or enhanced with increase or decrease in pH (Ying & Shang-Tian, 2004). Acidogenic process was highly active in the first 24h and became stable throughout the fermentation time, except at pH 6.8 the VFA production was in increment till 48h and had a sharp decrease at 72h but raised at 96h (Fig 6.2 a & c). The same was observed with 5g/L and 10 g/L $CaCO_3$, which could be attributed to efficient assimilation of acids to solvents at 72h.

Fig 6.2: Volatile Fatty Acids production during fermentation of *C. sporogenes* in biomass hydrolysates

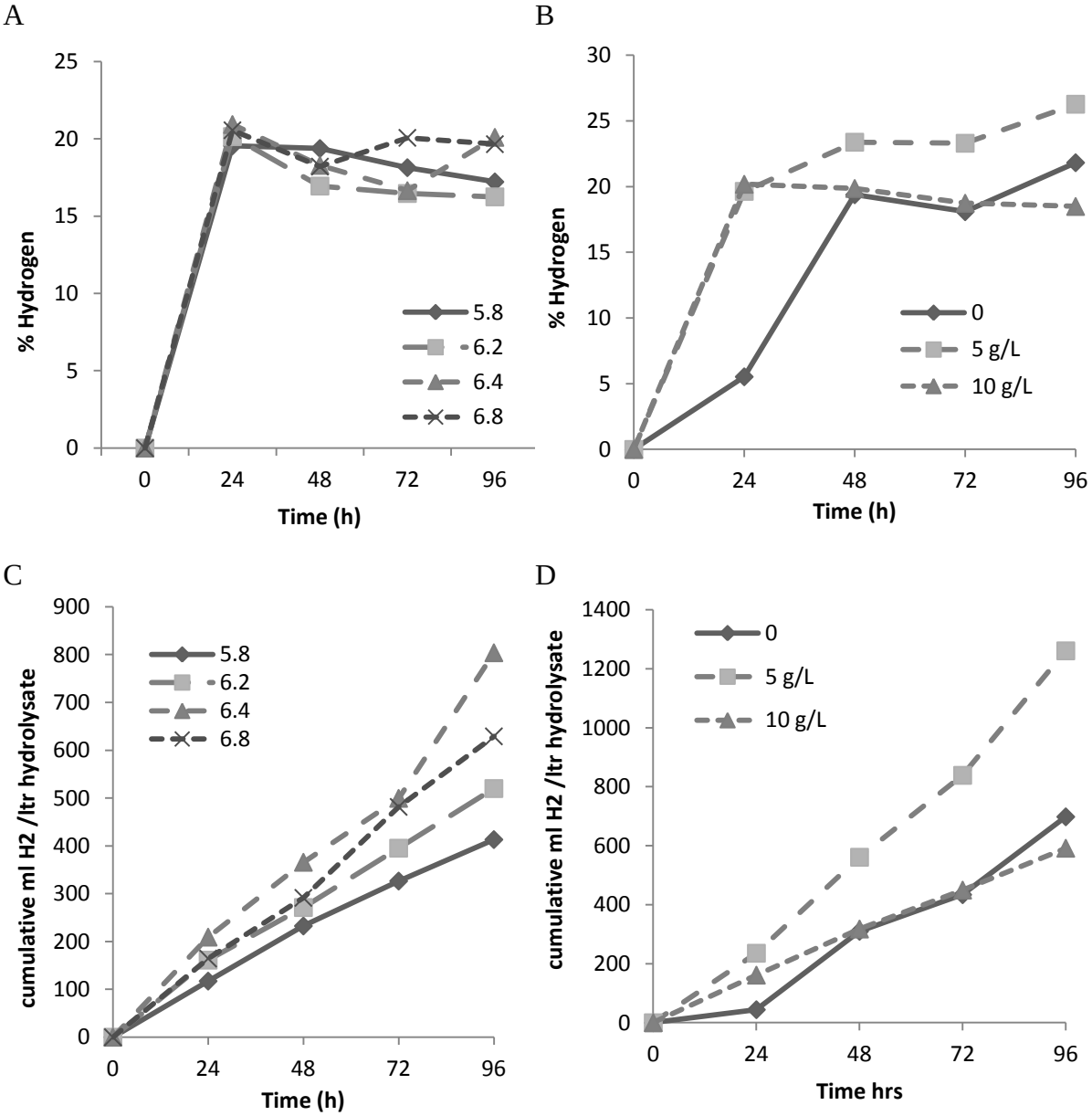


A: Butyric acid produced at different initial pH. B: Butyric acid produced with different $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$. C: Acetic acid produced at different initial pH. D: Acetic acid produced with different $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$.

Hydrogen production was in accordance with acids production and the highest percentage of hydrogen (20%) of total gas produced was at pH 6.4 and 6.8 (Fig 6.3a). Though, there was no considerable difference in the percentage of hydrogen produced, there was a notable difference in the total gas produced at different pH and that showed the marked variation when represented in terms of cumulative hydrogen (Fig 6.3c). The total hydrogen production increased with increase in pH till 6.4 but reduced at 6.8. Total hydrogen production at pH 6.4 continued till 96h and there was no much reduction in the gas production with respect to time (Fig 6.3c).

Optimal pH for hydrogen production varies with each species and strains. For *Clostridum beijerinkii* DSM 1820, optimum pH reported was 6.7, for *Clostridium pasteurinum,* it was 5.4, for *Clostridium butyricum* 5.1 etc (Masset et al, 2012). Hydrogen production varies with change in glucose concentration. In *Clostridium acetobutylicum* ATCC824 glucose fermentation, hydrogen production rate ranged from 680-1270 ml/ g glucose per liter of reactor (Zhang et al, 2006). There was increase in total gas production and percentage hydrogen production when $CaCO_3$ concentration was reduced to 5 g/L (1260 ml), but in the absence of $CaCO_3$ and with increase in $CaCO_3$ to 10 g/L the total hydrogen produced was 591 ml and 698 ml respectively (Fig 6.3b & d). Long acidogenic phase and higher acids production can be correlated with the increased hydrogen production with supplementation of 5 g/L $CaCO_3$. The favorability of 10 g/L $CaCO_3$ was towards solvents assimilation rather than hydrogen production.

Fig 6.3: Hydrogen production during fermentation of *C. sporogenes* in biomass hydrolysates



A) Percentage hydrogen produced at different initial pH, B) Percentage hydrogen produced with different $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$. C) Cumulative hydrogen produced at different initial pH. D) Cumulative hydrogen produced with different $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$.

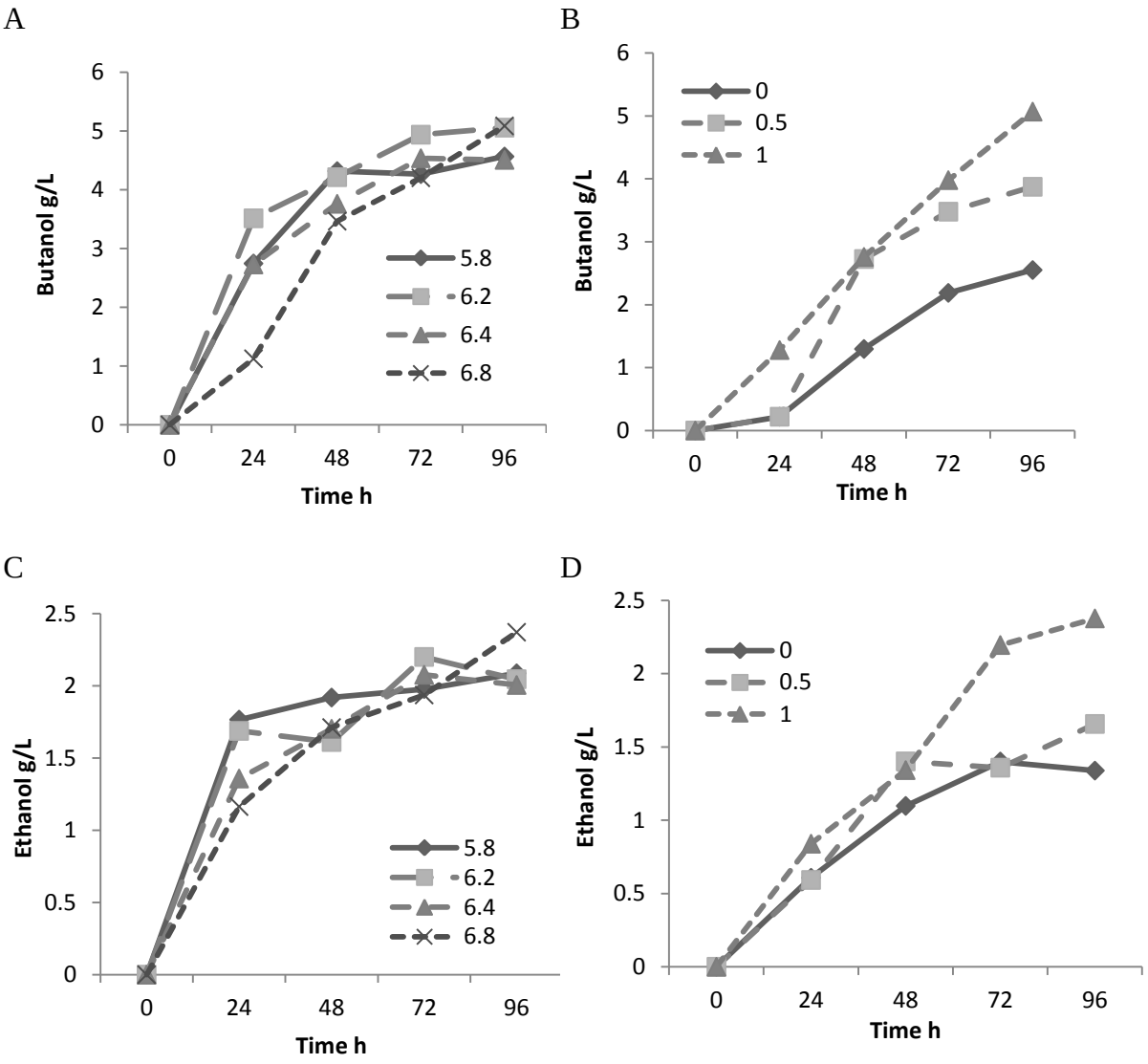
6.3.3. Solvents

Butanol and ethanol were the solvents produced by *C. sporogenes* BE01 and their ratio varies with the change in the external factors. Acetic acid and Butyric acid, produced in the acidogenic phase of the culture were assimilated at the later solventogenic phase of the culture (Lee at al, 2008). At 5.8 pH, though the rate of butanol and ethanol formation was high at first 48h and 24h respectively, solvent formation ceased later and total solvent produced was comparatively less (Fig 6.4 a & c). This could be due to lowered pH with VFA production and inefficient assimilation of VFA formed. At pH 6.2 and 6.4 relatively high solvent production was observed for the first 24h and 72h respectively. Further accumulation of solvents was not found from 72h to 96h. The efficient conversion of sugars and acids to solvents was achieved in a different mode by *C. sporogenes* BE01 at pH 6.8. The solvents formation in the first 24h was considerably low when compared to rest of the pH range, which signifies the acids accumulation did not lead to decrease in pH to the level where assimilation starts for solvents production, but the overall high solvent production 7.3 g/L was achieved with constant increase in solvent accumulation till 96h without attaining the saturation (Fig 6.4 a &c).

$CaCO_3$ was used to maintain the pH in the range by neutralizing organic acids formed during fermentation. $CaCO_3$ concentration was observed to effect the solvent production more than initial pH of the medium. Solvents production increased with increasing the concentration of $CaCO_3$ in the medium (Fig 6.4 b, d). This could be due to effect of calcium ions and its effective buffering action resulted in efficient VFA assimilation. Without $CaCO_3$supplementation, the total yield of solvents was just 3.8 g/L which could be due to lower glucose utilization rate and less VFA formation. Supplementation of 10 g/L $CaCO_3$ increases the total solvent production to 7.4 g/L.

Reduction in $CaCO_3$ concentration from 10 g/L to 5 g/L lead to 26% decrement in total solvents formed (Fig 6.4d).

Fig 6.4: Solvents production during fermentation of *C. sporogenes* in biomass hydrolysates



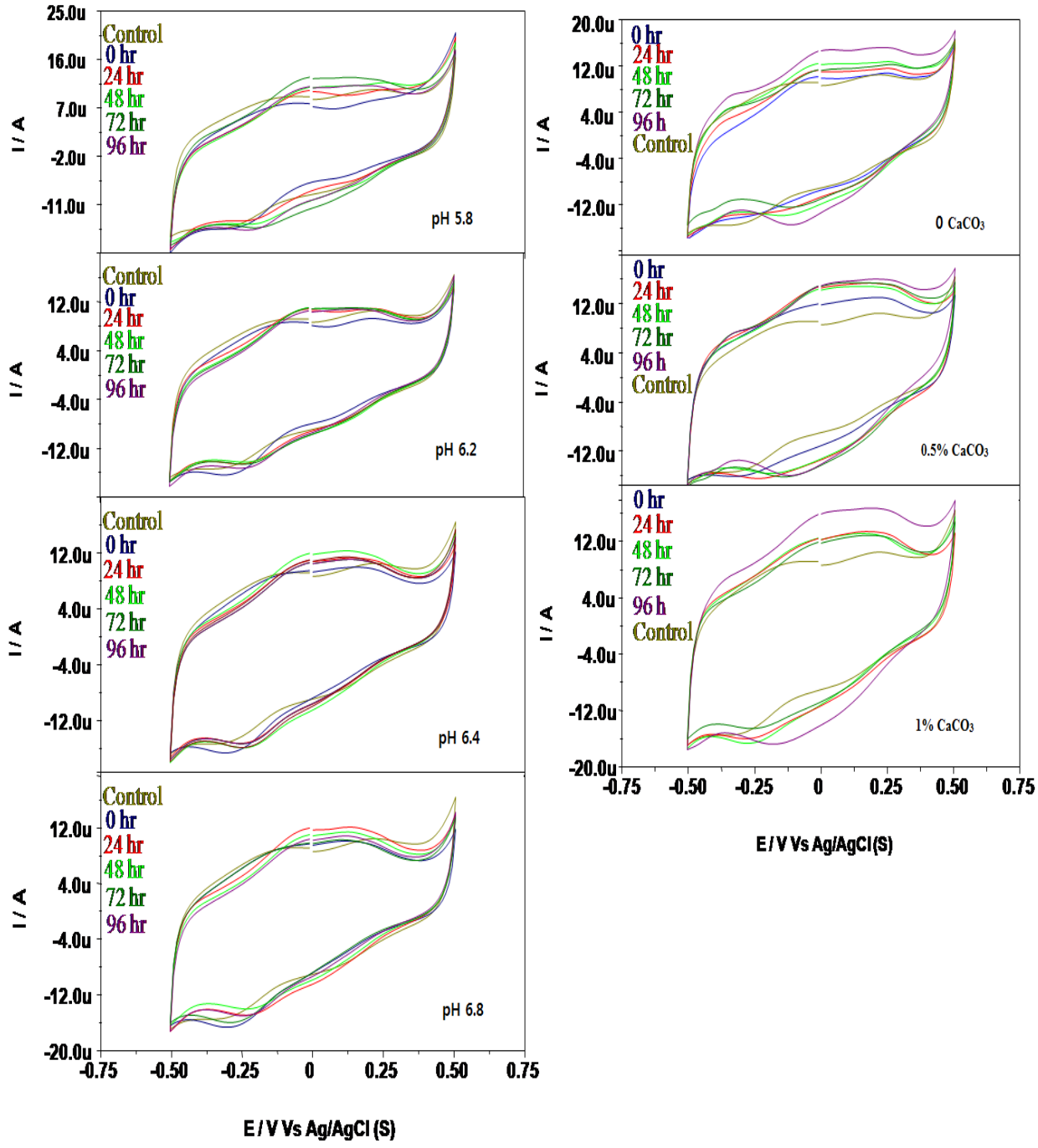
A) Butanol produced at different initial pH, B) Butanol produced with different $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$, C) Ethanol produced at different initial pH, D) Ethanol produced with different $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$.

6.3.4. Electro chemical analysis

With the function of difference in initial pH and $CaCO_3$ concentrations in the rice straw hydrolysate medium, there was variation in voltamogram peaks obtained and the change was with respect to time as well. Redox currents were found to be high with sterile rice straw hydrolysate alone, which signifies it as a potential substrate for bioelectric catalytic reactions. Redox currents were not differed predominantly and were mostly in the range of RC: -18 ± 3 μA to OC: 18 ± 4 μA. With the alterations in initial pH and with various $CaCO_3$ concentrations in the medium, the difference observed was marginal (Fig 6.5). The observations indicate that the catalytic activity was more or less same in all the conditions provided, but the difference was with the shift in metabolic activity and the mediators/electron carriers involved.

In all the different initial pH experiments except 5.8, Preliminary hours had shown oxido reduction potential for biological reaction of NAD^+ to NADH (E^0 values -0.32 V) and cytochrome complex (0.1 to 0.25 V) (Karp, 2009). In the later hours peaks for flavor-proteins (-0.2 V to -0.29 V), protein bound FAD (0.00 V to 0.1) and Iron-sulfur clusters were observed (Mayhew, 1999; Klaus-Heinrich, 2001) With the E^0 values obtained between -0.05 to -0.15, they can be attributed to Fe-S cluster of N2 [4Fe-4S cluster] associated with complex 1 (Ohnishi, 1998). Association of 4Fe-4S with Fe-Fe dehydrogenase enzyme for hydrogen production was well reported (Berto et al, 2012).

Fig 6.5: Cyclic voltamogram profiles recorded in various pH and $CaCO_3$ concentrations



Cyclic voltamogram profiles recorded in various pH and $CaCO_3$ concentrations:
0 - No $CaCO_3$, 0.5% - 5 g/L $CaCO_3$, 1% – 10 g/L $CaCO_3$.

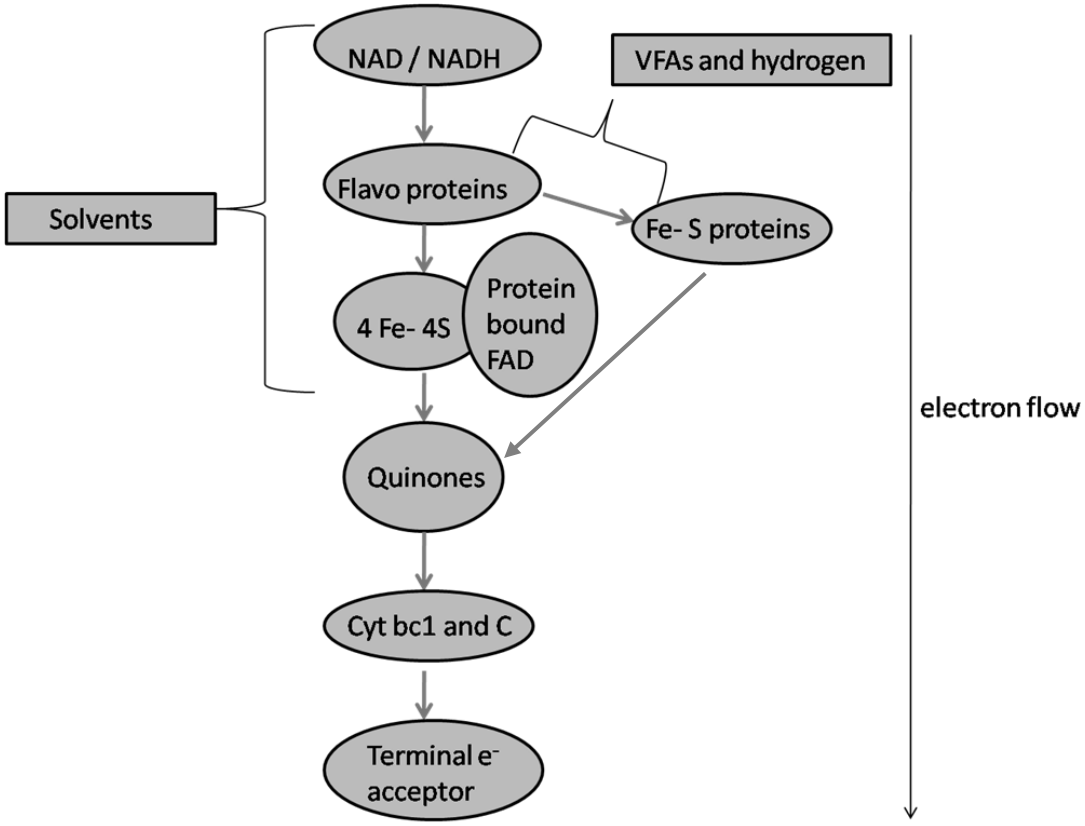
Interference of protein bound FAD increased with increase in pH and that can be attributed to reactions that occur in the presence of enzymes containing FAD as the prosthetic group. Flavoproteins are common intermediary species in the electron transport chain. Acyl- CoA dehydrogenases that are involved in metabolism of alkanoates belong to the family of flavo proteins (Neilson & Allard, 2012). Butyryl CoA dehydrogenase which catalyses the conversion of crotonyl CoA to butyryl CoA belongs to the class of short chain acyl CoA dehydrogenases (Boyton et al, 1996)

Peaks for Iron-sulfur clusters were not prominent in pH 5.8, but were observed at 24h in pH 6.2, extended to 24 and 72 h in pH 6.4 and at near neutral pH the peaks for iron-sulfur clusters were observed till 96h. There were reports on increased levels of Iron-sulfur clusters during solvent formation and furfural challenged cultures (Zhang & Ezeji, 2013). Though in bacteria their primary function is to mediate low potential electron transfer, their function is extended to several catalytic proteins.

Distinct peaks were observed with change in $CaCO_3$ concentrations in the medium. Peaks for Cytochrome bc1 were commonly observed throughout the fermentation, While NAD/NADH was in initial hours. The major difference was with frequency of peaks for protein bound FAD, flavoproteins and iron-sulfur clusters. Peaks were observed for all the mediators mentioned above with 10 g/L $CaCO_3$ containing medium. The presence of many electron transporters implies that the redox activity was occurring efficiently and this was also supported by high butanol production. While, in the absence of $CaCO_3$ peak for the common mediators Cytochrome bc1 and Iron-sulfur clusters were observed. Iron-sulfur clusters are low potential electron transporters and this could be the reason for comparatively less acids and solvents production. With 5 g/L $CaCO_3$ peaks for Cytochrome bc1, NAD/NADH, flavoproteins were found but protein bound FAD peaks were absent.

This results suggest that flavoproteins, protein bound FAD and Fe-S cluster were involved in electron transport system that might be facilitating a key reaction that result in higher butanol yield (Fig 6.6).

Fig 6.6: Electron transporters involved and possible electron flow during butanol , VFA and H_2 production by *C. sporogenes*



Electron transporters involved and possible electron flow during butanol, VFAs and hydrogen production from rice straw hydrolysate by *C. sporogenes* BE01.

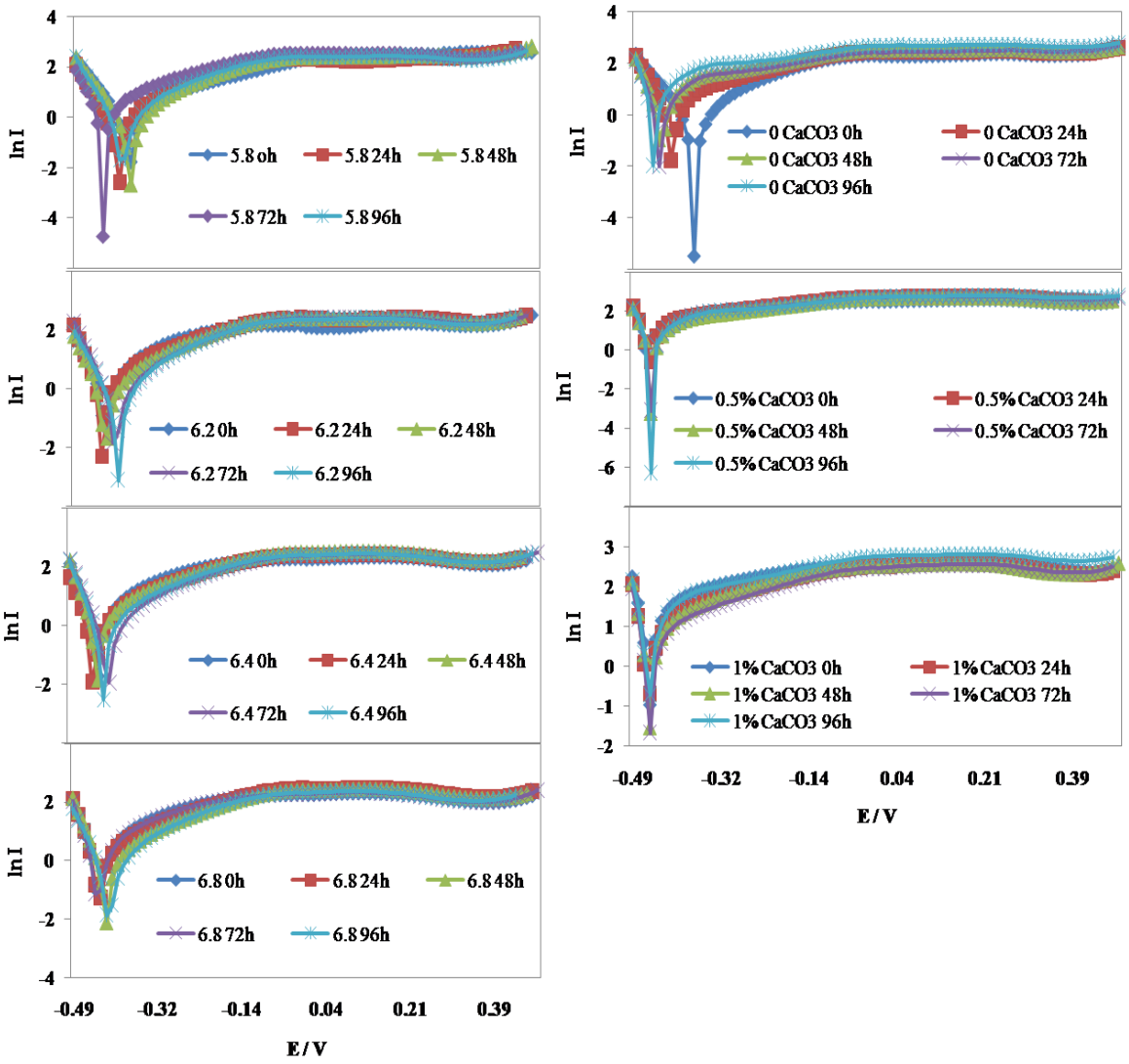
There are reports on flavin based electron bifurcation in Clostridia, that reduce low potential 4Fe-4S clusters of ferridoxin. This electron bifurcation system was reported to increase substrate level and electron transport phosphorylations. 4Fe-4S clusters, protein bound FAD, flavoproteins, NAD/NADH involvement is the characteristic of electron bifurcation system reported in few *Clostridium* species. The protein bound FAD could be the FAD bound to butyryl CoA dehydrogenase, which is reported to have 4Fe-

4S clusters (Chaudury, 2013). The Peak obtained at the reduction potential -0.015 also indicates the conversion of crotonyl CoA to butyryl CoA by butyryl CoA dehydrogenase (Loach, 1976). Electron transferring flavoprotein and butyryl CoA dehydrogenase couple the reduction of crotonyl CoA to butyryl CoA to the ferridoxin reduction with NADH (Chaudury, 2013). Calcium ions were reported to stabilize membrane bound proteins. The increased production of butanol with increase $CaCO_3$ could be attributed to any of these. These results present the possibility of electron transport bifurcation system in *C. sporogenes BE01*.

6.3.5. Bio-electro kinetic analysis

The rate of electron transfer to the electrode by oxidized and reduced species can be interpreted by Tafle plot. According to Tafle equation, when the over potential is large the reverse reaction rate is negligible. The slope of tafle plot reveals the value of electron transfer coefficient (Bard & Faulkner, 2000). Low tafle slope indicates the high current obtained at low overpotential. So, higher the slope higher the activation energy required, that indiactes the less favorability for the oxidation / reduction reaction (Mohan et al, 2013). The efficiency of the biorecator towards reduction or oxidation reactions and the favorability for the membrane associated biochemical redox reactions can be analyzed by the redox slopes obtained under each condition with respect to time. Oxidative tafle slope and reductive tafle slope were derived from the tafle plot obtained. Though there was variation in tafle slope obtained with respect to change in pH and $CaCO_3$ concentrations, in all the experimental conditions oxidative slope was higher than the reductive slope (Fig 6.7). This indicates that rice straw hydrolysate bioreactor with *C. sporogenes BE01* was more favorable for reduction metabolism.

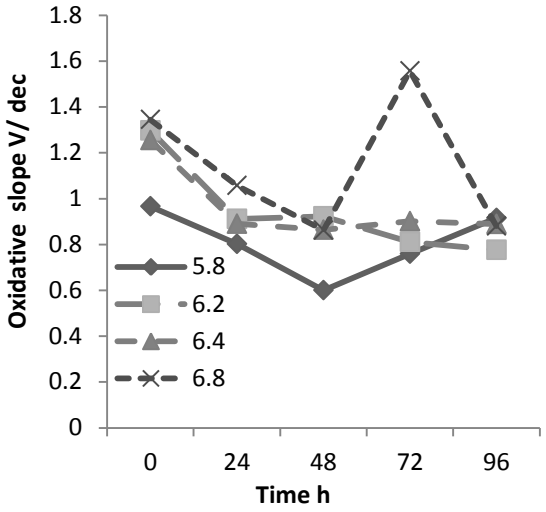
Fig: 6.7: Electro kinetic analysis in various pH and $CaCO_3$ concentrations based on Tafle plot.



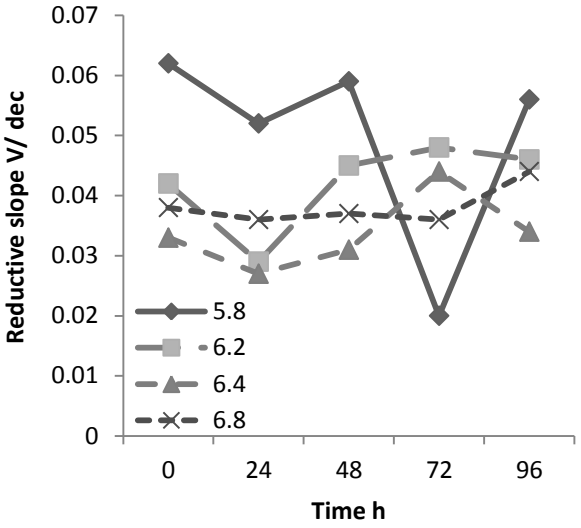
In relation to different initial pH experiments, oxidative slope increased with increase in pH and reductive slope decreased with increase in pH (Fig 6.8a & b). This indicates that, reduction reactions were favorable at pH 6.4 and 6.8 when compared to lower pH. In the absence of $CaCO_3$ oxidation slope was low but increased with increase in $CaCO_3$ concentration in the medium (Fig 6.8c). This suggests that addition of $CaCO_3$ was not favorable for oxidation reactions and in the case of reduction slopes, 5 g/L $CaCO_3$ showed comparatively less reduction potential followed by 10 g/L $CaCO_3$ and no

$CaCO_3$ supplementation (Fig 6.8d). Reduction in activation energy in the presence of $CaCO_3$ could be responsible for increased production of hydrogen, acids and solvents.

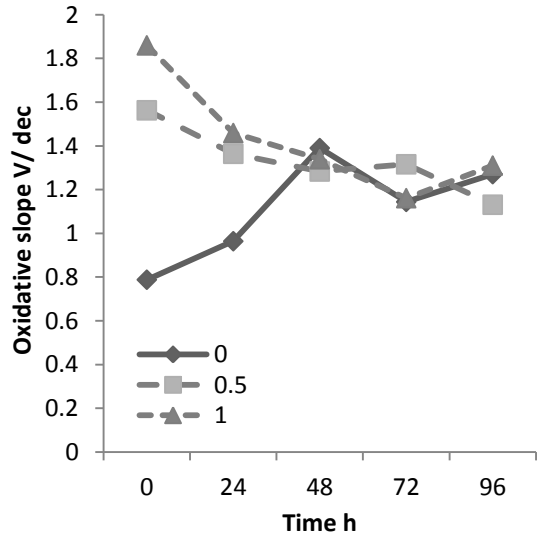
Fig 6.8: Oxidative and reductive slopes at different pH and calcium carbonate concentrations



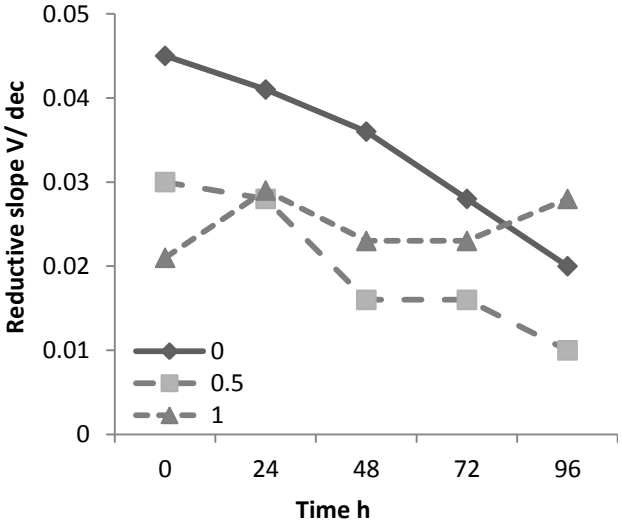
A) Oxidative slope at different initial pH



B) Oxidative slopes with different CaCO3 conc. – 0 –NO CaCO3, 0.5 – 5g/l CaCO3, 1-10g/L CaCO3



C) Reductive slope produced at different initial pH

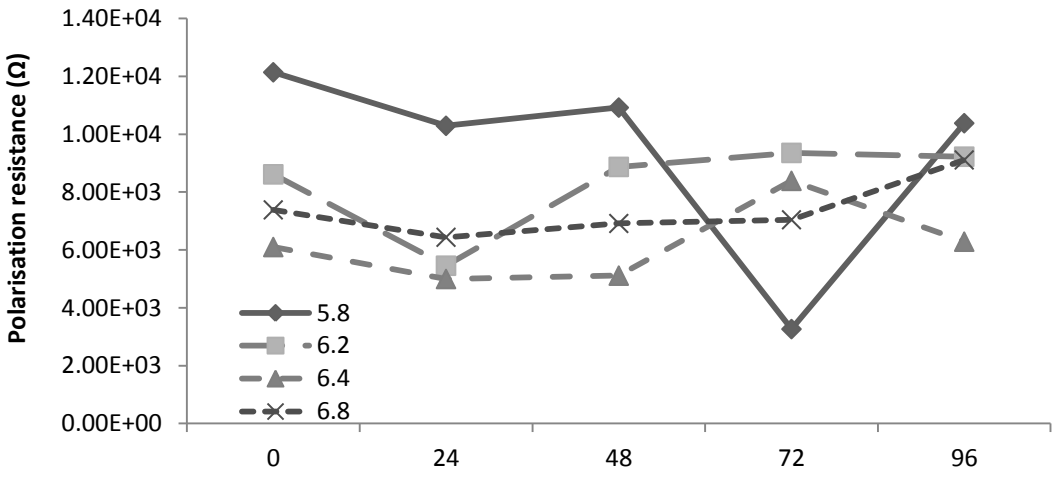


D) Reductive slopes with different CaCO3 concentrations, 0- No CaCO3, 0.5- 5g/L CaCO3, 1-10g/L CaCO3

Polarization resistance refers to the resistivity of the electrolytes around the electrode, this could be due to resistance of electron transfer by the microbe or the insulation effect of products formed on the electrode surface. For a reactor to be active in electron transfer and product formation, polarization resistance should be low. Fermentation with initial pH 6.4 showed less polarization resistance when compared with other pH range (Fig 6.9a). Polarization resistance was high in the reactor without $CaCO_3$ supplementation and decreased with supplementation of 5 g/L $CaCO_3$, but the resistance increased slightly with increased $CaCO_3$ supplementation (Fig 6.9b) as stated before it could be either due to resistance or insulation effect. Polarization effect increases when the products deposit in the reactor or on the electrode surface hinders the free electron transfer from microbes or electron transporters in the reactor to the electrode.

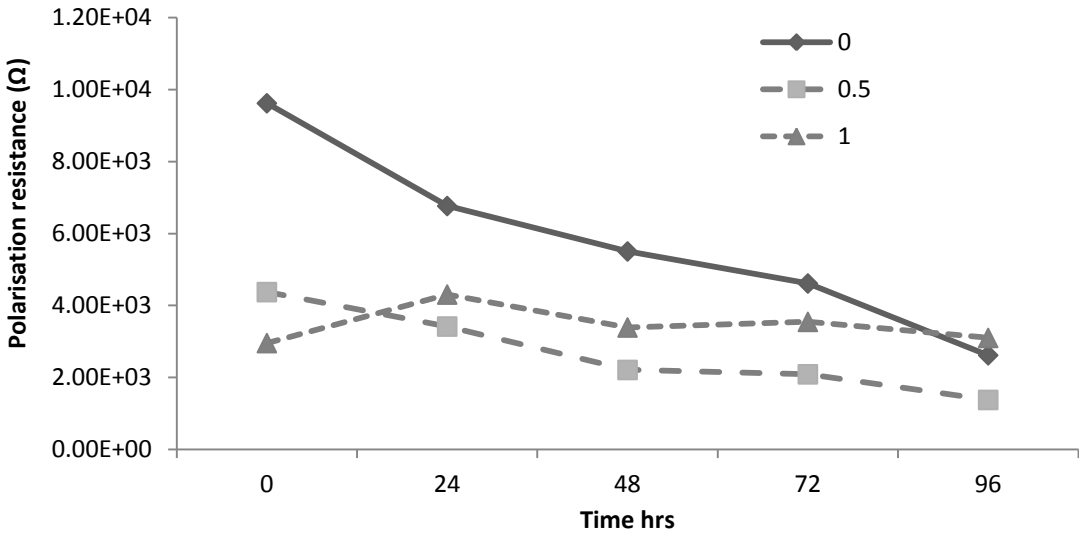
Fig 6.9: Polarization resistance at different pH and calcium carbonate concentrations

A



A) Polarization resistance at different initial pH.

B



B) Polarization resistance with different $CaCO_3$ concentrations: 0 - No $CaCO_3$, 0.5 - 5 g/L $CaCO_3$, 1 – 10 g/L $CaCO_3$.

6.4. Conclusion

C. sporogenes BE01 is capable of producing 7.0 g/L of VFAs and 1.2 L of hydrogen during butanol fermentation in rice straw hydrolysate with 30 g/L of glucose. 7.3 g/L of total solvents were produced of which 5 g/L was butanol. Butanol and ethanol production was in the ratio 7:3. Cyclic voltammetry analysis revealed that $CaCO_3$ supplementation stimulated electron transport system mediated by protein bound FAD and flavoproteins, that resulted in high solvent production. Fe-S clusters, Cyt bc1 and NAD/NADH were the common electron transporters involved during butanol fermentation. Presence of peak for the redox reaction of crotonyl CoA to butyryl CoA was found till 96h at 6.4 and 6.8 pH with 10 g/L $CaCO_3$ supplemented medium. This signifies that butyryl CoA dehydrogenase having FAD as the prosthetic group is stimulated or maintained active in the presence of 10 g/L $CaCO_3$ at near neutral pH.

Involvement of Fe-S clusters, NAD/NADH, protein bound FAD and flavo proteins during active butanol fermentation presents the possibility of electron bifurcation system mediated by FAD and 4Fe-4S clusters present in butyrl coA dehydrogenase complex. Tafle plot revealed that bioreactor with *C. sporogenes BE01* required less activation energy for reduction reactions making them more favorable than oxidation reactions. Further studies at proteomic and genetic levels are required to confirm the role of calcium on butyryl CoA dehydrogenase complex.

Chapter 7

Conversion of rice straw hydrolysate to butanol and volatile fatty acids by high cell density immobilized culture of *Clostridium sporogenes* BE01

7.1. Introduction

Anaerobic process of biobutanol fermentation by *Clostridium* species has challenges with attaining high cell densities, high productivity and several other factors that ultimately questions the feasibility of this bioprocess at industrial level. Low cell density is a common drawback of any anaerobic process and can be addressed by either biofilm reactors or immobilized cells (Zhao et al, 2013). Among several immobilization methods known, adsorption is considered efficient for microbial immobilization due to their easy access to substrates and easiness in handling. If the cells can adhere efficiently to the support, subsequently colonization on the support surface occurs and cell mass grows inside the reactor over time (Kilonzo & Bergougnou, 2012, Qureshi et al, 2005). *Clostridium* species are known biofilm formers and biofilm fermentation is a very common strategy being used for anaerobic digestion and fermentation.

Inert supports like polyurethane foam, nylon membranes, clay bricks, steel and aluminum mesh, etc are reported till date for microbial adsorption. Adsorption capacity and strength of binding should be considered for selecting suitable support material (Hsu et al, 2004). There is increase in use of tougher support materials and inorganic ceramic particles as support for immobilizing cells (Salter & Kell, 1991) Ceramic particles can be considered efficient for microbial adsorption due to their rigid and porous matrix. These ceramic particles can be made with required pore size and rigidity. Diversity in ceramics for their material, pore size and water absorption brings in options to choose or design

based on the requirements for microbial adsorption. The ideal immobilization support should have a rigid support structure and favorable mechanical properties to retain the cells adhered (Salter & Kell, 1991). Porosity is very important for nutrients diffusion, escape of gases produced during fermentation and to increase the surface area for cell adsorption. Pores provide a protected environment for cells to attach and grow with low shear forces inside the pore (Qureshi et al, 2005). Efficient immobilization is aided by correct choice of support material with required pore size (Bucke & Brown, 1983). Adsorption is also beneficial in sloughing of dead cells and by replacing with fresh cells during fermentation process.

Packed bed fermentation with immobilized cells is a vastly employed technique. High microbial cell densities in the reactor help in better conversion of substrates to products (Wang & Zhong, 2007). Fed batch fermentation and two-stage fermentation with the immobilized cells might improve the substrate to product conversion when compared to batch fermentation by efficient utilization and conversion of substrate to products.

In the current chapter, immobilization of a novel strain, *C. sporogenes* BE01 on to ceramic particles was studied in detail and the high cell density immobilized culture was tested for its efficiency in batch, fed batch and two stage fermentation of rice straw hydrolysate to butanol and VFAs.

7.2. Materials and methods

7.2.1. Static adsorption and dynamic adsorption

Adsorption studies were performed with cells grown to the stationary phase. Equal unit volumes of ceramic and polyurethane foam were added as carriers to the 48 h old culture medium or to the culture pellet suspended in sodium phosphate buffer and were incubated at 30 ºC for 5 h. Adsorbed cells adsorbed were quantified by an indirect method by pelleting the un-adsorbed cells followed by NaOH digestion. Total protein released out by NaOH digestion was estimated by Bradford's (1971) method and the corresponding biomass were calculated based on the standard graph plotted for biomass vs. protein. Control without adsorbent was placed to monitor cell growth during the incubation and to estimate the total biomass.

Dynamic adsorption was performed with continuous circulation of stationary phase cells for 5 to 6 h in a column packed with ceramic particles. Estimation of adsorbed cells was done as described above.

7.2.2. Static adsorption kinetics

Reversible first-order surface reaction was used to model the cell adhesion to the support matrix.

$$N + E \rightleftharpoons N - E \qquad (1)$$

Kinetics of forward reaction gives adsorption rate constant (K_a) and reverse reaction gives desorption rate constant (K_d). N is the total cell concentration, where E is the concentration of active adsorption sites on support. Complex of cell and support was represented as N-E. Cell adsorption rate in case of no diffusion limitation can be expressed as.

$$-\frac{dN}{dt} = K_a\,[N][E] \approx\ K_a\,[N][E_0] =\ K_a\,[N] \qquad (2)$$

Reaction time is denoted as 't' in the above equation, in our study it is incubation time for the adsorption to occur. Considering desorption rate negligible as adsorption dominates desorption, the equation can be reduced and integrated to the following equation.

$$\ln\left[\frac{N}{N_0}\right] =\ -K_a t \qquad (3)$$

Here, N_0 is the initial cell concentration. Adsorption rate constant (K_a) is determined by considering the adsorption following the first-order reaction kinetics. In this case K_a is the slope obtained by plotting $\ln\left[\frac{N}{N_0}\right]$ vs. time (Kilonzo & Bergougnou, 2012).

7.2.3. Fermentation with immobilized cells

Actively growing cells of *C. sporogenes* BE01 were circulated through the ceramic packed columns to obtain high cell density. Once the equilibrium was attained, circulation was switched to rice straw hydrolysate medium for fermentation and continued for 120 h at 37 ˚C for butanol production. Fermentation was also performed under static conditions with immobilized cells. Cells adsorption and biofilm formation was further confirmed by SEM analysis.

Batch, fed-batch and two-stage fermentations were tried with high-cell density immobilized cells of *C. sporogenes* BE01. In the case of batch fermentation, studies were done under static conditions, pulsed circulation with time interval of 2 h and continuous circulation of rice straw hydrolysate medium through packed bed reactor with immobilized cells. For fed batch fermentation, initially rice straw hydrolysate having 26

g/L of sugars was provided as substrate to the immobilized cells and later concentrated rice straw hydrolysate was fed to the reactor in two different feed rates, 5 g/L/24 h and 10 g/L/24 h to maintain the sugar concentration constant in the reactor throughout the fermentation.

In two-stage fermentation, shift from first stage to second stage was studied at 24 h, 48 h and 72 h for efficient solvents and VFAs formation. In the reactor for second stage, cells were maintained in clostridial form by refreshing the cells with fresh growth medium till the rice straw hydrolysate fermented for acids was added for assimilation to solvents. Based on our previous studies, in the first stage for acids fermentation, 5 g/L calcium carbonate was supplemented to rice straw hydrolysate and for second stage fermentation, fed batch fermentation and batch fermentation, 10 g/L calcium carbonate was supplemented for solvents production. Yeast extract (1.5 g/L) was added as nutrient source for all the fermentations mentioned.

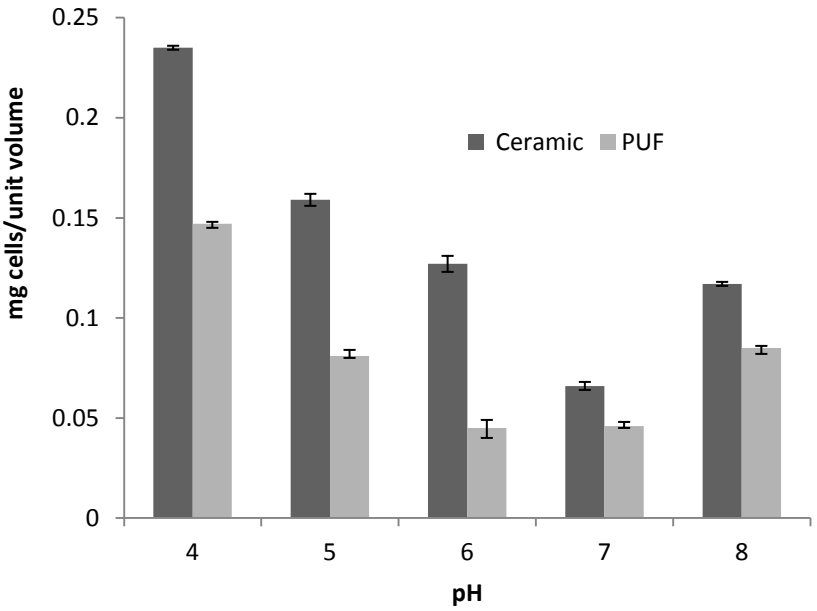
7.3. Results and discussion

7.3.1. Static adsorption and fermentation

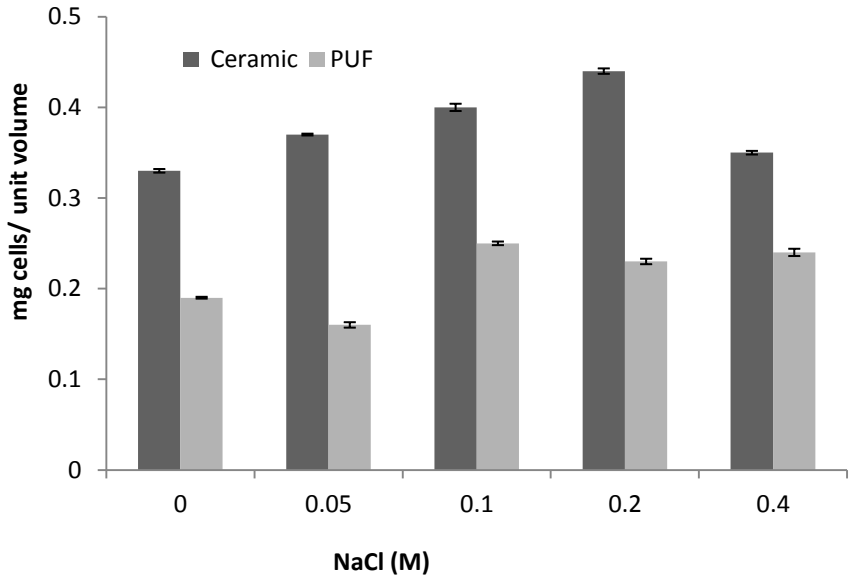
C. sporogenes BE01 was immobilized by simple passive adsorption method without addition of any covalent linking agents. Polyurethane foam (PUF), an inert support was compared against ceramic particles for static cell immobilization studies. Effect of pH and ionic strength on immobilization was evaluated as they play a key role in electrostatic interactions and cell adsorption onto support matrix. Adsorption of cells was found to be significantly pH dependent and was high in acidic pH. Adsorption was reduced drastically at neutral pH, but comparatively increased adsorption was found in

mild alkaline conditions (Fig 7.1a). Adsorption depends mainly on electrostatic interactions or repulsions between the support and cells. Hence, better adsorption can be achieved by altering the pH and by controlling the cell-support interactions. In ionic strength studies, there was increase in adsorption with increase in NaCl concentration from 0 to 0.2M, but decreased with further increase in ionic strength with NaCl (Fig 7.1b). Ion-bridging increases with increase in electrolyte concentrations and thus stabilize the cells attached to the support. Reduced adsorption beyond an electrolyte concentration could be due to destabilization of immobilized cells in presence of high ionic strength (Kilonzo & Bergougnou, 2012).

Fig 7.1: Effect of pH and salt concentration on adsorption of *C. sporogenes*



A) Effect of pH on adsorption of *C. sporogenes* BE01 to ceramic and PUF support



B) Effect of ionic strength on adsorption of *C. sporogenes* BE01 to Ceramic and PUF support.

Adsorption kinetics studied under the optimized conditions of pH and ionic strength revealed that the rate of adsorption (K_a) was better with ceramic support matrix in comparison to the commonly used polyurethane foam (Fig 7.2 & Table 7.1).

Fig 7.2: Kinetics of adsorption of *C. sporogenes* BE01 cells on ceramic and PUF support

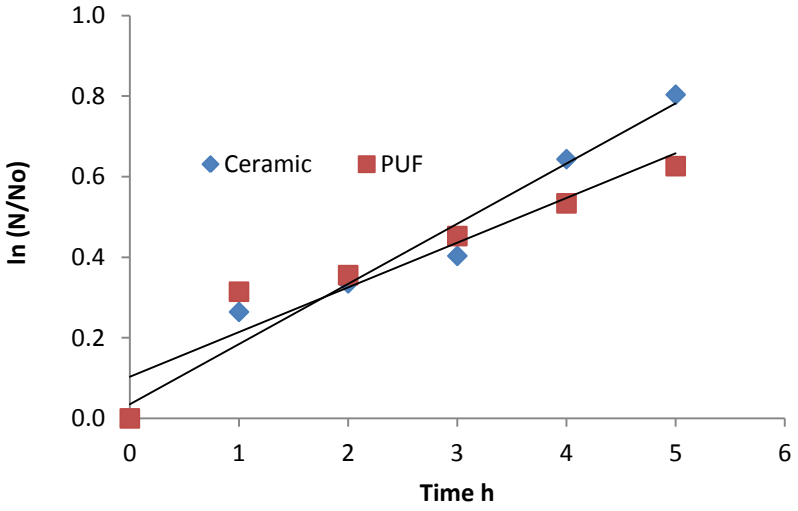
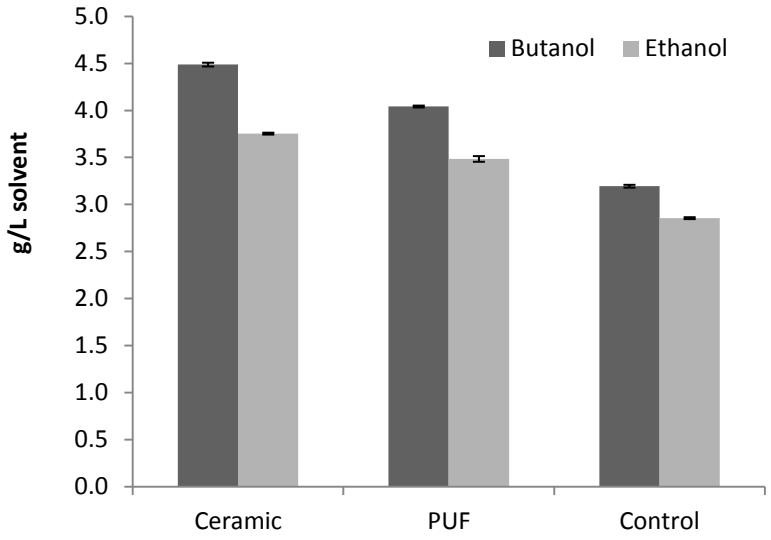


Table 7.1: Kinetics of *C. sporogenes* BE 01 cell adsorption on ceramic and polymeric matrices.

	PUF	**Ceramic**
Initial cell loading (mg/ml)	0.44	0.43
K_a (h^{-1})	0.111	0.149
% Adsorption	46.5	55.2

Cells immobilized onto ceramic particles and Polyurethane foam (PUF) was evaluated for fermentation under static conditions. Equal unit volumes of ceramic and poly urethane foam with cells adsorbed on them were inoculated in to 100 ml of rice straw hydrolysate medium. Enhanced production of butanol, ethanol and VFAs was observed with cells adsorbed on to ceramic particles, when compared to cells adsorbed on PUF and free cells (Fig 7.3). This could be due to higher quantity of cells adsorbed on to ceramic particles and better nutrient diffusion due to the porous nature of ceramic particles. Maximum butanol concentration of 4.5 g/L was achieved with cells adsorbed on to ceramic particles in rice straw hydrolysate having 30 g/L of sugars. Park et al (1989) reported butanol yields of 8.82 g/L from initial glucose concentrations of 60 g/L with *C. acetobutylicum* ATCC 824 immobilized onto natural sponge.

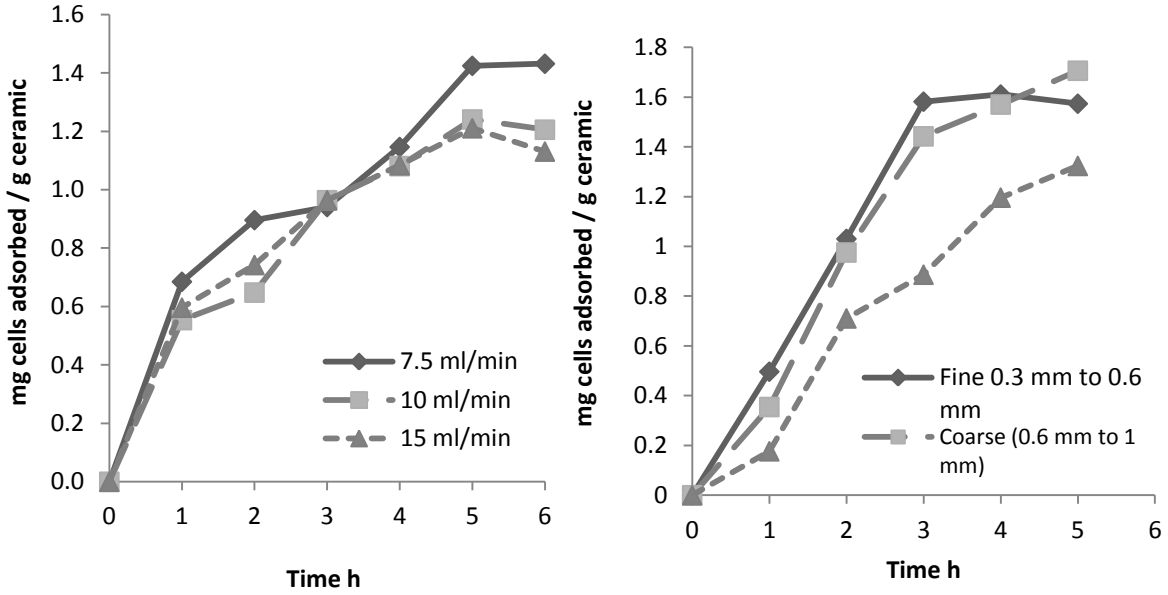
Fig 7.3: Solvents production from rice straw hydrolysate with immobilized cells of *C. sporogenes* BE01 on PUF and ceramic support



7.3.2. Dynamic adsorption and fermentation

Immobilization was carried out in a reactor packed with ceramic particles of various sizes. Ceramic particles were hammer milled to particle sizes in the range 0.3-1.5 mm. The mixed size ceramic particles were packed in the column and cells in stationary phase were circulated at three different flow rates (7.5, 10 and 15 ml/min). Immobilization was efficient with lower flow rates and decreased with increasing the flow rate (Fig 7.4a). Flow rate governs the rate of immobilization of cells onto surface and the flow rate at which cells are practically stationary with respect to the support surface is considered favorable (Kilonzo & Bergougnou, 2012).

Fig 7.4: Effect of flow rate and carrier matrix size on cell immobilization



A) Effect of flow rate on adsorption of cells

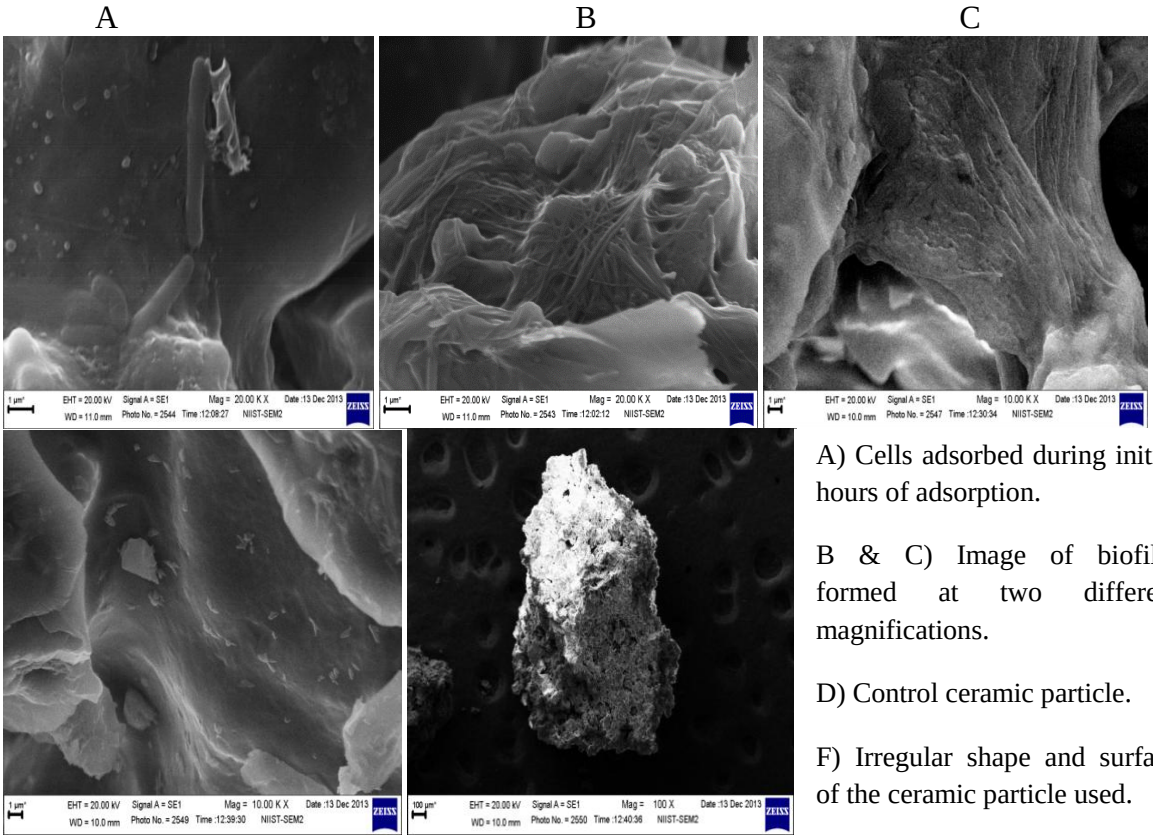
B) Effect of ceramic particle size on adsorption of cells

In order to investigate the effect of particle size of the support material on adsorption, ceramic particles were sieved to three different particle size ranges (0.3-0.6, 0.6-1.0 and 1.0-1.5 mm) and 48 h grown culture was circulated at the flow rate of 7.5 ml/min through the columns packed with ceramic particles for 6 h. Ceramic particles of size range 0.3 -0.6 mm and 0.6-1 mm was better for immobilization, where as larger size (1-1.5 mm) ceramic particles adsorbed lesser amount of cells (Fig 7.4b). Particle size of the support packing material influences the compactness of the column, surface area, porosity etc which in turn affects the cell adsorption and biofilm formation.

SEM analysis revealed the porous and rough surface of ceramic particle. Cells were initially adsorbed and when the circulation of fresh medium prolonged (120 h), biofilm formation was evident (Fig 7.5). These studies indicate the suitability of ceramic particles as a good support matrix for immobilization of cells and also as the support for

biofilm formation. Ceramic particles with high cell densities were used for butanol and VFA fermentation studies.

Fig 7.5: SEM images of cell adsorption on porous ceramic matrix



A) Cells adsorbed during initial hours of adsorption.

B & C) Image of biofilm formed at two different magnifications.

D) Control ceramic particle.

F) Irregular shape and surface of the ceramic particle used.

Packed bed fermentation with immobilized cells as represented in Fig 7.6 resulted in increased butanol and VFA production by circulating medium in pulse flow conditions when compared with continuous flow (Fig 7.7). There was no significant difference in production under static conditions (Fig 7.3) and pulse flow fermentation in a packed bed reactor. Hence, either of these methods can be used for fermentation with immobilized cells of *C. sporogenes* BE01.

Fig 7.6: Packed bed immobilization of *C. sporogenes* BE01 and fermentation for solvent production in rice straw hydrolysate medium

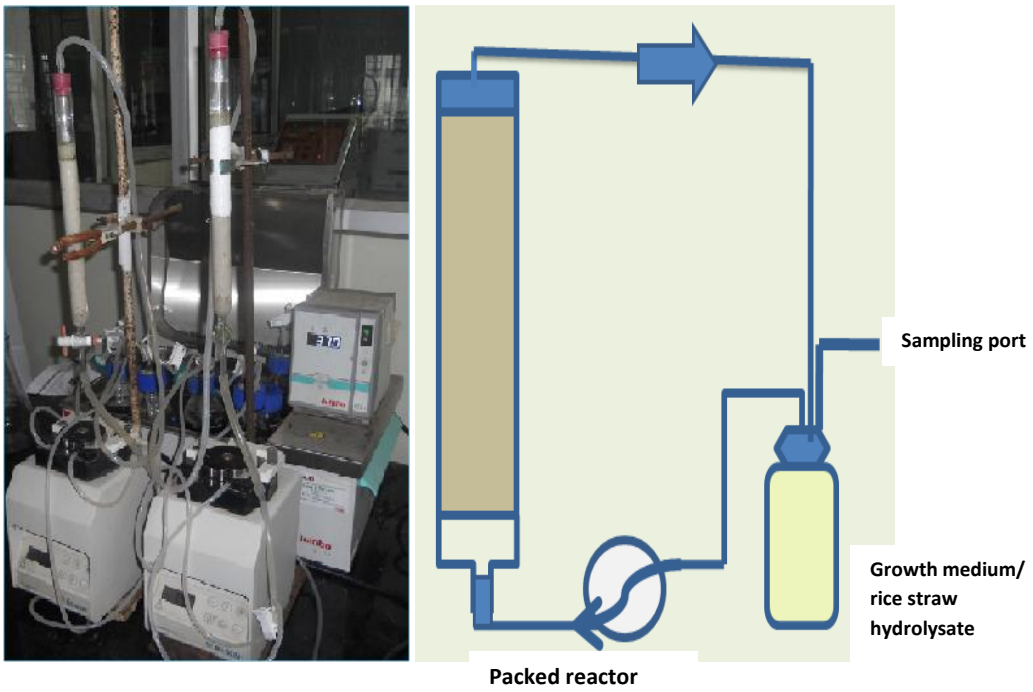
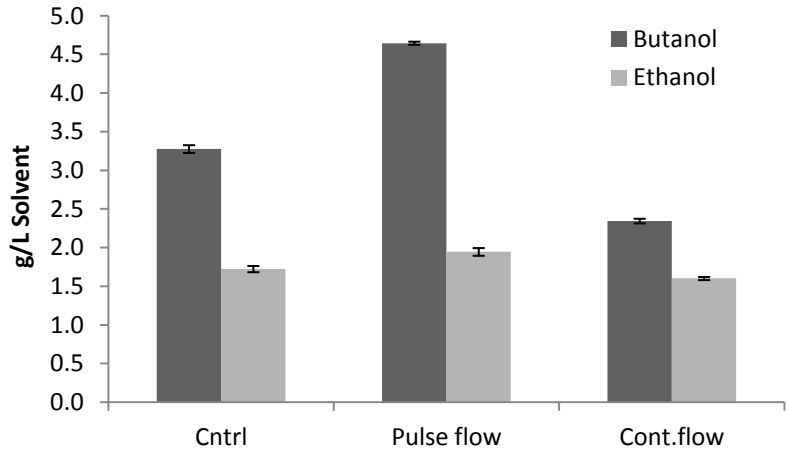


Fig 7.7: Packed bed fermentation with pulse and continuous circulation of rice straw hydrolysate medium



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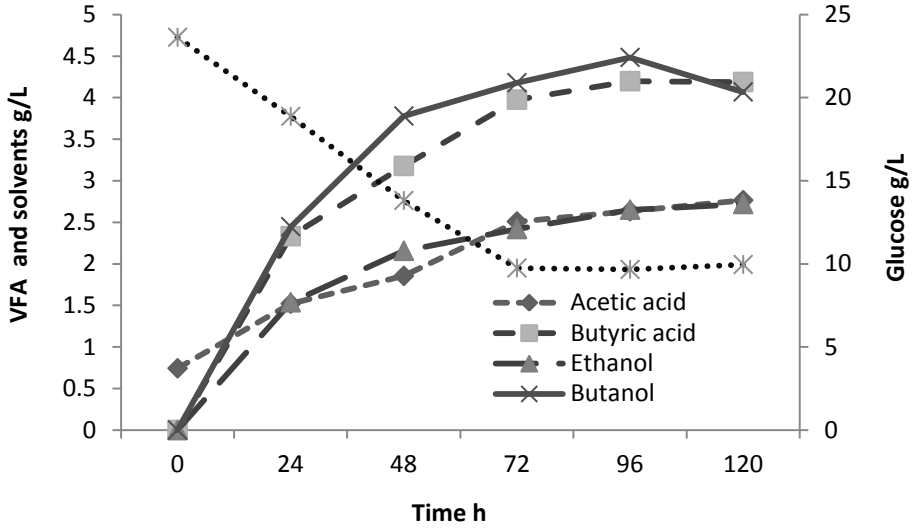
7.3.3. Batch and fed-batch fermentation

In batch fermentation with immobilized cells, samples collected every 24h were analyzed for glucose, VFAs and solvents. Glucose consumption was not efficient during fermentation and 10 g/L of glucose was left unutilized in the reactor. This could be due

to inhibition caused by high rate of VFA and solvents accumulation in the fermentation broth, in the first 24 h. Total solvents of 0.28 g/g and 0.29 g/g of VFAs were formed by *C. sporogenes* BE01 with the hydrolysate having 24 g/L of sugars. Butanol production was 4.5 g/L out of 7.2 g/L of total solvents produced. Butyric acid production was 4.20 g/L and acetic acid was 2.76 g/L (Fig 7.8).

Fig 7.8: Solvents and acids produced from rice straw hydrolysate in batch fermentation with immobilized cells of *C. sporogenes* BE01

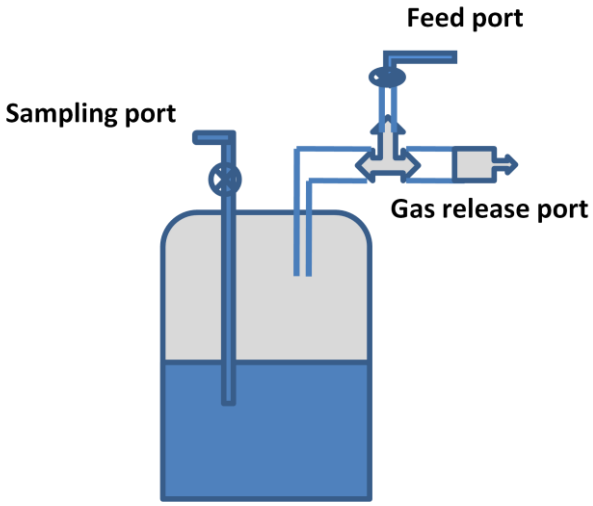


Clostridium acetobutylicum B3, immobilized by surface adsorption onto fibrous matrix and supplemented with methyl viologen for redox modulation improved butanol production to 15.6 g/L within 24h in repeated batch fermentation (Liu et al, 2013). *Clostridium tyrobutyricum* ATCC 25755 immobilized in a fibrous-bed reactor yielded 13.70 g/L and 0.46 g/g butyrate with 30 g/L initial glucose concentration in batch fermentation (Jiang et al, 2010).

As the overall production and conversion efficiencies of total solvents and VFAs were low because of less sugar concentration in the hydrolysate, fed batch fermentation was tried. To investigate the effect of sugar concentration on fermentation, hydrolysate

was fed to the reactor every 24 h at a feed concentration of 5 g/L and 10 g/L of glucose. Figure 7.9 represents the reactor setup for fed batch fermentation.

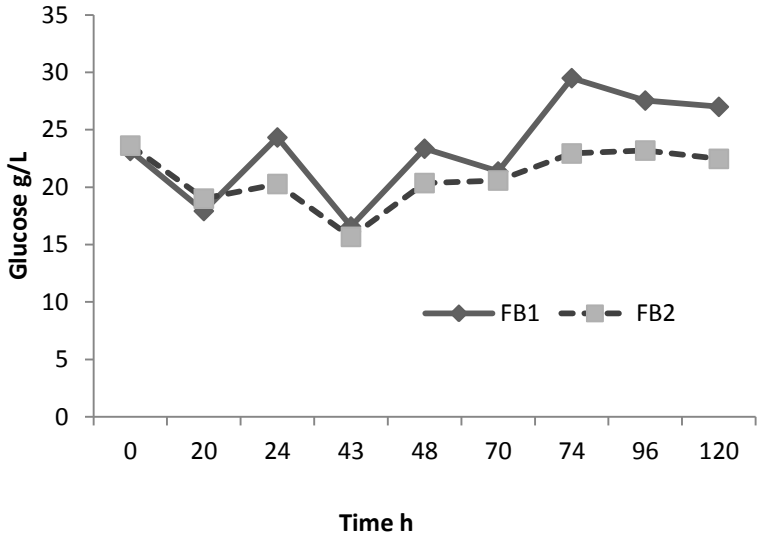
Fig 7.9: Reactor setup for fed batch fermentation with *C. sporogenes* using rice straw hydrolysate



Reactor consists of sampling port to collect samples at regular intervals and feed port for fed batch fermentation. It is equipped with gas release port to release the pressure during sampling and feeding the reactor.

Difference observed in solvents and VFAs production with two different feed rates was negligible and sugars provided were not utilized after 72h (Fig 7.10). Same was observed in batch fermentation, sugar utilization ceased at 72 h and as mentioned previously this could be due to inhibitory effect of solvents and VFAs.

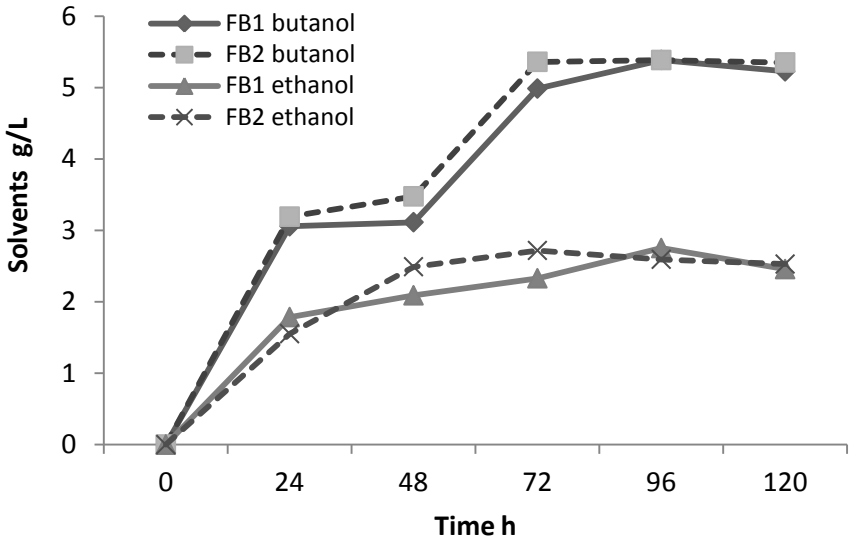
Fig 7.10: Glucose consumption during fed batch fermentation of rice straw hydrolysate using *C. sporogenes* BE01



Glucose feed rates FB1-10 g/L/24 h; FB2- 5 g/L/24 h

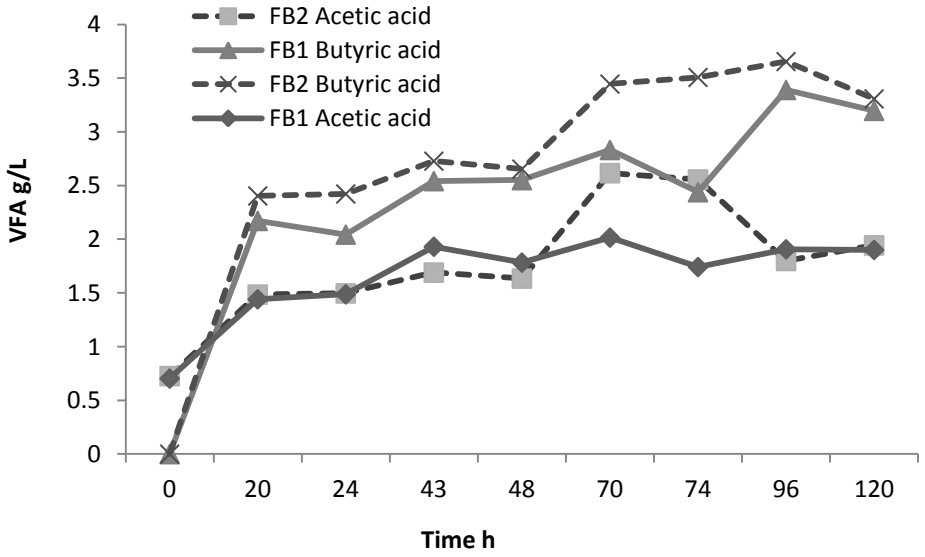
Fed batch fermentation resulted in increased production of solvents and VFAs, but with lower yields in terms of sugars fed to the reactor. Feed rate 5.0 g/L was comparatively more efficient when compared to 10 g/L feed rate in the production of total solvents (7.5 g/L) and VFA (5.2 g/L) with the yields of 0.26 g/g and 0.17 g/g respectively (Fig 7.11, 7.12) In fed-batch fermentation, with the concentrated hydrolysate, VFA and solvents production was not efficient due to the inefficient utilization of sugars by *C. sporogenes* BE01. Though the hydrolysate was treated with "Seralite" anion exchange resin for the removal of inhibitory organic acids, as described in Chapter 5, there could be other unknown inhibitory compounds accumulated by concentration of the hydrolysate, which could result in the reduced efficiency of the process.

Fig 7.11: Solvent production under fed batch fermentation using rice straw hydrolysate medium by *C. sporogenes* BE01



Feed rates FB1-10 g/L/24 h; FB2-5 g/L/24 h

Fig 7.12: VFA production under fed batch fermentation using rice straw hydrolysate medium by *C. sporogenes* BE01



Feed rates FB1-10 g/L/24 h; FB2-5 g/L/24 h

Comparison of batch and fed-batch fermentation resulted in an interesting observation that beyond 3.5 g/L of butyric acid and 2.5 g/L of acetic acid, there is decrement in the rate of VFA production and glucose utilization and even solvents formation was ceased at this point and became plateaued. It signifies the inhibitory effect of VFAs on *C. spororgenes* BE01. As *C. spororgenes* BE01 produces high levels of VFAs, the combined inhibitory effect of solvents and VFAs could be responsible for the stagnation of fermentation at 72 h. Fed batch fermentation by *C. beijerinckii* BA101 with a product recovery system resulted in 151.7 g butanol for 500 g glucose utilized (Ezeji et al, 2004)

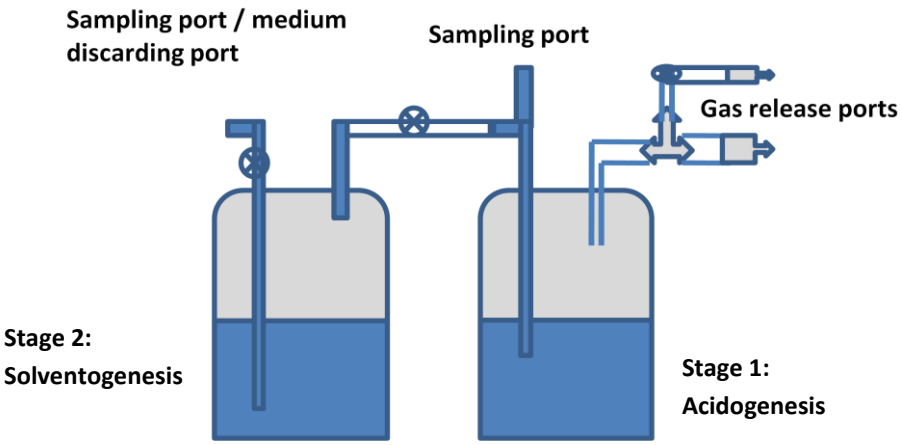
Considering the results obtained with batch and fed-batch fermentation, two-stage fermentation was tried to improve the VFAs and solvents production by efficient glucose utilization and acids assimilation.

7.3.4. Two-stage fermentation

In two-stage fermentation, with all the three time points of stage shift, there was efficient glucose utilization. Shift from first stage to second stage at 48 h and 72 h resulted in 99% sugar utilization. In the same manner, there was a considerable improvement in VFAs production and assimilation of solvents.

Two-stage fermentation is well reported for increased solvents yields. Usually the acids accumulated in the first stage were assimilated to solvents in the second stage fermentation (Fig 7.13)

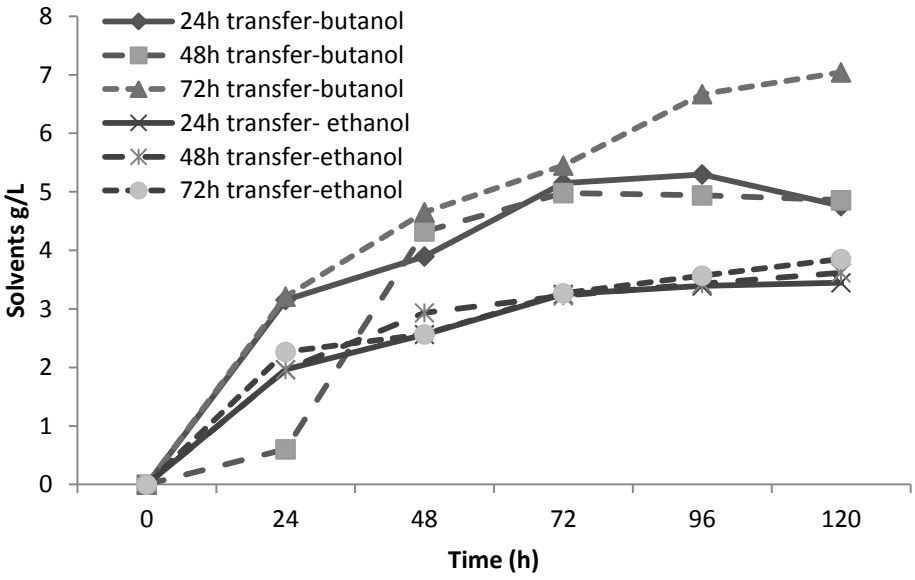
Fig 7.13: Reactor set up for two-stage fermentation by *C. sporogenes* BE01



First stage reactor consists of sampling port to collect samples at regular intervals and two gas release ports to withstand the pressure from both the reactors. Second stage reactor consists of medium discarding port to remove the growth medium and an outlet from first stage reactor for stage shift.

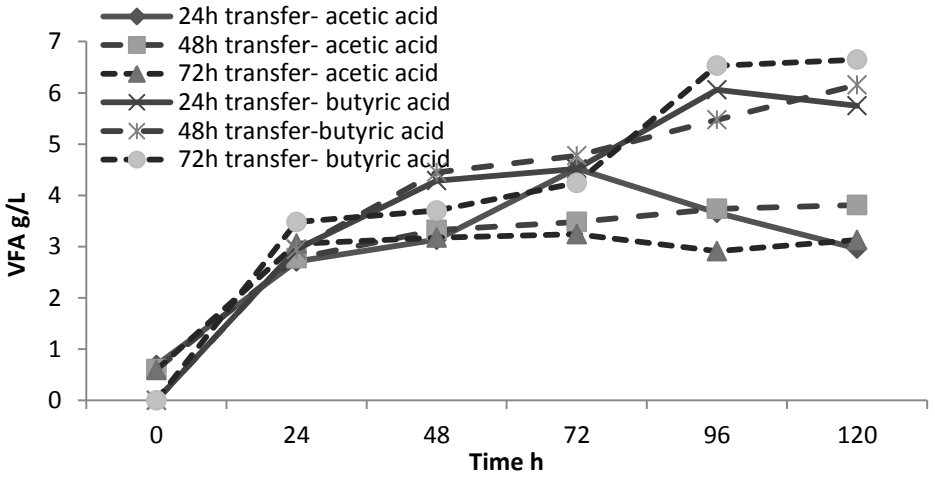
Comparing the stage shift at 24 h, 48 h and 72 h, there were variations in butanol production. Butanol production was increased to 7 g/L with stage shift at 72 h, but there was no marked difference in ethanol production (Fig 7.14)

Fig 7.14: Solvents production by *C. sporogenes* BE01 from rice straw hydrolysate in two-stage fermentation



In case of butyric acid and acetic acid production fluctuations were observed with respect to time and stage shift. The maximum butyric acid produced in batch and fed batch fermentation was 4.1 g/L and 3.5 g/L respectively, but in two stage fermentation it increased to 6.5 g/L and in case of acetic acid, the maximum production, 3.8 g/L was achieved (Fig 7.15). Increase in VFAs production even after the stage shift is contradictory, but from our previous results and the current results, we noticed that acids and solvents production goes together in fermentation in *C. sporogenes* BE01, in contrast to other solventogenic *Clostridium* species, where acids are produced and then assimilated to solvents. Hence decrease in acids accumulated in the reactor was not observed during solventogenesis, instead there was increase in both acids and solvents when the 72 h high cell density *C. sporogenes* BE01 fermented rice straw hydrolysate was added to the reactor with immobilized *C. sporogenes* BE01 cells in clostridial form. Total acids and solvents yield was 0.38 g/g and 0.42 g/g respectively. This signifies that there was not shift in fermentation from acidogenesis to solventogenesis, but transfer of fermented medium to the reactor with *C. sporogenes* BE01 immobilized and grown to clostridial stage had positive impact on VFAs and solvents production.

Fig 7.15: Solvents production by *C. sporogenes* BE01 from rice straw hydrolysate in two-stage fermentation



C. acetobutylicum DSM 792 immobilized column reactor system integrated with liquid-liquid extraction and two-stage fermentation with sugar mixture representative to lignocellulose biomass hydrolysates resulted in maximum solvent yield of 0.38 g/g (Bankar et al, 2013).

Comparative conversion efficiencies of all the fermentations tested in rice straw hydrolysate with the immobilized cells of *C. sporogenes* BE01 with respect to the initial sugar concentration is given in Table 7.2. It is very clear that two-stage fermentation was efficient for both acids and solvents production.

Table 7.2: Conversion efficiencies of various fermentations tested with immobilized cells of *C. sporogenes* BE01

		Butanol (g/g)	**Ethanol (g/g)**	**Acetic acid (g/g)**	**Butyric acid (g/g)**	**Total solvents (g/g)**	**Total VFA (g/g)**
Fed batch	10 g/L/24h	0.14	0.07	0.05	0.08	0.20	0.13
	5 g/L/24h	0.17	0.08	0.06	0.11	0.26	0.17
Two – stage	24h transfer	0.18	0.13	0.17	0.23	0.32	0.41
	48h transfer	0.19	0.14	0.15	0.24	0.33	0.38
	72h transfer	0.27	0.15	0.12	0.26	0.42	0.38
Batch	24 g/L	0.16	0.11	0.12	0.17	0.28	0.29

7.4. Conclusion

C. sporogenes BE01 could efficiently adhere and form biofilm on ceramic matrices. pH and ionic strength played a significant role in adsorption and maximum 55% of inoculated cells were adsorbed under static conditions with an adsorption rate of 0.149 mg/unit volume/h. In dynamic immobilization studies, maximum adsorption was obtained with 7.5 ml/min flow rate with ceramic particle size 0.6mm-1mm. SEM images confirmed adsorption and biofilm formation on ceramic particles.

Packed bed fermentation with pulse flow gave higher butanol production (4.8 g/L) when compared to continuous flow and static fermentation. In fed-batch fermentation, though butanol production was high at 5.3 g/L, yield was low when compared to batch fermentation, as sugar utilization was not efficient with fed batch fermentation. Two-stage fermentation was promising with maximum butanol production of 7 g/L and with increased yields of solvents and VFA in comparison with fed batch and batch fermentation. Sugars conversion to solvents was very efficient in two-stage fermentation and the highest yield was 0.42 g/g, whereas for VFA the total yield was 0.38 g/g. Solvents production can be further improved if the assimilation of VFA to solvents is made more efficient by reducing the toxicity of solvents by in situ stripping.

Chapter 8

Summary and Conclusion

Biobutanol has gained attention as liquid transportation fuel because of its similarity in properties to gasoline. It's high energy content, lower affinity to water, better blending capacities, made it more advantageous than ethanol. Butanol can be produced both petrochemically and fermentatively. Butanol production from sugars by using *Clostridium* species is an industrially well established process. The challenge of ABE fermentation is its low productivity, high cost of the substrate, product toxicity and its separation from fermentation broth. In order to overcome problems like substrate cost and availability, research is being focused on renewable agriculture residues as feed stock for biobutanol.

Rice straw is one of the most abundant lignocellulosic biomass available. It has high cellulosic and hemicellulosic content which can be hydrolyzed in to hexoses and pentoses by enzymes after efficient pretreatment. Hexoses can be readily utilized by Solventogenic *Clostridia* and pentoses can also be utilized, but at low rates. In ABE fermentation with known solventogenic Clostridial species, acetone is also produced in addition to butanol and ethanol. Acetone does not qualify as fuel and should be separated from the final products which results in a net low yield of solvents.

In our current study, a novel non-acetone forming butanol and ethanol producer was isolated and identified. Based on the 16s rDNA sequence BLAST and phylogenetic analyses, it was found to have high similarity with the reported hydrogen producing strains of *Clostridium sporogenes.* Biochemical studies revealed that it is lipase and protease positive. The lipolytic and proteolytic properties are the very important

characteristics of *Clostridium sporogenes.* Sugar utilization profile studies were positive for glucose, saccharose, cellobiose and weakly positive result to xylose.

Culture characteristics and butanol production was studied in glucose containing semi-defined P2 medium. Maximum specific growth rate (μ_{max}) was found to be 0.145 h^{-1} and saturation constant (K_s) was 17.65 g/L. *C. sporogenes* BE01 growth was inhibited by increased glucose concentrations and substrate inhibition constant (K_i) was 36.55 g/L. Butanol, ethanol and butyric acid also had inhibitory effect on growth rate of *C. sporogenes* BE01 with inhibition constants 5.57, 5.24 and 2.38 g/L respectively. Calcium carbonate, inoculum size and yeast extract were found to be significant parameters and central composite design depicted the interaction between pH and calcium carbonate. Single parameter optimization and multiple parameter optimizations resulted in increase in butanol production in modified P2 medium from 0.97 g/L to 4.5 g/L.

Rice straw hydrolysate with yeast extract and calcium carbonate was used as substrate for butanol fermentation without any supplementation of minerals and vitamins. Minerals supplementation to rice straw hydrolysate exhibited negative effect on both butanol production and sugar utilization. Butanol production increased from 3.43 g/l to 4.05 g/l by single parameter optimization of inoculum size and calcium carbonate. Dilute acid pretreatment and enzymatic hydrolysis of rice straw biomass resulted in acetic acid and formic acid generation in the hydrolysate. Presence of acetic acid and formic acid even at very low concentrations in the medium inhibited the growth of the *C. sporogenes BE01*. Inhibition constants (K_i) for acetic acid and formic acid were 1.6 g/L and 0.76 g/L respectively. Formic acid was found to be more inhibitory to the growth of *C. sporogenes BE01* than acetic acid.

Detoxification of rice straw hydrolysate was attempted with XAD 4, XAD 7, XAD 16 and Seralite 400 (anion exchange resin). XAD 4 was least efficient in the removal of acetic acid and formic acid. Though XAD 7, XAD 16 and Seralite 400 were more or less equally efficient in adsorbing acidic inhibitors, treatment of hydrolysate with Seralite 400, adsorbed less minerals from hydrolysate and supported marked increase in butanol production in comparison with other resins. Detoxified hydrolysate gave maximum butanol production of 5.6 g/L.

C. sporogenes BE01 could produce 7 g/L of VFAs and 1.2 L of hydrogen during butanol fermentation in rice straw hydrolysate with 30 g/L of glucose. Electro-chemical analysis by cyclic voltammetry revealed that $CaCO_3$ supplementation stimulated electron transport system mediated by protein bound FAD and flavoproteins. It also stimulated redox reaction of crotonyl CoA to butyryl CoA, which is mediated by butyryl CoA dehydrogenase. 4Fe-4S clusters were also prominent in the presence of calcium carbonate. Involvement of 4Fe-4S cluster, NAD/NADH, protein bound FAD and flavo proteins during active butanol fermentation presents the possibility of electron bifurcation system mediated by FAD and 4Fe-4S clusters present in butyrl CoA dehydrogenase complex. Fe-S clusters, Cyt bc1 and NAD/NADH were the common electron transporters involved during butanol fermentation. NAD/NADH was found in the first 24 h and depletion in the later hours indicated the start of solventogenesis. Electro kinetic analysis deduced that the rice straw hydrolysate system with *C. sporogenes* BE01 at near neutral pH was more favorable towards reduction reactions than oxidation reactions. Supplementation of calcium carbonate to rice straw hydrolysate decreased the polarization resistance for redox reactions.

Immobilization for high cell density fermentation was evaluated with ceramic particles and polyurethane foam as carrier matrices. *C. sporogenes* BE01 could

efficiently adhere to ceramic support with an adsorption rate (K_a (h^{-1})), 0.149. SEM analysis revealed that *C. sporogenes* BE01 was able to form biofilm on the ceramic particles in ~120 h. Batch and fed-batch fermentation with high cell density cultures of *C. sporogenes* BE01, resulted in a slightly improved butanol production. In Fed-batch fermentation -though butanol production increased, yield in terms of sugars fed to the reactor was less due to inefficient utilization of sugars from the concentrated hydrolysate. Two-stage, high cell density fermentation was effective and increased both VFA and solvent production to 10 g/L and 10.8 g/L respectively with the respective yields of 0.38 and 0.42 g/g sugar fed to the reactor.

8.2. Conclusions

This study demonstrated *C. sporogenes* BE01, an isolate from NIIST is having potential to compete with existing, well known butanol producers with the advantage of no acetone in the final solvent mixture. Rice straw hydrolysate is a potent source of substrate for butanol production by *C. sporogenes* BE01. Additional supplementation of vitamins and minerals were avoided by using rice straw hydrolysate as substrate. Its less growth, due to the inhibitors present in the hydrolysate and also inhibition by products resulted in less efficient conversion of sugars to butanol.

Calcium carbonate played an important role in improving the butanol production, by providing the buffering action during fermentation and stimulating the electron transport mediators and redox reactions favoring butanol production. Its capability to produce acetic acid, butyric acid and hydrogen in significant quantities during butanol production adds value to the conversion process of lignocellulosic biomass to butanol.

High cell density fermentation by immobilizing the cells on to ceramic particles improved the solvents and VFA production. Reduced sugar utilization from the concentrated hydrolysate could be due to accumulation of inhibitors in the hydrolysate during concentration. Two-stage fermentation was very efficient with immobilized cells and high conversions of sugars to solvents and VFAs were achieved. The information obtained from the study would be useful to develop a feasible technology for conversion of lignocellulosic biomass to biobutanol.

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APPENDIX 1

LIST OF ABBREVATIONS

#	Number
µ	Micron/ micrometer
µA	microampere
µg	microgram
µM	micromol
ABE	Acetone, butanol, ethanol
ANOVA	Analysis of Variance
asRNA	antisense Ribo Nucleic Acid
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CCD	Central Composite Design
CMM	Cooked Meat Medium
CoA	Coenzyme A
CSIR	Council of Scientific and Industrial Research
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
FAD	Flavin Adenine Dinucleotide
FID	Flame Ionisation Detector
FPU	Filter Paper Units
g	Grams
g/L	Grams/Liter
h	Hours
HMF	Hydroxy Methyl Furfurals
HPLC	High Performance Liquid Chromatography
ME	Minimum-Evolution
Min	Minutes
Ml/min	Milliliter/Minute
mM	Millimolar

mm	Millimtere
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced from of Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NIIST	National Institute for Interdisciplinary Science and Technology
NJ	Neighbour Joining
OD	Optical Density
ONPG	ortho-Nitrophenyl-β-galactoside
PCR	Polymerase Cahin Reaction
PDA	Photodiode Array
PUF	Poly Urethane Foam
RCB	Reinforced Clostridial Broth
rDna	Ribosomal Deoxy Ribonucleic Acid
RI	Refractive Index
rpm	Rotations per minute
rRna	Ribosomal Ribo Nucleic Acid
TCA	Tricarboxylic Acid
TGY	Tryptone, Glucose, Yeast Extract
UFLC	ULTRA Fast Liquid Chromatography
USA	United Sates of America
UV	Ultra Violet
UV-Vis	Ultra Violet-Visible
V	Volts
v/v	Volume/Volume
VFA	Volatile Fatty Acids
w/v	Weight/Volume
w/w	Weight/Weight

APPENDIX 2

LIST OF TABLES

APPENDIX 3

List of Figures