Sustainable production of biofuels from *Clostridium sporogenes* NCIM 2918: Process strategies and system biology approach

A Thesis

submitted for the partial fulfillment of the degree of

DOCTOR OF PHILOSOPHY

by

MEHAK KAUSHAL

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Under supervision of

Dr. Debasish Das

May 2018

Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Guwahati 781039, Assam, India

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DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India under the supervision of Dr. Debasish Das. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: May 2018 **Mehak Kaushal**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

CERTIFICATE

It is certified that the work described in this thesis entitled "**Sustainable production of biofuels from *Clostridium sporogenes* NCIM 2918: Process strategies and system biology approach**" by Miss Mehak Kaushal for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Date: May 2018 **Mehak Kaushal**

Abstract

Fossil fuels like petrol and diesel have been used for energy generation for more than a century. These fuels emit greenhouse gases such as CO_2, CH_4, NO_x into the environment thereby increasing content of these gases leading to global warming. Global warming coupled with depleting fossil fuel resources has warranted nations to produce fuels which are renewable and carbon neutral. Liquid biofuel (ethanol and butanol) production using *Clostridium* spp. has gained immense attention in recent times. However, it suffers from several bottlenecks which necessitates the development of a sustainable bioprocess through strain level as well as process level improvements.

Present study reports a non-acetone producing *Clostridium sporogenes* strain as a potential producer of liquid biofuels. Biochemical characterization, phylogenetic analysis and 16S rRNA gene sequence comparison of *Clostridium acetobutylicum* NCIM 2918 with other strains confirmed the inaccuracy in its taxonomic status and pointed towards reclassification as *Clostridium sporogenes* NCIM 2918. This will help in better understanding of the strain as a cell factory for industrial applications. The strain was cultivated under different carbon and nitrogen sources for a detailed characterization. Alcohol production was positively regulated by sorbitol and instant dry yeast as carbon and nitrogen sources respectively. Media optimization resulted in maximum butanol and ethanol titer (g L^{-1}) of 12.1 and 7.9 respectively. Depending on the combination of carbon sources, the organism was found to

manipulate its metabolism towards synthesis of either ethanol or butanol, thereby affecting the total alcohol titer. Amongst various dual substrate combinations, glucose-glycerol mixture in the ratio of 60:40 resulted in maximum butanol and ethanol titer (g L^{-1}) of 11.9 and 12.1 respectively with total alcohol productivity of 0.59 g L^{-1} h^{-1}. In the mixture, when pure glycerol was replaced with crude glycerol, butanol and ethanol titer (g L^{-1}) of 11.2 and 11.7 was achieved. Hence, the strain showed immense potential for biofuels production using cheaper substrate like crude glycerol as a carbon source.

Further in order to make the process even more economical attempt was made at replacement of pure glucose with another low-cost carbon source such as lignocellulosic hydrolysate. Three different biomass namely rice straw, sugarcane bagasse and areca nut husk were explored and maximum glucose was obtained from hydrolysis of rice straw. Optimization of hydrolysis conditions was carried out for rice straw and the hydrolysate thus obtained was then used to replace pure glucose in dual substrate blend of glucose-glycerol (60:40) while pure glycerol was replaced with hexane washed crude glycerol. Batch fermentation resulted in a total alcohol titer and productivity of 23.7 g L^{-1} and 0.52 g L^{-1} h^{-1} respectively. Batch fermentation coupled with gas stripping resulted in an improved alcohol titer of 26.4 g L^{-1} (butanol 13.6 g L^{-1} and ethanol titer 12.8 g L^{-1}) and an alcohol productivity of 0.69 g L^{-1} h^{-1} accompanied with complete utilization of carbon sources. To extend the cultivation period, the mode of fermentation was changed from batch fermentation to fed-batch. Fed-batch coupled to continuous gas stripping resulted in higher sugar consumption by the organism and a higher alcohol production of 44.4 g L^{-1} (butanol 21.5 g L^{-1} and ethanol 22.9 g L^{-1}) with an alcohol productivity of 0.62 g L^{-1} h^{-1} and yield of 0.37 alcohol per gram of sugar (glucose+glycerol).

Flux Balance Analysis (FBA) was performed to elucidate the reason behind substrate dependent modulation observed in alcohol biosynthesis using *Clostridium sporogenes* NCIM 2918. FBA was carried out for *Clostridium sporogenes* NCIM 2918 grown on sole glucose and glycerol or glucose-glycerol combinations at varied concentrations. During acidogenesis, glucose and glucose-glycerol combinations favored improved growth and butyric acid production. Glycerol fermentation was however marked by reduced growth and predominant ethanol synthesis. Further, with increase of glycerol fraction in glucose-glycerol blend, flux towards ethanol synthesis linearly increased with simultaneous decrease in butanol flux. Elevated ATP demand due to improved growth was satisfied by upregulated carbon flux towards butyric acid synthesis during both glucose and dual substrate fermentations. Possible repression of pyruvate carboxylase by glycerol resulting in downturn of carbon uptake flux towards TCA cycle through anaplerotic reaction may be responsible for reduced growth in glycerol fermentation. Ammonium acetate mediated induction of acetic acid utilization, during acidogenesis, led to excess acetyl-CoA generation and its subsequent metabolism to lesser reduced products, butyric acid or ethanol.

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CHAPTER 1

Introduction

1.1 Background and motivation

An ever increasing global demand for non-renewable energy resources such as coal, natural gas and oil irrespective of its depletion is proceeding at an alarming rate. Growing global population and industrialization has resulted in a constant pressure of climate change and has compelled researchers to search for alternative renewable energy resources. Liquid biofuels such as biobutanol and bioethanol have gained immense research attention because of their several inherent properties and advantages (Kumar et al., 2011). Blending gasoline with these liquid biofuels, will not only reduce greenhouse emissions, but also contribute towards global energy demand.

Clostridium spp. acetone-butanol-ethanol (ABE) fermentation of carbohydrates, a well explored biological process, was first demonstrated at the industrial scale during the start of the last century (Jones and Woods, 1986). However, in early 1960s the ABE process suffered a complete demise and it could not compete with the chemical production of the same products (Jones and Woods, 1986). After the oil crisis in 1970s, the process gained renewed interest and this led to the expansion of research towards biological production of solvents through ABE fermentation (Jones and Woods, 1986). Since this time, various researchers focused on gaining insights into this process and aimed at overcoming the various limitations it faces such as high feedstock cost, low yields, low productivity, low final product

titer, strain degeneration, acid crash, mixed product titers, low solvent tolerance of the bacteria and expensive product recovery (Sabra et al., 2014; Patakova et al., 2011).

Current research initiatives have focused on targeting the development of suitable producer strains via strain engineering (Jiang et al., 2009; Zheng et al., 2015) and on gaining in-depth understanding of the regulatory mechanisms involved in solvent biosynthesis (Kim et al., 2015; Kumar et al., 2013). All these technological progressions have been put forth to identify and develop a suitable cell factory for butanol production with enhanced productivity, high butanol selectivity, a wide range of substrate utilization capability and enhanced solvent tolerance (Green, 2011; Zheng et al., 2015; Gao et al., 2012). Green Biologics Ltd. (GBL) has developed a large collection of strains which were screened on the basis of their solvent production capacity and ability to utilize specific substrates (Green, 2011). Thus, it is imperative that novel commercially relevant and robust producer strains with capability of sustainable butanol production have to be identified and developed. A wide variety of carbohydrates such as hexose, pentose, disaccharides, sugar alcohols and polysaccharides etc. can be metabolized by *Clostridium* spp. to support its growth as well as ABE fermentation. Further, understanding the modulation in its central metabolism when exposed to different carbohydrates will be helpful in finding targets for metabolic and process engineering strategies to direct carbon and electron fluxes towards the desired pathways (Liao et al., 2016). System-level mathematical modeling approaches such as flux balance analysis can be used for basic understanding of clostridial physiology and also as a design tool assisting in rational metabolic engineering of strains for high biofuel production yields (Liao et al., 2016).

Further suitable process engineering approaches are required to develop an industrially viable process (Ni et al., 2012; Yadav et al., 2014b). Recent approaches

involve media engineering (Al-Shorgani et al., 2011; Li et al., 2014), multi substrate utilization (Wang et al., 2014; Yadav et al., 2014b), co-cultivation of producer strains (Li et al., 2013) and simultaneous recovery of the solvents from fermentation broth (Cai et al., 2017; Rochon et al., 2017; Yadav et al., 2014b). One of the most important factors governing the economic feasibility for biofuel production is the cost of substrates utilized which can account up to 50% of the overall cost (Yadav et al., 2014a). Therefore, use of cheaper substrates such as glycerol or lignocellulosic biomass is expected to decrease the production cost to a greater extent (Green, 2011). Lignocellulosic materials consisting of a variety of monosaccharides, represent the most abundant, sustainable and cost-effective biomass for biofuels production. Another possibility is the use of crude glycerol which is generated as a byproduct during both bioethanol and biodiesel production (Clomburg et al., 2013). Other than cheaper carbon substrates, low cost nitrogenous materials such as instant dry yeast, corn steep liquor, etc. have to be exploited to design an economical process for butanol production (Altaf et al., 2005).

Further ABE fermentation is severely affected due to severe solvent toxicity faced by the *Clostridium* spp. leading to termination of fermentation. An average butanol concentration tolerated by most wild type clostridial strains is approximately 13 g L^{-1} (García et al., 2011). Higher concentrations of approximately 20 g L^{-1} can be produced by mutant strains (Qureshi and Blaschek, 2001); however, the cost of separation of solvents by distillation still remains high (Patakova et al., 2011). Thus, methods to concentrate the solvents after the fermentation or during fermentation are also under consideration. If such methods are applied during fermentation it will provide an effective way to combat solvent toxicity, extend the production times ultimately resulting in high product titers and productivities. Product recovery

strategies such as liquid–liquid extraction, adsorption, pervaporation, reverse osmosis and aqueous two phase separation and gas stripping (Ezeji et al., 2010; Vane, 2008; Cai et al., 2017) are various methods to achieve increased yields. Furthermore to prolong the solvent production it is important to change the mode of operation from batch to fed batch or continuous fermentation coupled with product recovery strategy. Various researchers have demonstrated fed batch or continuous ABE fermentation process by maintaining sufficient amount of substrate in the fermentation broth and removal of solvents, hence overcoming substrate limitation and solvent toxicity simultaneously, allowing uninterrupted solvent production by the organism (Cai et al., 2017; Rochon et al., 2017; Cai et al., 2016).

Thus, ongoing research has outlined possible solutions to several major bottlenecks in the way of development of a commercial fermentation process. However, in order to attain sustainability of this technology more critical engineering innovation in terms of strain and process development are required along with dedicated efforts invested towards understanding the fundamental regulation and controls involved in alcohol metabolism. In view of these issues, the following objectives were framed to develop a feasible biofuel production process.

1.2 Objectives of the study

The present investigations are carried out on the sustainable production of biofuels utilizing *Clostridium sporogenes* NCIM 2918. The following objectives are based on process development strategies and a systems biology approach:

- ***Screen and characterize potential Clostridium sp. butanol producing strain.***
- ***Optimize media composition and process parameters for higher butanol titer.***

- ***Demonstrate multi product paradigm from Clostridium sporogenes and substrate dependent modulation of product composition.***
- ***Understand the regulation in substrate dependent modulation of growth and production of alcohols in Clostridium sporogenes NCIM 2918 through metabolic network reconstruction and flux balance analysis.***
- ***Demonstrate process and scaleup for production of sustainable biofuels via utilization of cheaper raw materials e.g. lignocellulosic biomass and glycerol.***

1.3 Approach

In the first step, six different clostridial strains were procured and screened for maximum butanol production. The best strain with inherent ability to accumulate maximum butanol was identified with, detailed characterization and evaluation. Combined biochemical and system biology approaches were employed to characterize the strain and to maximize alcohol production (Fig. 1.1).

Taxonomic dissection for clostridia based on phenotypic characterization was performed through biochemical analysis, followed by 16S rRNA gene sequencing and phylogenetic analysis. The biochemical approach further involved extensive characterization of the strain under different carbon and nitrogen sources for its ability to produce butanol. The preliminary screening experiments were followed by media optimization with an aim to maximize butanol titer. Characterization of the strain on sole carbon and nitrogen sources resulted in significant variation in butanol and ethanol titer. The results point towards possible substrate dependent modulation of alcohol production pathways in the strain. Therefore, in the next step, the organism was characterized on various dual carbon substrate combinations to assess their effect on butanol to ethanol ratio and in turn, change in total alcohol titer. Furthermore, for

the best dual substrate combination various cheap raw materials were screened to replace the respective sugars in the dual substrate blend.

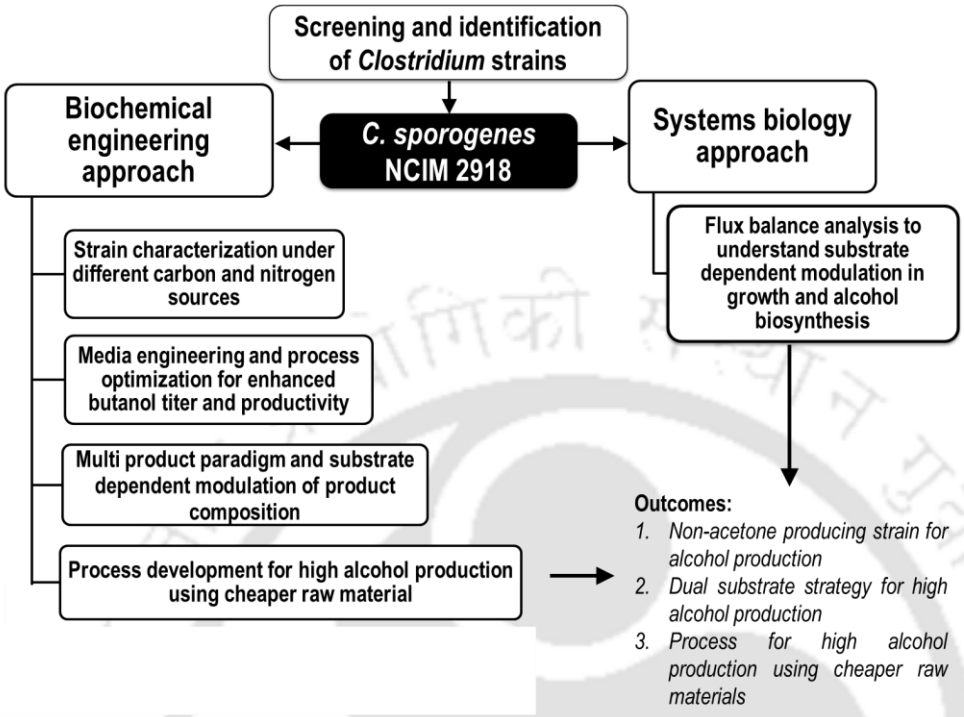

Fig. 1.1 Biochemical and system biology approaches employed to achieve high alcohol production and to understand the regulations involved in growth and alcohol biosynthesis for *Clostridium sporogenes* NCIM 2918

The process was demonstrated in batch fermentation followed by coupling of the process with product recovery strategy and change themode of fermentation from batch to fed batch. The system biology approach involved metabolic network reconstruction and flux balance analysis (FBA) to understand the modulation of carbon flux distribution towards alcohol biosynthesis with changing the type of carbon sources and their concentrations in a mixture. Further, a change in intracellular carbon flux distribution was predicted during metabolic shift from the acidogenic phase to the solventogenic phase. The final outcome of the present study was to design an efficient strategy for butanol-ethanol production with a non-acetone forming strain using cheaper raw material. The developed fed batch fermentation

process coupled with *in situ* product recovery system has shown potential for sustainable biofuel production.

1.4 Organization of the thesis

The thesis is presented in 8 chapters.

Chapter 1 presents a general introduction, objective and scope of the present work along with the approaches required to resolve the current issues. Chapter 2 provides a detailed review on the biology, biochemistry, physiology of clostridial systems along with present processes and advancements made in the field of biofuel production. This section highlights the major bottlenecks with the current technologies and methods to address the same. Chapter 3 deals with the screening of the most potential strain for biofuel production followed by its complete characterization under different physicochemical parameters aiding in correct identification of the strain. Chapter 4 involves the biochemical characterization of the selected strain under different carbon and nitrogen sources followed by media engineering and process optimization for maximum butanol production. Chapter 5 involves screening of various dual substrate combinations for developing a process engineering strategy for generation of high energy liquid biofuel production. Chapter 6 describes flux balance analysis (FBA) that was carried out for the strain to understand the modulation of carbon flux distribution towards alcohol biosynthesis with the change in type of carbon sources and their concentrations in a mixture. The analysis involved reconstruction of metabolic network followed by estimation of carbon fluxes via linear programming optimization. Chapter 7 describes the use of cheaper raw material such as lignocellulosic biomass and crude glycerol as a replacement to substrates used for batch and fed batch fermentation. Further, the fermentation strategy developed was coupled to a product recovery process resulting

in high biofuel production and productivity. Finally, chapter 8 covers the summary of the present study with the conclusions.

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CHAPTER 2

Literature Review

2.1 Fuel, crisis and the quest for renewable energy sources

Increasing global population coupled with increasing energy demand as well as increased industrialization and economic affluence has led to depleting fossil fuel inventories. In 2016, the global primary energy consumption was reported to be approximately 3300 million tonnes of oil equivalent (British Petroleum, 2017). According to British Petroleum (BP) Statistical Review of World Energy 2017, 85% of this primary energy consumption is fulfilled by non-renewable sources such as oil (33%), natural gas (24%) and coal (28%), respectively. While only a minimum share is contributed by renewable sources such as hydroelectricity, nuclear, wind and solar energy (Fig. 2.1). Aproximately 97 million barrel of oil is consumed to meet the global daily demand. In India approximately 4.6% of the global energy demand is utilized to sustain the economic growth (BP Statistical Review of World Energy 2017). The Indian scenario is not much different than global energy consumptionwith 57%, 30% and 6% being supported by coal, oil and natural gas respectively (Fig. 2.2). Based on the current fuel demand and supply statistics for India, it is predicted that these fuels will not last more than 50 years.

Another drawback faced by the use of these non-renewable sources is the enormous damage leading to climate change. Coal, oil and natural gas are classified

as non-carbon neutral fuels and utilizing them has caused the release of CO_2 and other anthropogenic greenhouse gases into the atmosphere.

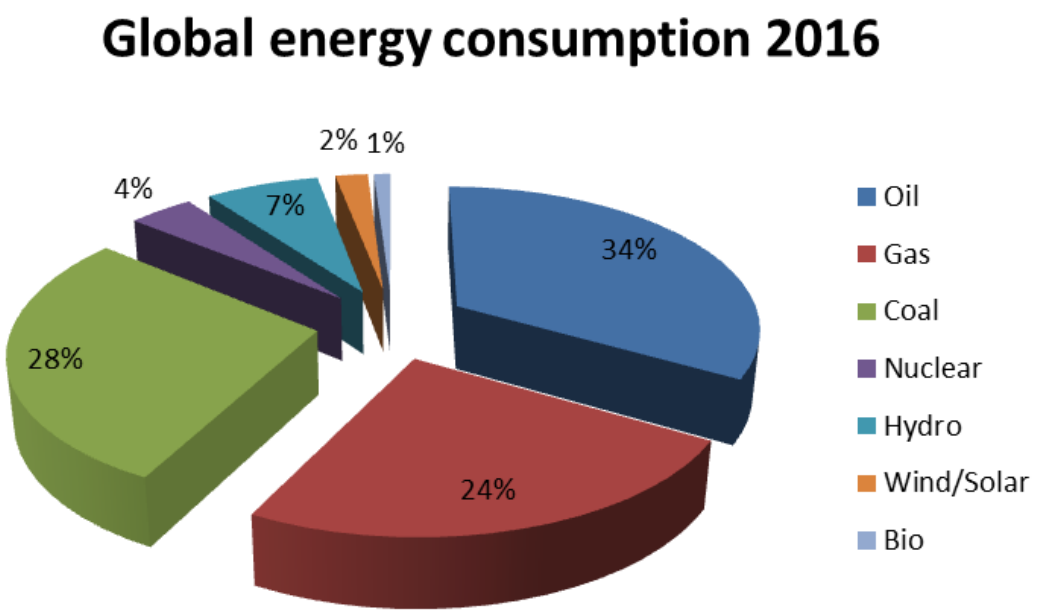

Fig. 2.1 Statistical distribution of the world's primary energy consumption by fuel (Source: BP Statistical review 2017)

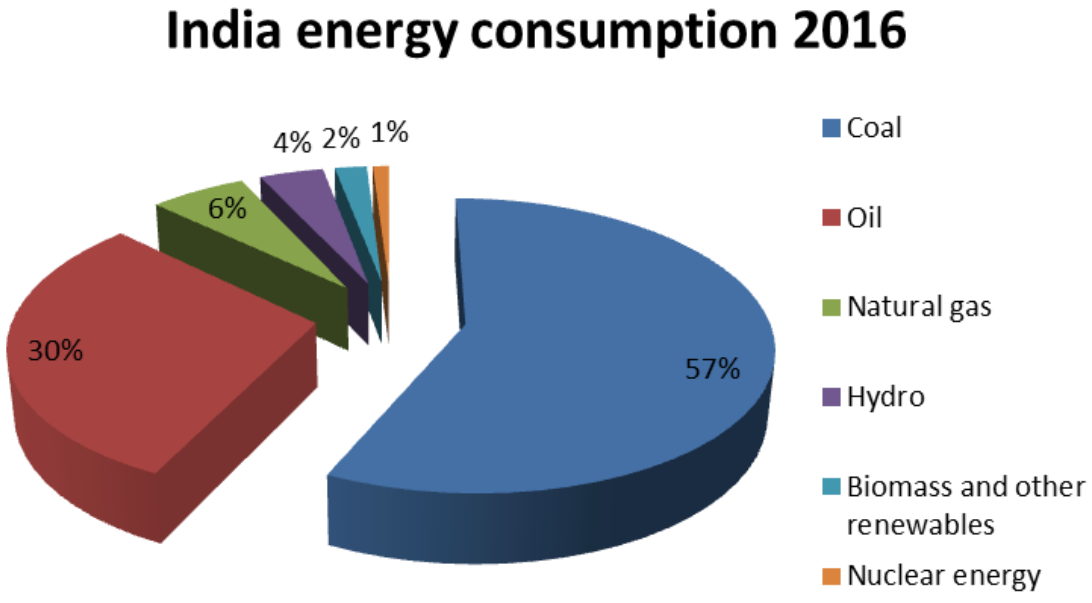

Fig. 2.2 Statistical distribution of the India's primary energy consumption by fuel (Source: BP Statistical review 2017)

This has led to the ever hovering danger of global warming and climate change. The world's CO_2 emissions are increasing at an alarming pace with ~ 40 % increase from

2000 to the 2016 (Fig. 2.3). India occupies the third place in the list of world highest CO_2 emitters with 6.8 % of the total contributed by it (BP Statistical report 2017). Increasing CO_2 emissions has altered the climate and this has caused a variety of effects on aquatic and terrestrial species. Developing carbon neutral fuels is expected reduce CO_2 emissions and alleviate climate problems. Alternate energy sources are expected to reduce CO_2 levels but also provide a replacement for fossil fuels (Brennan and Owende, 2010). Thus it is highly imperative that alternative energy sources are researched and developed so that a natural balance is maintained in the environment and world is saved from the catastrophe it is in line for. Alternate energy sources will not only provide assistance in maintaining normal levels of greenhouse gases but will also provide a sustainable fuel replacement (Brennan and Owende, 2010).

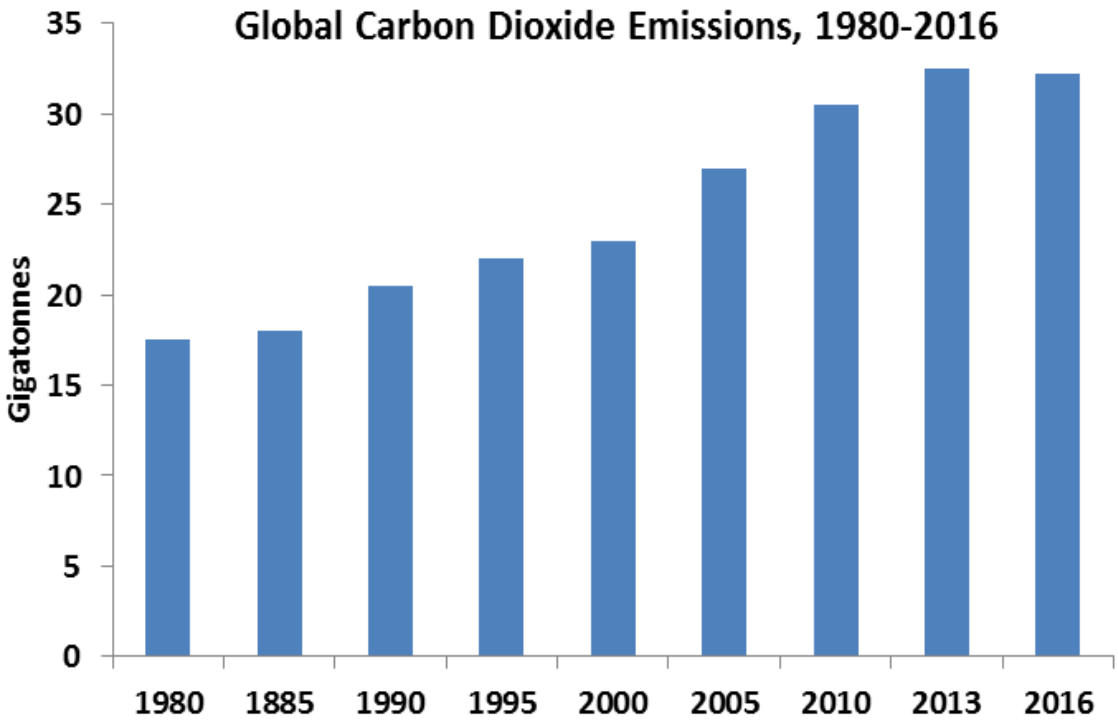

Fig. 2.3 CO_2 emission from the year 1980 to 2016 (Source: IEA, Key World Energy Statistics, 2017)

Energy production from biomass has gained immense interest during the recent years because a variety of low value agriculture residues and crops can be used to produce gaseous and liquid fuels such as hydrogen, methane, bioethanol,

biobutanol and biodiesel (Demirbas, 2008; Singh et al., 2010). Other feedstocks such as microalgae are utilized to produce biodiesel. Biofuels are characterized as first, second, third and fourth generation fuels on the basis of feedstocks used for production (Fig. 2.4).

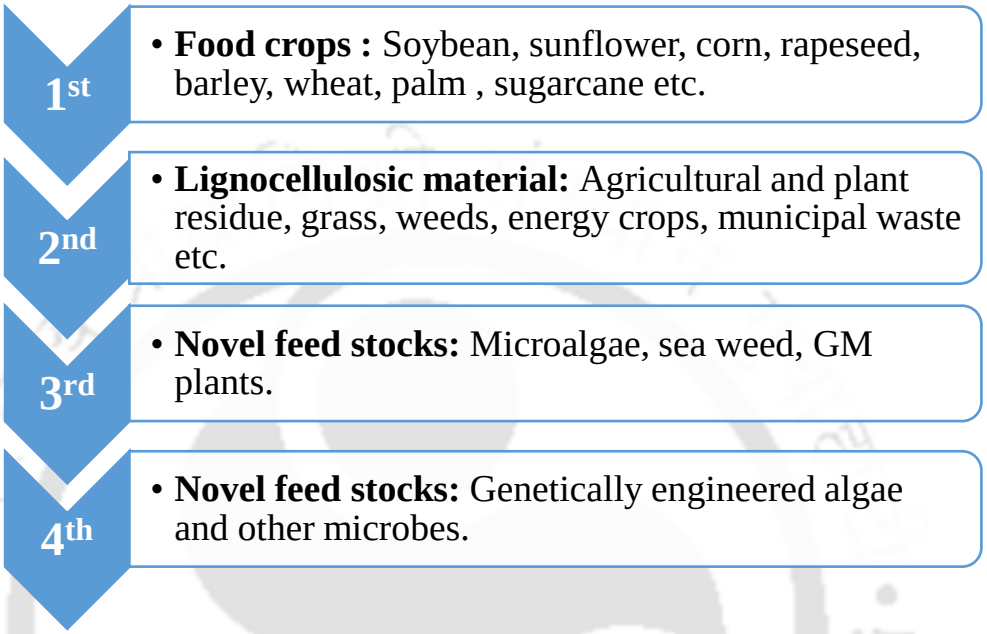

Fig. 2.4 Different generations of biofuel and their corresponding feed stocks (Source: Dutta et al., 2014)

First generation biofuels are produced utilizing food crops. This approach creates a competition between utilizing agriculture products for consumer food production versus energy chemicals for transportation (Dutta et al., 2014; Naik et al., 2010). Algae is the basis for third generation biofuel which does not compete for cropland; however this approach suffers from disadvantages such as dependence on environment, high energy consumption for cultivation (mixing and centrifugation), large amount of water for algae processing, contamination in open pond system and the high cost of photobioreactor, high production cost, environmental threat due to large-scale eutrophication or eco-toxicity during algae cultivation etc. (Dutta et al., 2014; Vassilev and Vassileva, 2016). Metabolically engineered microbes, yeast,

fungi, microalgae and cyanobacteria comprise the fourth generation of biofuel which is still at a nascent research stage. Second generation biofuels were introduced by using non-food crops or waste feed stocks such as agricultural residues, forest residues, municipal waste, sewage sludge, vegetative grasses, short rotation or energy crops as substrates for producing biofuel. Liquid biofuel generated using the inexpensive biomass consists of bioethanol and biobutanol both of which provide an efficient replacement to gasoline as a carbon neutral biofuel. Biobutanol and ethanol are produced along with acetone during ABE fermentation by *Clostridium* spp.

2.2 Biological production of butanol

2.2.1 History of ABE fermentation

First report of biological production of butanol from mixed bacterial culture dates back to 1861 which was reported by microbiologist Louis Pasteur (Dürre, 2007). However, acetone production during fermentation was discovered by Schardinger in 1905 (Dürre, 1998). At the end of the 19th century, the shortage of natural rubber directed research towards synthetic rubber production. Studies were undertaken under the supervision of Professor Perkins at Manchester University along with chemist Chaim Weizmann (Jones and Woods, 1986). Later in 1910, Strange and Graham Ltd.(England) recruited Perkins and Weizmann along with Fernbach and Schoen of the Institute Pasteur to develop the ABE process. After 1 year, Fernbach was successful in isolating a strain capable of fermenting potatoes into butanol but it was unable to utilize maize (Jones and Woods, 1986). Weizmann continued his research on the project at Manchester University although at this time, he did not work for Strange and Graham Ltd. Although Weizmann was a chemist but he trained himself in field of microbiology he was able to develop a remarkable breakthrough between 1912-1914 when he isolated new culture which now is designated as *Clostridium*

acetobutylium (Sauer et al., 2016). Theculture isolated by Weizmann proved itself to be better than the culture isolated by Fernbach in terms the butanol and acetone yield as well as the ability to utilize variety of starchy substances as substrates. The outbreak of First World War in 1914 evoked a great demand for acetone which was used as a solvent in manufacture of ammunitions. As a result of shortage in supply, the British government approached Strange and Graham Ltd. in 1915 but they were unable to meet the demands. After this, work of Weizmann caught attention of the British government who requested him to cooperate with other researchers. In March 1915, Weizmann was issued a patent for the Weizmann process (Poehlein et al., 2017). Pilot scale studies were performed by Weizmann and his team. In 1916, the British government took over the Strange and Graham Ltd. plant and Weizmann process was used in further production batches. This resulted in increased acetone production to about approximately 1,000 pounds per week. However, in 1916 due to the shortage of grains and other food, the fermentations process had to be shifted from England to Canada where it remained functional till 1918. A truce in November 1918 was marked with the shutdown of all acetone producing plants due to a lack of acetone demand.

During the production of acetone, butanol accumulated as a byproduct and later its use as a lacquer to give finish to automobile was initiated (Sauer et al., 2016). Commercial Solvents Corporation of Maryland obtained the global patent rights for Weizmann process. However, in 1923 bacteriophage infection became a major problem for the process resulting in decrease of fermentation yield by approximately 50%. In 1936, the Weizmann patent expired and since then use of molasses as a cheaper substitute when compared to previoussubstrates progressed rapidly (Moon et al., 2016). During this period a number of different strains were isolated and patented

by large producer firms. During the second World War AB fermentation was atop priority to fulfill acetone demand. In the early 1960s with the end of war, AB fermentation again failed to support itself and most of the major fermentation plants were shut down. During this time mothballing of existing production facilities was due to acute competition with chemically derived solvents and the increased cost for molasses which rendered the process uneconomical (Sauer et al., 2016). In the 1970s, development of the ABE process was revived due to the scarcity of oil, as an alternative to petrochemical fuel (Dürre, 2008).

2.2.2 ABE Clostridia fermentation

C. acetobutylicum is the most well studied *Clostridium* sp. at the genetic level and immensely explored for butanol production. Various clostridial species with attractive features have also been isolated and developed (Table 2.1). The selection of strains depends on the type of substrate used for fermentation and the ratio of solvents required at the end of the fermentation reaction.

Table 2.1 Wild-type butanol producing *Clostridium* spp.

Species	Main solvent products (Substrate)[a]	Solvents (g L^{-1})[b]	Reference
C. acetobutylicum	Butanol, acetone and ethanol (Glucose)	10.4 (B) 3.8 (A) 1.5 (E)	Huesemann et al., 2012
C. beijerinckii	Butanol, acetone and ethanol (Glucose)	15.2 (B), 5.44 (A) 0.41 (E)	Qureshi et al., 2010
C. saccharoper -butylacetonicum	Butanol, acetone and ethanol (Glucose)	16.2 (B), 7 (A) 1.0 (E)	Thang et al., 2010
C. saccharoper -butylicum	Butanol, acetone and ethanol (Glucose)	9.7 (B), 0.9 (A) 0.2 (E)	Berezina et al., 2009
C. pasteurianum	Butanol (Glucose)	6.9 (B)	Sabra et al., 2014
C. pasteurianum	Butanol and 1,3-propanediol (Glycerol)	13.9 (B) 5.3 (1,3-PDO)	Sabra et al., 2014
C. sporogenes	Butanol and ethanol (Glucose)	3.43 (B) 1.89 (E)	Gottumukkala et al., 2013
C. tetanomorphum	Butanol and ethanol (Glucose)	4.16 (B) 4.98 (E)	Gong et al., 2016
C. carboxidivorus	Butanol and ethanol (Carbon monoxide)	5.55 (B) 2.66 (E)	Fernández-Naveira et al., 2016
C. aurantibutyricum	Butanol, acetone and isopropanol (Glucose)	3.36 (B)	George et al., 1983

a-represents the substrates that are utilized by the organism which are denoted in the braces.

b-represents Acetone-A; Butanol-B and Ethanol-E

2.2.2.1 Clostridium acetobutylicum

Conventional ABE fermentation by *Clostridium acetobutylicum* results in the production of a blend of acetone, butanol and ethanol in a ratio of 3:6:1 (Jones and Woods, 1986) using glucose as carbon source. *C. acetobutylicum* is a gram-positive, endospore-forming obligate anaerobe which is able to utilize a variety of other carbon sources such as fructose, galactose, sucrose, mannose, starch, dextrin, lactose, mannitol, arabinose, xylose, and pectin (Jones and Woods, 1986). Typical glucose fermentation is biphasic exhibiting early acidogenesis phase followed by solventogenic phase (Patakova et al., 2011). Acetic acid and butyric acid are the key acids produced during the acidogenesis phase. When the concentration of acids reaches a threshold level, the organism starts to re-assimilate these acids into neutral

solvents such asbutanol, acetone and ethanol. This continues until the end of fermentation. Both the phases are accompanied by the release of hydrogen and carbon dioxide. The carbon flow from carbon source (glucose) through the main branches of the pathway leading to the formation of acids and solvents is shown in Fig. 2.5.

The formation of pyruvate through the glycolysis pathway is same as observed in various other organisms. Pyruvate is then cleaved by pyruvate-ferredoxin oxidoreductase enzyme in the presence of coenzyme A (CoA) resulting in formation of acetyl-CoA and reduced ferredoxin along with release of carbon dioxide. Acetyl-CoA is the central intermediate in the branched fermentation pathway which divert towards both acid and solvent production. Ferredoxin has a key role in electron distribution. Ferredoxin transfers electrons to the hydrogenase enzyme which diverts electrons to protons with a subsequent release of hydrogen gas. Two other important enzymes which play key role in the electron distribution include NADH ferredoxin oxidoreductase and NADPH ferredoxin oxidoreductase. NADH ferredoxin oxidoreductase performs the function of both oxidation and reduction of NAD by the exchange of electrons between NAD and ferredoxin (Gheslaghi et al., 2009). In comparison, NADPH ferredoxin oxidoreductase mainly functions for the generation of NADPH during the growth phase (Gheslaghi et al., 2009). This is the only possible mechanism to produce NADPH in *Clostridium* spp. including *C. acetobutylicum* as most of them lack the enzymes involved in oxidative pentose-phosphate pathway (Amador-Noguez et al., 2010; Crown et al., 2011).

The carbon flow from acetyl-CoA can be diverted to the formation of acetic acid through a single intermediate acetylphosphate. Butyric acid formation carbon flow is diverted through five other intermediates, acetoacetyl-CoA, 3-hydroxybutyryl CoA, crotonyl CoA, butyryl-CoA and butyrylphosphate as shown in Fig. 2.5. The

phosphate acetyltransferase and acetate kinase mediate the formation of acetate from acetyl-CoA while separate analogous enzymes (phosphate butyryltransferase and butyrate kinase) perform similar function for the formation of butyric acid from butyryl-CoA. The production of both the acids is accompanied with generation of ATP through the reaction catalyzed by acetate and butyrate kinase. These two enzymes used in producing both acids are also observed to exhibit reversible activity when the organism is exposed to acid toxicity it reutilizes the excess acids into solvents (Gheslaghi et al., 2009).

Acetyl-CoA and butyryl-CoA arekey intermediates during solvent production. Reduction of acetyl-CoA and butyryl-CoA by the action of aldehyde dehydrogenases results in formation of acetylaldehyde and butyraldehyde respectively. These two intermediates are further reduced into ethanol and butanol by alcohol dehydrogenase.

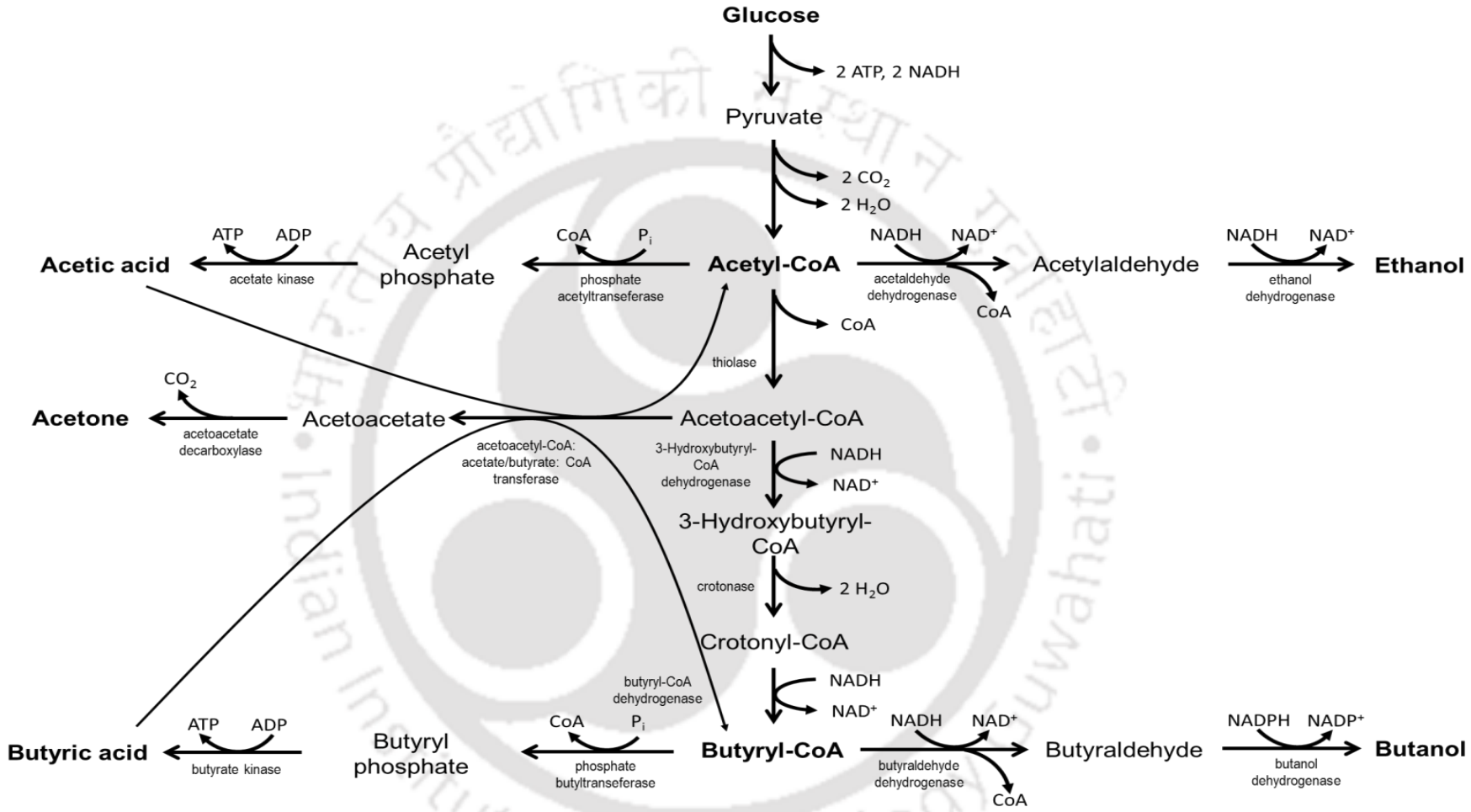

Fig. 2.5 Metabolic pathways in *C. acetobutylicum*

According to Jones and Woods (1986) acid re-assimilation occurs concurrently with sugar uptake. Hence, carbon flow from both pyruvate and the acid utilization pathway is diverted into solvent production. Further, the uptake of acetate and butyrate is directly coupled to the acetone production. This reaction is catalyzed by the enzyme acetoacetyl-CoA:acetate/butyrate:CoA transferase which utilizes acetate or butyrate as the CoA acceptor while converting acetoacetyl-CoA to acetoacetate. Acetoacetate is used to generate acetone in an irreversible decarboxylation reaction performed by acetoacetate decarboxylase enzyme. Hence, the uptake of acids is accompanied with formation of an equivalent amount of acetone (Jones and Woods, 1986). The solvent formation genes are present in form of sol operon consisting of *adhE–ctfA–ctfB* (encoding a bifunctional butyraldehyde/butanol dehydrogenase and the two subunits of CoA transferase), and an adjacent monocistronic *adc* operon (encoding acetoacetate decarboxylase). Both *sol* and *adc* operon reside on the megaplasmid pSOL1 (192,000 bp) which is indispensable for solvent formation and its loss leads to degeneration of the cells (Poehlein et al., 2017; Patakova et al., 2011).

2.2.2.2 Clostridium beijerinckii

Clostridium beijerinckii finds is named after the renowned Dutch microbiologist Martinus Beijerinck (Dürre, 2008). Since the discovery of biological production of butanol by numerous *Clostridium* strains various names have been assigned to butanol producing organisms (Dürre, 1998). An agreement was reached amongst the scientific community that all the strains producing acetone-butanol mixtures will be referred to as *C. acetobutylicum* while others producing acetone-butanol-isopropanol blends as *C. beijerinckii*. As a result, the well explored organism *C. acetobutylicum* NCIMB 8052 is now considered to belong to *C. beijerinckii*.

However, George et al. (1983) reported that several strains of *C. beijerinckii* did not produce isopropanol. Currently, *C. beijerinckii* is accepted as a broad group of clostridial strains which include both isopropanol producers and non-producers (Chen and Hiu, 1986; Hiu et al., 1987). A key difference is the presence of primary/secondary alcohol dehydrogenase enzyme which catalyzes the conversion of acetone to isopropanol (Bankar et al., 2014). Another important difference between *C. acetobutylicum* and *C. beijerinckii* is that the latter lacks the pSOL1 plasmid which encodes the solventogenic genes as in case of *C. acetobutylicum* (Milne et al., 2011). This restricts the phenomenon of degeneration observed in *C. acetobutylicum* when the organism loses its ability to produce solvents after repeated cell culture (Patakova et al., 2011). *C. beijerinckii* consists of a genome 50% larger than *C. acetobutylicum* although both have similar solvent forming profiles with solvent production in 3:6:1 ratio of acetone-butanol-ethanol (Milne et al., 2011). *C. beijerinckii* like *C. acetobutylicum* also performs a biphasic fermentation where acids are produced initially and re-assimilated into solvents during the later phase (Milne et al., 2011).

2.2.2.3 Clostridium saccharoperbutylacetonicum

Assingning the name *Clostridium saccharoperbutylacetonicum* is based on various characteristics it shows i.e. saccharin for sugar juice, per for throughout, butylum for butanol, acetonicus for acetonic and saccharoperbutylacetonicum representing the use of sugar for the production of a large amount of butanol and acetone (Keis et al., 2001). Hongo. (1960) in a U.S. (United States) patent assigned the name *C. saccharoperbutylacetonicum* to a new organism which he discovered to be distinct from other *Clostridium* spp. known at that time. This was further confirmed by Keis et al. (1995) using16S rRNA gene-sequence analysis and also by Johnson et al. (1997) employing DNA-DNA reassociation studies. Strain ATCC

27021, which is a derivative of the strain N1-4, was deposited in the American Type Culture Collection (ATCC) by Hongo and Murata and is considered the type strain for this species (Keis et al., 2001). *C. saccharoperbutylacetonicum* ATCC 27021 also contains a megaplasmid (136,188 bp) such as in *C. acetobutylicum*; however, the solventogenic genes are not contained within the DNA of this organism (Poehlein et al., 2017). The arrangement of sol operon genes in *C. saccharoperbutylacetonicum* is similar to that observed for *C. beijerinckii*. The operon consists of genes in the following order as NADH-dependent aldehyde dehydrogenase (*ald*), CoA transferase two subunits (*ctfA* and *ctfB*) followed by acetoacetate decarboxylase (*adc*). In terms of substrate utilization *C. saccharoperbutylacetonicum* is able to utilize cheaper molasses-based feedstocks and this results in superior solvent production properties when compared to *C. acetobutylicum* (Poehlein et al., 2017).

2.2.2.4 Clostridium saccharobutylicum

Clostridium saccharobutylicum like *C. saccharoperbutylacetonicum* was also assigned its name based on variety of characteristics. These characteristics include ability to consume saccharon, butylum for butanol and saccharobutylicum denoting utilization of sugar for the production of butanol. The *C. saccharobutylicum* strain earlier named as *'Clostridium saccharo-butyl-acetonicum-liquefaciens',* which was the first to be used at the commercial scale (Jones and Keis, 1995) was isolated and patented by Commercial Solvents Corporation. Keis et al. (2001) proposed to use a less cumbersome name *C. saccharobutylicum* for this species. Strain NCP 262 is considered as the type strain which was also deposited with DSMZ and the ATCC under accession numbers DSM 13864 and ATCC BAA-117 respectively. *C. saccharobutylicum* sol operon is localized in the chromosome and the solventogenic

genes are organized similarly to *C. beijerinckii* and *C. saccharoperbutylacetonicum* (Poehlein et al., 2013).

2.2.2.5 Clostridium aurentibutylicum

Clostridium aurentibutylicum is a, butyric acid-producing *Clostridium* which was isolated by Chaim Weizmann from South African *hibiscus* stems. The microorganism was described and given the current designation by Hellinger et al. (1947). The organism is able to utilize a variety of carbon sources such as glucose, sucrose, maltose lactose, galactose, xylose, starch, but not mannitol, glycerol and sorbitol. Glucose and maize fermentation results in formation of butyric and acetic acids followed by acetone, butanol, ethanol and isopropanol (Hellinger et al., 1947; George et al., 1983). *C. aurantibutyricum* strains contain plasmids which are 31,015 to 55,559 bp in size (Poehlein et al., 2017). The sol operon in *C. aurantibutyricum* is organized similar to *C. acetobutylicum* however it does not reside on megaplasmid as observed in case of latter.

2.2.2.6 Clostridium pasteurianum

A Russian microbiologist, Sergei Winogradsky, isolated *Clostridium pasteurianum* in 1890 and assigned thename in honor of Louis Pasteur (Winogradsky, 1894). This microorganism was the first discovered free nitrogen fixing anaerobe. The capability of a non-spore forming mutant of *C. pasteurianum* DSM to utilize glycerol as a sole carbon source resulting in 1-butanol production was reported by Dabrock et al. (1992). Later Biebl (2001) showed the same phenomena for the original strain *C. pasteurianum* DSM 525. The fermentation pattern is distinct when compared toother ABE producing bacteria. Glycerol is metabolized to DHA (dihydroxyacetone) and further to DHAP (dihydroxyacetone phosphate) by action of glycerol dehydrogenase and dihydroxyacetone kinase respectively. From the DHAP

node, the metabolism continues with standard glycolysis, while some glycerol is converted to 3-hydroxypropionaldehyde and further to 1, 3-propandiol (Dabrock et al., 1992; Biebl, 2001). Maximum butanol production followed by 1, 3-propandiol, ethanol, butyrate and acetate was reported by Biebl (2001). Similarly *C. pasteurianum* ATCC 6013 was also reported to produce butanol along with 1, 3-propandiol and ethanol by utilizing pure or crude glycerol (Taconi et al., 2009). Although this behavior was different from that observed for *C. acetobutylicum*, which cannot utilize glycerol as a sole carbon source, however glycerol utilization is reported only in a mixture containing both glucose and glycerol (Vasconcelos et al., 1994; Andrade and Vasconcelos, 2003).

2.2.2.7 Clostridium tetanomorphum

Clostridium tetanomorphum shows a different product profile compared to other well explored butanol producing species of *Clostridium*. This microorganism produces almost equimolar amounts of butanol and ethanol; while acids are reassimilated andno acetone is formed (Gottwald et al., 1984; Gong et al., 2016). Absence of the acetone synthesis pathway in *C. tetanomorphum* DSM665 was reported by Gong et al. (2016).

2.2.2.8 Clostridium sporogenes

Metchnikoff was the first to describe the type train DSM 795 of the species *Clostridium sporogenes* in 1908. This microorganism was isolated from human faeces and belongs to the proteolytic branch of clostridia. *C. sporogenes* use is mostly restricted to therapeutic application such as in the treatment of certain cancers (Poehlein et al., 2015a). Not many studies have been performed in utilizing this microorganism it as cell factory for biofuel production. *C. sporogenes* produces solvents such as ethanol and butanol but not acetone (Gottumukkala et al., 2013;

Ranganathan et al., 2010). This fermentation pattern which is similar to the product profile observed for *C. tetanomorphum*. The absence of acetone production was confirmed in work by Poehlein et al. (2015a). These researchers reported the genome for *C. sporogenes* DSM 795 which did not contain the acetone forming genes (CoA transferase and acetoacetate decarboxylase).

2.2.2.9 Clostridium carboxidivorans

Clostridium carboxidivorans is an acetogenic anaerobic bacterium, which is capable of utilizing CO, CO_2, and H_2 or syngas through the Wood-Ljungdahl (WL) pathway to produce biofuels (Liou et al., 2005). Acetic acid is produced in maximum concentration followed by butyric acid and hexanoic acid. Ethanol is the major alcohol produced during the fermentation along with butanol, and also some minimal amount of hexanol. Fermentation of CO: CO_2: H_2: N_2 mixture of gases in 2:2:1:5 ratio results in solvent production of hexanol, butanol and ethanol (H: B: E) in 1:2.5:6 ratio (Fernández-Naveira et al., 2017).

2.2.2.10 Clostridium drakei

This species was renamed as a result of studies conducted in the field of microbiology of acetogens by Harold L (Liou et al., 2005). Drake. This anaerobe is able utilize autotrophically H_2/CO_2 or CO, resulting in production of acetic and butyric acid along with ethanol, and butanol during the fermentation (Gößner et al., 2008).

2.2.2.11 Clostridium ragsdale

This acetogen has been reported to produce acetic acid and ethanol as major products during syngas (CO: CO_2: H_2) fermentation with a minimal amount of 0.47 g L^{-1} butanol (Kundiyana et al., 2010). However, very few studies have been ble to confirm the work reported by Kundiyana et al. (2010). In another study, Isom et al.

(2015) showed that *C. ragsdale* reduces fatty acids into primary alcohol such as propanol, butanol, pentanol, and hexanol.

2.2.2.12 Other butanol producing genera

Other genera whose members are also capable of producing butanol include *Butyribacterium* and *Thermoanaerobacterium* (Kerby and Zeikus, 1987; Fernández-Naveira et al., 2017). Amiri et al. (2016) reported that a *Nesterenkonia* sp. strain F isolated from Aran-Bidgol Salt Lake in Iran was able to produce acetone, butanol and ethanol under aerobic and anaerobic conditions.

2.2.3 ABE fermentation main bottlenecks

2.2.3.1 Taxonomic discrepancies

After the acceptance of *Clostridium* as a genus, a large number of clostridial strains showing typical characteristics such as rod shaped, anaerobic, spore forming, gram positive were included into this group. However, after the emergence of molecular tools such as 16S rRNA sequencing and DNA-DNA hybridization, the phylogenetic diversity that existed between the group members was established. Various groups have stressed on the fact that more detailed characterization studies consisting of biochemical studies, 16S rRNA sequencing, DNA-DNA hybridization and comparative whole genome analysis have to performed for the taxonomic dissection of these strains showing ambiguous properties and difficult to differentiate (Lawson et al., 1993; Stackebrandt et al., 1999; Cato and Stackebrandt, 1989; Collins et al., 1994; Lawson and Rainey, 2016; Wilkinson et al., 1995). A number of clostridial strains have been reclassified (Table 2.2) to more homologous groups. This correct identification will assist in better understanding and improvingthe strains for future biofuel applications.

Table 2.2 List of reclassified clostridial strains

Prior classification	New classification	Reference
C. pasteurianum NRRL B-598	*C. beijerinckii* NRRL B-598	Sedlar et al., 2017
C. acetobutylicum NCIMB 8052	*C. beijerinckii* NCIMB 8052	
C. kaneboi ATCC 824	*C. acetobutylicum* ATCC 824	
C. kaneboi DSM 1731	*C. acetobutylicum* DSM 1731	
C. acetobutylicum NRRL B591	*C. beijerinckii* B591	
C. acetobutylicum NRRL B597	*C. beijerinckii* B597	Keis et al., 2001
C. butanologenum ATCC 6014	*C. beijerinckii* ATCC 6014	
C. acetobutylicum B596	*C. beijerinckii* B596	
C. acetobutylicum NCP 249	*C. saccharobutylicum* NCP 249	
C. acetobutylicum NCP 262	*C. saccharobutylicum* NCP 262	

2.2.3.2 Substrate cost

Substrate cost is an important factor and can constitute approximately 50% of the overall process economics (Yadav et al., 2014; Sabra et al., 2014). Although ABE can be produced from both edible and non-edible feedstock, in order to develop an economical process,cheap feedstocks must be sourced to alleviate issues related to*food* versus *fuel* production from crops such a corn and wheat (Algayyim et al., 2018). This problem can be addressed by isolating new wild type clostridial strains which can utilize cheaper raw material or genetically modifying current biofuel producing cell factories. However the latter raises the question of the stability of the recombinant strains. An alternate approach involves developing a variety of inexpensive raw materials which can be utilized on an industrial scale.

Lignocellulosic raw material

Lignocellulosic biomass being an abundant global carbon feedstock provides a renewable cheap carbon source for ABE fermentation (Zhang et al., 2007). Various

lignocellulosic biomass explored for biofuel production include forest residues, energy crops, agriculture residues, industrial waste, municipal solid waste and liquid waste etc. (Table 2.3). Lignocellulosic biomass is comprised of cellulose, hemicellulose, and lignin polymers. Cellulose is a polymer of glucose while hemicellulose is a polymer of mostly five carbon sugar such as xylose, mannose and arabinose (Rajendran et al., 2017). Lignin consists of phenylpropanoid units and behaves like a shield coveringthe sugar polymer matrix. This complex structure provided the plant tissue strength and resistance against insect and microorganisms (Isikgor et al., 2016). The composition of the three polymers in the lignocellulosic complex varies with the type of biomass and cultivation conditions. Hence, individual pretreatment methods must be developed to target cellulose and hemicellulose as wellas the pretreatment process may vary with the biomass composition (Seidl and Goulart, 2016). Different pretreatment methods used including physical, physicochemical, thermochemical and biological perform various functions such as decreasing the degree of polymerization, reducing the crystalline structure of cellulose, reducing the particle size for efficient enzyme treatment, improved surface area, altering the lignin structure, cellulose hydrolysis and hemicellulose hydrolysis (Rajendran et al., 2017). Physical methods include extrusion, freezing, irradiation, mechanical commination, microwave, sonication etc. (Kumar et al., 2009; Rajendran et al., 2017). Physicochemical pretreatment includes processes such as steam explosion, ammonia fiber explosion and carbon dioxide explosion while thermochemical methods include alkali treatment, acidic treatment, organosolvent treatment, ozonolysis and oxidative delignification (Kumar et al., 2009; Putro et al., 2016).

Biological treatment involves using microorganisms such as brown, white or soft rot fungi to degrade cellulose, hemicellulose or lignin (Galbe and Zacchi, 2007). Amajor disadvantage of the biological method is that the process is time consuming with treatment times reaching weeks to months. Hence biological methods are uneconomical for biofuel production (Rajendran et al., 2017). Otherbiological pretreatment consist of employing fungal hydrolyzing enzymes such as cellulase, hemicellulase and xylanase etc. Each pretreatment method has its own pros and cons and hence, no universal method can be applied to all biomass. Researchers have coupled more individual aggressive methods with alkaline and acid treatment to improve sugar hydrolysis rates and ultimately reducing the pretreatment times (Moradi et al., 2013). These aggressive methods include combining steam explosion withacid pretreatment (Ranjan et al., 2013), alkaline with hydrothermal pretreatment (Grimaldi et al., 2015), supercritical CO_2 with steam explosion pretreatments (Alinia et al., 2010), liquid hot water plus aqueous ammonia (Kim and Lee, 2006) and as well as organosolv and biological pretreatments (Monrroy et al., 2010).

Table 2.3 Biofuel production from various lignocellulosic materials

Microorganism	Substrate	Pretreatment and hydrolysis methods	Reference
C.saccharoperbutyl -acetonicum N1-4	Palm oil mill effluent	1% sulphuric acid and enzymatic hydrolysis	Shorgani et al., 2012
C. saccharobutylicum DSM 13864	Corncob	0.5 mol L^{-1} NaOH and enzymatic hydrolysis	Gao and Rehmann, 2014
C. beijerinckii NRRL B-466	Brewery liquid waste, starch industry wastewater and apple pomace ultrafiltration sludge	Dilute sulphuric acid	Maiti et al., 2016
C. sporogenes BE01	Rice straw	Dilute acid+Enzyme	Gottumukkala et al., 2013
C. acetobutylicum ATCC 824	Corn stover	Alkali extrusion +Enzyme	Zhang et al., 2014
C. acetobutylicum ATCC 824	Switch grass	Hydrothermolysis +Enzyme	Liu et al., 2015
C. saccharobutylicum DSM 13864	Switch grass	Alkali+Enzyme	Gao et al., 2014
C. saccharobutylicum DSM 13864	Corn stover	Alkali+Ionic liquid+Enzyme	Ding et al., 2016
C. acetobutylicum ATCC 824	Bamboo	Acid+Enzyme	Kolawole et al., 2016
C. acetobutylicum GX01	Sugarcane bagasse	Alkali+Enzyme	Pang et al., 2016
C. acetobutylicum DSM 1731	Barley straw	Acid+Enzyme	Yang et al., 2015
C. acetobutylicum CH02	Sugarcane bagasse	Diluted acid+aqueous ammonia+Enzyme and diluted acid+oxidat ammonolysis+Enzyme	Li et al., 2017
C. acetobutylicum NRRL B-591	Sweet sorghum bagasse	Acetone +Enzyme	Jafari et al., 2016
C. acetobutylicum DSM 1731	*S. schwerinii*	Acid+Enzyme	Yang et al., 2017
C. beijerinckii CC101	Jerusalem artichoke stalk	Alkali+H_2O_2 (oxidative pretreatment)+ Enzyme	Xue et al., 2017

Hence, the key hindrance in utilizing lignocellulosic material lies in its complex structure. In order to overcome this recalcitrance, efficient and cost effective pretreatment methods have to be developed along with in-depth understanding of the available biomass composition.

Crude glycerol

Recently, crude glycerol a byproduct obtained from biodiesel production has received much attention as an alternative substrate for biofuel production (Da Silva et al., 2009; Biswas et al., 2017). Since glycerol has a higher degree of reduction per carbon (number of available electrons per unit of carbon) compared to glucose or xylose, it provides advantage when considering the production of fuels and reduced chemicals (Clomburg and Gonzalez, 2013). *C. pasteurianum* is known to metabolize glycerol through the DHA pathway (Clomburg and Gonzalez, 2013); however, *C. acetobutylicum* cannot use glycerol as a sole carbon source (Vasconcelos et al., 1994). Biebl (2001), reported glycerol fermentation *C. pasteurianum* resulted in 17 g L^{-1} butanol along with 1, 3-propanediol, butyric and acetic acids and ethanol. Taconi et al. (2009) showed that *C. pasteurianum* ATCC 6013 was successful in utilizing biodiesel-derived crude glycerol resulting in a butanol maximum yield of 0.30 gram per gram of crude glycerol. A mutant strain *C. pasteurianum* MBEL_GLY2 was cultivated in a continuous fermentation mode resulted in an improved butanol production (10.8 g L^{-1}) compared to that obtained with the parent strain (Malaviya, et al., 2012). Further Khanna et al. (2013) used immobilized cells of *C. pasteurianum* for converting crude glycerol to biofuel. These studies point towards the possible use of crude glycerol as a low-cost, renewable feedstock for butanol production. However, a key issue with the efficient utilization of crude glycerol is the presence of impurities such as methanol, salts (in the form of potassium chloride or sulfate), heavy metals

and fatty acids that were not transesterified could hinder clostridial growth with subsequent prolonged batch time and low productivity (Venkataramanan et al., 2012). The inhibitory effect of each individual impurity on microbial growth has been examined and strategies have been developed to remove chemicalsin crude glycerol (Venkataramanan et al., 2012). Therefore, it is important that strains which have the natural ability to utilize crude glycerol are isolated or wild type strains are genetically modified for efficient crude glycerol conversion into biofuel.

Marine macroalgae (seaweeds)

Different types of marine macroalgae such as green, red, and brown macroalgae have gained attention during the recent years as a substrate for third generation biofuels. These feedstocks are known to have high a carbohydrate composition coupled with a low lignin content as well as cultivation do not require arable land (Ohgren et al., 2007; Wargacki et al., 2012). Various researchers have explored the potential of using marine macroalgae for biofuel production; however, these studies have reported low yields (Huesemann et al., 2012; Kim et al., 2013; Potts et al., 2012; van der Wal et al., 2013). The key reason for this is that clostridia is unable to ferment sugars such as L-rhamnose, 3,6-anhydro-L-galactose (AHG), and 4-deoxy-L-erythro-5-hexoseulose (DEH) (van der Wal et al., 2013; Wargacki et al., 2012; Yun et al., 2015). Hence for the efficient utilization of macroalgal sugars, current clostridia strains have to be genetically engineered to provide a synthetic platform for biofuel production.

Syngas

Syngas is mostly composed of mixtures containing CO, CO_2, and H_2. The concentration of each gas in the syngas mixture varies according to the feedstock and the gasification parameters. Several industrial waste gases comprise of syngas and can

be used as a carbon source for biofuel production (Weissermel and Arpe, 2008). This method not only provided an inexpensive feedstock for biofuel production but also incurred environmental benefits. Acetogens are the anaerobic microorganisms that have the natural capability to utilize these gases for growth and product formation. Carbon monoxide can be used both as a carbon and energy source while carbon dioxide can only be a carbon source with requirement for hydrogen gas as an energy source. This uptake of syngas takes place through the Wood-Ljungdahl (WL) pathway resulting in production of acids and solvents. The first step in WL pathway is the conversion of CO or CO_2 + H_2 into acetyl-CoA which behaves like the branch point for all the acid (acetic, butyric and hexanoic acid) and alcohol (ethanol, butanol and hexanol) synthesis pathway (Drake et al., 2008). Clostridial spp. that are known to ferment syngas include *C. glycolicum* (Küsel et al., 2001), *C. ljungdahli* (Köpke et al., 2010), *C. autoethanogenum* (Guo et al., 2010) and *C. carboxidivorans* (Bruant et al., 2010; Fernández-Naveira et al., 2017). *C. carboxidivorans* has been reported to produce a mixture of ethanol, butanol and hexanol (Fernández-Naveira et al., 2017); however, *C. ljungdahlii* and *C. autoethanogenum* lack the butanol synthesis genes and hence, do not produce butanol using syngas (Guo et al., 2010; Köpke et al., 2010).

2.2.3.3 Low titer, yield and productivity

Forlow value high volume products, achieving a high titer is essential for commercial scale production of liquid biofuels. However ABE fermentation suffers from low product titer along with low product yield and productivity as key limitations. These limitations can be overcome by either strain or process improvement. Before metabolic flux analysis and metabolic engineering tools were available, most studies were focused mainly on improvement by optimizing various fermentation parameters such as medium composition, feeding control, pH control

and cell density (Moon et al., 2016). Various process strategies have been explored in the past and recent studies are also focused on achieving desired improvements in product tier, yield and productivity.

Batch, fed-batch and continuous mode of fermentation

Modes of fermentation can be classified based on the following substrate feeding conditions: batch, fed-batch and continuous. In batch fermentation, inoculum andmedia components are filled into the reactorto initiate the reaction with process parameters being monitored over time. The processis is terminated with the cessation of metabolic activity, depletingsubstrate levels and maximum product formation. However during batch fermentation, *Clostridium* spp. suffers from solvent toxicity which results in termination of the reaction (Moon et al., 2016). One approach to improve solvent titer is to integrate the process with a product recovery strategy. However, coupling the process with product recovery causes substrate limitation and restricts the production time. To overcome this problem, changing the fermentation feed mode can assist with increasing the product yield. Fed batch fermentation is used when high initial substrate levels can inhibit microbial growthand hence cause substrate inhibition. Media components fed at regular intervals and coupled with product recovery leads to considerably improvement in solvent titer, yield and productivity. In continuous fermentation, the media components' feeding is accompanied by continuous product removal such that the volume of the fermentation remains constant. This mode of fermentation is able to aid in reducing the lag phase. However,batch and fed batch conditions; however suffers from limitations such as cell wash out, cell degeneration, high water requirement and waste water generation (Ezeji et al., 2007; Gottumukkala et al., 2017; Ezeji et al., 2005).

Co-cultivation and multistage fermentation strategy

Co-culture fermentations are used when the characteristics of two different organisms are explored in a single fermentation process. For instance, when a product forming organism lacks the enzymes to metabolize a specific substrate, this organism can be co-cultivated with another organism capable of utilizing the substrate. Nakayama et al. (2011) reported the co-cultivation of cellulose-utilizing *C. thermocellum* strain together with a butanol-producing *C. saccharoperbutylacetonicum* strain using cellulose was able to yield 5.8 g L^{-1} of butanol. Abd-Alla and El-Enany (2012) demonstrated that anaerobic environment can be achieved during fermentation by co-cultivation of *Bacillus subtilis* and *C. acetobutylicum*. Further Li et al. (2013) explored the characteristics of two *Clostridium* spp. in a single fed batch fermentation reactor. These organisms including *C. tyrobutyricum* produced butyric acid while *C. beijerinckii* converted butyric acid into butanol with a yield reaching 12.8 g L^{-1}. Synergism between *C. acetobutylicum* and *Saccharomyces cerevisiae* demonstrated improved growth and production. Luo et al. (2015) attributed to better amino acid utilization, increased substrate utilization and improved energy cofactors generation. Since *Clostridium* is known to undergo two distinct metabolic stages, use of multistage fermentation is a rational approach. Mutschlechner et al. (2000) conducted two-stage continuous fermentation where in the first stage *C. beijerinckii* was grown acidogenically at rapid rate and then transferred to a second stage vessel where the conditions simulating solventogenesis were established. This strategy which resulted in overall solvent productivity of 0.27 g L^{-1} h^{-1} over 1600 h of operation depicts the stability of organism over long fermentation times. Similarly, Ramey. (1998) employed two stage fermentation strategy utilizing two different strains of *Clostridium* spp. First the

Clostridium strain was cultivated for maximum acid production and then this media was transferred to a second reactor containing media for solventogenic growth in the second phase using *Clostridium* spp. The second strain was uniquely characterized by the fast conversion of acids to solvents coupled with the carbon source not converted to ancillary products.

Immobilization and cell recycling

Immobilized culture systems have several advantages (Jones and Woods., 1986) over process utilizing suspended cells. These advantages include (1) cells remain separated from the product which assisted in easy product separation, (2) high cell concentration maintained during fermentation, (3) reduced loss due to cell washout, (4) no product inhibition and cells viability is maintained, (5) higher productivity and (6) compatibility with different reactor configurations. Some demerits which restrict using this strategy are mass transfer limitations, reduced cell activity due to immobilization, cells leakage and owing to the gas formation during fermentation, interactions between the cells and the substrate are restricted (Kourkoutas et al., 2004; Bickerstaff, 1997). Several immobilization supports have been explored in the literature such as κ-carrageenan, calcium alginate beads, PVA, tygon rings, fibrous matrix, chitosan, beechwood shaving, coke, bonechar, sponge, clay brick, ceramic beads, corn stalks, wood pulp, corn stover, sugarcane bagasse etc. (Moon et al., 2016; Sarchami et al., 2016; Gottumukkala et al., 2017). An ABE productivity of 15.8 g $L^{-1}h^{-1}$ was detected using *C. beijerinckii* BA101 attached to clay bricks in a packed bed reactor operating under continuous flow conditions (Qureshi et al. 2000). In a work conducted with wood pulp toimmobilize *C. acetobutylicum* DSM 792, an ABE productivity of 13.7 g L^{-1} h^{-1} was reported at a dilution rate of 1.9 h^{-1} (Survase et al., 2012). Similarly, ABE productivity of 11.3

g L^{-1} h^{-1} was attained using chemically modified sugarcane bagasse to support the immobilization of *C. acetobutylicum* (Kong et al. 2015).

Cell recycling is another method employed toachieve high cell concentration. In this method the cells are concentrated by filtering the fermentation broth through a porous membrane. The filtered cells are recycled back to the reactor while solvents are recovered from the permeate by suitable methods. Using this strategy various researchers (Malaviya et al., 2012; Chang et al., 2011; Zheng et al., 2013) have been successful in achieving high cell density resulting in increased solvent productivity. Malaviya et al. (2012) were able to achieve a butanol productivity of 7.8 g L^{-1} h^{-1} during glycerol fermentation by *C. pasteurianum* mutant compared to 0.43 g L^{-1} h^{-1} which wasobserved when the same fermentation was performed without cell recycling. Similarly Zheng et al. (2013) achieved double butanol productivity of 1.22 ± 0.26 g L^{-1} h^{-1} compared to 0.529±0.206 g L^{-1} h^{-1} for suspended cells during glucose-xylose continuous fermentation.

Multi-substrate fermentation

Since the overall cost of the fermentation process is highly dependent on the substrate cost, an important consideration in developingindustrial strains are their ablityto utilize a broad range of carbon sources from low-value sources. For instance, lignocellulosic biomass is a complex polymer made of varying composition of three other polymers cellulose, hemi-cellulose and lignin. Hydrolysis of cellulose and hemicellulose polymers in lignocellulosics results in a mixture of hexose and pentose sugars (Zaldivar et al., 2001). Hence efficient utilization both hexose and pentose sugar by clostridial strains is important for obtaining high yields (Lee et al., 2016). However, most of the clostridial strains suffer from carbon catabolite repression (CCR) and they are unable to utilize other sugars in the presence of glucose. To

overcome this problem researchers have isolated novel strains capable of simultaneous fermentation of mixed sugars. Xin et al. (2014) reported a wild type *Clostridium* sp. strain BOH3 which is capable of producing 11.7 g L^{-1} butanol by simultaneous utilization of glucose and xylose from a horticultural waste cellulosic hydrolysate. Also strains have been genetically modified to operate with mixed sugar feedstocks. Transaldolase enzyme which catalyzes the conversion of glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate into erythrose 4-phosphate and fructose 6-phosphate in the pentose phosphate pathway was overexpressed in *C. acetobutylicum* (Gu et al., 2009). These researchers reported improved xylose utilization in a sugar mixture. Further, Ren et al. (2010) silenced the CcpA gene which is responsible for CCR in *C. acetobutylicum* and this resulted in similar consumption of both glucose (33 g L^{-1}) and xylose (30 g L^{-1}). Another potential avenue is the co-utilization of lignocellulosic biomass and crude glycerol in single fermentation system. Sabra et al. (2014) demonstrated that *C. pasteurianum* DSMZ 525 was capable of utilizing glucose and glycerol simultaneously with enhanced growth and acid production. Mixed substrate fermentation using biomass hydrolysate and glycerol resulted in 17 g L^{-1} butanol with a maximum productivity rate of 1.1 g L^{-1} h^{-1}. In another study, dual substrate process resulted in enhanced butanol production of 17.75 g L^{-1} when glucose was fed during glycerol fermentation (Yadav et al., 2014). This study used a novel strain *C. acetobutylicum* KF158795 which is only strain of *C. acetobutylicum* known to utilize glycerol as sole carbon source. Kao et al. (2013) investigated the sequential or simultaneous addition of glucose and glycerol and observed glycerol was the more efficientcarbon source forbutanol production. The substrates were replaced with cheaper sources such as sugarcane bagasse hydrolysate and crude glycerol and a *C. pasteurianum* CH4 culture produced 11.8 g

L^{-1} butanol at a productivity of 0.14 g L^{-1} h^{-1} which was greater than cultures fed with onlyglycerol. Ahn et al. (2011) reported the co-utilization of lactic acid and glycerol in thin stillage (byproduct of ethanol industry) by *C. pasteurianum* DSM 525. These researchers reported lactic acid present in the thin stillage was usefull as a buffering agent by the organism. A yield of 0.32–0.44 g butanol per g glycerol consumedwas higher than previously reported yields of 0.14–0.31 g butanol per g glycerol (Biebl., 2001).

2.2.3.4 Low biofuel selectivity

Another key limitation that ABE fermentation suffers from is the low butanol selectivity in the final product mixture. Acetone, which comprises 30% of the product mix, does not have biofuel value such as butanol and ethanol. Hence, in order to improve the biofuel selectivity, researchers have isolated novel non-acetone producing strains. Natural non-acetone forming butanol producers reported include *C. sporogenes* BE01 (Gottumukkala et al., 2013), *C. pasteurinum* CH4 (Kao et al., 2013), *C. pasteurinum* DSM 525 (Sabra et al., 2014; Ahn et al., 2011), *C. acetobutylicum* KF158795 (Yadav et al., 2014) and *C. tetanomorphum* DSM 665 (Gong et al., 2016). Further studies have also been performed on knocking down the acetone forming pathway in known clostridial strains. Jiang et al. (2009) disrupted the acetoacetate decarboxylase genes in *C. acetobutylicum*; however, these researchers could not achieve much success since acetone formation is necessary for cytosolic detoxification from a proton gradient caused by carboxylic acids. In an alternate approach acetone was converted into iso-propanol which is a fuel additive by genetically engineering the strain with the iso-propanol forming pathway resulted in a 20.4 g L^{-1} mixture consisting of isopropanol, butanol and ethanol (Lee et al., 2012). In another study,an asporogenous strain of *C. acetobutylicum* M5 was altered using the

adhE1gene (Sillers et al., 2008) and the adhE1-ctfAB operon (Lee et al., 2009) while *C. acetobutylicum* DG1 was engineered by overexpressing adhE2 gene (Fontaine et al., 2002). These strains showed higher butanol selectivity and produced butanol and ethanol fuel mixture without acetone. However, the recombinant strains developed suffered from low solvent production and hence, they were attractive by improving alcohol forming ability. Further, incorporation of artificial electron carriers (methyl viologen or neutral red) in the fermentation medium have been reported to successfully reinforce the carbon flux towards butanol production, although their use is restricted due to high cost (Du et al., 2015).

2.2.3.5 Solvent toxicity

Distillation is used for product purification; however, owing to the low alcohol titers this process is energy inefficient (Gottumukkala et al., 2016). The key reason for the low product titer obtained with using clostridial strains is solvent toxicity.Butanol is more toxic to microbial growth when compared to ethanol or acetone. Most wild type *Clostridium* strains have butanol tolerance less than 13 g L^{-1} and hence, they fail to produce titers above this threshold limit. One solution to this limitation is the isolation of solvent tolerant strains or chemically mutating and/or adapting current strains to high butanol concentrations (Gottumukkala et al., 2016). However, the threshold limit of tolerance can only be increased up to a certain limit. Another possible solution which has proved to be highly efficient in recent years is the integration of fermentation process with a product recovery strategy which is aimed at product removal when the toxic limit is reached (Ezeji et al., 2007; Xue et al., 2014b; Lee et al., 2016; Gottumukkala et al., 2016). Liquid-liquid extraction and adsorption techniques require selectingan extractant or adsorbent respectively for product removal which is not harmful to the cells and does not remove media nutrients (Ezeji

et al., 2007; Xue et al., 2014b). Further, in the case of adsorption, a highly efficient desorption process has to be developed to achieve high butanol titer recovery. Pervaporation is a promising technique which involves product recovery on a hydrophobic organic or inorganic membrane where the product diffuses and is desorbed by vaporization and finally condensed to achieve the concentrated product (Xue et al., 2014b). This process is advantageous because it has no harmful effects on the culture and there is no uptake of media nutrients (Lee et al., 2016); however, the process suffers from bottlenecks such as high membrane cost and membrane fouling (Gottumukkala et al., 2016). Gas stripping, another method for product recovery, involves using acarrier gas such as N_2 or CO_2 to strip solvents. This exhaust gas is condensed and recycled back to the fermenter. Gas stripping has several advantages such as no harmful effect to the cells, ease of operation, low operational cost, and no loss of any media components from the fermentation broth (Ezeji et al., 2005; Xue et al., 2014a). Reduced efficiency of stripping for low solvent containing culture broth is a key disadvantage. Coupling recovery strategieswith the fermentation process will not only assist in mitigating solvent toxicity but also aid in increasing the solvent titer, solvent productivity and concentrating the solvent mix for further distillation.

2.2.3.6 Understanding of regulatory mechanism still in nascent stage

Clostridial ABE fermentation is characterized by a remarkable shift in metabolic machinery and cellular organization from the acidogenic to solventogenic phase. To understand possible complex regulations that determine the transition between these two distinct metabolic states, mathematical models are employed for generating of testable hypotheses. The crosslinks between different levels of regulation in cells is not completely understood. Even for *C. acetobutylicum* which is the most studied *Clostridium* sp. contradictory experimental and theoretical results

have createda void in our current understanding of the ABE metabolic network structure. While for others *Clostridium* spp. research is still at a nascent stage. Thus, for overcoming current gaps in in theoretical as well as experimental knowledge we need a collaborative approach between the modelers and experimentalists.

The first clostridial model developed by Papoutsakis. (1984) was focused on fermentation equations for butyric acid bacteria. This was followed by an extended stoichiometric model developed by Desai et al. (1999). The first complete genome sequence of *C. acetobutylicum* ATCC 824 published by Nolling et al. (2001), is an important resource for developing various *in silico* genome-scale metabolic models and in turn for designing various metabolic engineering strategies (Lee et al., 2008; Senger and Papoutsakis, 2008). In 2008, two different research groups published the first two genome-scale metabolic models for *C. acetobutylicum* (Lee et al., 2008; Senger and Papoutsakis, 2008). Senger and Papoutsakis (2008) developed a genome-scale metabolic network using 422 intracellular metabolites, 552 reactions and 80 membrane transport reactions while Lee et al. (2008) employed 502 reactions and 479 metabolites. Both groups provided different explanations for using an incomplete TCA cycle. Senger and Papoutsakis (2008) tried to resolve by incorporation of urea cycle whereas Lee et al. (2008) suggested an active reductive pathway between pyruvate and α-ketoglutarate. However, within the next 2 years, researchers have been focused on resolving these discrepancies in the current/prevailing notion of the clostridial TCA cycle (Amador-Noguez et al., 2010; Crown et al., 2011). Amador-Noguez et al. (2010) employed ^{13}C-labeling, mass spectrometry coupled with quantitative flux modeling and proposed a complete but bifurcated TCA existed in *C. acetobutylicum*. These researchers suggested that the oxidative and reductive branch were disconnected thus resulting in succinate excretion. Similar conclusions regarding

a broken TCA cycle were put forwardby Crown et al. (2011). They proposed a putative *Re*-citrate synthase (CS) was active in the conversion of citrate to α-ketoglutarate with no carbon exchange between α-ketoglutarate and fumarate. Using the work reported by Senger and Papoutsakis (2008), Crown et al. (2011) and Amador-Noguez et al. (2010), another extended model for *C. acetobutylicum* with a complete TCA cycle was developed by McAnulty et al. (2012).

Later in 2014 contrary to previous two studies (Amador-Noguez et al., 2010; Crown et al., 2011), Au et al. (2014) proposed an incomplete TCA cycle operating in the oxidative direction, with no prominent flux between α-ketoglutarate and succinyl-CoA or succinate and fumarate. Dash et al. (2014) developed a second-generation genome-scale model of *C. acetobutylicum* using work reported by Au et al. (2014). This model contained all the relevant genes involved in fatty acid, purine, pyrimidine and cobalamin synthesis along various thermodynamically unfavourable reactions resulting in a network 3 fold in size when compared to earlier models (Senger and Papoutsakis., 2008; Lee et al., 2008). Finally in a recent study an even larger model with 20% more genes than the model reported by Dash et al. (2014) was developed by Yoo et al. (2015). The Yoo et al. (2015) model made a combined use of transcriptomic, proteomic and fluxomic data. However, since the CoA transferase gene which is responsible for converting of succinyl-CoA to succinate has not been identified in the *C. acetobutylicum* genome, key questions related to formation of succinyl-CoA and succinate and their interconverion still remain unanswered.

Over the years genome scale metabolic model have been developed *Clostridium* spp., *C. cellulolyticum* (Salimi et al., 2010), *C. thermocellum* (Roberts et al., 2010), *C. beijerinckii* (Milne et al., 2011), *C. ljungdahlii* (Nagarajan et al., 2013) and *C. butyricum* (Bermudez et al., 2017). The genome for other potential *Clostridium*

spp. which can be useful for biofuel production include *C. saccharobutylicum* (Poehlein et al., 2013), *C. saccharoperbutylacetonicum* (Poehlein et al., 2014), *C. pasteurianum* (Poehlein et al., 2015b), *C. sporogenes* (Poehlein et al., 2015a) and *C. tetanomorphum* (Gong et al., 2016). These organisms have been sequenced; however, genome scale models are yet to be developed. Additionally, a study involving re-sequencing of *C. acetobutylicum* ATCC 824 (Ehsaan et al., 2016) have found certain discrepancies in the earlier sequencing results (Nölling et al., 2001). However, the impact of these differences on reconstruction metabolic network for *C. acetobutylicum* remains unexplored.

Along with GSM, alternate network analysis methods such as elementary mode analysis (EMA) and flux balance analysis (FBA) have also been developed to analyzing small scale metabolic network and designing strains (Millat et al., 2017). These mathematical models have been used by various researchers for simulating fermentation process under altered environmental conditions; aiding in elucidating and predicting cellular phenotypes and their interactions with metabolism (Kumar et al., 2014; Sharma et al., 2017; Gallardo et al., 2016; Li et al., 2014). Since *Clostridium* is able to metabolize a broad spectrum of carbon substrates and survive in different environmental conditions, these models will provide biological hypotheses for versatile phenotypes when limited experimental evidence is available. Kumar et al. (2014) used elementary mode analysis as a tool to quantitate metabolic fluxes in *C. acetobutylicum* under unstressed and stressed conditions (e.g. a sudden change of external pH and addition of exogenous acids). Similarly the FBA method was used by Sharma et al. (2017) to study the effect of ultrasound on biohydrogen production by *C. pasteurianum* during glycerol fermentation while Gallardo et al. (2016) used it to analyze the *C. acetobutylicum* response to external electron supply. Further in another

study, metabolic flux analysis was used to elaborate on the impact of higher butanol to acetone ratios was when feeding a cassava or corn-based substrate for *in-situ* extractive fermentation by *C. acetobutylicum* (Li et al., 2014).

2.3 Overview

Over the past 100 years, significant progress in the field of biofuel production using *Clostridium* spp has been observed. Though the maximum titer and productivity achieved is still restricted to 25 g L^{-1} (Lepiz-Aguilar et al., 2013) in batch mode fermentation and a productivity of 10.7 g L^{-1} h^{-1} (Jang et al., 2013) in continuous mode. Many gaps are yet to be filled towards completely understanding *Clostridium* metabolism including the TCA cycle. Many new species are still at a nascent research stage in terms of bioprocess development, system biology studies and metabolic engineering. However, persistent efforts towards developing sustainable biofuel production processes which will aid in alleviating the environmental risks and social repercussions associated with replacing fossil fuels. Sustainable biofuel production would require a combination of different approaches that includes (1) identifying potential biofuel cell factories with favorable characteristics such as a broad range of substrate utilization, high tolerance, high biofuel selectivity and production etc., (2) selecting of suitable cost effective substrates such as lignocellulosic biomass, crude glycerol or syngas and developing methods for maximum sugar hydrolysis or undertaking inhibitor studies for removing toxic substrates, (3) optimization of fermentation media and process parameters for the selected cell factories and (4) developing cost effective and efficient solvent removal strategies coupled with the fed-batch or continuous biofuel production processes. Additionally, using a systems biology approach to understand regulations at different cellular levels as a response to varied metabolic stages or environmental conditions will aid in the rational

engineering of clostridial strains and fermentation processes for maximum biofuels production.

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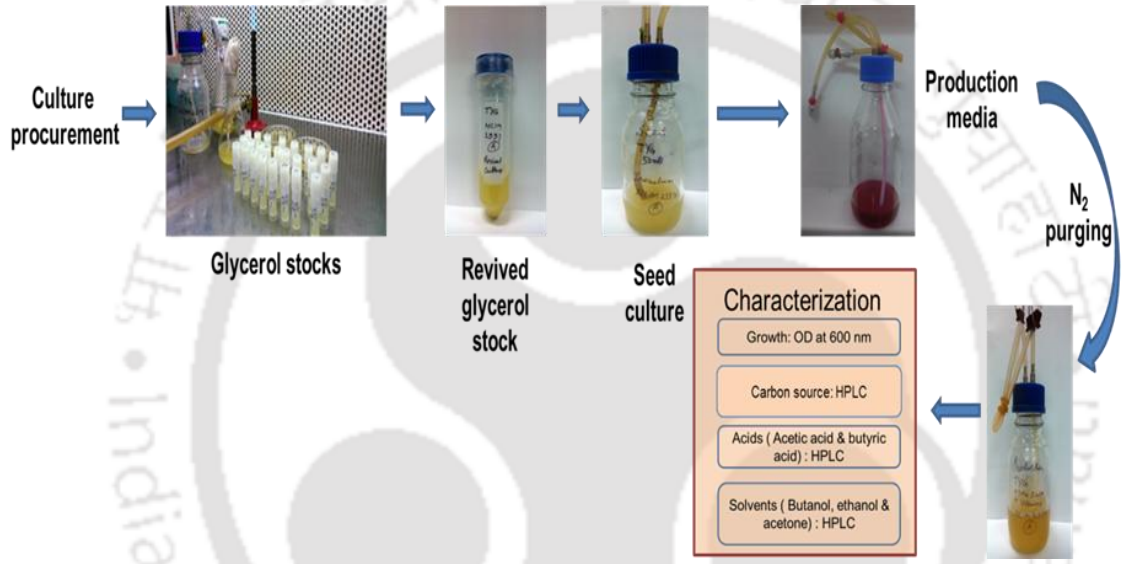
CHAPTER 3

Screening and characterization of potential butanol producing *Clostridium* sp. strains.



Culture procurement and screening of clostridial strains for high biofuel production followed by biochemical characterization

3.1 Background and motivation

Increasing demand and depletion of conventional fossil fuels has encouraged research activities to identify alternate renewable biofuels. Butanol has gained immense attention among the researchers because of several inherent properties and advantages (Kumar et al., 2011). Butanol incomparison to ethanol, has 25% higher energy content owing to a reduced structure, low volatility, less corrosiveness and can be used in the present engine models without any additional modifications (Zheng et al., 2015). With a gasoline-like octane rating, butanol can be blended with existing gasoline at much higher proportions than ethanol and has wide range of other

applications in the synthesis of different value added chemicals and biomolecules (Garcia et al., 2011; Yadav et al., 2014). Butanol production from sugars through anaerobic ABE (acetone, butanol, and ethanol) fermentation by *Clostridium* spp. has been thoroughly researched. However, several bottlenecks with using commercial scale fermentation process associated with high production cost, low productivity, solvent selectivity, end product has hindered the overall process development (Karimi et al., 2015).

Current research initiatives have targeted the development of suitable producer strains via strain engineering (Jiang et al., 2009; Zheng et al., 2015) and in-depth understanding of the regulatory mechanisms involved in solvent biosynthesis (Kim et al., 2015; Kumar et al., 2013). All these technological progressions have been put forth to identify and develop a suitable cell factory for butanol production with enhanced productivity, high butanol selectivity, wide range of substrate utilization capability and enhanced solvent tolerance (Green, 2011; Zheng et al., 2015; Gao et al., 2012). For instance, a glycerol utilizing *C. acetobutylicum* KF158795 was identified for butanol production among twenty different anaerobic bacteria screened by Yadav et al. (2014). Similarly a non-acetone producer, pentose utilizing *C. sporogenes* BE01 was isolated from contaminated cooked meat medium which showed maximum butanol titer of 5.52 g L^{-1} in rice straw hydrolysate (Gottumukkala et al., 2013). Green Biologics Ltd. (GBL) has developed a large collection of strains which were screened on the basis of their solvent production capacity and ability to utilize specific substrate (Green, 2011). Thus, it is imperative that novel producer strain with capability of sustainable butanol production have to be identified and developed.

Further, *Clostridium* belongs to the heterogeneous group of rod shaped, gram positive and spore forming anaerobes (Riley, 2012; Jones and Woods, 1986). This genus is polyphyletic in nature lacking phenotypically coherent characteristics such as variation in proteolytic properties, spore forming capability, variable gram staining, anaerobic growth conditions and diverse substrate utilization (Riley, 2012; Jones and Keis, 2005). These discrepancies in the phenotype based categorization have been a source of misclassification of its representatives (Sedlar et al., 2017; Lawson et al., 2016; Rainey and Lawson, 2016; Moon et al., 2008). In particular, for strains with such ambiguous properties, a holistic characterization approach needs to be undertaken. This uniform and precise nomenclature will assist in developing strain specific techniques for future therapeutic and industrial applications. To this end, six different strains were screened for butanol production in the present study. For the most potential strain, taxonomic dissection was performed on the basis of phenotypic characterization through biochemical analysis, followed by 16S rRNA gene sequencing and phylogenetic analysis.

3.2 Materials and methods

3.2.1 Organism, growth and maintenance conditions

Four strains of *Clostridium acetobutylicum* (NCIM 2337, 2841, 2877 and 2918) and two *Clostridium beijerinckii* strains (NRRL B-593 and B-592) were procured from National Collection of Industrial Microorganisms (NCIM), India and Northern Regional Research Laboratory (NRRL), in the United States of America (USA) respectively, and stored as glycerol stocks at -80°C (Table 3.1). The glycerol stocks were revived in test tubes containing 15 mL TYG media which comprises (in g L^{-1}) tryptone 30, glucose 20, yeast extract 10, cysteine hydrochloride 0.05 (Annous and Blaschek, 1990) and incubated at 37°C in a static incubator under anaerobic

condition. The anaerobic condition was maintained by intermittent purging of oxygen free nitrogen (99.99%, Assam Air products, Guwahati, India) at regular time intervals while the anaerobicity was confirmed by addition of a redox indicator dye resazurin at 1 g L^{-1}. After 24 h of growth, 10 mL of the revived culture was transferred to 100 mL TYG media in 500 mL air tight glass bottles and incubated under same conditions for the development of seed culture. The seed culture was grown to obtain biomass equivalent of absorbance 3.0 at 600 nm and 10% (v/v) was used as inoculum for the production media in all experiments unless otherwise mentioned. All the chemicals were purchased from Himedia, India unless otherwise mentioned.

Table 3.1 List of strains procured for screening of best butanol producer strain

S.No.	Strain name	Strain No.	Source
1.	*C. acetobutylicum*	NCIM 2337	NCL, Pune
2.	*C. acetobutylicum*	NCIM 2841	NCL, Pune
3.	*C. acetobutylicum*	NCIM 2877	NCL, Pune
4.	*C. acetobutylicum*	NCIM 2918	NCL, Pune
5.	*C. beijerinckii*	B-593	NRRL, USA
6.	*C. beijerinckii*	B-592	NRRL, USA

3.2.2 Screening and selection of potential butanol producing strain

Bacterial strains have different nutritional requirements and also the production potential of a strain is specific to particular medium composition (Ezeji et al., 2005). Therefore the above mentioned six strains (Section 3.2.1) were screened for their butanol biosynthesis potential in two different production media compositions: (i) P2 medium and (ii) P2TY medium. The reason for selection of the first media, was its widespread use for cultivation of *clostridium* spp. especially for solvent production. This media was first described by Monot et al. (1982) where he optimized the concentration of all the media components including the trace elements

for maximum solvent production. P2 medium comprises (in g L^{-1}) glucose 50.0, K_2HPO_4 0.50, KH_2PO_4 0.50, $MgSO_4.7H_2O$ 0.20, $MnSO_4.H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, NaCl 0.01, CH_3COONH_4 3.22, para-amino-benzoic acid 0.01 and biotin 0.001 (Monot et al., 1982). However, when the six strains were cultivated on P2 media, most of the strains showed decreased growth compared to seed media. It was interesting to notice the key difference between TYG and P2TY was the absence of an organic nitrogen source. Thus, the second media, P2TY was prepared by supplementing the P2 medium with tryptone 30.0 g L^{-1} and yeast extract 10.0 g L^{-1} (Annous and Blaschek, 1990). The fermentation batch was carried out for 120 h at 37°C in static condition and samples were drawn every 6 h for analysis of biomass growth, pH change, substrate utilization and product formation. The media and the strain exhibiting highest butanol titer were selected for further characterization.

3.2.3 Biochemical characterisation of potential butanol producing clostridial strain

The selected strain was phenotypically characterized in presence of twenty four different carbon sources which included three pentoses (xylose, ribose and arabinose), seven hexoses (dextrose, galactose, fructose, sodium gluconate, glucuronic acid, citrate and mannose), four disaccharides (lactose, sucrose, cellobiose and maltose), five sugar alcohols (sorbitol, mannitol, xylitol, myo-inositol and glycerol) and five polysaccharides (starch, cellulose, pectin, xylan and dextrin) in 500 mL glass bottles. The growth of the organism was characterized in TYG media with glucose being replaced with respective carbon sources such that an equimolar concentration (0.6 M) of carbon was maintained. The revived cultures were centrifuged at 5,000 × g for 10 min at 4°C and pellet was re-suspended in the media containing different carbon sources. This was conducted to avoid any residual glucose

from the revival media to be carried forward into test media. Samples were drawn for analysis of substrate utilization, growth and product formation (acid and solvent) for all the carbon sources while exhaust gas analysis was carried out for glucose fermentation. The exhaust of the glass bottle was connected to a 2.5 L tedlar bag (Sigma, Germany) for collection and analysis of the gaseous products of fermentation. Further, detailed characterization was conducted through biochemical tests including motility, catalase test, oxidase, Voges Prokauer, urease utilization, indole production, lipase, growth at different initial pH (5, 5.5, 6, 6.5 and 7), Nagler, gelatin hydrolysis, hydrogen sulfide production, milk coagulation, nitrate reduction, and lecithinase test according to the standard methods described in Bergey's Manual of Determinative Bacteria (Buchanan and Gibbons, 1974). For the purpose of comparison during biochemical characterization, biochemical analysis data for *C. acetobutylicum* MTCC 11274, equivalent to type strain *C. acetobutylicum* DSM 792, was taken from experiments conducted by a lab colleague. The data is only used for reference and is not part of this thesis.

3.2.4 Identification of the producer strain

The cells of the selected strain were disrupted and the genomic DNA was isolated using the GeneJET™ Genomic DNA Purification Kit (Fermentas K0721, EU). The purified genomic DNA was used as the template to amplify the 16S rDNA sequence using bacterial universal primers (27f and 1492r). The amplified sequence was further separated using gel electrophoresis and gel elution kit (Sigma, USA). Sequencing was performed using ABI PRISM 3700 DNA sequencer (Applied Biosystems, USA). Similarity to sequence was determined using BLAST-NCBI. The homologous genes having sequence similarity not less than 90% to the 16S rRNA sequence of NCIM 2918, were retrieved and used for phylogenetic analysis.

Construction of phylogenetic tree was carried out using MEGA 6 software, commonly used for statistical analysis of molecular evolution (Larkin et al., 2007). ClustalW, a progressive algorithm present in MEGA 6, was used for multiple sequence alignment (Chaichoompu et al., 2006). ClustalW algorithm consists of 3 stages: In stage 1, pairwise alignment of all the sequences is carried out in order to construct a distance matrix based on the percentage of mismatches of each pair of sequences. In stage 2, the guide tree is constructed based on the distance matrix using a neighbor joining algorithm (Saitou and Nei, 1987). The principle of this method is to find pairs of operational taxonomic units (OTUs = leaves of the tree) or neighbours that minimize the total branch length of the tree. Neighbours (N) are defined as a pair of OTUs connected through a single interior node. Among these possible pairs of OTUs, we choose the one that gives the smallest sum of branch lengths. This pair of OTUs is then regarded as a single OTU, and the next pair of OTUs that gives the smallest sum of branch lengths is again chosen. This procedure is continued until all N - 3 interior branches are found. In stage 3, the sequences are progressively aligned according to the guide tree. Once the tree was constructed, the evolutionary distances (in number of base substitutions per site) were calculated using Maximum Composite Likelihood method. All abstruse positions were removed and a total of 636 positions were utilized in the final set. To estimate the reliability of the phylogenetic tree bootstrap method was used. No. of Bootstrap replicates were set to an integer between 100 and 2,000. Bootstrap analyses (1,000 replicates in %) is shown next to the branches and the taxon name starts with the gene accession number. Generally a bootstrap value of more than 70% is considered as significant.

3.2.5 Analysis of growth, substrate utilization and formation of acids & solvents

A known volume of sample was collected and centrifuged at 10,000 × *g* for 10 min at 4 ºC to obtain pellet and supernatant. While the pellet was used for optical density measurement, the supernatant was utilized to obtain pH, substrate utilization profile and dynamic changes in organic acid (acetate, butyrate) and solvent (acetone, ethanol and butanol) concentration. The pellet was washed twice and re-suspended in saline (0.85% w/w, NaCl) to measure growth in terms of optical density at 600 nm using UV-visible spectrophotometer (Cary 50, Varian, Australia). The composition of the exhaust gas was determined by gas chromatography (GC Dhruva, Chromatography and Instruments Company, Gujarat, India) equipped with a thermal conductivity detector (TCD) and molecular sieve 5A packed column. Nitrogen gas was used as the carrier gas at a flow rate of 30 mL min^{-1}. Column, detector and injector all were kept at 30°C. The substrates, organic acids and solvents were measured in high performance liquid chromatograph (HPLC, LC-20AD, Shimadzu, Japan) equipped with a Rezex ROA column (300 × 7.8 mm, Phenomenex, USA). The minimum detection limits for substrates, acids and solvents were 0.1g L^{-1}, 0.04 g L^{-1} and 0.06 g L^{-1} respectively. Organic acids were quantified in ultra-violet (UV) detector at 210 nm whereas substrates and solvents were quantified in refractive index detector (RID) at 37°C. The mobile phase 0.005 N H_2SO_4 (98%, Merck, Germany) was used at a flow rate of 0.5 mL min^{-1} and the column oven temperature was kept at 28 °C. Samples were filtered with 0.2 µm filters and 10 µL of sample was used for analysis. Correlation standard curves were

obtained (Appendix). All the experiments were conducted in triplicates and the values are measured as mean ± standard error.

3.3 Results and discussion

3.3.1 Screening and selection of potential butanol producer strain

Clostridium strains procured from different culture repositories were screened for butanol production in two different media compositions reported in the literatures (Monot et al., 1982; Annous and Blaschek, 1990). All the six strains showed butanol production in P2TY media with the maximum butanol titer of 3.72 ± 0.11 g L^{-1} obtained from NCIM 2918 followed by NCIM 2337 (Fig. 3.1). To the best of our knowledge, there is no reporting of butanol production from the strain NCIM 2918 in the literature. The other four strains showed a butanol production in the range of 1 g L^{-1} to 2 g L^{-1} when using the P2TY media (Fig. 3.1). The P2 media failed to induce butanol in all of the strains except NCIM 2918 and B-593 while only a negligible amount was observed in NCIM 2337. This observation supports the fact that presence of organic nitrogen sources is a necessary nutritional requirement governing the growth of such fastidious organisms (Welsh et al., 1987). Therefore, P2TY media and the strain NCIM 2918, exhibiting highest butanol titer, were selected for further experiments.

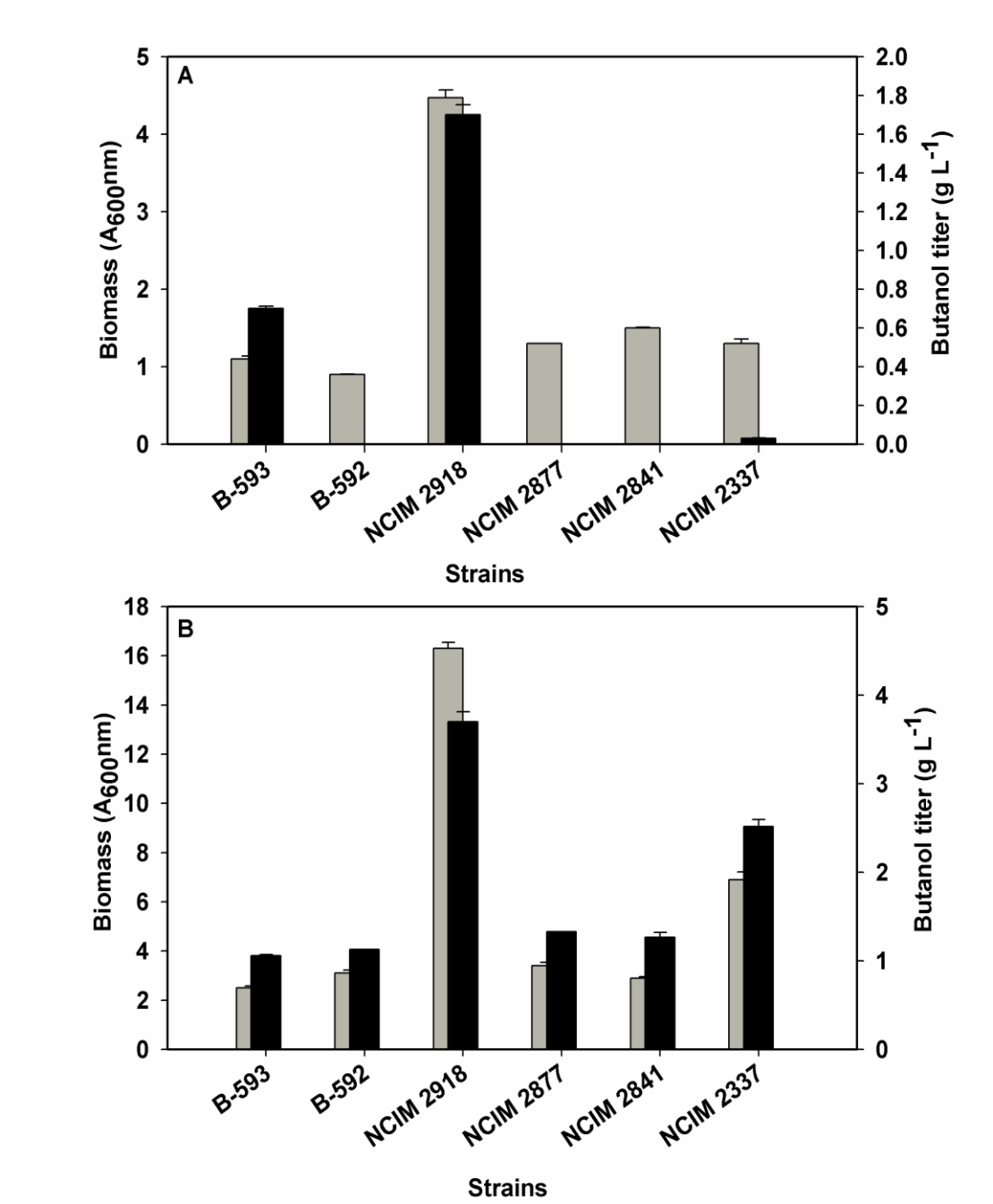


Fig. 3.1 Screening of six *Clostridium* strains for growth (grey bar) and butanol (black bar) production in (A) P2 and (B) P2TY medium

3.3.2 Biochemical analysis, 16S rDNA sequencing and phylogenetic analysis

The absence of acetone production during the screening of NCIM 2918 led the need for its identification using detailed biochemical analysis, 16S rDNA sequencing and phylogenetic analysis. Detailed characterization of NCIM 2918 and its

comparison with *C. acetobutylicum* MTCC 11274 revealed several differences in the phenotypic traits (Table 3.2).

Key difference was the lack of acetone formation in NCIM 2918 which is a major product in ABE fermentation by *C. acetobutylicum.* Positive test for lipase, gelatin liquefaction and H_2S production indicated towards the dissimilarities in phenotypic behavior between NCIM 2918 and *C. acetobutylicum* MTCC 11274 (Table 3.2). Further the strain NCIM 2918 was capable of performing Stickland fermentation involving coupled oxidation and reduction of amino acids to organic acids unlike *C. acetobutylicum* MTCC 11274 which has an obligatory requirement of carbohydrates for growth (Table 1). NCIM 2918 was unable to utilize mannitol and starch, but it could ferment glycerol and sorbitol as sole carbon sources (Table 3.2). On the contrary, *C. acetobutylicum* MTCC 11274 exhibited a completely opposite utilization pattern (Table 3.2). The inability to utilize mannitol and starch by NCIM 2918 was found to be another key phenotypic trait distinguishing the strain from *C. acetobutylicum* strains which are reported to utilize these substrates (Keis et al., 2001). A positive lipase activity which is reported to be a characteristic feature of *C. sporogenes* and *C. botulinum* distinguishes them from other *Clostridium* species (Cowan and Steel, 1993).

Table 3.2 Characteristics of strain NCIM 2918 and *C. acetobutylicum* MTCC 11274

Phenotypic trait	Strain NCIM 2918	Strain MTCC 11274[c]
Cell morphology	Rods	Rods
Spore induction	+	+
Motility	+	+
Oxidase test	-	-
Catalase test	-	-
Urease production	-	-
Indole production	-	-
Voges Proskauer test	-	-
Lipase production	+	-
Milk Coagulation test	Curd	Curd
Proteolytic[a]	+	-
Nitrate reduction test	-	-
Nagler test (lecithinase)	-	-
Hydrogen production	+	+
H_2S production	+	-
Gelatin liquefaction	+	-
Temperature	37	37
pH range	5.5-7	5.0 -7
Fermentation end products[b]	B,E, Aacid & Bacid	A,B,E, Aacid & Bacid
Utilization of different carbon sources		
Dextrose	+	+
Galactose	+	+
Fructose	+	+
Sodium gluconate	+	+
Mannose	-	+
Lactose	+	+
Sucrose	+	+
Maltose	+	+
Xylose	+	+
Ribose	-	-
Arabinose	-	+
Starch	-	+
CMC	-	-
Dextrin	-	+
Sorbitol	+	-
Mannitol	-	+
Glycerol	+	-
Cellobiose	-	+
Xylitol	+	-
Xylan	+	+

Pectin	-	+
Inositol	+	-
Citrate	-	-
Glucuronic acid	+	-

+ represents positive; - represents negative; a-represents that the proteolytic test was conducted in a media devoid of any carbohydrates as carbon and energy source; b-represents the A-acetone, B-butanol, E-ethanol, Aacid-Acetic acid, Bacid-butyric acid; c- Biochemical analysis data for *C. acetobutylicum* MTCC 11274 was taken from experiments conducted by lab colleague for comparative purpose only

As NCIM 2918 gave positive lipase activity, it was speculated that the culture could either be *C. botulinum* or *C. sporogenes*. Test for lecithinase activity via egg yolk emulsion reaction is specifically used to differentiate between *C. botulinum* and *C. sporogenes* (Cowan and Steel, 1993). In this test, *C. botulinum* a positive reaction with lecithinase results into precipitation, whereas *C. sporogenes* is lecithinase negative (McClung and Toabe, 1947). In this study, NCIM 2918 gave a negative reaction with lecithinase depicting the strain to be possibly *C. sporogenes*. Lipase and lecithinase assay together with ability to ferment glycerol but not starch suggested that culture could be *C. sporogenes*. This is in support of the study by Princewill et al. (Princewill, 1978) where it has been reported that more than 90 % of *C. sporogenes* strains tested positive for glycerol utilization and negative for starch. The growth phase of NCIM 2918 fermentation was accompanied with gas formation up to 10.0 L L^{-1} media which extended up to 48 h with no gas accumulation during the late stationary phase. An analysis of gas composition showed hydrogen and carbon dioxide concentrations of 0.43 and 0.19 L L^{-1} respectively of the total gas produced (10 L). Gottumukkala et al. (2015) has also reported hydrogen production from another strain, *C. sporogenes* BE01. The strain was unable to grow at initial media pH 5 while growth was observed when the initial media pH 5.5 was used. Further, maximum growth was observed at pH 6 and 6.5 while further increase in pH

(pH 7) lead to decreased growth. Hence, it was concluded that the optimum pH for the growth of NCIM 2918 lies between 6-6.5.

Analysis of the 16S rRNA gene sequences showed that the strain NCIM 2918 shares only 93% similarity with *C. acetobutylicum* ATCC 824 (type strain) while similarity to *C. sporogenes* strain DSM 795, which serves as the type strain for this species, is as high as 99% (Table 3.3 and Fig. 3.2). The results identified the strain as *C. sporogenes* and further phylogenetic analysis confirmed the species to be *C. sporogenes* and not *C. acetobutylicum* (Table 3.3 and Fig. 3.2). The sequence was submitted to GenBank under the accession number KT441028. Whole genome sequencing of *C. sporogenes* DSM 795 has also revealed the absence of acetone producing genes CoA transferase and acetoacetate decarboxylase in their genome (Poehlein et al., 2015) which supports the present finding. The analyses demonstrate undisputable evidences that *C. acetobutylicum* NCIM 2918 was misclassified hence, we suggested reclassification as *C. sporogenes* NCIM 2918.

Table 3.3 Sequence similarity between the 16S rRNA genes of NCIM 2918 and representative species of the genus *Clostridium*

Species	Strain	Sequence similarity (%) to *C. acetobutylicum* NCIM 2918
C. sporogenes	DSM 795 (T)	99
C. sporogenes	McClung 2004	99
C. sporogenes	DSM 29422	99
C. botulinum	ATCC 25763(T)	99
C. botulinum	ATCC 3502	99
C. acetobutylicum	ATCC 824 (T)	93
C. acetobutylicum	DSM 1731	93
C. pasteurianum	DSM 525 (T)	93
C. pasteurianum	JCM 1408	93
C. butyricum	ATCC 19398 (T)	91
C. butyricum	JCM 1391	91
C. beijerinckii	DSM 791 (T)	92
C. beijerinckii	NCIMB 8052	92

C. saccharobutylicum	P262 (T)	92
C. saccharobutylicum	NCP195	92
C. saccharoperbutylacetonicum	N1-4 (T)	92
C. saccharoperbutylacetonicum	N1-504	92
C. cellulovorans	DSM 3052 (T)	90
C. tetanomorphum	DSM 4474 (T)	93
C. tyrobutyricum	ATCC 25755 (T)	92
C. ljungdahlii	ATCC 55383 (T)	91

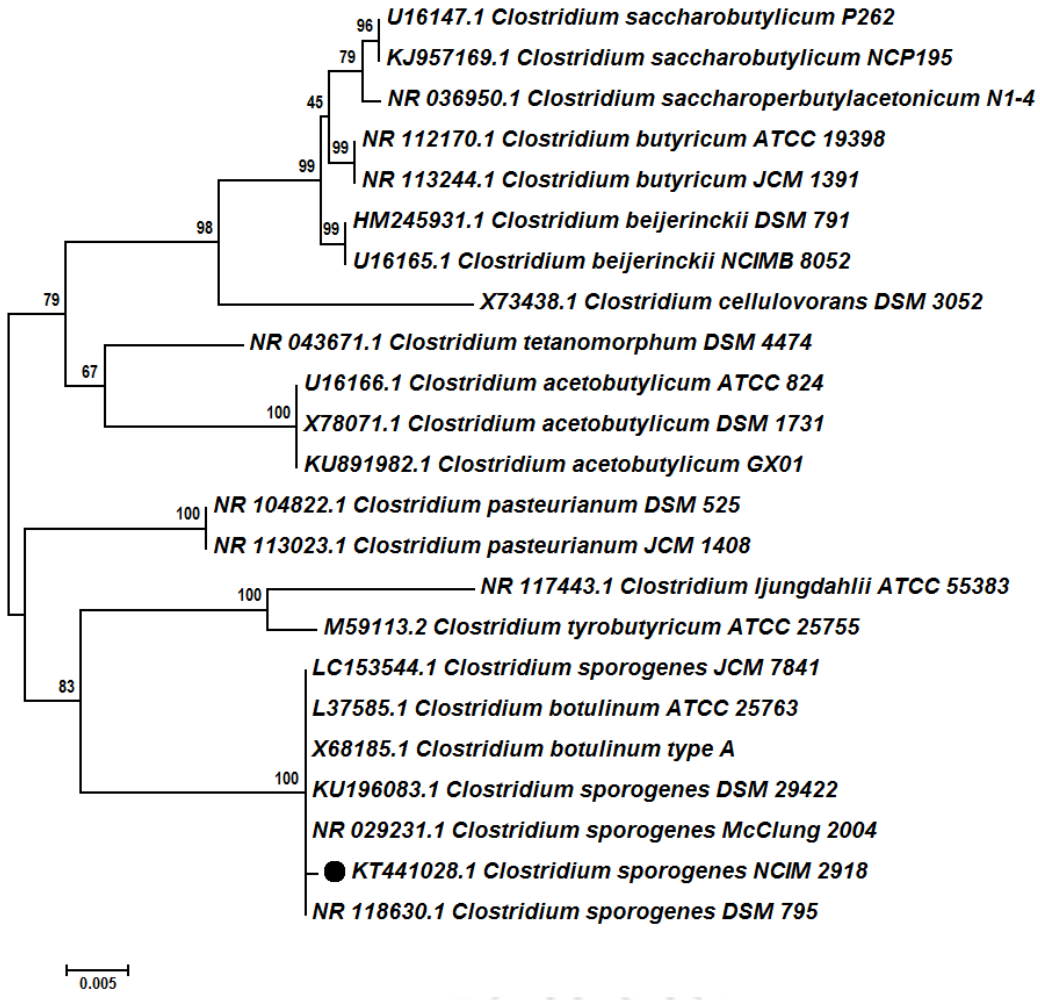


Fig. 3.2 Phylogenetic tree inferred using 16S rRNA sequence of *C. sporogenes* NCIM 2918 along with other *Clostridium* strains having sequence similarity not less than 90%. The strain reported in the present study is marked with a closed circle

Based on our results NCIM, India has changed the taxonomic status of the strain in their database. From here on the strain will be referred to as *C. sporogenes*

NCIM 2918. This reclassification lays a platform for reaching a genetic and molecular understanding of the strain for both academic and commercial purpose.

3.4 Conclusions

Six different clostridial strains were procured and screened for butanol production. NCIM 2918 showed highest butanol titer of 3.7 g L^{-1}. Thus, NCIM 2918 was chosen for further identification and characterization. Biochemical characterization, phylogenetic analysis and 16S rRNA gene sequence comparison of *Clostridium acetobutylicum* NCIM 2918 with other strains confirmed the inaccuracy in its taxonomic status and pointed towards reclassification as *C. sporogenes* NCIM 2918. This will assist in better understanding the strain as a cell factory for industrial applications.

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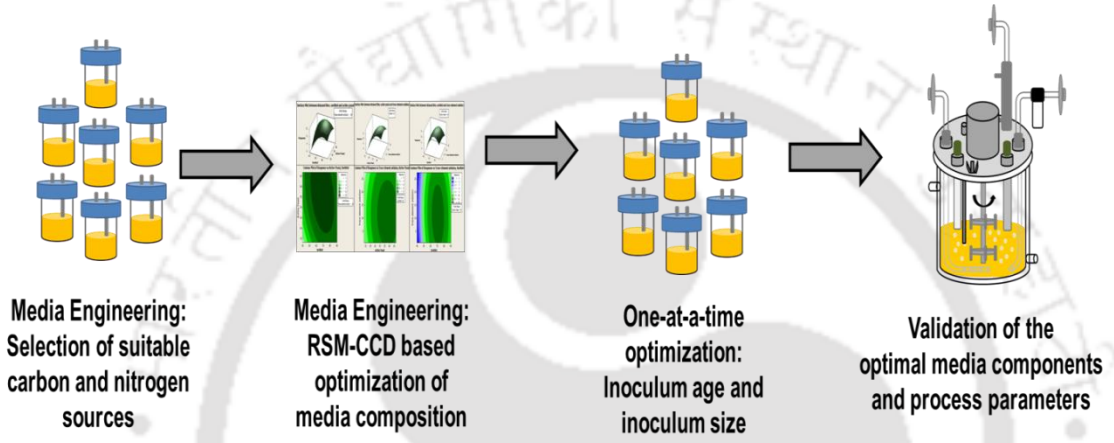
CHAPTER 4

Optimization of media composition and process parameters for higher butanol titer



C. sporogenes NCIM 2918 was evaluated by exposure to different carbon sources and nitrogen sources followed by media and process optimization

4.1 Background and motivation

Increasing environmental concerns and the need for alternate fuel sources have motivated the researchers around the world to invest their intellectual expertise into the development of renewable biofuel processes which show viability at the industrial scale. Liquid biofuels such as butanol and ethanol have provided an alternative to the use of non-renewable sources and at the same time providing zero carbon fuel (Kumar et al., 2011). However industrial production suffers from various limitations such as low product titer, yield, productivity, high substrate cost and solvent toxicity (Karimi et al., 2015). Hence, suitable process engineering approaches are under investigation to design and develop a feasible process (Ni et al., 2012; Yadav et al., 2014b). The

recent approaches involve media engineering (Al-Shorgani et al., 2011; Li et al., 2014), multi substrate utilization (Wang et al., 2014; Yadav et al., 2014b), co-cultivation of producer strains (Li et al., 2013) and simultaneous recovery of the solvents from fermentation broth (Ezeji et al., 2003; Ezeji et al., 2007; Yadav et al., 2014b). An important factor governing the process economic feasibility is the 50% overall cost due to substrates utilized for butanol production (Yadav et al., 2014a). Therefore, use of cheaper substrates such as glycerol or lignocellulosic biomass is expected to decrease the production cost to a greater extent (Green, 2011). Other than cheaper carbon substrates, low cost nitrogenous materials such as instant dry yeast, corn steep liquor, etc. need to be considered in the design of an economical process for butanol production (Altaf et al., 2005). Further *Clostridium* strains are known to show modulation during growth and product formation in response to substrate changes (Welsh et al., 1987; Al-Shorgani et al., 2011). For instance, Sabra et al., (2014) cultured *C. pasteurianum* DSMZ 525 on glucose with maximum biomass formation (13.2 g L^{-1}), butanol production (6.9 g L^{-1}) and no 1,3-propanediol production while in comparison, when the same strain was grown on glycerol as the sole carbon source, the organism modulated its metabolism towards high butanol formation (13.9 g L^{-1}) followed by 1,3-propanediol (5.3 g L^{-1}) and decreased biomass (3.2 g L^{-1}). Hence, it is important that before process development extensive characterization of newly identified strains is undertaken. This approach will assist in finding the best substrates for maximum butanol production as well as the concentration which can be further optimized using statistical tools. The study was conducted because of a lack of data for *C. sporogenes* NCIM 2918 nutrient requirements for maximum butanol formation and also on the relationship between medium compositions and product formation. Response surface methodology is used

to study the effect of individual media components and the combined effect as a result of their interaction among themselves (Baş and Boyacı, 2007). This method has several advantages over conventional one factor at a time optimization approach including less time consuming, less labor intensive, minimum experiments are required to be conducted saving on the cost of experimentation and a large amount of data can be generated (Wani et al., 2012). The mathematical model shows the effect of individual factor along with binary combination of parameters on the defined objective function (Baş and Boyacı, 2007; Wani et al., 2012). The three key steps involved are as follows: initial experimentation to determine the individual parameter concentrations, use of these levels to formulate an experimentation design which is performed and validated. Finally, the generation of response surface and contour plots of response versus parameters to obtain the optimum concentrations of parameters for maximization or minimization of the defined objective function (Baş and Boyacı, 2007). In the present study, a novel strain *C. sporogenes* NCIM 2918 was explored as a potential cell factory for butanol production. The strain was evaluated using different carbon and nitrogen sources followed by media optimization with an aim to maximize butanol titer. Finally, a process was demonstrated in an automated bioreactor for the twin alcohol production with a total alcohol titer which demonstrated the potential of the strain under consideration as one amongst the highest alcohol producers reported in literature.

4.2 Materials and Methods

4.2.1 Organism, growth and maintenance conditions

C. sporogenes NCIM 2918 was stored as glycerol stocks at -80°C. The glycerol stocks were revived in test tubes containing 15 mL TYG media (Annous and Blaschek, 1990) which comprises (in g L^{-1}) of tryptone 30.0, glucose 20.0, yeast

extract 10.0 and cysteine hydrochloride 0.05. This was followed by incubation at 37°C in a static incubator under anaerobic growth conditions. An anaerobic atmosphere was maintained by intermittent purging with pure nitrogen (99.99%, Assam Air products, Guwahati, India) at regular time intervals while the anaerobicity was confirmed by addition of a redox indicator dye resazurin at 1 g L^{-1}. After 24 h of growth, 10 mL of the revived culture was transferred to 100 mL TYG media into, taken in 500 mL air tight glass bottles and incubated under similar conditions for developing the seed culture. The seed culture was grown untill a biomass equivalence of absorbance 3.0 at 600 nm was obtained and 10% (v/v) was used as inoculum for the production media (P2) in all experiments unless otherwise stated. The initial pH of the production media was kept between 6-6.5, which was left uncontrolled during the fermentation. All the chemicals were purchased from Himedia, India unless otherwise mentioned.

4.2.2 Characterization of the strain under different carbon and nitrogen sources for butanol production

The strain was characterized in presence of different carbon and nitrogen sources to study their effect on growth and alcohol production. The study was conducted in P2 media (Monot et al., 1982) which comprises (in g L^{-1}) of the following: glucose 50.0, tryptone 30.0, yeast extract 10.0, K_2HPO_4 0.50, KH_2PO_4 0.50, CH_3COONH_4 3.22, para-amino-benzoic acid 0.01, biotin 0.001 plus atrace element of 10 mL L^{-1} containing $MgSO_4.7H_2O$ 0.20, $MnSO_4.H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, NaCl 0.01. The effect of carbon sources was studied by substituting glucose in the P2 media with equimolar carbon concentration (1.51 M) of seventeen different carbon sources. These included three pentoses (xylose, ribose and arabinose), five hexoses (dextrose, galactose, fructose, sodium gluconate and mannose), three

disaccharides (lactose, sucrose and maltose), three sugar alcohols (sorbitol, mannitol and glycerol) and three polysaccharides (starch, cellulose and dextrin). The carbon source which supported maximum butanol and ethanol production was selected and used for all subsequent characterization and optimization experiments. The effect of nitrogen sources was further studied by replacing tryptone and yeast extract in P2 media with eight different organic nitrogen sources containing equimolar nitrogen concentration of 0.31 M. These included tryptone, yeast extract, peptone, protease peptone, beef extract, urea, instant dry yeast (IDY) and casein acid hydrolysate. The best nitrogen source resulting maximum alcohol production was selected and used in further experiments. Inorder to avoid any influence of residual carbon and nitrogen sources on alcohol production, 10% (v/v) of the inoculum was centrifuged at 5,000 $\times g$ for 10 min at 4°C and the cells were resuspended in the production medium containing carbon and nitrogen sources. The fermentation batch was carried out in 500 mL glass bottles for 120 h at 37°C in static condition and samples were drawn every 6 h and analyzed for biomass growth, pH change, substrate utilization and product formation.

4.2.3 Maximization of butanol titer via media and inoculum optimization

Central composite design (CCD) based response surface methodology (RSM) was employed to optimize the initial concentration of carbon source, nitrogen source and trace elements in the range as depicted in Table 4.1.

Table 4.1 Actual levels of the selected media components for the butanol production from *C. sporogenes*

Media Components	Parameter levels used in CCD for optimization				
	-α	**-1**	**0**	**1**	**+α**
X_1 Sorbitol (g L^{-1})	39.7	50	65	80	90.2
X_2 Instant dry yeast (g L^{-1})	33.18	40	50	60	66.82
X_3 Trace element (mL L^{-1})	0.55	6	14	22	27.45

A 3 factor 5 level CCD was formulated (Table 4.2) using MINITAB (Version 16.1.1, Minitab Inc., USA) with 6 replicates at the center point making a total of 20 experiments. All the experiments were performed in triplicates and mean value of butanol titer was considered as the response. The results were analyzed via considering the linear, quadratic and interaction effects between the selected medium components and butanol titer which is mathematically expressed as in Eq. (4.1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (4.1)$$

Where, Y is the model predicted response (butanol titer in g L^{-1}), X_i and X_j are the concentration of the medium components (i and j varies from 1 to 3), k is the total number of parameters, β_0 is a constant, β_i, β_{ii} and β_{ij} are the regression coefficients for linear, quadratic and interaction functions respectively. The experimental data obtained from the CCD were fitted with the polynomial equation and the significance of the predicted model parameters were determined through an analysis of variance (ANOVA) and statistical regression analysis. Validation of the optimized concentration of the media components was performed by comparing the experimental data with the predicted values.

4.2.4 Effect of inoculum age and inoculum size on butanol

The effect of inoculum age on butanol production of the strain was studied by inoculating the production media with the seed culture obtained from different time

points 6, 12, 18 and 24 h. A constant inoculation volume of 10% (v/v) was used in all these experiments and samples were removed at regular time intervals to monitor butanol production. Inoculum at the harvest age which supported the maximum butanol production was then used in different sizes of 5, 10, 15, 20, 25 and 30 (%, v/v) to screen for the best inoculum size. These experiments were conducted with incubation temperature and initial pH of culture set at 37°C and 6.5 respectively, without using agitation in 500 mL air tight bottles.

4.2.5 Evaluation of the strain in 3.0 L bioreactor using optimized media and inoculum

Butanol production was evaluated in a 3.0 L automated bioreactor (Bio Console ADI 1025, Applikon Biotechnology, Holland) with 1.0 L optimized media. Fermentation was performed for 120 h at 37°C with constant agitator speed of 300 rpm and initial pH of 6.5. Anaerobiosis was maintained by flushing Nitrogen before inoculation. Samples were collected every 6 h for estimation of optical density, pH, residual sugar, acids and solvents in the fermentation broth.

4.2.6 Evaluation of the strain for solvent tolerance

The butanol tolerance limit of the strain was determined by supplementing different concentration of butanol (5, 10, 15 and 20 g L^{-1}) in the optimized medium obtained in Section 4.2.3. The cells in the solventogenic phase of the growth (24 h of growth) were harvested and re-suspended in their respective fresh production media with 0-20 g L^{-1} butanol. Samples were removed every 6 h and analyzed for microbial growth and butanol production.

4.2.7 Analysis of growth, substrate utilization and formation of acids & solvents

A known volume of sample was collected and centrifuged at 10,000 × *g* for 10 min at 4ºC to obtain pellet and supernatant. While the pellet was used for optical density measurement, the supernatant was utilized to obtain pH, substrate utilization profile and dynamic changes in organic acid (acetate, butyrate) and solvent (acetone, ethanol and butanol) concentration. The pellet was washed twice and re-suspended in the saline (0.85%, w/w of NaCl) to measure biomass growth in terms of optical density at 600 nm using UV-visible spectrophotometer (Cary 50, Varian, Australia). In case of fermentation using IDY as nitrogen source, biomass was estimated by filtering the fermentation broth through 1.2 µm membrane filter to separate the yeast cells from bacteria and filtrate was then used as above for optical density determination (Nobile, 1967). The substrates, organic acids and solvents were measured in high performance liquid chromatograph (HPLC, LC-20AD, Shimadzu, Japan) equipped with a Rezex ROA column (300 × 7.8 mm, Phenomenex, USA). Organic acids were quantified in ultra-violet (UV) detector at 210 nm whereas substrates and solvents were quantified in refractive index detector (RID) at 37°C. The minimum detection limits for substrates, acids and solvents were 0.1 g L^{-1}, 0.04 g L^{-1} and 0.06 g L^{-1} respectively. The mobile phase 0.005 N H_2SO_4 (98%, Merck, Germany) was used at a flow rate of 0.5 mL min^{-1} and the column oven temperature was kept at 28 °C. Samples were filtered with 0.2 µm filter and 10 µL of sample was used for analysis. Correlation standard curves were obtained (Appendix). All the experiments were conducted in triplicates and the values are measured as mean ± standard error.

4.3 Results and Discussion

4.3.1 Characterization of the strain under different carbon and nitrogen sources

Modulation of carbon and nitrogen sources resulted in varied growth and butanol production in *Clostridium* sp. (Welsh et al., 1987; Al-Shorgani et al., 2011). Dearth of literature detailing the nutritional requirements for the growth of *C. sporogenes* NCIM 2918 led to the screening of suitable carbon and nitrogen sources. To that end, seventeen different carbon sources were screened for growth and alcohol production by NCIM 2918 (Fig. 4.1 A). Among the carbon sources screened, glucose supported the maximum growth followed by sorbitol, maltose, xylose and fructose. Even though the organism was able to grow in all the carbon sources, carbon utilization was not observed in case of ribose, arabinose, mannitol, dextrin, cellulose and starch. The growth of the organism in these carbon substrates was therefore attributed to the utilization of tryptone and yeast extract which can be considered as the source of both carbon and nitrogen (Bapat et al., 2006).

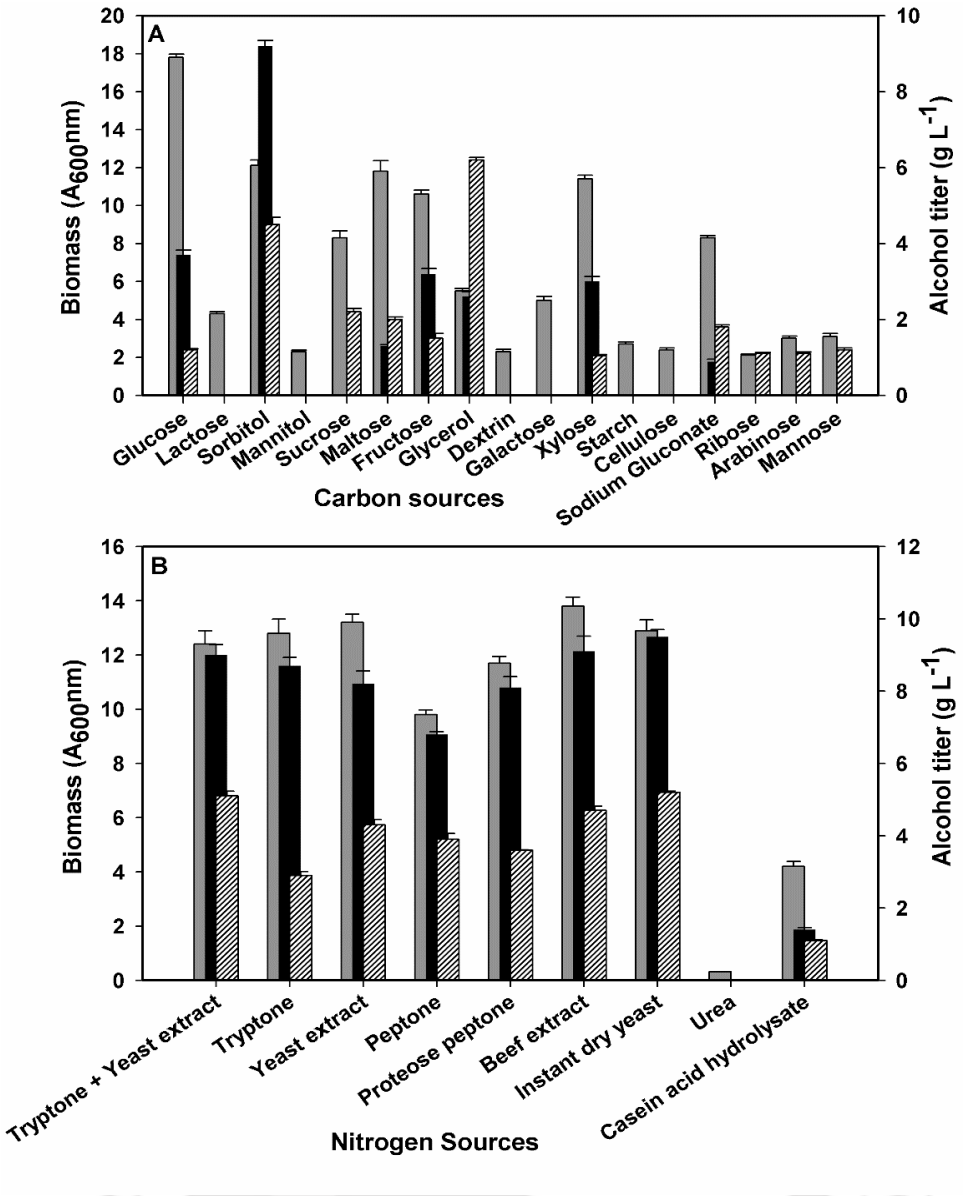


Fig. 4.1 Effect of different (A) carbon and (B) nitrogen sources on growth, butanol and ethanol production in *C. sporogenes* NCIM 2918. Growth, butanol titer and ethanol titer is represented by grey, black and striped bar respectively

The total alcohol production was observed to be maximum at a concentration of 13.7 g L^{-1} in case of sorbitol which was 2.79 folds higher than that obtained with glucose feed (Fig. 4.1 A). Use of more-reduced sugars such as sorbitol resulted in the formation of additional reducing equivalents and in turn, higher amount of reduced end products such as butanol and ethanol were produced. This redistribution of the fermentation pattern towards reduced products assisted the organism in maintaining

its redox balance by utilizing excess reducing factors such as NADH and NADPH. Alterations in the availability of reducing factors resulted in the shift of metabolic flux towards different end products in the wild-type *Clostridium* sp. strain BOH3 (Li et al., 2014). Glycerol, another sugar alcohol which also resulted in increased total alcohol titer (8.8 g L^{-1}) when compared with glucose (Fig. 4.1 A) confirmed the requirement of higher levels of NAD(P)H for increased production of alcohols. *C. pasteurianum* (Malaviya et al., 2012) and *C. acetobutylicum* (Yadav et al., 2014) have already been shown to use glycerol which corroborates with the current findings. Fructose, xylose, sodium gluconate and maltose supported total alcohol production in the range of 2-5 g L^{-1} while other carbon sources did not support butanol production. The ability of the organism to utilize xylose opens a route for using cheap lignocellulosic material as substrates for alcohol production (Gottumukkala et al., 2013). However, owing to the maximum total alcohol titer obtained, sorbitol was selected as the carbon source for further experiments. Among the eight organic nitrogen sources screened, utilization of IDY resulted in highest total alcohol titer of 14.7 g L^{-1} (Fig. 4.1 B) which was marginally higher when compared to that obtained using tryptone and yeast extract mixture. Except urea, all other nitrogen sources resulted in growth and solvent production (Fig. 4.1 B). IDY, besides classified as a good source of nitrogen also provides vitamins, proteins and other nutrients which are essential for the microbial growth (Sridee et al., 2011). Several industrial fermentation processes use IDY and corn steep liquor as the major nitrogen sources for the growth of various fastidious organisms (Altaf et al., 2005; Sridee et al., 2011). Thus, IDY was selected as the nitrogen source because of its low cost (Altaf et al., 2005) when compared to other organic sources of nitrogen. The absence of acetone production was observed for all the carbon and nitrogen sources screened. Further, the concentration of sorbitol, IDY

and trace elements were optimized using CCD-RSM approach followed by optimization of inoculum age and size.

4.3.2 Optimization of butanol production by response surface methodology

A CCD was constructed with 20 experiments to optimize the concentration of sorbitol, IDY and trace elements which resulted in a wide range of butanol titer from 6.93 ± 0.26 g L^{-1} to 11.92 ± 0.37 g L^{-1}. Maximization of butanol titer was used as the objective function in the CCD-RSM based optimization. The experimental data obtained were tabulated along with the corresponding RSM predicted butanol titer (Table 4.2). RSM based model construction yielded a polynomial Eq. (4.2) which correlated the medium parameters with the predicted response of butanol titer.

$$Y = -50.31 + 1.074\,X_1 + 0.86\,X_2 + 0.137\,X_3 - 0.006X_1^2 - 0.005X_2^2 - 0.004X_3^2 - 0.004\,X_1X_2 - 6.9 \times 10^{-4}X_1X_3 - 9.98 \times 10^{-4}X_2X_3 \quad (4.2)$$

Where, Y is the predicted butanol titer (g L^{-1}) and X_i (i = 1-3) representing the medium components sorbitol, IDY and trace elements respectively.

Table 4.2 Central composite design matrix of different media components used in RSM with corresponding experimental and predicted measurements of the response (butanol titer, g L^{-1})

Std. Order	Sorbitol X_1	Instant dry yeast X_2	Trace elements X_3	Experimental Butanol Titer	Predicted Butanol titer
1	50	40	6	6.93	6.71
2	80	40	6	10.13	10.12
3	50	60	6	10.33	9.51
4	80	60	6	11.39	10.67
5	50	40	22	6.54	6.61
6	80	40	22	9.51	9.69
7	50	60	22	10.01	9.39
8	80	60	22	10.67	10.21
9	39.7	50	14	4.93	5.55
10	90.2	50	14	8.82	9.11
11	65	33.18	14	8.72	8.38
12	65	66.82	14	9.93	11.18
13	65	50	0.55	10.11	10.83
14	65	50	27.45	10.19	10.37
15	65	50	14	11.34	11.28
16	65	50	14	10.58	11.28
17	65	50	14	11.32	11.28
18	65	50	14	11.33	11.28
19	65	50	14	11.92	11.28
20	65	50	14	11.31	11.28

Significance of the obtained data was analyzed through ANOVA and the results which are tabulated in Table 4.3 shows that the model is significant with *p*-value less than 0.05. The regression analysis which shows a correlation coefficient (R^2) of 0.91 indicates that out of 20 experiments only 9% could not be fitted by the model. A normal distribution of residuals ensures an adequate fit of the model with the experimental data (Fig 4.2). Among the three variables, linear and quadratic effect of sorbitol and instant dry yeast except for trace elements were significant while there was no significant interaction between the carbon, nitrogen and trace elements in the concentration ranges used for the optimization (Table 4.3) (Fig.4.3).

Table 4.3 Analysis of variance for the quadratic regression model obtained from CCD-RSM employed in optimization of media components for the butanol production from *C. sporogenes* NCIM 2918

Source[#]	DF	Seq SS	Adj SS	Adj MS	F	p-value*
Regression	9	57.97	57.97	6.44	11.66	0
Linear	3	25.00	25.00	8.33	15.08	0
X_1	1	15.30	15.30	15.30	27.68	0
X_2	1	9.44	9.44	9.44	17.09	0.002
X_3	1	0.26	0.26	0.26	0.47	0.507
Square	3	30.38	30.38	10.13	18.33	0
X_1*X_1	1	25.81	28.08	28.08	50.82	0
X_2*X_2	1	3.74	4.06	4.06	7.34	0.022
X_3*X_3	1	0.83	0.83	0.83	1.5	0.249
Interaction	3	2.58	2.58	0.86	1.56	0.26
X_1*X_2	1	2.53	2.53	2.53	4.58	0.058
X_1*X_3	1	0.05	0.05	0.05	0.1	0.759
X_2*X_3	1	0.00	0.00	0.00	0	0.976
Residual Error	10	5.53	5.53	0.55		
Lack-of-Fit	5	4.57	4.57	0.91	4.77	0.056
Pure Error	5	0.96	0.96	0.19		
Total	19	63.49				

* p-value higher than 0.05 shows insignificance

 # X1, X2, X3 represents the media components sorbitol, instant dry yeast and trace elements respectively

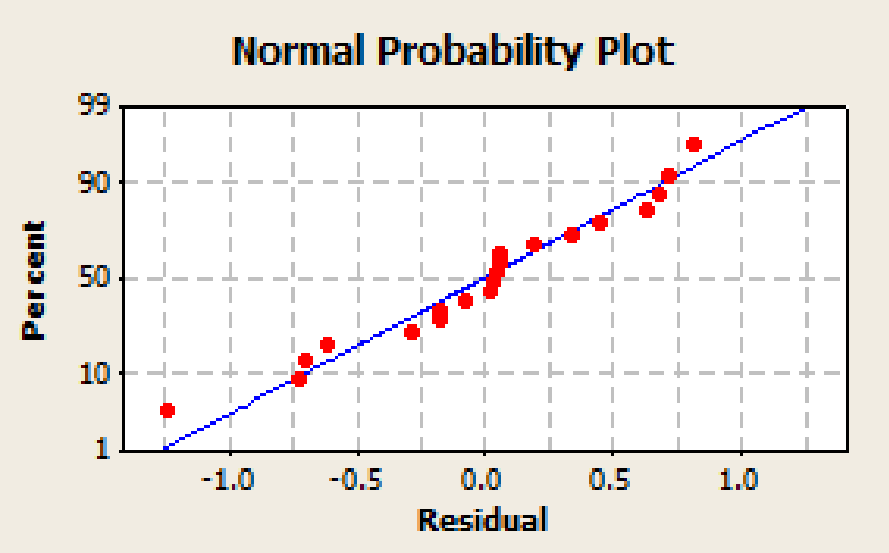


Fig. 4.2 Normality plot of residuals.

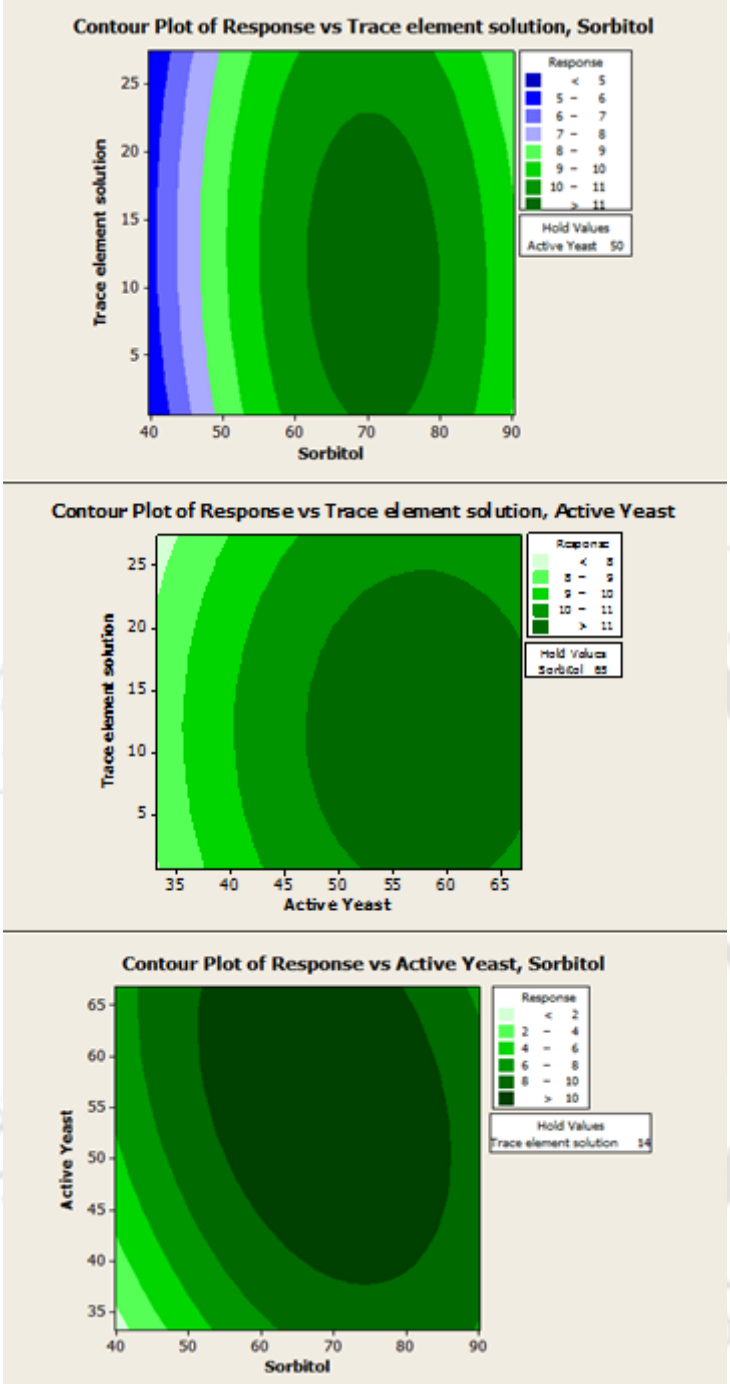


Fig. 4.3 Effect of design factors on the response variable.

A maximum butanol concentration of 11.7 g L^{-1} was predicted by RSM with sorbitol concentration of 68.8 g L^{-1}, IDY of 56.6 g L^{-1} and trace elements 11.1 mL L^{-1} (Table 4.4) (Fig. 4.4). Validation of medium composition predicted by RSM was performed in triplicates and the butanol titer was found to be 11.97 ± 0.13 g L^{-1} which

was similar to the predicted values. In comparison to the un-optimized media composition, RSM optimized media composition has shown 25% higher butanol titer. Increased sorbitol concentration was predicted as lower carbon substrate concentration supports only the first acidogenic phase of fermentation resulting in eventual carbon depletion during solventogenic phase (Monot et al., 1982). Similar to the present observation, increase of glucose concentration from 20 to 80 g L^{-1} increased butanol production from 1.85 to 13.03 g L^{-1} in the strain *C. acetobutylicum* (Wang et al., 2014).

Table 4.4 Comparison of butanol and ethanol production titer from *C. sporogenes* NCIM 2918 grown in un-optimized media and CCD-RSM based optimized media composition

Media Components (g L^{-1})	**Un-optimized Medium**	**CCD-RSM Optimized Medium**
Instant dry yeast	52	56.6
Sorbitol	50	68.8
K_2HPO_4	0.5	0.5
KH_2PO_4	0.5	0.5
$MgSO_4.7H_2O$	0.2	0.22
$MnSO_4$.H2O	0.01	0.011
$FeSO_4$.7H2O	0.01	0.011
NaCl	0.01	0.011
CH_3COONH_4	3.22	3.22
para-amino-benzoic acid	0.01	0.01
Biotin	0.001	0.001
Predicted butanol titer	ND	11.7
Experimental butanol titer	9.5	11.97 ± 0.13
Experimental Ethanol titer	5.2	7.92 ± 0.27
% Increase in butanol[a]	ND	25
% Increase in ethanol[a]	ND	52
% Increase in total alcohol titer[a]	ND	35

a – represents the percentage increase of the solvent titer (butanol, ethanol and total alcohol titer) in the optimized medium as compared to the un-optimized medium under flask conditions; ND – Not determined

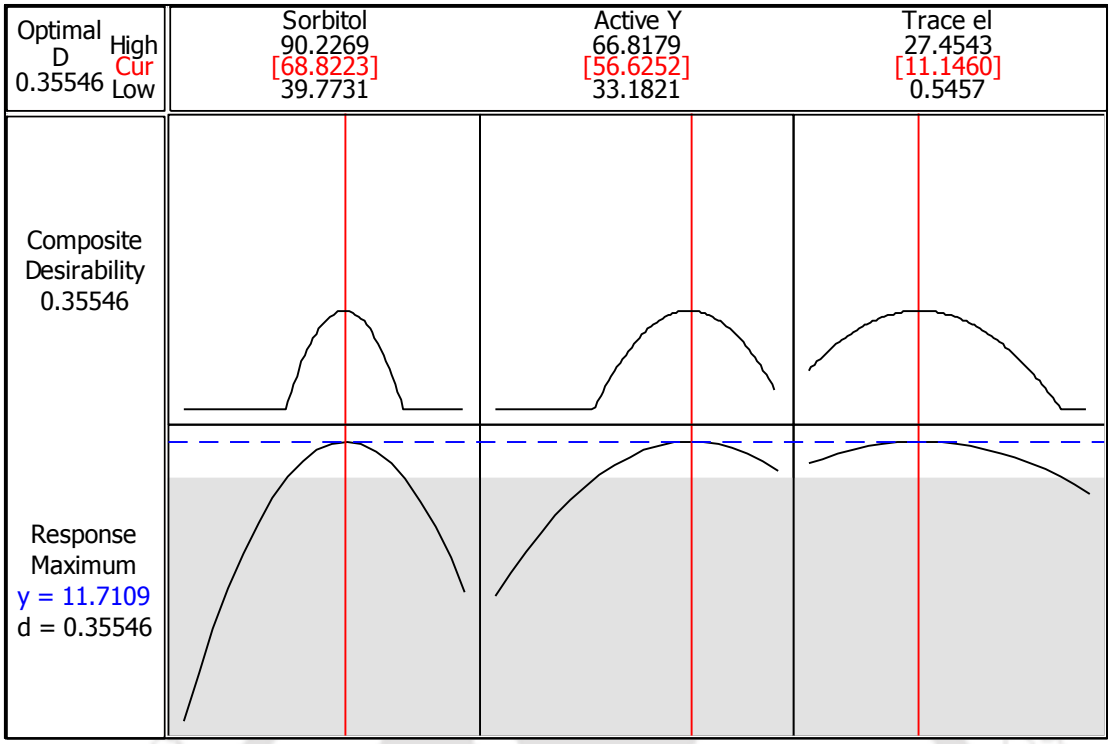


Fig. 4.4 Optimality plot to locate optimum factor levels for maximized response.

4.3.3 Optimization of inoculum age and size for maximum butanol production

Inoculum age and the inoculum size play a key role in governing the growth and production of secondary metabolites in any fermentation process. Therefore, effect of these two parameters on butanol production was determined for the strain NCIM 2918. In the first step, the inoculum age was optimized by transferring inoculum from four different time points to the production media. Age of the inoculum did not affect the final titer of butanol when considered for early time points of fermentative cycle (Fig. 4.5 A). However, the later stage of inoculum at 18 and 24 h resulted in a negative effect on butanol production and growth.

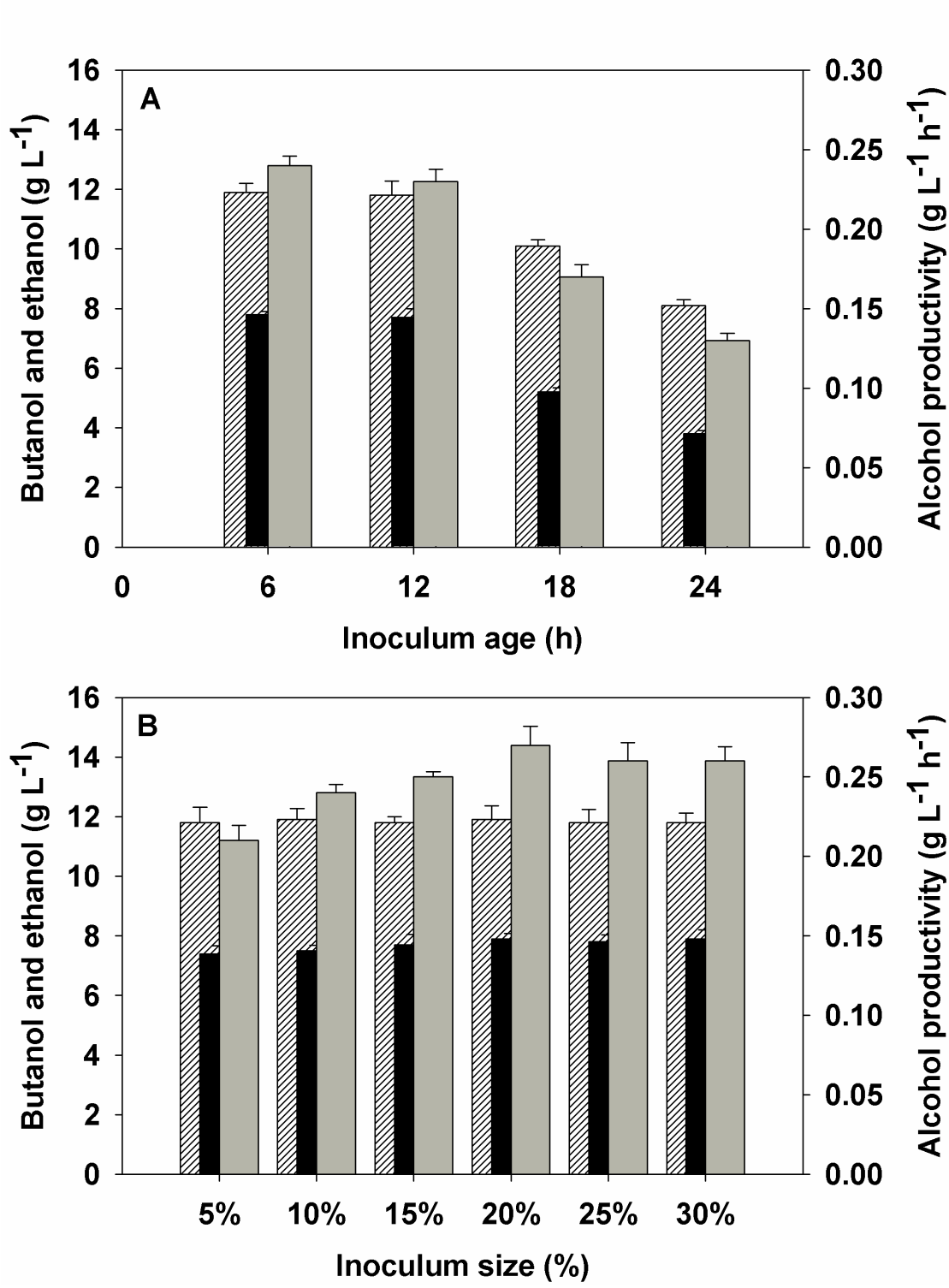


Fig. 4.5 Effect of (A) inoculum age and (B) inoculum size on butanol titer (stripped bar), ethanol titer (black bar) and total alcohol productivity (grey bar) of the strain *C. sporogenes* NCIM 2918

Microscopic examination of the cells in a field microscope at all stages of the seed, showed actively growing motile cells at 6 h and 12 h when compared to other stages

(results not shown). Thus, the 6 h grown inoculum was selected for further experimentation to optimize the inoculum size (Fig. 4.5 A). In case of inoculum size, no significant increment in butanol titer was observed with increasing the inoculum size. However, the butanol productivity increased linearly with increasing theinoculum size up to 20% (v/v) with no further increase in butanol productivity when the inoculum size was increased beyond 20% (v/v) (Fig. 4.5 B). A similar trend which was reported with increasing inoculum size from 5 to 15% (v/v) in *C. acetobutylicum* showed an optimum inoculum size of 16.2% (Razak et al., 2013). In general, increase in inoculum size increases the initial number of cells and the initial concentration of acids in the production medium which in turn assists in the early initiation of the solventogenic phase and subsequently, increasing the butanol productivity. This can be justified by the fact that acids produced in seed culture behave as precursors for solvent production as reported by Jones and Woods (1986). Hence, a 6 h old 20% (v/v) of inoculum was selected as the optimized inoculum for use in further experiments.

4.3.4 Production of butanol in automated bioreactor using sorbitol and IDY

The production potential of NCIM 2918 was evaluated in a 3.0 L automated bioreactor using optimized media and inoculum conditions (Fig. 4.6). The additional mixing condition provided in the reactor with agitation at 300 rpm resulted in 25% higher biomass (15 A_{600} nm) when compared to the biomass obtained in flask conditions (Fig. 4.6 A). The butyric acid concentration which increased exponentially during the acidogenic phase of fermentation followed by its utilization during the solventogenic phase was also marked by a dynamic change in the broth pH (Fig. 4.6 A and 4.6 B). Acetic acid on the other hand was consumed from beginning of the

fermentation. However, no significant difference in the butanol titer (12.17 ± 0.36 g L^{-1}) or butanol productivity (0.19 g L^{-1} h^{-1}) was observed when the cells were grown in the bioreactor when compared to the flask conditions (Fig. 4.6 C).

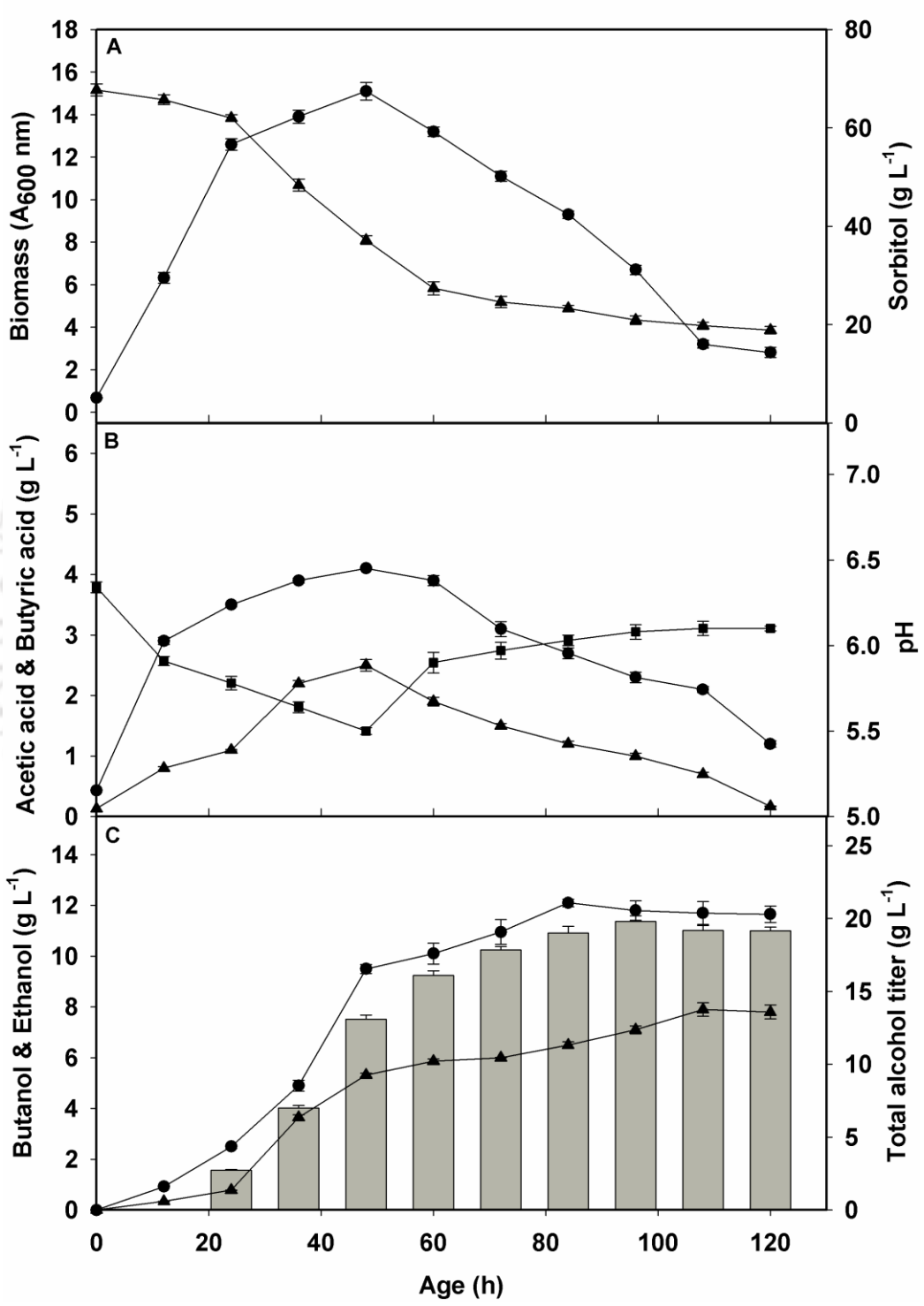


Fig. 4.6 Dynamic profiles for (A) growth (●) and sorbitol (▲) utilization; (B) pH (■), acetic acid (●) and butyric acid (▲); (C) butanol (●), ethanol (▲) and total solvent (grey bar) production of *C. sporogenes* NCIM 2918 under batch mode of fermentation

The butanol tolerance experiment was conducted to ascertain whether similar butanol titer in both shake flask and the reactor was due to the inherent capacity of the strain or a result of solvent toxicity. When the cell was exposed to 5 and 10 g L^{-1} butanol, the maximum biomass was reduced by 27% and 70% in comparison to the unstressed cells whereas, the culture failed to initiate growth in 15 and 20 g L^{-1} butanol concentration (Fig. 4.7 A). Similarly, Gao et al. (2012) reported that wild type *C. acetobutylicum* ATCC 824 failed to grow when exposed to 14 g L^{-1} butanol. When comparing the butanol profile of cells exposed to 5 and 10 g L^{-1} butanol, an increased butanol level of 4.2 g L^{-1} (total 9.6 g L^{-1}) was observed at the low initial concentration while in the second case, the butanol level did not increase (Fig. 4.7 B). This shows that the production was terminated after attaining a particular limit even if the cells are able to survive after exposed to a therehold level of the solvent. Thus, the critical butanol tolerance lies between 10 to 15 g L^{-1} which is also evident from the restriction of maximum butanol titer achieved in the reactor conditions at 12.1 g L^{-1} (Fig. 4.6 C). The ethanol and total alcohol titer was observed to be 7.92 ± 0.27 g L^{-1} and 20 ± 0.38 g L^{-1} (Fig. 4.6 C) under the optimized growth conditions which was 52% and 35% higher than the un-optimized conditions. The total alcohol titer obtained from NCIM 2918 was compared with various other wild type *Clostridium* strains (Table 4.5). The *C. saccharoperbutylacetonicum* N1-4 and *C. acetobutylicum* KF158795 exhibited a high butanol production of 16.7 g L^{-1} and 13.57 g L^{-1}, respectively. However, owing to their low ethanol production (Thang et al., 2010; Yadav et al., 2014a) a total alcohol titer of 17.61 g L^{-1} and 17.2 g L^{-1} could only be achieved for the two strains, respectively. The NCIM 2918 strain with the highest alcohol titer it is considereda potential cell factory for biofuel production.

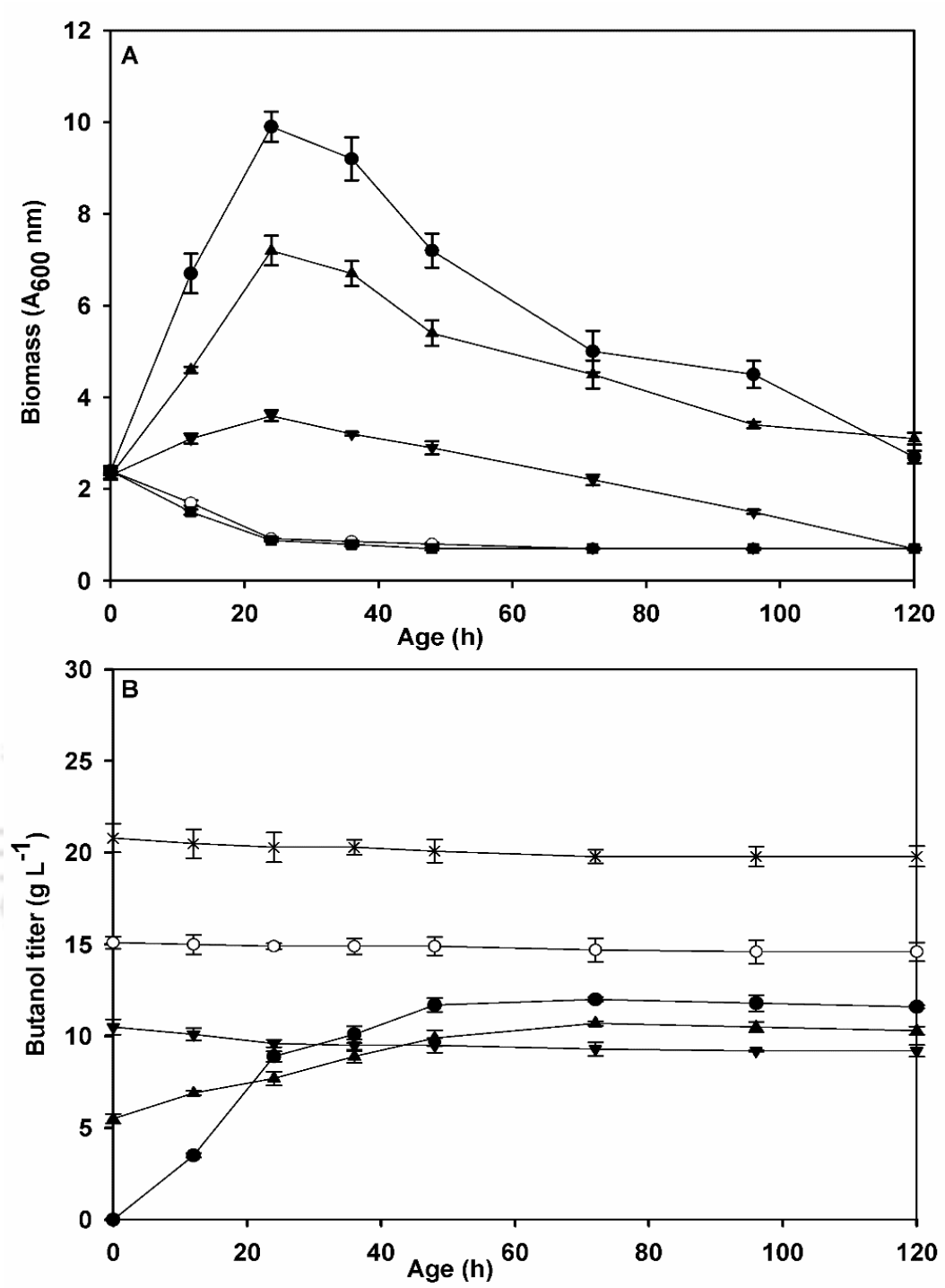


Fig. 4.7 Butanol tolerance assay: Dynamic profiles of (A) growth and **(**B**)** butanol production of *C. sporogenes* NCIM 2918 cultured in optimized medium with different concentrations of butanol (g L^{-1}): 5 (▲), 10 (▼), 15 (○) and 20 (■). Control (●) symbolizes culture not exposed to exogenous butanol stress

Table 4.5 Comparison of butanol, ethanol and total alcohol titer produced by different *Clostridium* sp. under batch conditions in the presence of different substrates

Microorganism	Substrate ($g\ L^{-1}$)	Butanol titer ($g\ L^{-1}$)	Ethanol titer ($g\ L^{-1}$)	Total alcohol titer ($g\ L^{-1}$)	Reference
C. sporogenes NCIM 2918	**Sorbitol 68.8**	**12.1**	**7.9**	**20**	**This study**
C. acetobutylicum KF158795	Glycerol 45	13.57	4.04	17.61	Yadav et al., 2014a
C.saccharoperbutylacetonicum N1-4	Glucose 65.9	16.2	1	17.2	Thang et al., 2010
C. beijerinckii BA101	Glucose 59.7	13.7	0.5	14.2	Ezeji et al., 2007
C.beijerinckii P260	Glucose 62	12.7	0.6	13.3	Qureshi et al., 2007
C. beijerinckii BA101	Glucose 59.2	11.9	0.5	12.4	Ezeji et al., 2003
C. acetobutylicum ATCC 824	Xylose 90	10.8	0.5	11.3	Sun and Liu., 2012
C. pasteurianum ATCC 6103	Glycerol 91	10	0.6	10.6	Malaviya et al, 2011
C. acetobutylicum ATCC 824	Glucose 70	8.1	1.8	9.9	Razak et al., 2013
C. saccharobutylicum DSM 13864	Fructose 40	9.1	0.4	9.5	Ni et al., 2012
C. saccharobutylicum DSM 13864	Sucrose 40	8.4	0.5	8.9	Ni et al., 2012
C. saccharobutylicum DSM 13864	Glucose 40	7.8	0.4	8.2	Ni et al., 2012

Maximum butanol, ethanol and solvent yields of 0.61, 0.65 and 1.26 mol mol^{-1} of sorbitol were achieved, respectively, during the fermentation. This observation was similar to the yield 0.66 mol mol^{-1} glucose for *C. beijerinckii* BA101 reported by Ezeji et al. (2003) and larger than the 0.45 mol mol^{-1} glucose (Wang et al., 2014) reported for *C. acetobutylicum* ATCC 824. Further process development with *in situ* solvent removal strategies and by modulating the operational mode of fermentation from batch to fed-batch or continuous is expected to increase the butanol titer to higher levels. The acidogenic growth phase of the fermentation was accompanied with gas formation which extended up to 48 h with no gas accumulation during solventogenesis. Thus, multiproduct paradigm and the ability to utilize cheaper substrates have evolved into considering using *C. sporogenes* NCIM 2918 in full-scale production.

4.4 Conclusions

C. sporogenes NCIM 2918 produced butanol with a titer of 12.1 g L^{-1} and a significant amount of ethanol (7.9 g L^{-1}) was observed when grown on sorbitol and IDY. Glycerol and xylose utilizing ability of the strain holds great promise for the conversion of biodiesel industry by product and lignocellulosic-derived feedstocks to n-butanol. This study is particularly significant due to limited literature availability on exploring *C. sporogenes* as a cell factory for biofuel production in terms of both bioprocess development and metabolic engineering.

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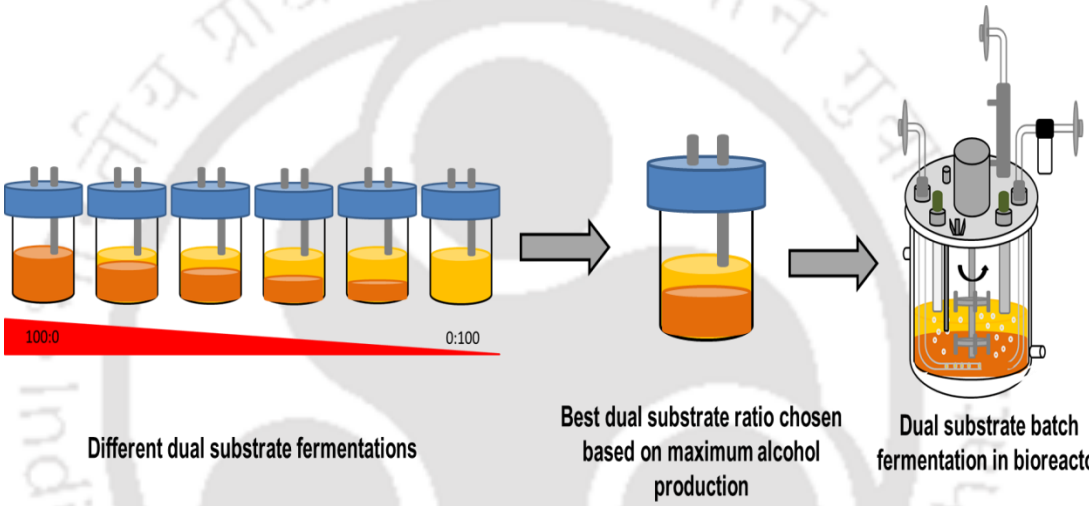
CHAPTER 5

Demonstrate multi product paradigm from *Clostridium sporogenes* and substrate dependent modulation of product composition



Examining the effects of various dual substrate combinations and different compositions of particular dual substrate on growth and product formation in *C. sporogenes* NCIM 2918

5.1 Background and motivation

The global demand for fossil fuels is increasing at an alarming pace. Undiscerning fossil fuel combustion has resulted in climatic change and this has compelled a global search for alternative renewable energy sources. Biomass is one such source of both liquid and gaseous fuels. Liquid biofuels such as ethanol and butanol, when blended with gasoline, have the potential to reduce greenhouse emissions, besides combating issues associated with increasing energy demand. Acetone-butanol-ethanol (ABE) fermentation of carbohydrates is a well explored biological process using *Clostridium* sp. However, regardless of the above mentioned

driving factors, ABE fermentation from *Clostridium* sp. faces various limitations, which include high feedstock cost, low yields, low productivity, low final product titer, low solvent tolerance of the bacteria and expensive product recovery (Sabra et al., 2014).

Substrate cost which accounts for approximately 50% of the total expense of fermentative alcohol production determines the economic viability of the process at the industrial level (Yadav et al., 2014; Sabra et al., 2014). A wide variety of carbohydrates such as hexose, pentose, disaccharides, sugar alcohols and polysaccharides etc. can be metabolized by *Clostridium* sp. to support growth as well as ABE fermentation. Few studies have been carried out to examine the effects of substrate modulation on product formation in *Clostridium* sp. For instance, *C. pasteurianum* CH4 has been reported to efficiently produce H_2 along with butyrate when grown on glucose or sucrose (Lee et al., 2004; Lo et al., 2008). The same strain produced butanol followed by 1, 3-propanediol (1, 3-PDO) and ethanol as the major products when glycerol was used as carbon source (Moon et al., 2011). An improved butanol production from 11 g L^{-1} to 13.3 g L^{-1} was reported for the same strain under a dual-substrate feed combination of glucose and glycerol as the carbon sources (Kao et al., 2012). Similarly, dual substrate fermentation of *C. pasteurianum* DSMZ 525 achieved a maximum butanol productivity of 0.96 g L^{-1} h^{-1} and a butanol titer of 21 g L^{-1} (Sabra et al., 2014). Xin et al. (2016) studied the cumulative effect of hexose and pentose on glycerol metabolism by *Clostridium diolis* strain DSM 15410. They reported 19% and 22% improvement in 1, 3-PDO titer and yield, respectively, with mixed substrate fermentation when compared to sole glycerol fermentation. These studies indicate microbes capable of utilizing multiple substrates are desirable for

commercial scale production of fuels and continuous efforts on identifying such strains is necessary to attain process sustainability.

The current study reports modulation of carbon flux distribution towards the production of butanol and ethanol in a non-acetone forming strain such as *C. sporogenes* NCIM 2918. Characterization of the strain on the sole carbon and nitrogen sources resulted in significant variation in butanol and ethanol titer which was reported in studies described in Chapter 4 (Section 4.3.1). These results point towards a possible substrate dependent modulation of alcohol production pathways in the strain. Therefore, in the next step of these studies, the organism was characterized based on various dual carbon substrate combinations. The study is conducted to assess their effect on butanol to ethanol ratio and in turn, change in total alcohol titer. The highest butanol and ethanol titer of 11.9 g L^{-1} and 12.1g L^{-1}, respectively, was achieved when a combination of glucose and glycerol was used in the ratio of 60:40. Finally, a process was demonstrated in 3 L bioreactor on dual substrate combination of glucose and crude glycerol, as a low cost carbon source from the biodiesel industry. The total alcohol titer of 22.9 g L^{-1} (butanol, 11.2 g L^{-1} and ethanol, 11.7 g L^{-1}) was observed to be the highest amongst the reported literatures, when the cultures were fed on crude glycerol alone or in combination with other substrates.

5.2 Materials and methods

5.2.1 Organism, growth and maintenance conditions

C. sporogenes NCIM 2918 was stored as glycerol stocks at -80°C. The glycerol stocks were revived in test tubes containing 15 mL TYG media (Annous and Blaschek, 1990) which comprises (in g L^{-1}) of tryptone 30.0, glucose 20.0, yeast extract 10.0 and cysteine hydrochloride 0.05. This was followed by incubation at 37°C in a static incubator under anaerobic growth conditions. The anaerobic

atmosphere was maintained by intermittent purging of pure nitrogen (99.99%, Assam Air products, Guwahati, India) at regular time intervals while the anaerobicity was confirmed by addition of a redox indicator dye resazurin at 1 g L^{-1}. After 24 h, 10 mL of the revived culture was transferred to 100 mL TYG media, placed into 500 mL air tight glass bottles, and incubated under similar conditions for the development of a seed culture. The seed culture was cultivated until a biomass equivalence at an absorbance 3.0 at 600 nm was obtained and 20% (v/v) was used as an inoculum for the production media (P2) in all experiments. The initial pH of the production media was kept between 6-6.5, which was left uncontrolled during the fermentation. All the chemicals were purchased from Himedia, India unless otherwise mentioned.

5.2.2 Screening of suitable dual substrate combination(s) for maximum alcohol production

Five different dual substrate combinations (sorbitol-xylose, glucose-xylose, sorbitol-glycerol, glucose-glycerol, and xylose-glycerol) were examined to identify the best carbon source blend towards maximum alcohol (butanol plus ethanol) production. Mixtures of the two substrates in ratios of 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100 were used as the carbon source for fermentation such that the total mole of carbon was equal to the molar of carbon present in the optimized media as that obtained in the Chapter 4 (Section 4.3.2). All the carbon sources were autoclaved separately and added aseptically in the desired ratio. In order to avoid any influence of residual carbon and nitrogen sources on alcohol production, a 20% (v/v) inoculum was centrifuged at 5,000 × g for 10 min at 4°C and the cells were resuspended in the production medium containing the respective carbon and nitrogen sources. The fermentation batch was performed for 120 h at 37°C in static condition and samples

were drawn every 6 h for analysis of biomass growth, pH change, substrate utilization and product formation.

5.2.3 Process Demonstration for alcohol production using a dual substrate combination of glucose and crude glycerol in bioreactor

Dual substrate characterization studies of the strain resulted in a combination of glucose-pure glycerol in a ratio of 60:40 as the best carbon substrate blend which provided a maximum total alcohol titer. Based on these studies, futher work was proposed to assess whether the organism can utilize crude glycerol as sole carbon source. Hence, the pure glycerol in the above substrate blend can be replaced with the crude glycerol which can give us the opportunity to reduce cost of the substrate. To that end ability of NCIM 2918 to utilize crude glycerol was tested by replacing carbon source (sorbitol) in the optimized media obtained in Chapter 4 (Section 4.3.2) with varying concentrations of crude glycerol (10, 20, 30, 40 and 50 g L^{-1}). Crude glycerol (65%, w/w) was produced (Fig. 5.1) from sunflower oil obtained from our hostel waste (Anand and Saxena, 2012). Absolute glycerol content present in per liter solution of crude glucerol was determined and the same was taken into account while preparing media irrespective of the impurities.

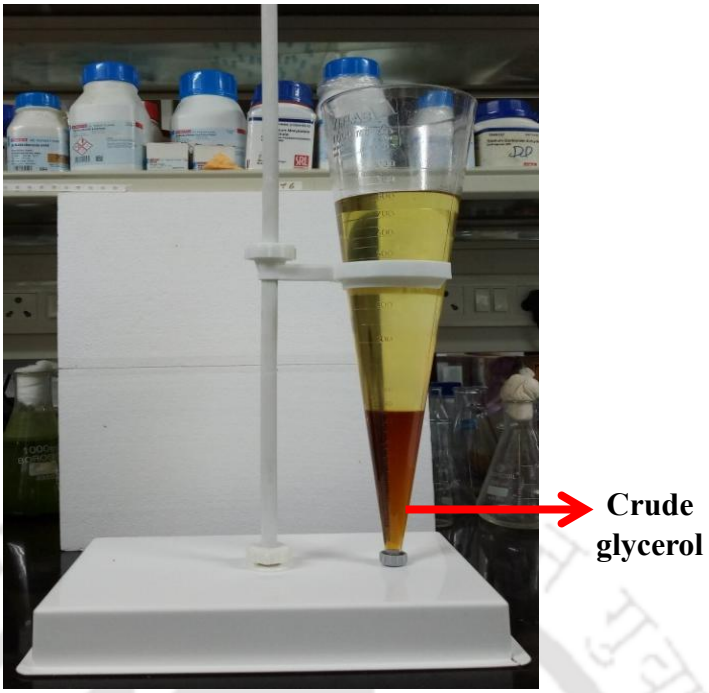


Fig. 5.1 Crude glycerol preparation using sunflower oil from hostel waste (Anand and Saxena, 2012)

Following the characterization experiments, crude glycerol was substituted for pure glycerol in the optimized ratio for glucose-pure glycerol obtained previously in Section 5.2.2. Batch fermentation was carried out in a 3.0 L automated bioreactor (Bio Console ADI 1025, Applikon Biotechnology, Holland) with 1.0 L of the optimized media. Fermentation studies were performed for carried out for 120 h at 37°C with constant agitation speed of 300 rpm and initial pH of 6.5 under anaerobic condition which was maintained by flushing N_2 before and after inoculation. Liquid samples were removed every 6 h to determine the optical density, pH, residual sugar, acids and solvents in the fermentation broth.

5.2.4 Analysis of growth, substrate utilization and formation of acids & solvents

A known volume of sample was collected and centrifuged at 10,000 $\times g$ for 10 min at 4ºC to obtain pellet and supernatant. While the pellet was used for optical density measurement, the supernatant was utilized to obtain pH, substrate utilization

profile and dynamic changes in organic acid (acetate, butyrate) and solvent (acetone, ethanol and butanol) concentrations. The pellet was washed twice and re-suspended in a NaCl solution (0.85%, w/w) to measure biomass growth in terms of optical density at 600 nm using UV-visible spectrophotometer (Cary 50, Varian, Australia). In the case of fermentation using IDY as the nitrogen source, the quantity ofbiomass was estimated by filtering the fermentation broth through 1.2 µm membrane filter to separate the yeast cells from the bacteria cells and the filtrate was subsequently used for optical density determination (Nobile, 1967). The substrates, organic acids and solvents were measured in high performance liquid chromatograph (HPLC, LC-20AD, Shimadzu, Japan) equipped with a Rezex ROA column (300 × 7.8 mm, Phenomenex, USA). Organic acids were quantified in ultra-violet (UV) detector at 210 nm whereas substrates and solvents were quantified in refractive index detector (RID) at 37°C. The minimum detection limits for substrates, acids and solvents were 0.1g L^{-1}, 0.04 g L^{-1} and 0.06 g L^{-1} respectively. The mobile phase 0.005 N H_2SO_4 (98%, Merck, Germany) was used at a flow rate of 0.5 mL min^{-1} and the column oven temperature was kept at 28 °C. Samples were filtered with 0.2 µm filter and 10 µL of sample was used for analysis. Correlation standard curves were obtained (Appendix). All the experiments were conducted in triplicates and the values are measured as mean ± standard error.

5.3 Results and discussion

5.3.1 Substrate dependent modulation of product formation during mono substrate fermentation

Change in individual carbon substrate, butanol to ethanol ratio significantly affected the fermentation. While sorbitol and glucose favour the production of butanol over ethanol (Fig. 5.2), a completely reverse trend was observed when glycerol was

used as the sole carbon source (Fig. 5.4). While various types of aldehyde/alcohol dehydrogenases are actively involved in butanol and ethanol biosynthesis in *Clostridium* sp., variation in their specificity towards a particular type of alcohol, order of expression and substrate preference was observed for these enzymes (Dai et al., 2016). For instance, iron-containing alcohol dehydrogenase enzyme encoded by *SMB_P058* gene and NAD(P)H dependent butanol dehydrogenase were found to exhibit higher specificity for ethanol over butanol (Dai et al., 2016). In a similar study, higher initial concentration of glycerol negatively regulated the synthesis of 1,3-propanediol with concomitant up-regulation of butyrate synthesis pathways. This study demonstrated that while some of the proteins in butyrate synthesis pathways were up-regulated under the influence of higher concentrations of glycerol, the expression levels of proteins involved in the reductive pathway of glycerol to 1,3-propanediol was negatively affected (Gungormusler-Yilmaz et al., 2014). Therefore, we hypothesize that while use of glycerol as a carbon source might have up-regulated the ethanol specific enzymes, sorbitol and glucose on the other hand overexpressed the enzymes specific to butanol synthesis. An equal distribution of carbon flux towards butanol and ethanol biosynthesis pathway was however, observed in case of xylose as carbon source (Fig. 5.2).

5.3.2 Substrate dependent modulation of butanol to ethanol ratio and total alcohol titer during dual substrate fermentation

The organism was further characterized for its growth and ability to produce total alcohol under five different combinations of dual substrates. This study also intended to determine if variation in ratio of dual substrates has any significant effect on distribution pattern of butanol and ethanol. All the dual and mono substrate fermentations were performed by replacing sorbitol in the optimized media described

in Chapter 4 (Section 4.3.2) with sole carbon source or with different dual substrate ratios. This was done such that the total molar carbon concentration remained equivalent to that of 68.8 g L^{-1} sorbitol as obtained in Chapter 4 (Section 4.3.2).

5.3.2.1 Characterization of the strain under dual substrate combination of sorbitol-xylose and glucose-xylose

The maximum butanol and ethanol titer of 3.7 g L^{-1} & 2.6 g L^{-1} and 11.9 g L^{-1} and 7.9 g L^{-1}, respectively ,were achieved when the culture was fed glucose or sorbitol as sole carbon source and IDY as nitrogen source. When xylose was used along with sorbitol or glucose, the increase in xylose concentration negatively regulated alcohol titer for both sorbitol-xylose and glucose-xylose combination (Figs. 5.2 A and 5.2 B). Hence, supplementation of xylose along with sorbitol or glucose resulted in significant reduction in alcohol titer and productivity in comparison to the mono substrate (sorbitol or glucose) fermentation (Table 5.1). However, glucose and xylose are the two major components found in the lignocellulosic hydrolysates. The study demonstrated that the co-utilization of both substrates would further provide an opportunity to explore different lignocellulosic biomass feedstocks for biofuel production. Acheiving the optimal dual substrate ratio blend from two feedstocks which can provide a maximum total alcohol titer is an issue for further consideration in full-scale facilities. In the present study, the organism was characterized in terms of growth and alcohol production under various concentration ratios of the dual substrates.

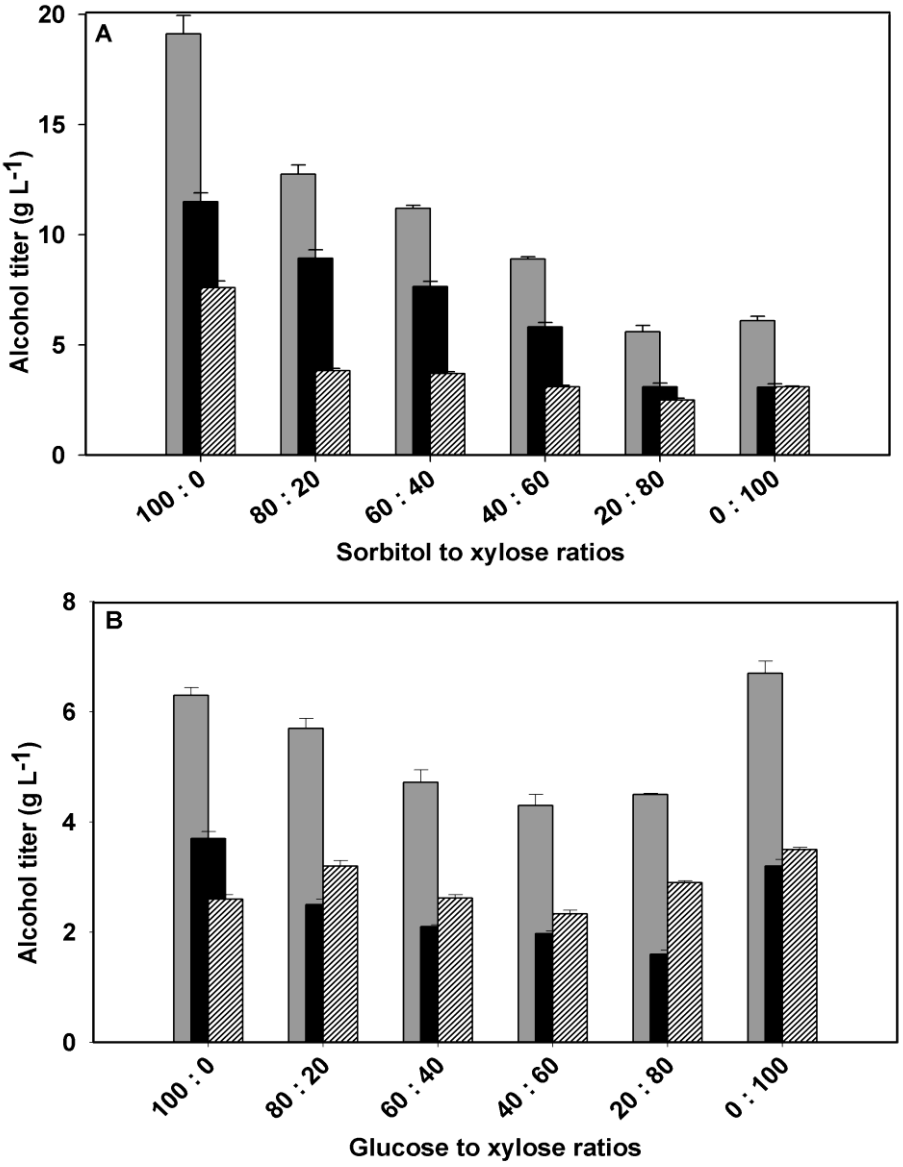


Fig. 5.2 Effect of dual substrate combination with different concentration ratios on total alcohol titer, butanol titer and ethanol titer in *C. sporogenes* NCIM 2918. (A) sorbitol-xylose and (B) glucose-xylose with varied concentration ratio. Total alcohol titer, butanol titer and ethanol titer is represented by grey, black and striped bar respectively

Table 5.1 Comparison of alcohol production titer in the presence of mono and dual substrate combination from *C. sporogenes* NCIM 2918

Mono/dual substrate	Substrate consumed (g)		Butanol yield ($g\ g^{-1}$)	Ethanol yield ($g\ g^{-1}$)	Biomass (A_{600} nm)	Alcohol productivity ($g\ L^{-1}\ h^{-1}$)
Sorbitol	48.9		0.24	0.16	12.3	0.3
Glycerol	38.9		0.10	0.41	6.6	0.54
Xylose	26.9		0.11	0.13	13.5	0.13
Glucose	33.2		0.11	0.07	16.4	0.21
Sorbitol:Xylose	**Sorbitol**	**Xylose**				
80:20	39.9	6.2	0.19	0.08	13.3	0.2
60:40	32.9	8.9	0.18	0.08	12.5	0.17
40:60	26.8	11.6	0.15	0.08	12.6	0.14
20:80	14.9	20.6	0.08	0.07	13.7	0.08
Glucose:Xylose	**Glucose**	**Xylose**				
80:20	22	1.2	0.10	0.13	13.1	0.2
60:40	23	3.3	0.07	0.09	12.6	0.18
40:60	22.7	4.2	0.07	0.08	12.1	0.16
20:80	15.1	9.1	0.06	0.11	12.3	0.17
Sorbitol:Glycerol	**Sorbitol**	**Glycerol**				
80:20	26.3	15.2	0.13	0.31	11.9	0.33
60:40	7.6	28.3	0.10	0.44	9.2	0.35
40:60	4.3	32.6	0.11	0.46	8.1	0.37
20:80	1.7	40.9	0.09	0.41	7.8	0.52
Xylose:Glycerol	**Xylose**	**Glycerol**				
80:20	24.1	14.4	0.08	0.29	13.2	0.44
60:40	13	24	0.09	0.42	10.4	0.48
40:60	4.1	31.5	0.10	0.45	9.8	0.46
20:80	2.1	38.5	0.09	0.41	8.3	0.52
Glucose:Glycerol	**Glucose**	**Glycerol**				
80:20	36.1	14.7	0.19	0.12	11.5	0.55
60:40	35.9	22.2	0.20	0.20	9.9	0.59
40:60	28.9	25	0.15	0.23	9.2	0.55
20:80	14.8	26.8	0.17	0.32	8.5	0.52

Maximum individual as well as total alcohol titer (Fig. 5.2 A & 5.2 B) and productivity (Table 5.1) were obtained when the xylose fraction was kept lowest at 20% of the total sugar concentration (sorbitol/glucose: xylose ratio of 80:20) in both combination feeds. This reduced total alcohol titer could be attributed to the organism

subjected to carbon catabolite repression (CCR) where presence of a preferred carbon source restricts the utilization of the presence of other carbon sources in the sugar mixture (Jiang et al., 2014).

A similar phenomena was observed in *C. acetobutylicum* ATCC 824 (Jiang et al., 2014) when grown in different glucose to xylose ratios. The total alcohol production declined from 11.1 g L^{-1} (glucose: xylose of 2:1) to 1.6 g L^{-1} (glucose: xylose of 1:5). Interestingly, a butanol to ethanol ratio of 2.3:1 (Fig. 5.3 A) was observed at a 80:20 sorbitol to xylose mixture which was higher even in comparison to respective mono substrate fermentation. This might be attributed to elevated negative regulation of ethanol biosynthesis pathway in comparison to butanol pathway under the influence of lower fraction of xylose. However, repressive effect of xylose towards ethanol biosynthesis in comparison to butanol became less prominent with the increase in xylose fraction in the sugar mixture (Fig. 5.3 A). In case of glucose-xylose mixture, no significant pattern in butanol to ethanol ratio was observed with the change in composition of the sugar mixture (Fig. 5.3 B).

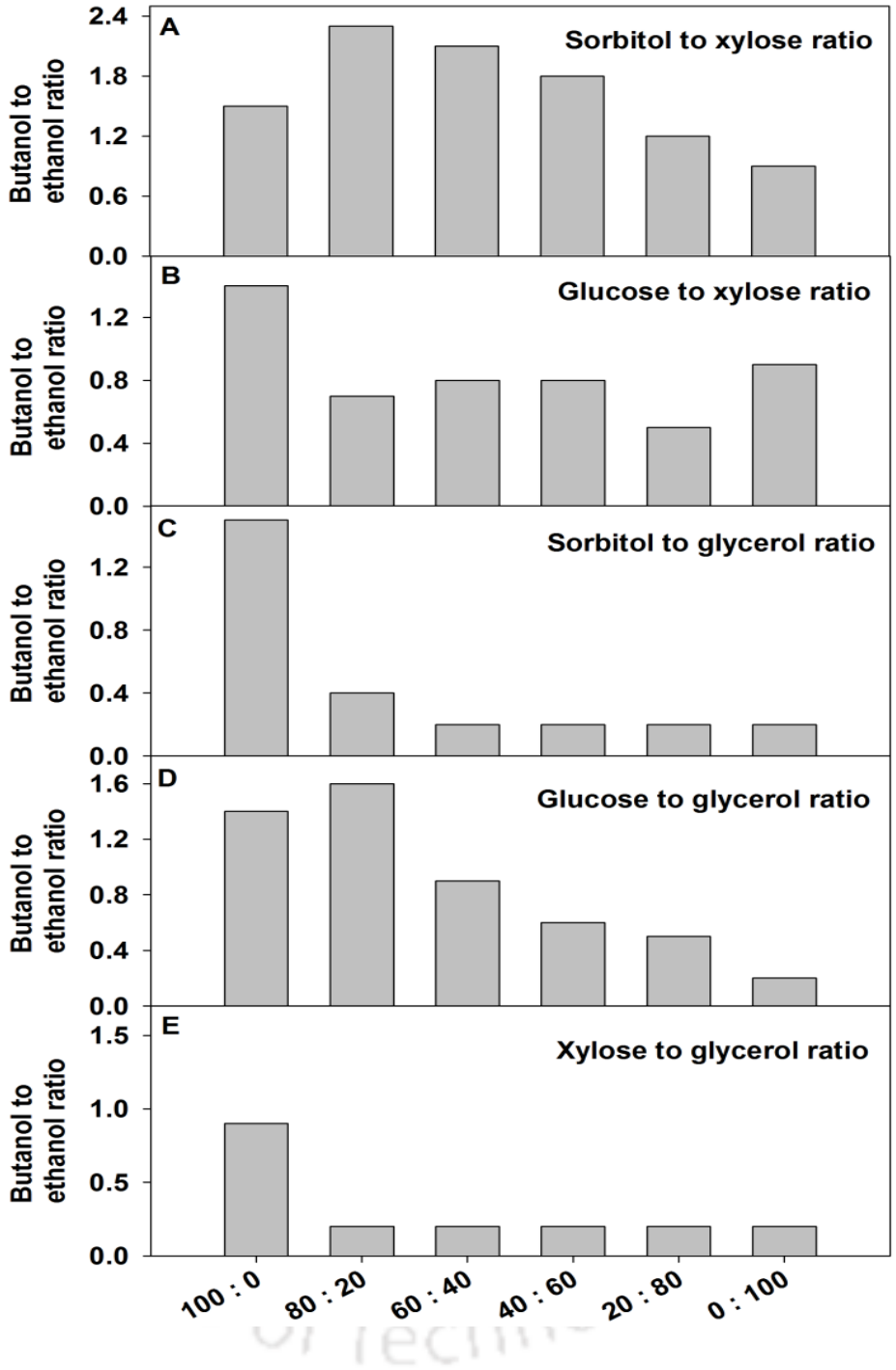


Fig. 5.3 Modulation of butanol to ethanol ratio under the influence of various dual substrate combinations and different compositions of particular dual substrate

5.3.2.2 Characterization of the strain under dual substrate combination of glucose-glycerol, sorbitol-glycerol and xylose-glycerol

Many *Clostridium* species such as *C. pasteurianum* (Malaviya et al., 2012; Ahn et al., 2011; Sarchami et al., 2016), *C. acetobutylicum* (Yadav et al., 2014)

or *C. tetanomorphum* (Panitz et al., 2014) have a natural ability to utilize glycerol and this provides a possible route for glycerol conversion to alcohols. In the present study, we tried to evaluate the response of our organism NCIM 2918 in terms of its ability to produce different alcohols and their distribution pattern when glycerol is supplemented with other carbon sources. In view of this, the organism was grown on three different dual substrate combinations where sorbitol or glucose or xylose was used as second carbon source along with glycerol. Further, for each of this combination the dual carbon substrates were used at different concentration ratios ranging from 100:0 (only sorbitol or glucose or xylose) to 0:100 (only glycerol). Comparisons of individual and total alcohol titer are presented in Figs. 5.4 A, 5.4 B and 5.4 C. Note all the carbon substrate screening experiments (Chapter 4, Section 4.2.2) were conducted with tryptone and yeast extract as the sole nitrogen sources. All the dual substrate characterization studies which were performed with IDY contained the best nitrogen source after screening different nitrogen substrates. Using a dual substrate combination of sorbitol and glycerol, did not increase the total alcohol titer significantly in comparison to mono substrate (sorbitol or glycerol) fermentation (Fig. 5.4 A). While changes in their concentration ratio altered the total alcohol titer in the range of 19 g L^{-1} to 21.6 g L^{-1} (Fig. 5.4 A), the alcohol productivity was significantly improved from 0.33 g L^{-1} h^{-1} to 0.52 g L^{-1} h^{-1} with changing the sorbitol-glycerol ratio from 80:20 to 20:80 (Table 5.1). However, butanol and ethanol titer was significantly varied when the sorbitol and glycerol ratio was modulated (Fig. 5.4 A). For instance, ethanol production was elevated with concomitant decrease in the butanol production when the glycerol concentration was increased in the sugar mixture (Fig. 5.4 A). Hence, supplementation of glycerol resulted in redirection of the carbon flux from butanol to the ethanol biosynthesis pathway. Further, growth of the organism on

sorbitol-glycerol ratio of 0:100 (glycerol as the sole carbon source) and IDY as the nitrogen source resulted in a total alcohol titer of 20.1 g L^{-1}. This is a significant improvement which when compared with the growth on glycerol as carbon source and tryptone-yeast extract as nitrogen source demonstrated a lower total alcohol titer of only 8.8 g L^{-1}. This total alcohol titer of 20.1 g L^{-1} with a productivity of 0.54 g L^{-1} h^{-1} in batch fermentation was found to be higher (Table 5.2) than that demonstrated for various other strains reported using pure glycerol (Malaviya et al., 2012; Yadav et al., 2014; Panitz et al., 2014; Sarchami et al., 2016). The organism was further characterized on a dual substrate combination of glucose-glycerol at different concentration ratio (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100). Amongst all the substrate combinations used in the present study, glucose-glycerol combination with a ratio of 60:40 was found to yield the highest total alcohol titer of 24 g L^{-1} with butanol (11.95 ± 0.37 g L^{-1}) and ethanol (12.16 ± 0.5 g L^{-1}) at a ratio of 1:1 (Fig. 5.4 B). For any industrially relevant fermentation process, the primary criterion towards maximization of the product titer is to remove any possible substrate limitation which may exist (Palabhanvi et al., 2016).

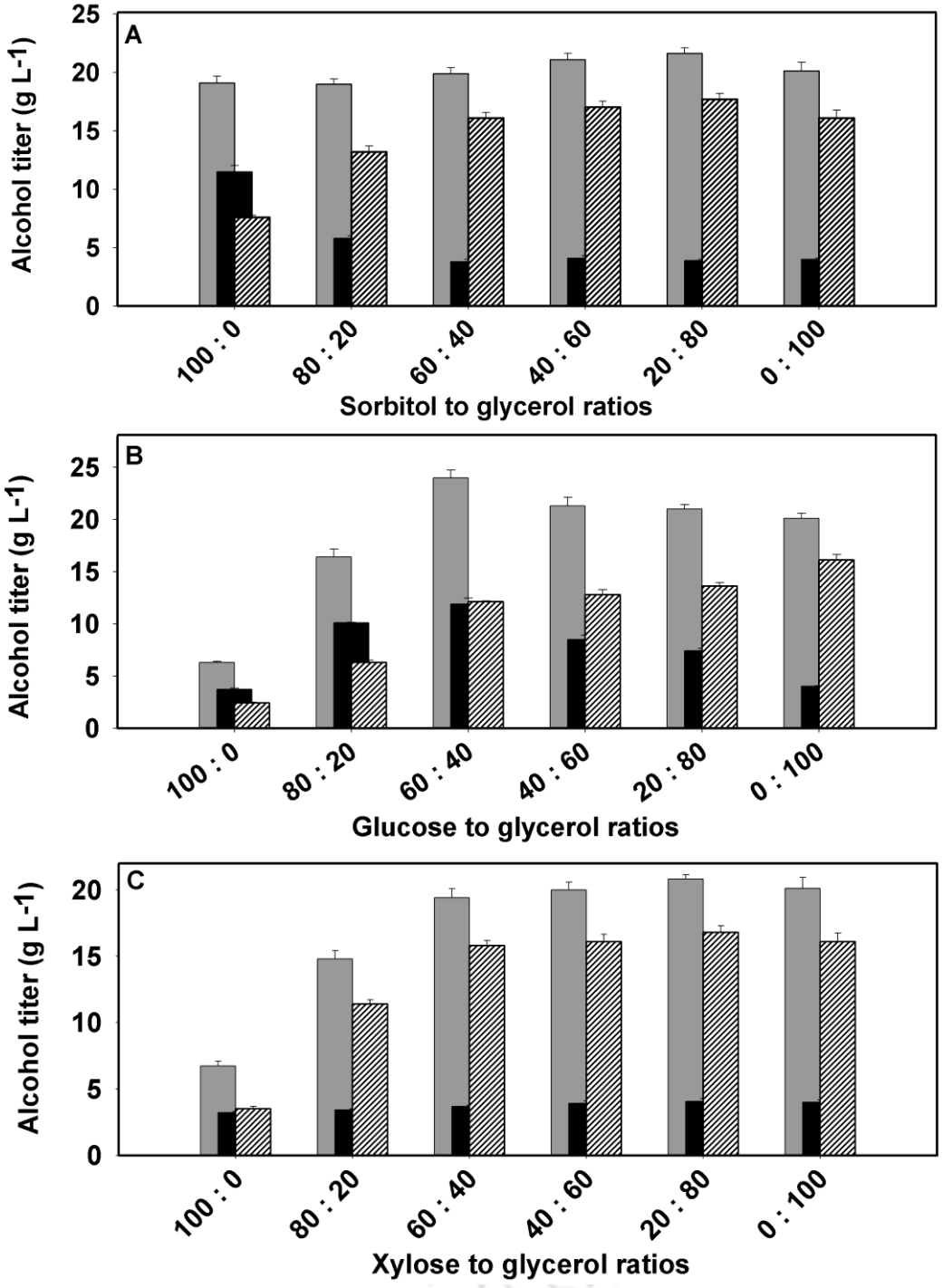


Fig. 5.4 Effect of dual substrate combination with different concentration ratios on total alcohol titer, butanol titer and ethanol titer in *C. sporogenes* NCIM 2918. (A) sorbitol-glycerol, (B) glucose-glycerol ratios and (C) xylose-glycerol with varied concentration ratio. Total alcohol titer, butanol titer and ethanol titer is represented by grey, black and striped bar respectively

In the present study, the organism did not suffer any substrate limitation while grown on a feed containing a glucose-glycerol ratio of 60:40 and hence, the total alcohol titer reached a maximum (Fig. 5.5 B). However, for other combinations

either glucose or glycerol was exhausted within 24-48 h of fermentation resulting in lower product titer (Fig. 5.5 A, 5.5 C and 5.5 D). A maximum total alcohol productivity 0.59 g L^{-1} h^{-1} when utilizing a glucose-glycerol feed ratio of 60:40. *C. pasteurianum* CH_4 was reported to produce butanol with a maximum titer of 13.2 g L^{-1} however, the productivity of 0.19 g L^{-1} h^{-1} (Kao et al., 2012) was lower than the butanol productivity of 0.37 g L^{-1} h^{-1} (Table 5.2). Supplementing glycerol with glucose significantly influenced the production of individual as well as total alcohol. For instance, with increasing the glycerol concentration in the mixture of glycerol and glucose, the ethanol titer increased linearly.

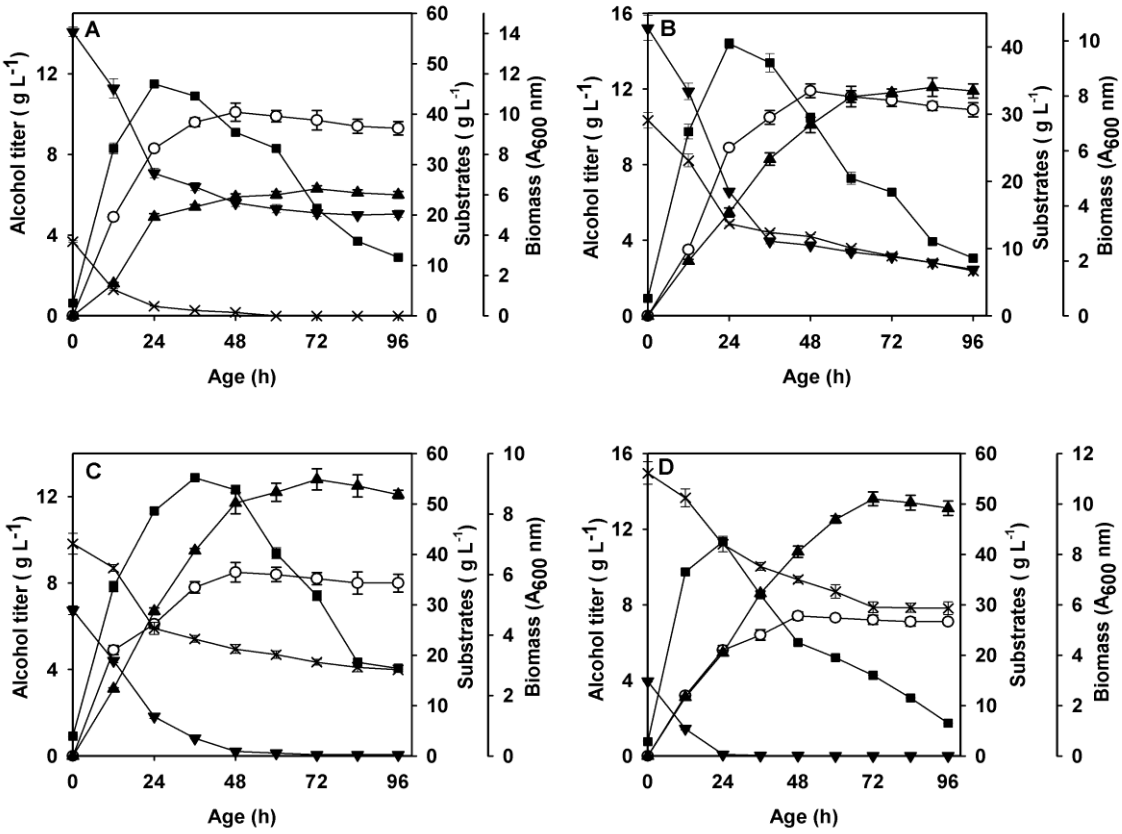


Fig. 5.5 Dynamic profiles for glucose (▼) utilization, glycerol (×) utilization, butanol (○) production, ethanol (▲) production and biomass (■) formation by *C. sporogenes* NCIM 2918 under dual substrate batch fermentation using glucose-pure glycerol blend in a ratio of (A) 80:20, (B) 60:40, (C) 40:60 and (D) 20:80.

The butanol titer increased until the glycerol concentration reached 40% of the total carbon substrate concentration and subsequently, decreased thereafter with further increasing theglycerol concentration (Fig. 5.4 B). While grown on a dual substrate mixture of glucose and glycerol, *C. pasteurianum* DSMZ 525 produced 1, 3-PDO and butanol which followed similar fermentation pattern like ethanol and butanol respectively as observed in the present study (Sabra et al., 2014). The butanol to ethanol ratio reached a maximum when glycerol was supplemented with glucose in a ratio of 20:80 (Fig. 5.3 D). Further increasing the glycerol fraction in the sugar mixture resulted in a linear decline in the butanol to ethanol ratio (Fig. 5.3 D). This suggests that a positive interaction effect towards butanol specificity might have occurred when glycerol was supplemented with glucose at a ratio of 20:80. Under these conditons, glucose, a simple sugar, served as a growth supporting carbon source while glycerol served as a NADH supplier for the solventogenic pathway. When grown on a mixture of glucose plus glycerol with a feed ratio of 60:40, the organism produced ethanol or butanol with a maximum yield of 0.2 g g^{-1} of glucose plus glycerol. This value of individual alcohol yield was found to be similar (0.23 g g^{-1} glucose plus glycerol) to that reported for *C. pasteurianum* DSMZ 525 when grown on glucose-glycerol blend in a ratio of 1:1 (Sabra et al., 2014).

Table 5.2 Comparison of butanol, ethanol and total alcohol titer produced by different *Clostridium* sp. under batch conditions using pure glycerol

Microorganism	Substrate ($g\ L^{-1}$)	Butanol titer ($g\ L^{-1}$)	Ethanol titer ($g\ L^{-1}$)	Total alcohol titer ($g\ L^{-1}$)	Reference
C. sporogenes NCIM 2918	**Glucose (42.2) + Glycerol (28.7)**	**11.9**	**12.1**	**24**	**Present study**
C. pasteurianum DSMZ 525	Glucose (50) + Glycerol (50)	21.1	ND	ND	Sabra et al., 2014
C. sporogenes NCIM 2918	**Glycerol (67.1)**	**4**	**16.1**	**20.1**	**Present study**
C. pasteurianum DSM 525	Glycerol (60)	17	ND	ND	Biebl et al., 2001
C. acetobutylicum KF158795	Glycerol (45)	13.57	4.04	17.61	Yadav et al., 2014
C. tetanomorphum GT6	3% (v/v) glycerol	11.5	2	13.5	Panitz et al., 2014
C. pasteurianum DSM 525	Glycerol (50)	12.3	1-2	13.3-14.3	Sarchami et al., 2016
C. pasteurianum CH4	Glucose (20) + Glycerol (60)	13.2	ND	ND	Kao et al., 2012
C. pasteurianum CH4	Butyrate (6) + Glycerol (60)	12.1	ND	ND	Kao et al., 2012
C. pasteurianum ATCC 6103	Glycerol (91)	10	0.6	10.6	Malaviya et al., 2012

ND-not defined

Comparison of butanol, ethanol and total alcohol titer produced by different *Clostridium* sp. under batch conditions using pure glycerol is shown in Table 5.2**.** The organism was further evaluated for its ability to produce alcohols when xylose was added as a co-substrate to glycerol (Fig. 5.4 C). The highest total alcohol titer of 20.8 g L^{-1} was obtained when xylose and glycerol was used with a ratio of 20:80 (Fig. 5.4 C). However, with the increase in glycerol concentration the ethanol titer was increased gradually, while butanol titer remained unchanged (Fig. 5.4 C). Irrespective of the composition, the butanol to ethanol ratio in case of a dual substrate mixture of sorbitol-glycerol and xylose-glycerol was found to be similar to that observed for the mono-substrate glycerol feed (Fig. 5.3 C and 5.3 E). This dominating effect of glycerol over xylose and sorbitol point towards possible up-regulation of ethanol specific enzymes by glycerol. A similar phenomena were reported for *Clostridium diolis*, where production of 1,3 propandiol is highly favored over butyrate in presence of feeds containing only glycerol or a dual substrate feed containg xylose and glycerol (Xin et al., 2016).

5.3.3 Alcohol production under dual substrate combination of glucose and crude glycerol in bioreactor

Characterization of the strain under all possible dual substrate combinations resulted in highest total alcohol titer of 24 g L^{-1} with an ethanol to butanol ratio of 1:1 when cultivated with a feed containing glucose and glycerol. Therefore, combination of glucose plus glycerol with a ratio of 60:40 was found to be best dual substrate combination. Further, the same alcohol titer can be achieved when pure glycerol was replaced with crude glycerol. This particular experiment was carried out in order to evaluate the performance of microorganism when using crude glycerol, a by-product of the biodiesel industry. Also reduced biofuel production has been reported for

microorganisms fed with crude glucerol ontaining inhibitors such as methanol, ash, sodium or potassium ions, free fatty acids and triglycerides (Venkataramanan et al., 2012; Khanna et al., 2013). Based on these studies, the organism was characterized using different concentration of crude glycerol ranging from 10 g L^{-1} to 50 g L^{-1} (Fig. 5.6 A).

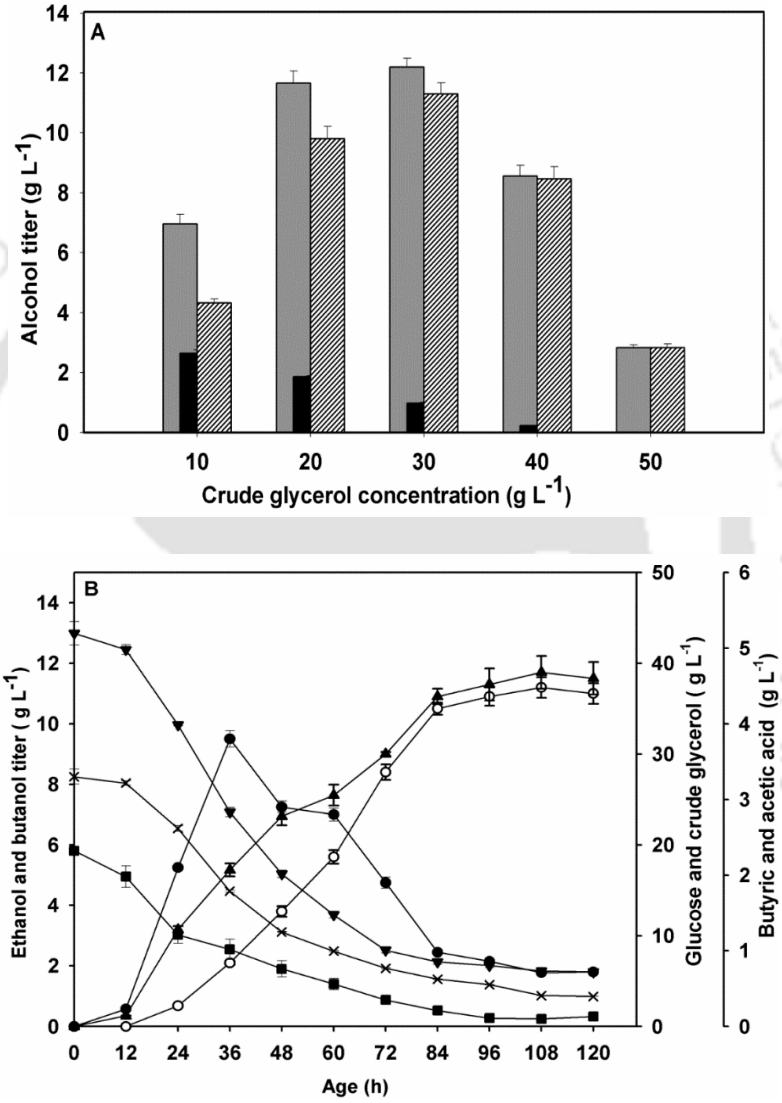


Fig. 5.6 (A) Effect of varying crude glycerol (10-50 g L^{-1}) concentration on butanol titer (black bar), ethanol titer (stripped bar) and total alcohol titer (grey bar) of strain *C. sporogenes* NCIM 2918. (B) Dynamic profiles for utilization of glucose (▼) utilization, crude glycerol (×) and production of butanol (○), ethanol (▲), acetic acid (■) and butyric acid (●) by *C. sporogenes* NCIM 2918 under dual substrate fermentation using glucose-crude glycerol blend in a ratio of 60:40.

With increasing the crude glycerol concentration from 10 g L^{-1} to 30 g L^{-1}, a substantial increase in the ethanol titer coupled with a concomitant decrease in the

butanol titer was observed thus resulting in increased net total alcohol titer. However, further increase in crude glycerol concentration, impeded the biofuel production yield. *C. pasteurianum* is well known for its ability to utilize crude glycerol for the butanol production (Gallardo et al., 2014; Sarchami et al., 2016). Khanna et al. (2013) reported a maximum butanol concentration of 8.8 g L^{-1} produced by *C. pasteurianum* when 25 g L^{-1} crude glycerol was used and any further increase in the substrate concentration resulted in decreasing the product yield. In the next step, batch fermentation in 3 L automated bioreactor was performed with a supplementation of dual substrate consisting of glucose and crude glycerol. The crude glycerol concentration of at 27.5 g L^{-1}, a level less than 30 g L^{-1}, was the optimal concentration for alcohol production based on previous experiments reported in this thesis. NCIM 2918 produced 11.24 ± 0.33 g L^{-1} butanol and 11.77 ± 0.54 g L^{-1} ethanol at the end of fermentation, with a residual glucose and glycerol concentration of 5.9 and 6.1 g L^{-1} respectively (Fig. 5.6 B). The alcohol productivity for the glucose-glycerol dual fermentation was reduced from 0.59 g L^{-1} h^{-1} for pure glycerol to 0.23 g L^{-1} h^{-1} for crude glycerol while not much significant difference was observed in the final product titer. A similar pattern of pronounced lag time in *C. pasteurianum* ATCC 6013 which was reported by Taconi et al. (2009) when crude glycerol was employed to replace pure glycerol could be attributed to the presence of impurities. Hence further studies should be performed to analyze the effect of impurities in the biodiesel-derived crude glycerol on the growth of NCIM 2918. Eliminating the impurities would result in a fermentation profile similar to that of pure glycerol. Comparing the butanol and ethanol production performance of various strains utilizing crude glycerol as a sole carbon source or as a co-substrate in batch fermentation is shown in Table 5.3.

Table 5.3 Comparison of butanol, ethanol and total alcohol titer produced by different *Clostridium* sp. under batch conditions using crude glycerol

Microorganism	Substrate ($g\ L^{-1}$)	Butanol titer ($g\ L^{-1}$)	Ethanol titer ($g\ L^{-1}$)	Total alcohol titer ($g\ L^{-1}$)	Reference
C. sporogenes NCIM 2918	**Glucose (43.3) + Crude Glycerol (27.5)**	**11.2**	**11.7**	**22.9**	**Present study**
C. pasteurianum CH4	Acid-pretreated bagasse (25) and crude glycerol (60)	11.8	1.5	13.3	Kao et al., 2012
C. pasteurianum DSM 525	Crude glycerol (50)	12.3	1-2	13.3-14.3	Sarchami et al., 2016
C. sporogenes NCIM 2918	**Crude glycerol (30.2)**	**0.98**	**11.3**	**12.2**	**Present study**
C. pasteurianum DSM 525	Crude glycerol (42.86)	9.75	1.07	10.82	Gallardo et al., 2014
C. pasteurianum DSM 525	Crude glycerol (30)	9.05	0.6	9.65	Johnson and Rehmann, 2016
C. pasteurianum MTCC 116	Crude glycerol (25)	8.8	ND	ND	Khanna et al., 2013
C. pasteurianum ATCC 6013	Crude glycerol (25)	7.8	ND	ND	Taconi et al., 2009
C. pasteurianum DSM 525	Thin stillage glycerol (20)	6.2–7.2	ND	ND	Ahn et al., 2011

ND-not defined

NCIM 2918 is potential cell factory candidate for biofuel production because it was able to produce the highest alcohol titer. Further, process development with *in situ* solvent removal strategies and modulating the bioreactor operational mode is expected to increase the butanol titer to higher levels.

5.4 Conclusions

Substrate dependent modulation of the ethanol-butanol ratio was observed for non-acetone producing *C. sporogenes* NCIM 2918. A dual substrate combination of feeding glucose-glycerol mixture showed production of butanol 11.9 g L^{-1} and ethanol 12.1 g L^{-1} in 1:1 ratio. Further, evaluation of the strain with crude glycerol as a replacement to pure glycerol in the glucose-glycerol ratio resulted in a butanol and ethanol titer reaching levels of 11.2 g L^{-1} and 11.7 g L^{-1}, respectively. Thus, the strain exhibits potential for utilization of cheaper substrates to produce biofuel.

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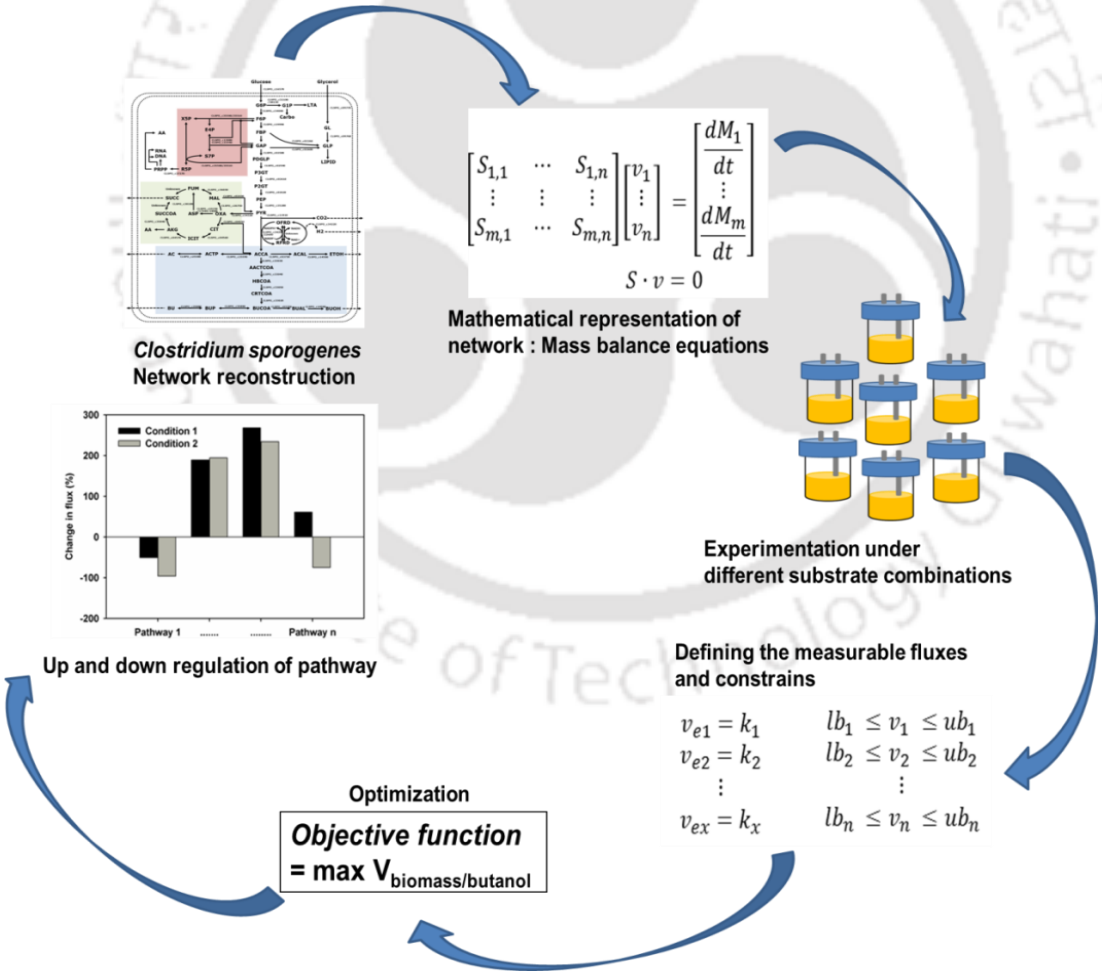
CHAPTER 6

Understand the regulation in substrate dependent modulation of growth and production of alcohols in *Clostridium sporogenes* NCIM 2918 through metabolic network reconstruction and flux balance analysis



Flux balance analysis to understand the regulations of carbon partitioning and alcohol metabolism of *C. sporogenes* NCIM 2918 under different substrate combinations

6.1 Background and motivation

Increasing global energy consumption has raised alarms regarding demand and supply of energy resources accompanied by environmental concerns and energy security. The International Energy Agency has reported a rise of approximately 47.7% in fuel consumption and an approximate 55.8% increase in CO_2 emissions over the past two decades (IEA Statistics, 2016). Such startling statistics have driven a global search for sustainable alternative energy sources. One such prospective option is using biofuels such as ethanol and butanol. Simultaneous production of butanol and ethanol is performed by the bacteria in the *Clostridium* genus with *C. acetobutylicum* as the most widely employed during fermentation (Durre 2007; Lee et al., 2008; Jones and Woods, 1986). However, lower butanol titers with limited ethanol production and narrow substrate utilization range of the existing *Clostridium* strains have motivated the search for novel cell factories. Substrate dependent modulation of butanol and ethanol production in non-acetone forming strain *Clostridium sporogenes* NCIM 2918 was reported in Chapter 5 (Section 5.3). The data indicate changes in the substrate combinations and variation in the dual feed concentration significantly affected not only on the butanol and ethanol distribution pattern, but also on the total alcohol titer. Despite the extensive amount of work in the field of medical microbiology for therapeutic applications (Poehlein et al., 2015), exploiting *C. sporogenes* in biofuel research still remains at a very nascent stage. Moreover, the alcohol titers required to achieve industrial feasibility has not been attained due to solvent toxicity and in part due to competition for electrons amongst the pathways for major reduced end products (Claassen et al., 2011). Therefore, understanding the modulation in its central metabolism when exposed to different carbohydrates will be helpful in finding

targets for metabolic and process engineering strategies to direct carbon and electron fluxes towards the preferred pathways.

Fluxes, the outcome of non-linear interactions among genes, proteins and metabolite concentrations (Sauer, 2006) are considered to be the fundamental determinants of the metabolic phenotype under a given condition (Stephanopoulos, 1999). Therefore, while gene expression data or protein levels or metabolite concentrations alone cannot be the reliable indicators of metabolic phenotype (Sauer, 2006), quantification of carbon flux distribution in the metabolic network is crucial to understand the complex interplay between genotypic alterations and the corresponding phenotypic responses (Shastri and Morgan 2005; Boyle and Morgan 2009). Estimation of carbon fluxes by Flux Balance Analysis (FBA) is achieved by maximizing or minimizing a biologically relevant objective with pseudo steady state approximation for the metabolites (Orth et al., 2010). Since, the first genome-scale metabolic model of *E. coli* appeared (Edwards and Palsson, 2000), similar reconstructions have also been reported for six different *Clostridium* spp. namely *C. acetobutylicum, C. beijerinckii, C. butyricum, C. cellulolyticum, C. ljungdahlii* and *C. thermocellum* (Lee et al., 2008; Senger and Papoutsakis, 2008; Salimi et al., 2010; Roberts et al., 2010; Cai et al., 2010; Milne et al., 2011; Nagarajan et al., 2013; Dash et al., 2016). However, as there are very few reports on *C. sporogenes* as a cell factory for biofuel production, there have been no attempts at modeling its metabolism.

In the present study, FBA was performed to understand the modulation of carbon flux distribution towards alcohol biosynthesis with the change in type of carbon sources and their concentrations in a feed mixture. Further, a change in intracellular carbon flux distribution was predicted during metabolic shift from acidogenic phase to solventogenic phase. The analysis involved reconstruction of

metabolic network followed by estimating the carbon fluxes via linear programming optimization. Validation of the model was performedby comparing the experimental values obtained for a specific growth rate or carbon flux towards butanol formation to the corresponding model predicted values.

6.2 Materials and methods

6.2.1 Organism, growth and maintenance conditions

C. sporogenes NCIM 2918 was stored as glycerol stocks at -80 °C. Revival of the glycerol stock and seed preparation was performed as previously described in Chapter 5. The anaerobic condition was achieved and maintained by purging nitrogen (99.99%, Assam Air products, Guwahati, India) prior to inoculation and at regular intervals respectively. Resazurin (1 g L^{-1}), a redox indicator dye, was used to ensure anaerobic condition throughout the fermentation. A 6 h old 20% (v/v) seed culture was used as inoculum for batch cultivation of the organism. Experiments were performed in an optimized P2 media (Monot et al., 1982) comprising of (in g L^{-1}) instant dry yeast 56.6, K_2HPO_4 0.50, KH_2PO_4 0.50, CH_3COONH_4 2.2, para-amino-benzoic acid 0.01, biotin 0.001, $MgSO_4.7H_2O$ 0.22, $MnSO_4.H_2O$ 0.011, $FeSO_4.7H_2O$ 0.011, NaCl 0.011. A carbon source blend was added to the media in six different glucose-glycerol ratios of 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100 in such a manner that the total initial carbon content was equal to 2.26 M as prescribed for the optimized media in Chapter 4 (Section 4.3.2). Carbon source(s) were autoclaved separately and added aseptically in the desired ratio. Prior to inoculation, the seed culture was centrifuged at 5,000 $\times g$ for 10 min at 4°C in order to remove any residual carbon which might alter the composition of the carbon source(s). Batch fermentation was performed at 37°C in under static conditions and sampling was performed at regular intervals for analysis of biomass, substrate utilization and

product formation. The initial pH of the production media was kept between 6-6.5, which was left uncontrolled during the fermentation. All the experiments were conducted in duplicate and the values were measured as mean ± standard error. All the chemicals were purchased from Himedia, India unless otherwise mentioned.

6.2.2 Flux balance analysis

The FBA involved reconstructing the metabolic network containing essential biochemical reactions followed by a mathematical representation of the stoichiometric coefficients for each reaction in the form of a matrix designated as the stoichiometric model. These coefficients impose constraints on the fluxes through the metabolic network. The stoichiometric model was then solved using linear programming (Muthuraj et al., 2013) with a suitable objective function. A mass balance was performed for all the metabolites involved in the various reactions with the exception of protons (Montagud et al., 2010) and water molecules (detailed list is provided in the reaction list in appendix). The FBA was based on pseudo steady state assumption (Manish et al., 2007) for the metabolites (Eq. (6.1)) where the metabolic model was converted to the stoichiometric model as follows (Stephanopoulos, 1999).

$$S.v = 0 \qquad (6.1)$$

S was the stoichiometric matrix of dimension $m \times n$ containing stoichiometric coefficient of m metabolites in n reactions. v was the flux vector that corresponded to the flux of n reactions (Orth et al., 2010; Hendry et al., 2016).

In the present study, the dimension of the stoichiometric matrix was 152 x 206 with 54 (n-m) as the degree of freedom (f). Since the number of unknowns are greater than the degree of freedom (n>f), the system is underdetermined and hence, necessitates the use of linear optimization to solve the matrix. *Clostridium* spp. are known to exhibit a biphasic fermentation comprising of an early acidogenic phase

marked by fast growth rate of the organism and formation of acids predominantly, followed by a solventogenic phase marked with slower growth rate, re-assimilation of acids and subsequent solvent production. With the aim of capturing changes in the flux distribution during the metabolic shift from acidogenesis to solventogenesis, the FBA was performed using data collected at 7 h and 18 h intervals of cultivation, respectively. Flux analysis at 7 h was performed using *maximization of biomass* (M_μ) since this time pont corresponds to the exponential growth phase of the fermentation. While *maximization of butanol* (M_B) was used as an objective function at 18 h as this time point falls under the solventogenic phase of fermentation when the organism is producing maximum alcohol. For the flux analysis in the acidogenic phase, the model inputs were the experimentally determined uptake rates for glucose, glycerol, acetic acid and butyric acid plus the formation rate of acetic acid, butyric acid, succinic acid, ethanol and butanol. For analysis in the solventogenic phase, the glucose, glycerol, acetic acid and butyric acid uptake rates and the specific growth rate and formation rate of acetic acid, butyric acid and ethanol were considered as model inputs. Of the total 15 exchange reactions, 10 were experimentally determined and these fixed flux values were used as input for the model. For other exchange compounds, the lower and upper flux values were provided. The FBA was performed for both acidogenic and solventogenic phase while growing only on glucose, only glycerol and mixtures containing glucose plus glycerol at different concentration ratios. An inbuilt routine in MATLAB (MATHWORK, Natick, MA) '*fmincon*' was used to solve the stoichiometric model based on linear programming optimization.

6.2.3 Reconstruction of metabolic network

C. sporogenes NCIMB 10696 genome annotated in the KEGG database was used as the primary source for reconstruction of the metabolic network for *C.*

sporogenes. Nevertheless, other databases such as MetaCyc (Caspi et al., 2012) and SEED (Overbeek et al., 2005) were used for enzymes that were not annotated in KEGG. Finally, the BLAST searches were undertaken for enzymes which were not present in any of the above databases. In spite of yielding low scores, some of these enzymes were added to the network for gap filling. A schematic representation of the major pathways in *C. sporogenes* is shown in Fig. 6.1.

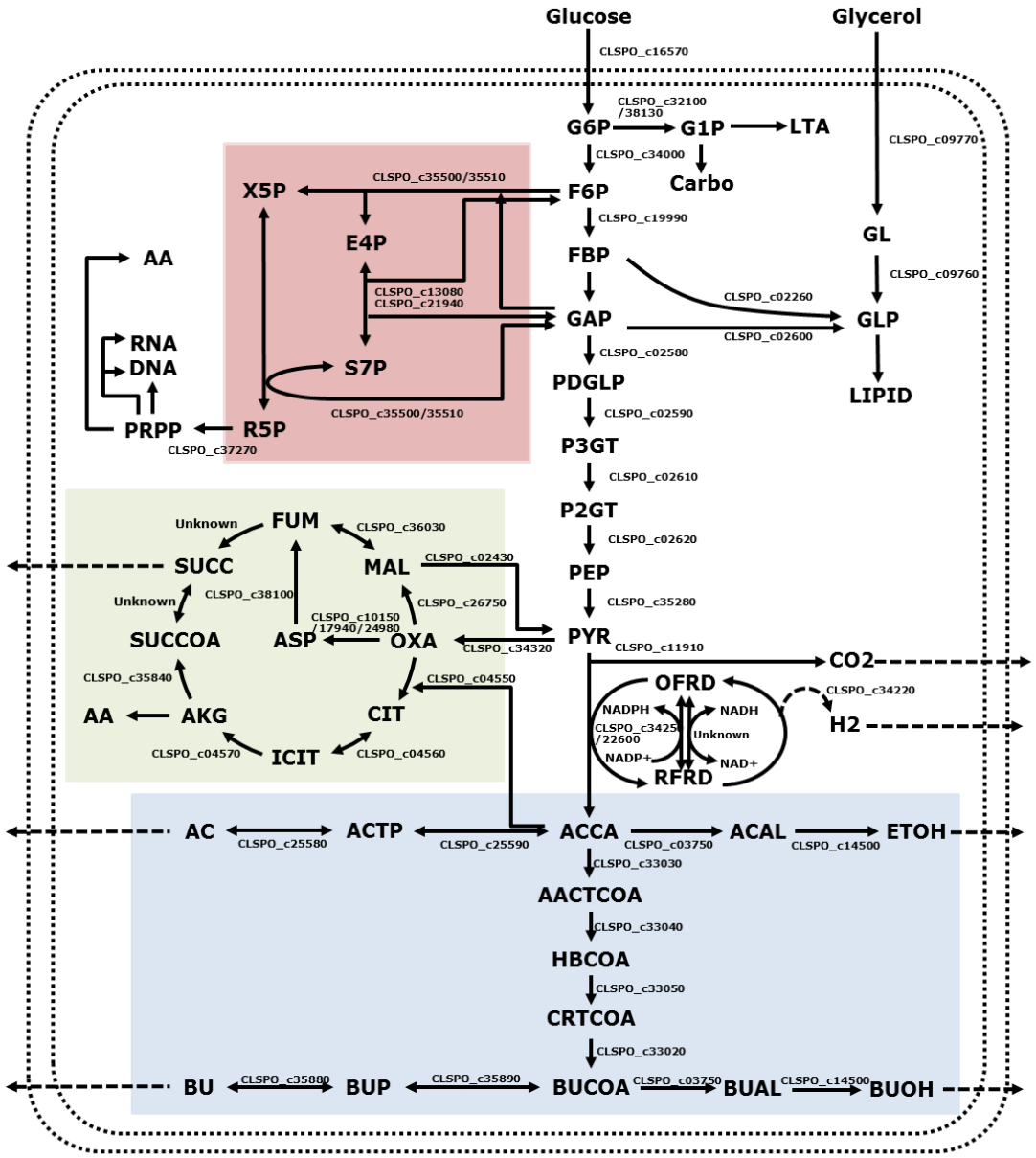


Fig. 6.1 Central metabolic pathway in *C. sporogenes*. Genes whose products were identified are labeled with the corresponding gene locus while genes with no locus are the ones whose products were not identified

Table 6.1 and Table 6.2 enlists all the manually added reactions referred from various reports on other clostridial spp. and identified by BLAST analysis. In the present study, the FBA was performed with glycerol as a sole carbon source or in combination with glucose. Therefore, the pathway for glycerol metabolism was also included in the metabolic network from the annotations given in MetaCyc database. *C. sporogenes*, like other clostridial spp. uses the glycolytic pathway to catabolize glucose conversion to pyruvate which is subsequently converted into acetylCoA and CO_2, a reaction catalyzed by pyruvate: ferredoxin oxidoreductase (Poehlein et al., 2015). This conversion of pyruvate to acetyl-CoA is also linked with H_2 production which is significant in maintaining redox balance in clostridial spp. The enzymes involved with catalyzing the oxidation of ferredoxin coupled with reduction of $NAD(P)^+$ and H_2 production include ferredoxin: NAD(P)H oxidoreductase and hydrogenase respectively (Jones and Woods, 1986). Although ferredoxin: NADH oxidoreductase is not annotated in clostridial genome databases, it is postulated to be a key enzyme in the redox balancing pathway (Yoo et al., 2015). Therefore, this reaction based on work by Gheshlaghi et al. (2009) was considered reversible and incorporated into the model. Enzymatic analyses of *C. pasteurianum*, *C. butyricum* and *C. acetobutylicum* have reported activities for both NADH: ferredoxin oxidoreductase and ferredoxin: NAD reductase which catalyze reduction of ferredoxin by NADH and the reverse reaction *i.e.*, the reduction of NAD by ferredoxin respectively (Gheshlaghi et al., 2009).

Table 6.1 Enzymes added in the amino acid biosynthesis pathways

S. No	Missing EC number	Enzyme name	Reaction	Reference
1	2.7.2.8	Acetylglutamate kinase	ATP + N-Acetyl-L-glutamate <=> ADP + N-Acetyl-L-glutamate 5-phosphate	Dash et al., 2014
2	1.2.1.38	N-acetyl-gamma-glutamyl-phosphate reductase	N-Acetyl-L-glutamate 5-semialdehyde + Orthophosphate + NADP+ <=> N-Acetyl-L-glutamate 5-phosphate + NADPH + H+	Dash et al., 2014
3	2.6.1.11	Acetylornithine transaminase	N-Acetylornithine + 2-Oxoglutarate <=> N-Acetyl-L-glutamate 5-semialdehyde + L-Glutamate	Dash et al., 2014
4	2.3.1.35	Glutamate N-acetyltransferase	N-Acetylornithine + L-Glutamate <=> L-Ornithine + N-Acetyl-L-glutamate	Senger and Papoutsakis, 2008
5	2.7.7.4	Sulfate adenylyltransferase	ATP + Sulfate <=> Diphosphate + Adenylyl sulfate	Dash et al., 2014
6	1.8.99.2	Adenylyl-sulfate reductase	Sulfite + Acceptor + AMP <=> Adenylyl sulfate + Reduced acceptor	Dash et al., 2014
7	2.6.1.17	Succinyldiaminopimelate transaminase	N-Succinyl-LL-2,6-diaminoheptanedioate + 2-Oxoglutarate <=> N-Succinyl-2-L-amino-6-oxoheptanedioate + L-Glutamate	Yoo et al., 2015
8	1.1.1.86	Acetohydroxy acid isomeroreductase	(R)-2,3-Dihydroxy-3-methylbutanoate + NADP+ <=> (S)-2-Acetolactate + NADPH + H+	Dash et al., 2014
9	2.3.3.13	2-isopropylmalate synthase	alpha-Isopropylmalate + CoA <=> Acetyl-CoA + 3-Methyl-2-oxobutanoic acid + H2O	Dash et al., 2014
10	4.2.1.33	3-isopropylmalate dehydratase	alpha-Isopropylmalate <=> 2-Isopropylmaleate + H2O	Dash et al., 2014

11	1.1.1.85	3-isopropylmalate dehydrogenase	4-Methyl-2-oxopentanoate + CO2 <=> (2S)-2-Isopropyl-3-oxosuccinate	Dash et al., 2014
12	4.1.3.27	Anthranilate synthase	Chorismate + L-Glutamine <=> Anthranilate + Pyruvate + L-Glutamate	Dash et al., 2014
13	2.4.2.18	Anthranilate phosphoribosyltransferase	N-(5-Phospho-D-ribosyl)anthranilate + Diphosphate <=> Anthranilate + 5-Phospho-alpha-D-ribose 1-diphosphate	Dash et al., 2014
14	5.3.1.24	Phosphoribosylanthranilate isomerase	N-(5-Phospho-D-ribosyl)anthranilate <=> 1-(2-Carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate	Dash et al., 2014
15	4.1.1.48	Indole-3-glycerol phosphate synthase	1-(2-Carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate <=> Indoleglycerol phosphate + CO2 + H2O	Dash et al., 2014
16	4.2.1.20	Tryptophan synthase	L-Serine + Indole <=> L-Tryptophan + H2O	Dash et al., 2014
17	1.3.1.12	prephenate dehydrogenase	Prephenate + NAD+ <=> 3-(4-Hydroxyphenyl)pyruvate + CO2 + NADH + H+	Dash et al., 2014
18	4.2.1.91	arogenate dehydratase	L-Arogenate <=> L-Phenylalanine + H2O + CO2	Senger and Papoutsakis, 2008

Table 6.2 Enzymes added in the different biosynthesis pathways other than amino acid biosynthesis

S. No	Missing EC no.	Enzyme name	Reaction	Pathway	Reference
1	1.3.5.4	Fumarate reductase	'Fum + RFRD => OFRD + Suc'	TCA	Amador-Noguez et al., 2011
2	2.3.3.1	Putative *Re*-citric synthase	'OXA + ACCA => Cit + CoA'	TCA	Crown et al., 2011; BLAST Analysis
3	6.2.1.5	Succinyl-coenzyme A synthetase	'SCOA + ADP + Pi --> Suc + CoA + ATP'	TCA	Dash et al., 2014
4	2.7.4.6	Nucleoside-diphosphate kinase	ATP + UDP <=> ADP + UTP' CDP + ATP <=> CTP + ADP'	Nucleic acid biosynthesis	Dash et al., 2014
5	2.7.7.27	Glucose-1-phosphate adenylyltransferase	ATP + D-Glucose 1-phosphate <=> Diphosphate + ADP-glucose	Carbohydrate biosynthesis	Muthuraj et al., 2013
6	2.4.1.21	ADP-glucose synthase	ADP-glucose + Amylose <=> ADP + Amylose	Carbohydrate biosynthesis	Muthuraj et al., 2013
7	2.4.1.18	Amylose isomerase	Amylose <=> Starch	Carbohydrate biosynthesis	Muthuraj et al., 2013
8	2.3.1.38	Acetyl coenzyme A-	Acetyl-CoA + Acyl-carrier protein <=> CoA +	Fatty acid	Senger and

		acyl-carrier-protein transacylase	Acetyl-[acyl-carrier protein]	biosynthesis	Papoutsakis, 2008
9	3.1.3.27	phosphatidylglycerophosphatase	Phosphatidylglycerophosphate + H2O <=> Phosphatidylglycerol + Orthophosphate	Fatty acid biosynthesis	BLAST Analysis
10	1.18.1.3	NAD+-ferredoxin reductase	2 Reduced ferredoxin + NAD+ + H+ <=> 2 Oxidized ferredoxin + NADH	Fatty acid degradation	Yoo et al., 2015
11	1.5.1.3	Dihydrofolate reductase	Tetrahydrofolate + NADP+ <=> Dihydrofolate + NADPH + H+	One carbon pool by folate	BLAST Analysis
12	2.7.7.39	glycerol-3-phosphate cytidylyltransferase	CTP + sn-Glycerol 3-phosphate <=> Diphosphate + CDP-glycerol	Glycerophospholipid metabolism	Yoo et al., 2015

A lack of the oxidative phosphorylation pathway, it has been assumed that the main function of NADPH: ferredoxin oxidoreductase is to generate NADPH (Jungermann et al., 1973). Taking this hypothesis into account, along with observation of low specific activity of NADPH: ferredoxin oxidoreductase (Gheshlaghi et al., 2009), the directionality of reactions involved in the oxidation of ferredoxin coupled with reduction of NADP+ was considered irreversible (Fig. 6.1). The solventogenic phase in clostridia encompasses solvent production and acid reutilization, which in case of *C. acetobutylicum* is coupled with acetone formation (Jones and Woods, 1986). The genome of *C. sporogenes* which lacks the sequence encoding for both the enzymes, acetoacetyl-CoA: acetate/butyrate: CoA transferase and acetoacetate decarboxylase, which is able to catalyze acid reutilization and acetone formation respectively (Poehling et al., 2015). However during batch fermentation acid reutilization was observed while acetone formation was not detected. It was hypothesized that in case of *C. sporogenes*, acetic acid and butyric acid reutilization take place only via phosphotransacetylase-acetate kinase (PTA-AK) and phosphotransbutyrylase-butyrate kinase (PTB-BK) mediated pathway respectively. Therefore, the enzymes for the PTA-AK and PTB-BK pathways were considered reversible (Fig. 6.1). The final reactions towards alcoholic products were catalyzed by alcohol dehydrogenase though contrasting results have been put forth regarding its cofactor (NADH or NADPH) requirements (Gheshlaghi et al., 2009). Substrate requirement for NADH generation is equal to that for NADPH formation (Villadsen et al., 2011). Therefore, the overall substrate consumed for alcohol production using either of the cofactors is same. Taking this into consideration and for the purpose of simplification, we have considered in the proposed work a common representative (NADH) for both NADH and NADPH.

The pentose phosphate pathway (PPP) is an important part of clostridial metabolism because it provides essential precursors such as ribose-5-phosphate and erythrose-4-phosphate for nucleotide and amino acid biosynthesis. The production of ribose-5-phosphate can be achieved via oxidative PPP from glucose-6-phosphate (G6P) or nonoxidative PPP from fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (GAP). ^{13}C based studies using *C. acetobutylicum* revealed the absence of PPP oxidative route (Crown et al., 2011). Consistent with above results we also observed the lack of oxidative pentose phosphate pathway enzyme homologues in the *C. sporogenes* genome and hence the oxidative reactions were not included in the metabolic network. The PPP reaction scheme is mediated through the non-oxidative route catalyzed by transketolase and transaldolase enzymes. Clostridial energy generation is mediated through substrate level phosphorylation as these microbes lack electron transport chain. Therefore, the absence of oxidative phosphorylation renders the main objective of the tricarboxylic acid (TCA) cycle as production of precursors for amino acids synthesis. Thus modulation of flux across the TCA cycle is an important indicator of the growth of the organism and how the organism shifts its metabolism during the solventogenic phase of fermentation from growth to alcohol formation. *C. sporogenes* also exhibited a broken TCA cycle, which was evident from the missing annotations for citrate synthase, succinyl-CoA synthetase and the fumarate reductase enzyme. ^{13}C labeling experiments utilizing *C. acetobutylicum* by Amador-Noguez et al. (2010) and Crown et al. (2011) have shown the existence of a bifurcated TCA cycle coupled with succinate secretion. The ortholog (CAC0970) to a *Re*-face stereospecific citrate synthase of *C. kluyveri* was found in *C. acetobutylicum* ATCC 824 (Crown et al., 2011) which catalyzes condensation reaction of oxaloacetate and acetyl-CoA to citrate. A BLAST search of the *C. sporogenes*

NCIMB 10696 genome with the nucleotide sequence of CAC0970 showed 74% similarity suggest the presence of a similar ortholog in *C. sporogenes* (Locus tag CLSPO_c04550). Enzymes catalyzing subsequent TCA cycle reactions are annotated in the genome except succinyl-CoA synthetase and fumarate reductase and therefore, the reactions involving them were added in the model based on literature. Directionality of fumarate reductase catalyzed reaction was considered irreversible (Fig. 6.1) based on the study by Crown et al. (2011). On the contrary, in the current study, succinyl-CoA synthetase was considered reversible (Fig. 6.1) since, the rate of succinate secretion in the fermentation broth was very low. According to Senger and Papoutsakis (2008) and Au et al. (2014), this could be attributed to the reconversion of succinate to succinyl-CoA. The genes encoding CoA transferase catalyzing this reconversion is yet to be identified in clostridial spp. (Au et al., 2014). Beside the TCA cycle, a few more non gene associated reactions belonging to key biosynthetic pathways of amino acids (Table 6.1), nucleic acids, fatty acids and carbohydrates were also included in the network (Table 6.2). Dihydrofolate reductase (DHFR) is another important enzyme in the folate cycle responsible for DNA synthesis but its sequence is not annotated in any of the three databases. The DHFR gene sequence in *C. botulinum* showed a 91% similarity against the genomic DNA of *C. sporogenes* NCIMB 10696. Similar sequence hit was observed for fatty acid synthesis enzyme phosphatidylglycerophosphatase. The key difference between the constructed model and the published models of other *Clostridium* spp. is that this is the first model mimicking BE (Butanol-Ethanol) fermentation. The earlier published models addressed the following processes: ABE (Acetone-Butanol-Ethanol) fermentation in case of *C. acetobutylicum* (Lee et al., 2008; Senger and Papoutsakis, 2008) and *C. beijerinckii* (Milne et al., 2011), ethanol fermentation in case of *C. butyricum* (Cai et

al., 2010), *C. cellulolyticum* (Salimi et al., 2010), *C. thermocellum* (Roberts et al., 2010), and *C. ljungdahlii* (Nagarajan et al., 2013) and 1,3–propanediol fermentation in case of *C. pasteurianum* (Sarma et al., 2017).

6.2.4 Biomass composition

The biomass composition was based on an optimized equation developed for *C. acetobutylicum* (Senger and Papoutsakis, 2008) assumed the structure contained the following constitutents: DNA, RNA, protein, carbohydrate, lipid, peptidoglycan and lipoteichoic acid. The equation for protein, DNA and RNA composition was adopted from Lee et al. (2008). The lipid and peptidoglycan compositions were maintained the same as reported by Dash et al. (2014) for *C. acetobutylicum* with hexadeconic acid selected as the fatty acid representative (Senger and Papoutsakis, 2008). The carbohydrate composition was based on glucose-1-phosphate (G1P) which was assumed similar across the species (Muthuraj et al., 2013) while equation for the cell wall and lipoteichoic acid formation were adopted from McAnulty et al. (2012) and Senger and Papoutsakis (2008), respectively. The details regarding biomass composition of *C. spororgenes* used in the present study is provided in the annexure.

6.2.5 Measureable external fluxes

In order to simulate the microbial growth, certain additional constraints such as maximum nutrient consumption rate and product formation rate were included in the model. The biomass growth was measured in terms of optical density (OD) at 600 nm using UV–visible spectrophotometer (Cary 50, Varian, Australia) as described in Chapter 3 (Section 3.2.5). The OD was converted in to dry cell weight (DCW) using the calibration equation $1\ OD = 0.328\ g\ L^{-1}$ DCW. The substrates, organic acids and solvents were measured using a high performance liquid chromatograph (HPLC, LC-20AD, Shimadzu, Japan) as described in Chapter 3 (Section 3.2.5). Correlation

standard curves were obtained and provided in the Appendix. ATP utilization was considered in the following two forms: Growth associated (GA) and non-growth associated (NGA) maintenance energy. The GA maintenance energy, required during biomass synthesis, was maintained constant as 40 mmol ATP g^{-1} DCW (Lee et al., 2008; Senger and Papoutsakis, 2008). The NGA value for maintenance energy was determined by fitting the model predicted fluxes to experimental data.

6.3 Results and discussion

6.3.1 Substrate dependent modulation of butanol to ethanol ratio and total alcohol titer

In Chapter 5, substrate dependent modulation of butanol to ethanol ratio and total alcohol titer was observed for the strain *C. sporogenes* NCIM 2918 when grown on glucose or glycerol as sole carbon source or in combination with varied glucose-glycerol ratio. Based on these observations, experiments were designed to examine the flexibility of the metabolic network when the organism is grown on different carbon sources. This is particularly important in view of the critical balance of the cofactor ratios such as that of NADH/NAD+, NADPH/NADP+ and Fdred/Fdox, as optimal allocation of these resources eventually ascertains the fate of the final products. To address this objective, batch fermentation studies were performed with samples removed at shorter time intervals to capture the transient changes in the metabolism with the changes in metabolic phase during the fermentation process (Fig. 6.2). When grown on glucose as sole carbon source (Fig. 6.2 A), production of butyric acid (6.9 g L^{-1}) was dominant, followed by butanol (4.6 g L^{-1}) and ethanol (3.2 g L^{-1}). Interestingly, a completely different product distribution pattern was observed when glycerol was used as the sole carbon source (Fig. 6.2 F).

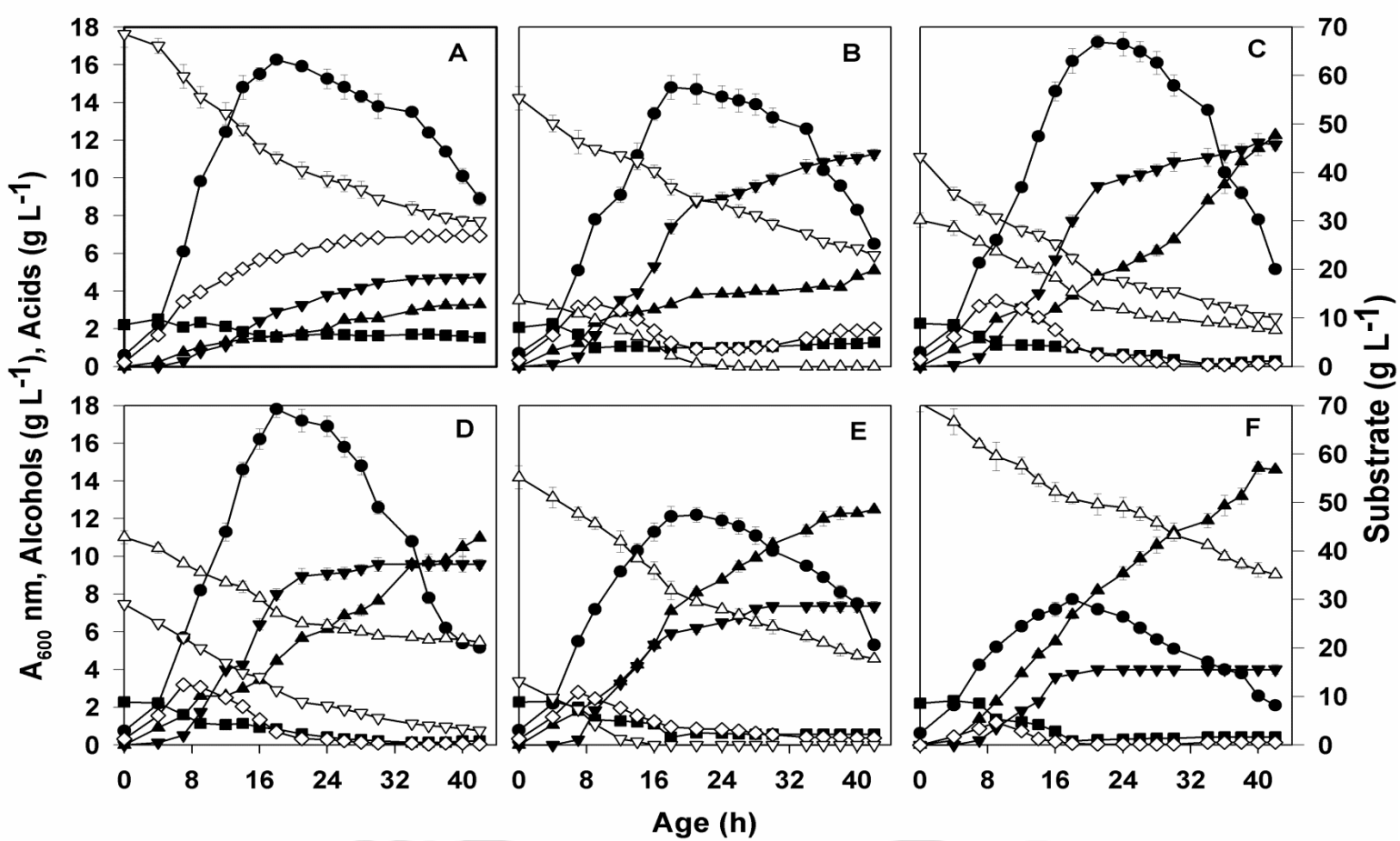


Fig. 6.2 Dynamic profiles for glucose (▽) utilization, glycerol (△) utilization, acetic acid (■), butyric acid (◊) production, butanol (▼) production, ethanol (▲) production and biomass (●) formation by *C. sporogenes* NCIM 2918 under dual substrate batch fermentation using glucose-glycerol blend in a ratio of (A) 100:0, (B) 80:20, (C) 60:40, (D) 40:60, (E) 20:80 and (F) 0:100

Ethanol synthesis was significantly elevated with a titer of 14.7 g L^{-1} followed by butanol (4 g L^{-1}). An important note to consider is that supplementation of glycerol to glucose in different ratios resulted in significant variation of the butanol and ethanol titers. For instance, with the increase in glycerol concentration in the sugar mixture, production of ethanol was upregulated while, the butanol titer increased up to a glucose-glycerol ratio of 60:40 and decreased thereafter (Fig. 6.2 B-E). Further, with change in substrate composition, the total alcohol titer was variable from 7.9 g L^{-1} to 24 g L^{-1}, with a maximum obtained for the glucose to glycerol ratio of 60:40. These results point towards the existence of inherent metabolic versatility and adaptability of the organism in response to the change in substrate composition. In order to understand the underlying regulation governing this metabolic flexibility experiments were conducted by employing flux balance analysis for different glucose-glycerol combinations.

6.3.2 Flux distribution of C. sporogenes NCIM 2918 when grown on glucose, glycerol and glucose-glycerol dual substrate combination

The FBA was performed to determine the distribution of the intracellular carbon flux at 7 h of cultivation on glucose, glycerol and glucose-glycerol mixture (Fig. 6.3 A, 6.4 A and 6.5 A). Among the different glucose-glycerol ratios studied, the highest individual and total alcohol titer was achieved for 60:40 ratio. Therefore, in order to compare the flux distribution under dual substrate to that of single carbon source, a glucose-glycerol ratio of 60:40 was considered. Comparison of the model predicted and the experimentally obtained specific growth rates at 7 h of cultivation

exhibited a similarity of 90.6%, 91.6% and 91.3% for glucose, glycerol and glucose-glycerol (60:40) mixture , respectively (Table 6.3).

Table 6.3 Comparison of model predicted and experimentally determined specific growth rates (h^{-1}) and butanol flux (mmol g^{-1} h^{-1}) for five different glucose-glycerol ratios

Glucose-glycerol ratio	Specific growth rate (h^{-1})[a]		Butanol flux (mmol g^{-1} h^{-1})[b]	
	Experimental	Predicted	Experimental	Predicted
100:0	0.34	0.37	0.64	0.70
80:20	0.33	0.35	3.33	3.59
60:40	0.32	0.35	2.90	3.08
40:60	0.32	0.35	2.03	2.19
20:80	0.31	0.33	1.10	1.20
0:100	0.24	0.25	0.43	0.47

a-Objective function *maximization of biomass* was used for the flux analysis at 7 h of growth

b-Objective function *maximization of butanol* was used for the flux analysis at 18 h of growth

In the case of growth on glucose, the flux distribution in the central metabolic pathway initiated with glucose uptake with the first catabolic flux partitioning taking place at the G6P node. From the G6P node while majority of the incoming flux (98.96%) was directed toward glycolysis pathway (Fig. 6.3 A), the remaining flux was channeled toward G1P, which was further bifurcated into the major cell wall constituent of lipoteichoic acid (LTA) and storage carbohydrates (Muthuraj et al., 2013). Next, the carbon flux partitioning occurring at the F6P node between glycolysis and pentose phosphate pathway (PPP) (Fig. 6.3 A) mostly provided biosynthetic precursors for the synthesis of nucleic acids and amino acids. ^{13}C metabolic flux analysis of *C. acetobutylicum* ATCC 824 showed a similar flux bifurcations at the F6P node (glycolysis—98 ± 0.1% and PPP—0.9 ± 0.1%) for growth on glucose (Au et al., 2014). The flux distribution at 7 h of cultivation on glycerol began with fixing glycerol in the form of glycerone (GL) which was subsequently converted in to glycerone phosphate (GLP) (Fig. 6.4 A).

From the GLP node, the carbon flux was further diverted into the following three branches: glycolysis (via the GAP node), lipid biosynthesis and the gluconeogenesis pathways (Fig. 6.4 A). During glycerol metabolism, of total flux at the pyruvate node (93.8%), 3.66 % was streamed into the TCA cycle while remaining 86.25% towards the acetyl CoA formation node. Further from the acetyl CoA node (ACCA), a maximum flux (48.91%) was diverted for ethanol synthesis, followed by 16.48% towards butyryl CoA formation node. At the butyryl CoA (BUCOA) node, the flux was partitioned between butyric acid (9.87%) and butanol formation (6.6%). For dual substrate combination of glucose and glycerol, the total carbon uptake was divided into 34.61% glucose and 65.40% glycerol (Fig. 6.5 A). Of the total carbon uptake, the glucose flux was distributed between polysaccharide formation (0.66%), PPP (0.94%) and glycolysis (32.51%) while glycerol flux bifurcated into lipid formation (1.30%) and glycolysis (64.10%). The combined flux (128.19%) from both glucose and glycerol uptake coincided at the GAP node which was further streamed in to the glycolysis pathway. Pyruvate metabolism had an input flux of 125.73% which fed the TCA cycle with 5.65% flux through the anapleurotic reaction while 115.11% was diverted to acetyl CoA (ACCA) synthesis. Further, from the ACCA node, a maximum flux (50.08%) was diverted for BUCOA formation node, followed by 23.29% towards ethanol synthesis. At the BUCOA node, the flux was partitioned between butyric acid (38.03%) and butanol formation (12.05%). This carbon partitioning observed at the ACCA and BUCOA node was similar to that observed for glucose. During glucose metabolism, 1.87% and 9.22% of the initial carbon uptake flux was fed into TCA cycle (Fig. 6.5 A) via ACCA and pyruvate (anaplerotic reaction) node respectively.

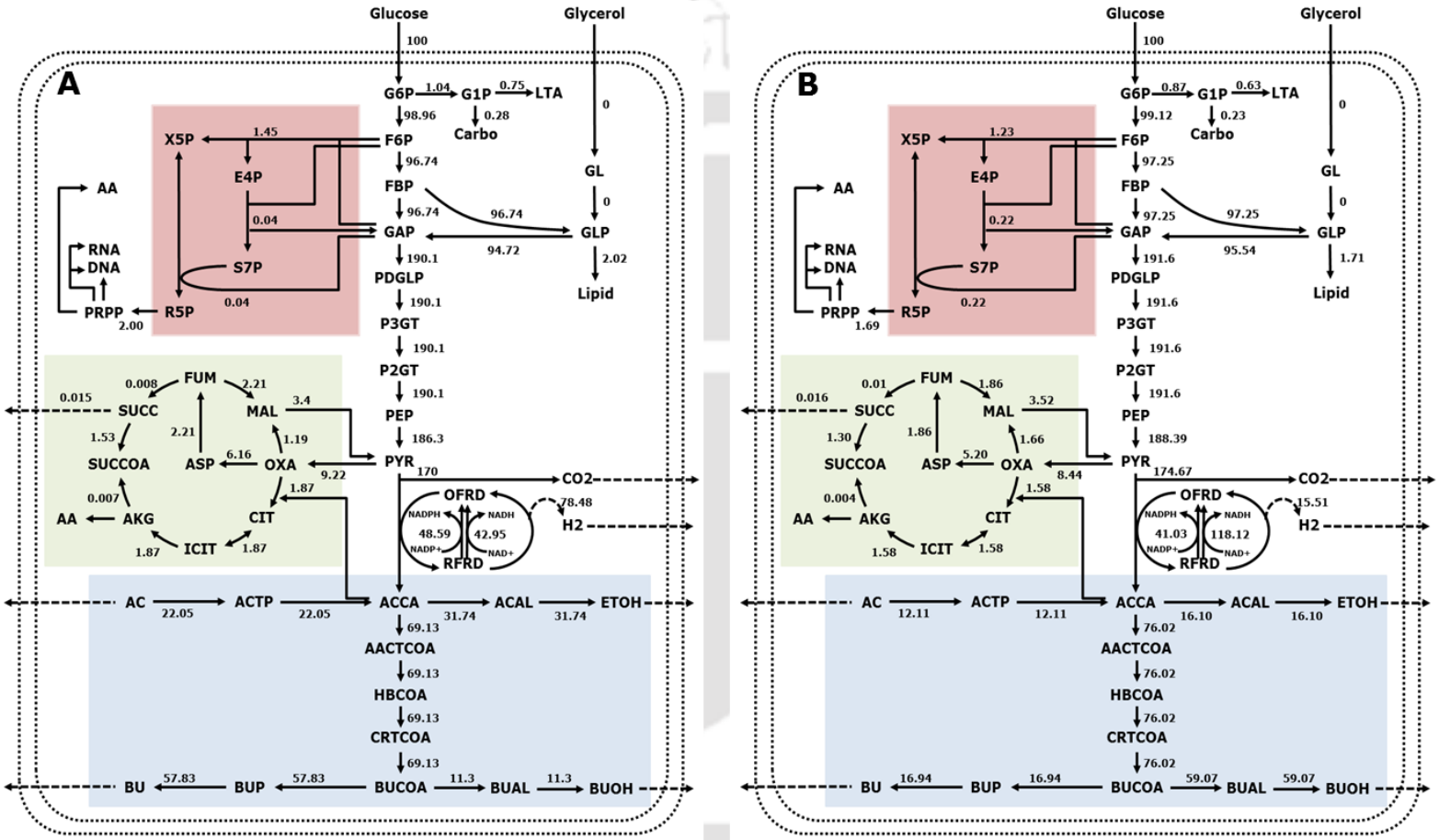


Fig. 6.3 Distribution of carbon fluxes under glucose cultivation (A) at 7 h with maximization of biomass as objective function and (B) at 18 h with maximization of butanol as objective function. All the flux values were normalized to 100 mmol glucose assimilated and were measured in mmol g^{-1} DCW h^{-1}

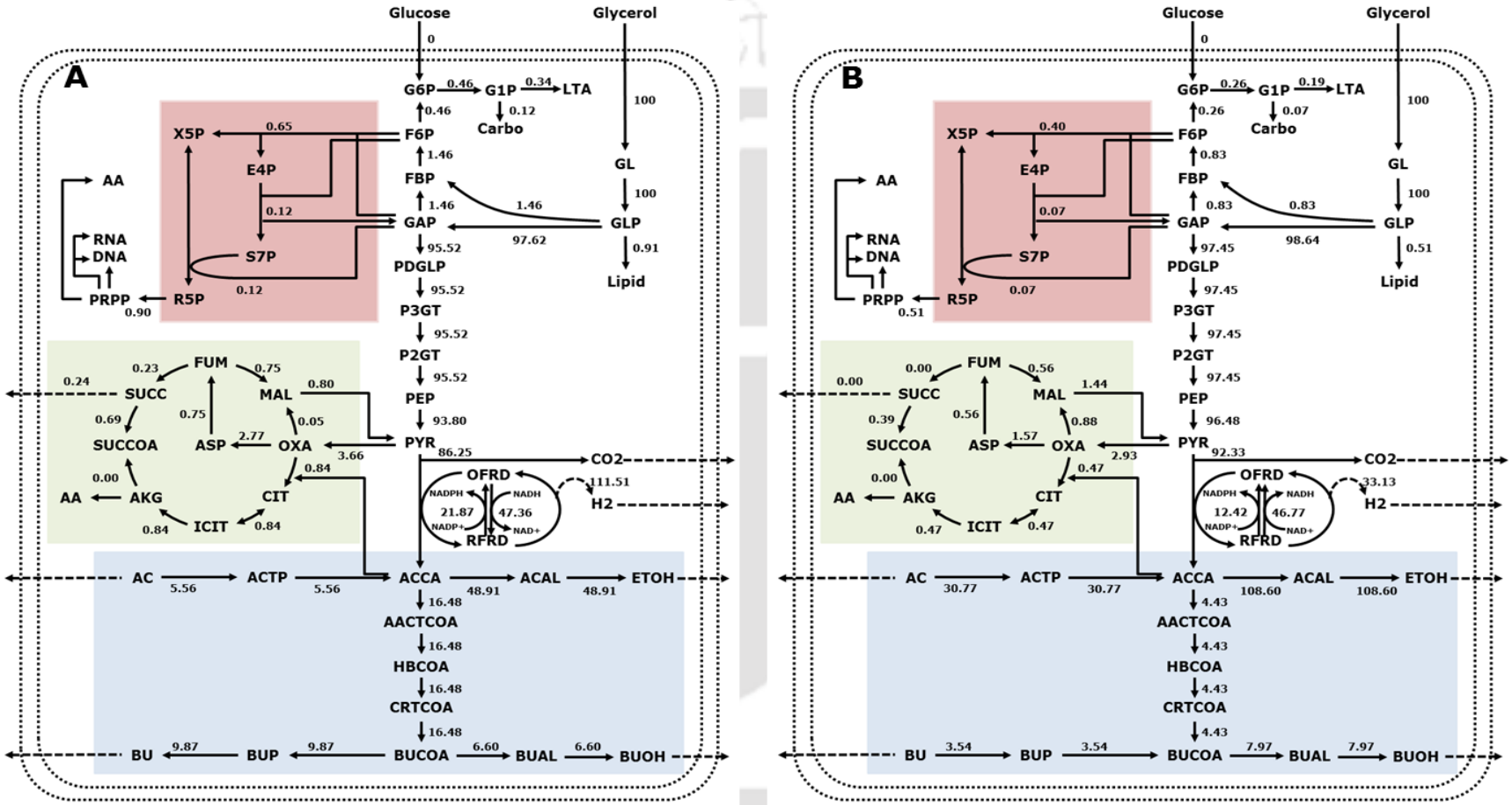


Fig. 6.4 Distribution of carbon fluxes under glycerol cultivation (A) at 7 h with maximization of biomass as objective function and (B) at 18 h with maximization of butanol as objective function. All the flux values were normalized to 100 mmol glycerol assimilated and were measured in mmol g^{-1} DCW h^{-1}

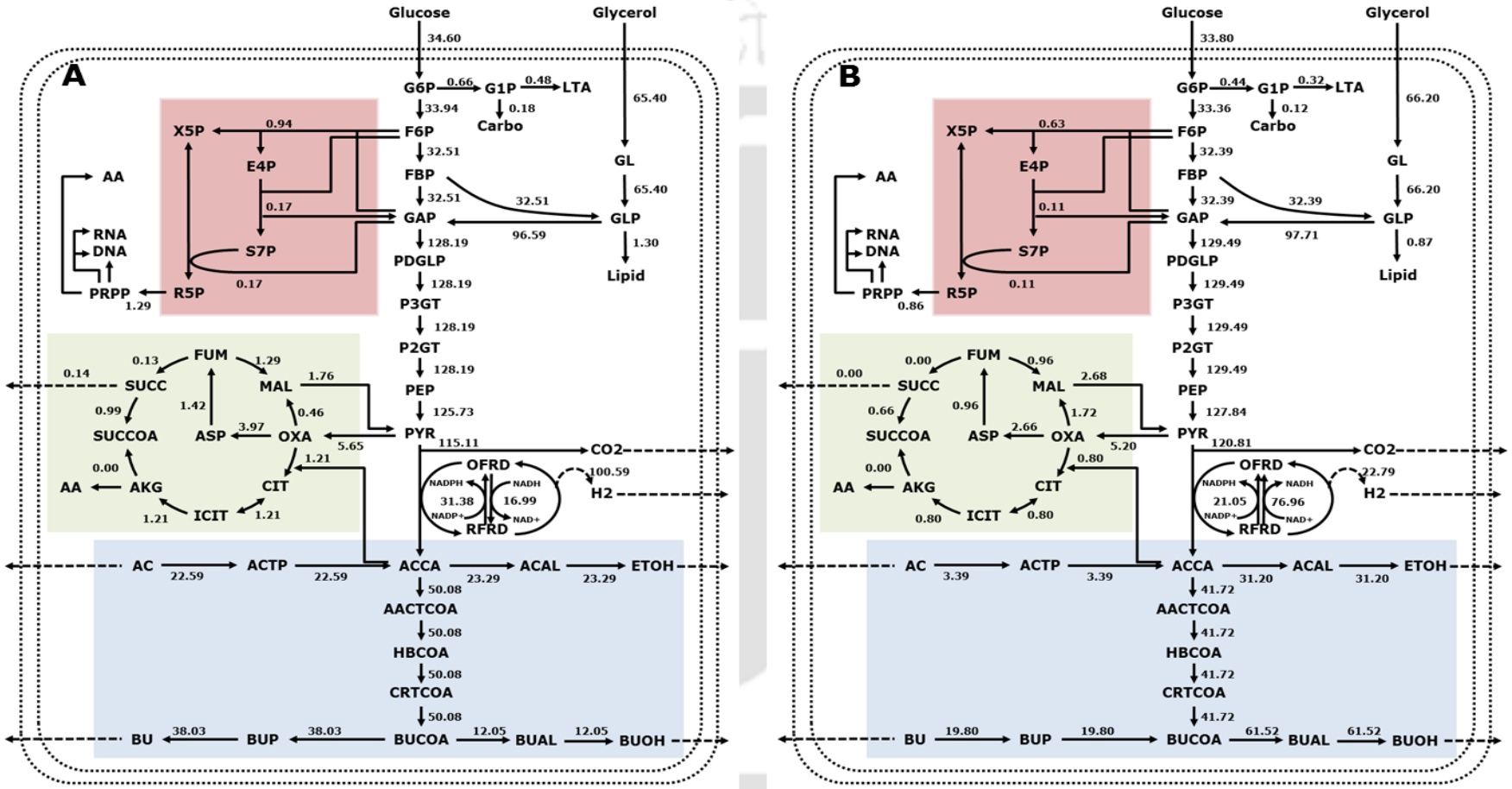


Fig. 6.5 Distribution of carbon fluxes under dual substrate batch fermentation using glucose-glycerol blend in a ratio of 60:40 (A) at 7 h with maximization of biomass as objective function and (B) at 18 h with maximization of butanol as objective function. All the flux values were normalized to 100 mmol total carbon substrate (glucose plus glycerol) assimilated and were measured in mmol g^{-1} DCW h^{-1}

While both oxidative and reductive branch of the TCA cycle was found to be active, they were weakly connected as observed from insignificant flux (≤ 0.01) between α-ketoglutarate and succinyl-CoA or between succinate and fumarate (Fig. 6.3 A). The fate of succinate, whether it was secreted into media or converted into succinyl-CoA, depends upon the succinyl-CoA requirement for growth as part of lysine and methionine biosynthesis (Au et al., 2014). During glucose metabolism, the small flux (1.53%) diversion towards succinyl-CoA and a minimal carbon flux of <0.1% directed towards succinate secretion (Fig. 6.3 A), is in agreement with the work reported by Au et al. (2014). The lack of oxidative phosphorylation (Crown et al., 2010; Amador-Noguez et al., 2010) narrows the role of TCA cycle as producer of precursors of important metabolites (Lee et al., 2016). This is achieved by independently operating oxidative and reductive branches (Au et al., 2014). In the oxidative branch, α-ketoglutarate a key metabolite is involved in the biosynthesis of various amino acids (Nelson and Cox, 1994). The reductive branch operates from oxaloacetate to fumarate via aspartate exclusively as part of arginine biosynthesis (Au et al., 2014). Unlike glucose fermentation the reductive branch of the TCA cycle in case of glycerol was connected with the succinate synthesis pathway with 0.23% flux contributing towards the conversion of fumarate to succinate (Fig. 6.4 A). The TCA cycle flux distribution under glucose-glycerol dual substrate fermentation followed a similar pattern as glycerol metabolism with a flux (>0.1) in all the branches with the exception of a zero flux value for the reaction catalyzing the conversion of α-ketoglutarate to succinyl-CoA (Fig. 6.5 A).

The presence of glucose as sole carbon source or in a dual substrate mixture resulted in improved growth of the organism when compared to glycerol alone. This improved growth is linked to the elevated demand for ATP which in turn is satisfied

by the upregulated carbon flux towards the butyric acid synthesis pathway as an additional source of ATP along with glycolysis (Fig. 6.3 A and 6.5 A, Table 6.4). Interestingly, when grown on glycerol, the organism showed distinct phenotypic response in terms of reduced growth and butyric acid formation (Fig. 6.4 A). In a recent study, the enzyme pyruvate carboxylase was shown to be repressed in *C. pasteurianum* with increasing the glycerol concentration in a dual substrate combination of glucose-glycerol (Sabra et al., 2016). The anaplerotic reaction catalyzed by this enzyme behaves as the supply line of carbon flux from glycolysis to TCA cycle. Therefore, downregulated expression of this enzyme in presence of glycerol results in the downturn of the carbon uptake flux towards TCA cycle which in turn negatively affected microbial growth. In the present study, a similar downregulation (Fig. 6.10 A) in carbon flux towards the anaplerotic reaction was observed in case of glycerol (Fig. 6.4 A and 6.4 B) when compared to glucose (Fig. 6.3 A and 6.4 B) or a mixture of glucose-glycerol (Fig. 6.5 A and 6.5 B). Further, a reduced butyric acid flux in case of glycerol may be attributed to this reduced growth resulting in a lower ATP demand (Table 6.4). In all three substrate combinations, while the ethanol flux was substantial, flux towards butanol formation was minimal in the early phase (7 h) of the fermentation. Addition of acetic acid was reported to alter expression level or cause inactivity of the enzymes responsible for acetic acid production in *C. acetobutylicum* (Kumar et al., 2014). Hence, presence of acetic acid in the form of ammonium acetate in the production media might be the reason for early induction of acetic acid utilization with no flux in acetic acid production for all three substrate combinations (Fig. 6.3 A – 6.5 B and Fig. 6.10 G). Formation of ACCA via utilization of acetate is uncoupled with the formation of NADH unlike the ACCA generated through glycolysis.

Table 6.4 ATP balance for glucose, glucose-glycerol (60:40) ratio and glycerol

Pathways	Glucose[a]		Glucose:Glycerol (60:40)[a]		Glycerol[a]	
	M_μ 7h[b]	M_B 18h[c]	M_μ 7h[b]	M_B 18 h[c]	M_μ 7h[b]	M_B 18 h[c]
ATP balance						
Glycolysis	179.64	182.64	186.84	191.06	189.33	193.60
Glycerol metabolism	0.00	0.00	-65.38	-66.20	-100.00	-100.00
Pyruvate metabolism	-0.96	-1.24	-0.35	-1.18	-0.04	-0.62
Acidogenesis	35.78	4.84	15.44	-23.20	4.31	-34.31
TCA Cycle	-10.76	-9.75	-6.65	-5.87	-4.36	-3.33
Pentose phospate pathway	-2.00	-1.69	-1.29	-0.87	-0.90	-0.51
Nucleic acid metabolism	-18.28	-17.97	-10.79	-11.56	-6.81	-6.56
Amino acid metabolism	-29.32	-25.43	-18.71	-13.55	-12.84	-7.93
Aminosugar metabolism	-0.71	-0.60	-0.46	-0.31	-0.32	-0.18
Carbohydrate metabolism	-0.28	-0.24	-0.18	-0.12	-0.13	-0.07
Fattyacid biosynthesis	-15.06	-12.72	-9.73	-6.53	-6.78	-3.85
Lipid biosynthesis	-0.79	-1.39	-0.25	-1.35	-0.02	-0.74
Maintenance Energy	-0.92	-1.32	-0.42	-1.25	-0.04	-0.62
DNA biosynthesis	0.00	0.00	0.00	0.00	0.00	0.00
RNA biosynthesis	-0.39	-0.33	-0.25	-0.17	-0.17	-0.10
Protein biosynthesis	-42.92	-36.25	-27.72	-18.60	-19.33	-10.98
Peptidoglycan biosynthesis	-0.71	-0.60	-0.46	-0.31	-0.32	-0.18
Biomass	-92.32	-77.97	-59.63	-40.00	-41.57	-23.61

a Cofactor balance obtained for energy yielding and consuming pathways for glucose, glucose-glycerol (60:40) ratio and glycerol. The flux (mmol g-1 h-1) values are expressed per 100 mmol glucose, 100 mmol total carbon substrate (glucose plus glycerol) and 100 mmol glycerol consumed for glucose, glucose-glycerol (60:40) ratio and glycerol respectively. b-Cofactor balance obtained using *maximization of biomass* as objective function at 7 h (Mμ7). c Cofactor balance obtained using *maximization of butanol* as objective function at 18 h (MB18)

Table 6.5 Balance for the cofactors NADPH and NADH for glucose, glucose-glycerol (60:40) ratio and glycerol

Pathways	Glucose[a]		Glucose:Glycerol (60:40)[a]		Glycerol[a]	
	M_μ 7h[b]	M_B 18h[c]	M_μ 7h[b]	M_B 18 h[c]	M_μ 7h[b]	M_B 18 h[c]
NADPH balance						
Aminoacid metabolism	-30.20	-25.50	-19.50	-13.08	-13.60	-7.72
Aminosugar metabolism	-0.24	-0.20	-0.15	-0.10	-0.11	-0.06
Fattyacid biosynthesis	-15.06	-12.72	-9.73	-6.53	-6.78	-3.85
Lipid biosynthesis	-2.02	-1.71	-1.31	-0.88	-0.91	-0.52
Ferredoxin-NADP+ reductase	46.22	39.04	29.86	20.03	20.81	11.82
One carbon pool by folate	1.30	1.10	0.84	0.56	0.59	0.33
NADH balance						
Glycolysis	190.06	191.60	128.19	129.49	95.52	97.46
Glycerol metabolism	0.00	0.00	65.38	66.20	100.00	100.00
Pyruvate metabolism	3.40	3.52	1.76	2.68	0.80	1.45
Acidogenesis	-138.26	-152.05	-100.17	-83.45	-32.97	-8.87
Solventogenesis	-85.78	-150.09	-70.49	-185.32	-110.92	-233.07
TCA Cycle	0.69	-0.08	0.74	-0.91	0.80	-0.40
Nucleic acid metabolism	0.90	0.76	0.58	0.39	0.41	0.23
Amino acid metabolism	1.11	0.94	0.72	0.48	0.50	0.28
Fattyacid biosynthesis	-15.06	-12.72	-9.73	-6.53	-6.78	-3.85
Ferredoxin-NAD+ reductase	42.95	118.12	-17.00	76.96	-47.37	46.77

a Cofactor balance obtained for energy yielding and consuming pathways for glucose, glucose-glycerol (60:40) ratio and glycerol. The flux (mmol g-1 h-1) values are expressed per 100 mmol glucose, 100 mmol total carbon substrate (glucose plus glycerol) and 100 mmol glycerol consumed for glucose, glucose-glycerol (60:40) ratio and glycerol respectively. b-Cofactor balance obtained using *maximization of biomass* as objective function at 7 h (Mμ7). c Cofactor balance obtained using *maximization of butanol* as objective function at 18 h (MB18)

Hence, the organism chose to metabolize this excess ACCA to lesser reduced products such as butyric acid (in high glucose containing blend) or ethanol (in high glycerol containing blend) as compared to butanol (Jones and Woods, 1986). The metabolism of glycerol resulted in producing an excess NADH pool when compared to glucose. Therefore, in case of glucose, the additional requirement of NADH was satisfied through the NAD: ferredoxin oxidoreductase reaction (Fig. 6.3 A, Table 6.5). While, in case of glycerol or glucose-glycerol mixture, NADH: ferredoxin oxidoreductase mediated transfer of electrons from NADH to reduced ferredoxin balanced the surplus NADH generated through glycolysis (Fig. 6.4 A and 6.5 A, Table 6.5). This reduced ferredoxin in turn was used to generate molecular hydrogen by the action of hydrogenase. Whereas, during glucose fermentation, reduced carbon flux through NADH: ferredoxin oxidoreductase explains the decreased hydrogen flux compared to glycerol and glucose-glycerol mixture at 7 h of fermentation (Fig. 6.10 H). This observation is supported by the work reported by Trchounian et al. (2017) where an increase in specific activity of hydrogenase enzyme was reported when *C. beijerinckii* DSM 791 cells were grown on glycerol when compared to glucose, as the sole carbon source.

6.3.3 Changes in flux distribution during metabolic shift from acidogenic phase to solventogenic phase

Changes in the intracellular flux distribution during a metabolic shift from the acidogenic phase to the solventogenic phase was captured by performing the FBA at 18 h of growth and compared with the flux values at 7 h. The model predicted flux towards butanol formation was in reasonable agreement with the corresponding experimental values at 18 h of cultivation (Table 6.3). Unlike *C. acetobutylicum*, which exhibits typical biphasic fermentation, *C. sporogenes* NCIM 2918 showed

mixed mode of fermentation without distinct acidogenic and solventogenic phase. However, this attribute, which differentiates 18 h of cultivation from that of 7 h, was different distribution pattern of carbon flux towards synthesis of alcohols and acids. For instance, while grown on glucose, cultivation at 18 h was marked with significantly elevated carbon flux towards butanol synthesis with concomitant decrease in carbon flux towards production of ethanol, acids and growth when compared to 7 h (Fig. 6.3 A and 6.3 B, Fig. 6.6 A). Similar results were reported by Amador-Noguez et al. (2011) for *C. acetobutylicum* showed that quantitative flux modeling was carried out to understand the transition from acidogenesis to solventogenesis. On the contrary, completely different product distribution pattern was observed during a metabolic shift from 7 h of fermentation to 18 h when grown on glycerol (Fig. 6.4 A and 6.4 B). At 18 h, the organism showed a divergence in carbon flux from growth to ethanol synthesis pathway without any significant difference in the flux for butanol synthesis (Fig. 6.4 A, 6.4 B and 6.6 C). In case of glucose-glycerol mixture, a significant upregulation in alcohol biosynthesis flux was observed at 18 h of fermentation when compared to 7 h (Fig. 6.5 A and 6.5 B and 6.6 B). A striking difference noted during metabolic shift from 7 h to 18 h of cultivation was observed for the glucose fed cultures when compared to glycerol and sugar mixture was the absence of butyric acid reutilization (Fig. 6.10 F). In various *Clostridium* spp. anabolic reactions such as biosynthesis of lipids, amino acids, fatty acids and amino sugars are catalyzed by the NADPH dependent enzymes (Jones and Woods, 1986). In order to fulfill this requirement of NADPH, ferredoxin NADP+ reductase enzyme reaction was highly active during the early phase (7 h) of growth on all substrate combinations (Fig. 6.3 A, 6.4 A, 6.5 A and Table 6.5).

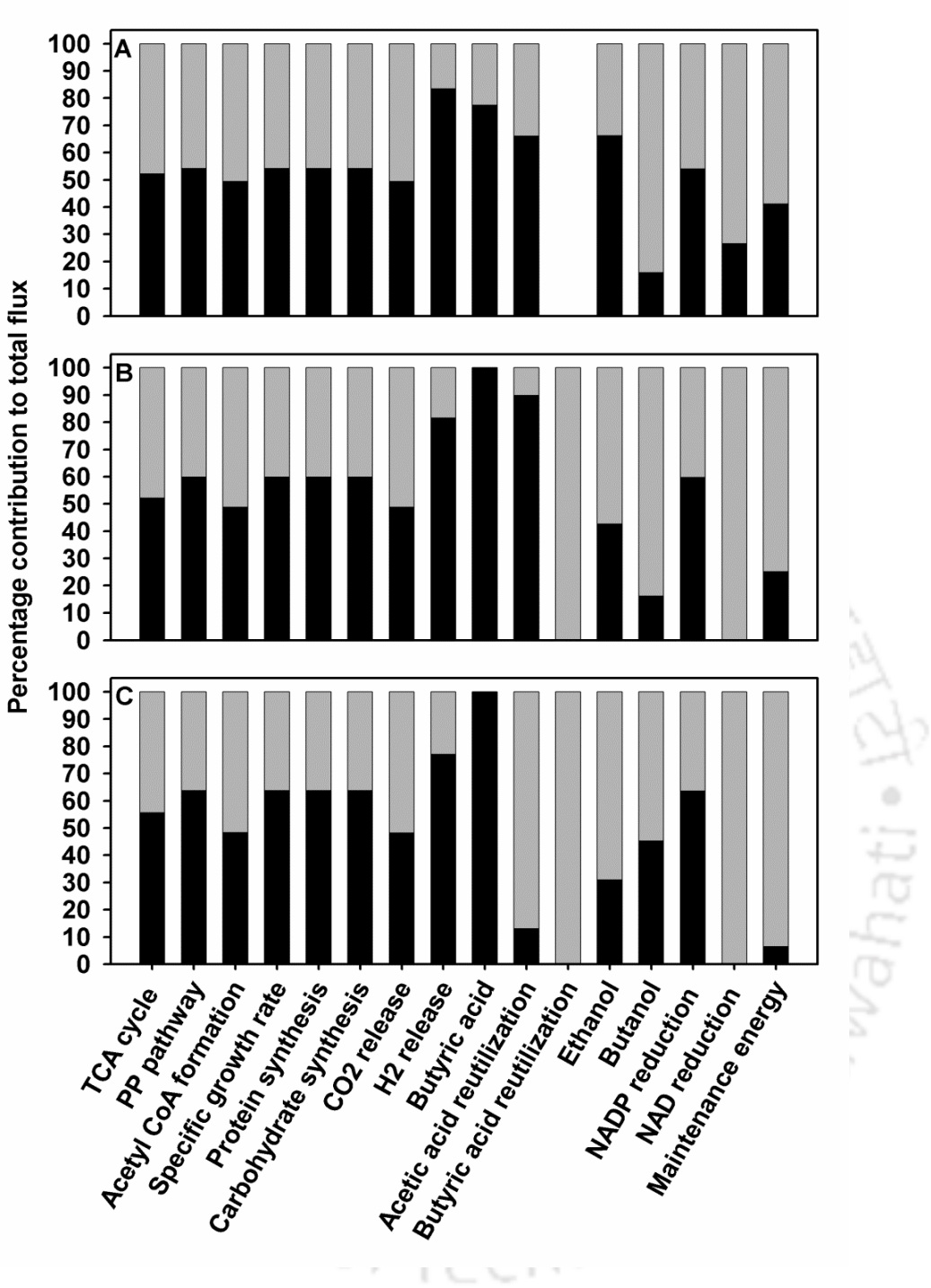


Fig 6.6 Percentage contribution of normalized flux values at 7 and 18 h to total flux (7 h and 18 h cumulative) is shown for (A) glucose, (B) glucose-glycerol (60:40) ratio and (C) glycerol. Black and gray bars indicate flux estimates at 7 h with the objective function maximization of biomass and 18 h with maximization of butanol respectively. The flux (mmol g^{-1} h^{-1}) values were normalized to 100 mmol glucose, 100 mmol total carbon substrate (glucose plus glycerol) and 100 mmol glycerol consumed for glucose, glucose-glycerol (60:40) ratio and glycerol respectively

However, at the latter phase of fermentation (18 h), the flux via this reaction decreased due to reduced growth (Fig. 6.3 B, 6.4 B, 6.5 B and Table 6.5). Conversely,

the substantial increase in the NAD+ reductase flux (Table 6.5) could be due to the higher requirement of NADH towards elevated synthesis of butanol and/or ethanol was mediated by NADH-dependent enzymes in the later phase of the fermentation. At 18 h of fermentation, the decreased hydrogen flux which was observed when compared to 7 h for all feed conditions under consideration (Fig. 6.3 A-6.5 B and Fig. 6.6) could be attributed to increased alcohol formation flux. This increase in alcohol formation behaves as a sink for NADH and hence, eliminates the need of the NADH: ferredoxin oxidoreductase pathway to balance the NADH surplus. An increase in the NGA maintenance energy was observed at 18 h when compared to 7 h of growth for glucose, glycerol and glucose-glycerol (Fig. 6.3 A-6.5 B and Fig. 6.6). This requirement of higher NGA maintenance energy in the later phase of growth may be attributed to the various cellular maintenance operations essential for cell survival and adaptation to butanol stress conditions (Muthuraj et al., 2013).

6.3.4 Flux distribution under different glucose-glycerol ratio in dual substrate cultivation

The flux distribution for the six different glucose-glycerol ratios is shown in Fig 6.3 A-6.5 B and Fig. 6.7 A-6.9 B. In the growth phase (7 h), with an increase in the glycerol fraction in the mixed substrate feed, the linear increase in flux directed towards succinate secretion was coupled with a downfall in butyric acid formation (Fig. 6.5 B). This increase in succinate flux may be attributed to the reduced flux towards the conversion of succinate to succinyl CoA. This in turn was a result of a lower flux in lysine and methionine biosynthesis pathway owing to decreased biomass formation. As mentioned earlier, a reduction in the butyric acid flux (Fig. 6.10 C) with an increase in glycerol concentration may be attributed to this reduced growth resulting in lower ATP requirement.

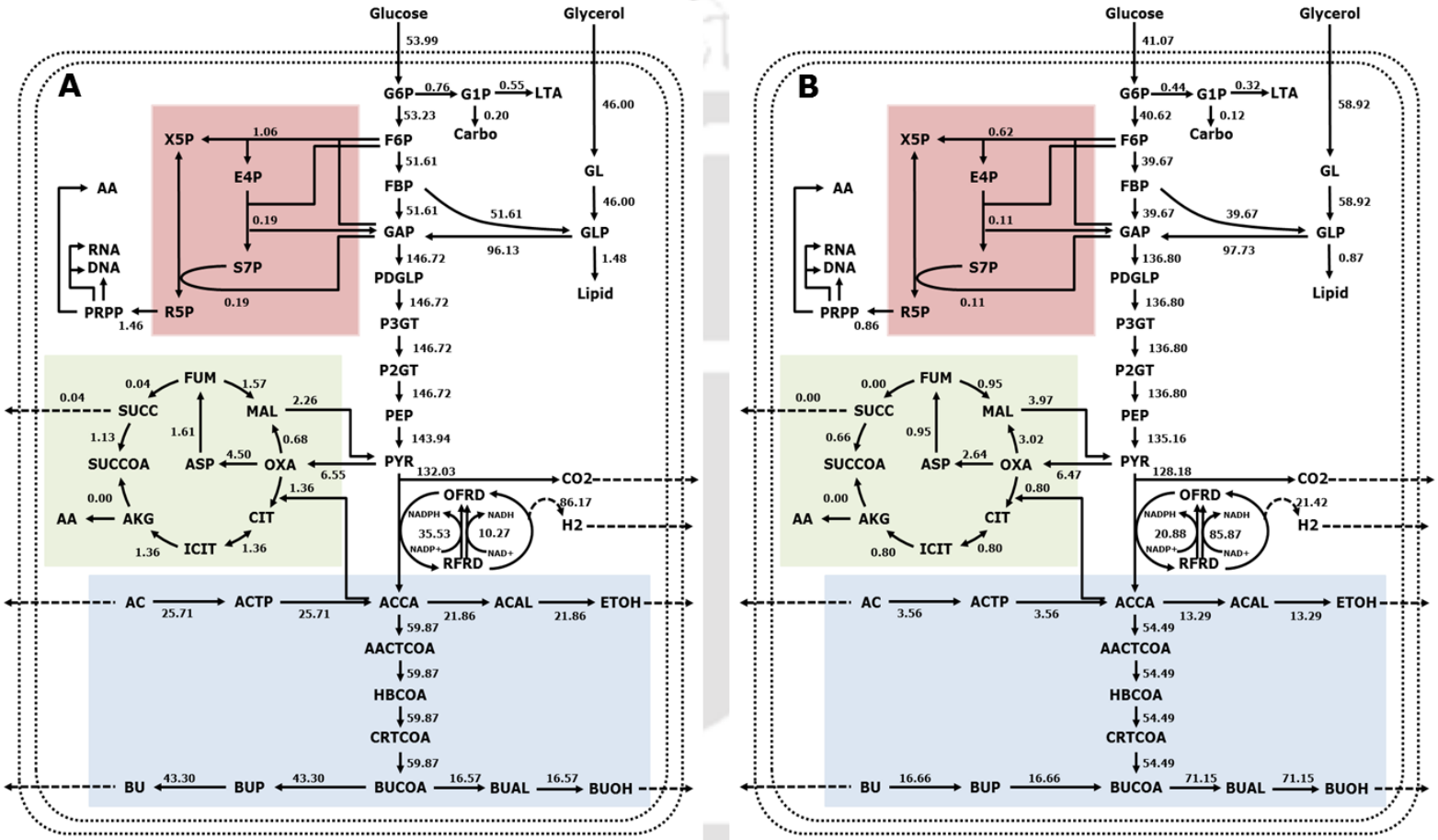


Fig. 6.7 Distribution of carbon fluxes under dual substrate batch fermentation using glucose- glycerol blend in a ratio of 80:20 (A) at 7 h with maximization of biomass as objective function and (B) at 18 h with maximization of biomass as objective function. All the flux values were normalized to 100 mmol total carbon substrate (glucose plus glycerol) assimilated and were measured in mmol g^{-1} DCW h^{-1}

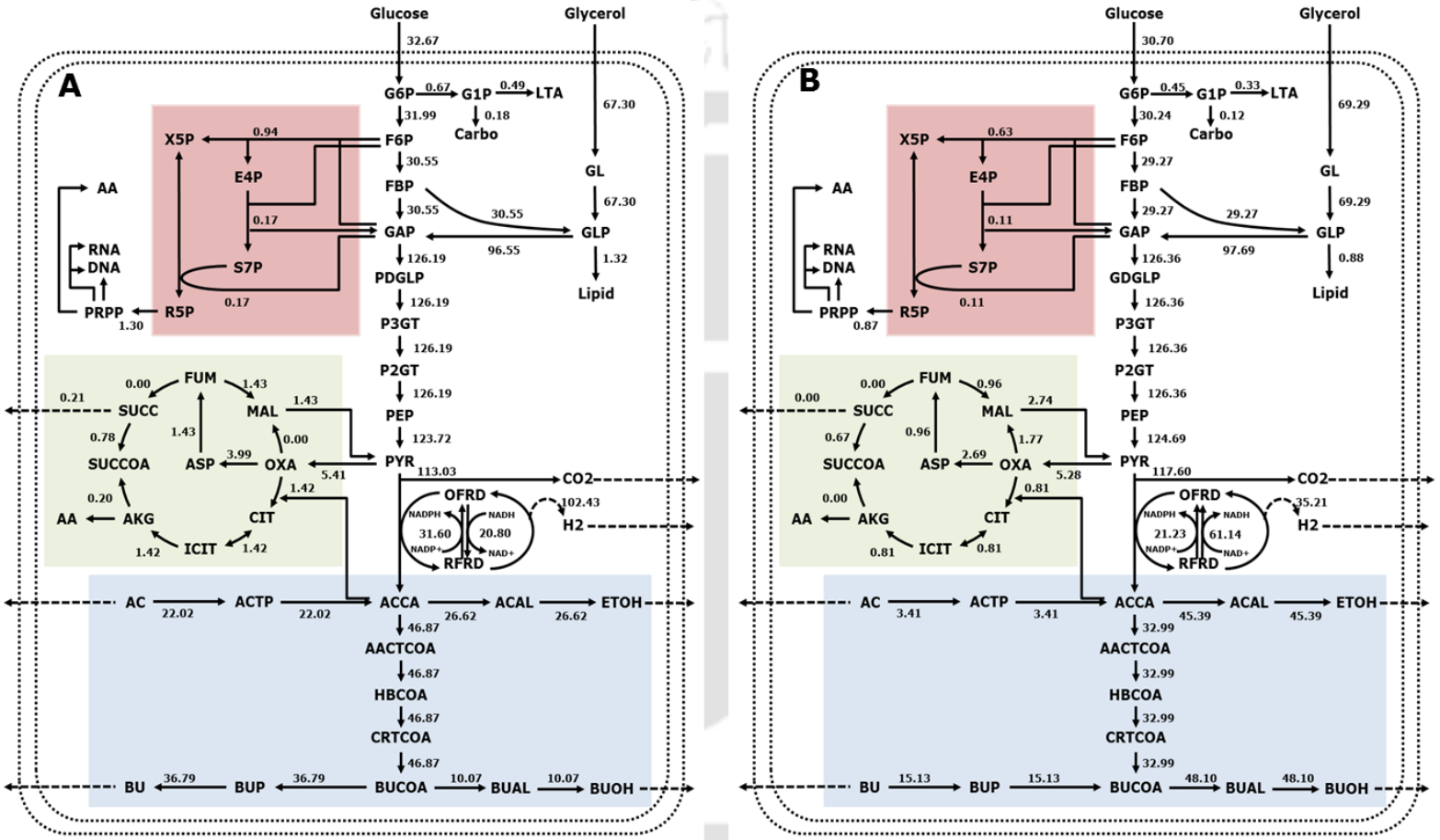


Fig. 6.8 Distribution of carbon fluxes under dual substrate batch fermentation using glucose- glycerol blend in a ratio of 40:60 (A) at 7 h with maximization of biomass as objective function and (B) at 18 h with maximization of biomass as objective function. All the flux values were normalized to 100 mmol total carbon substrate (glucose plus glycerol) assimilated and were measured in mmol g^{-1} DCW h^{-1}

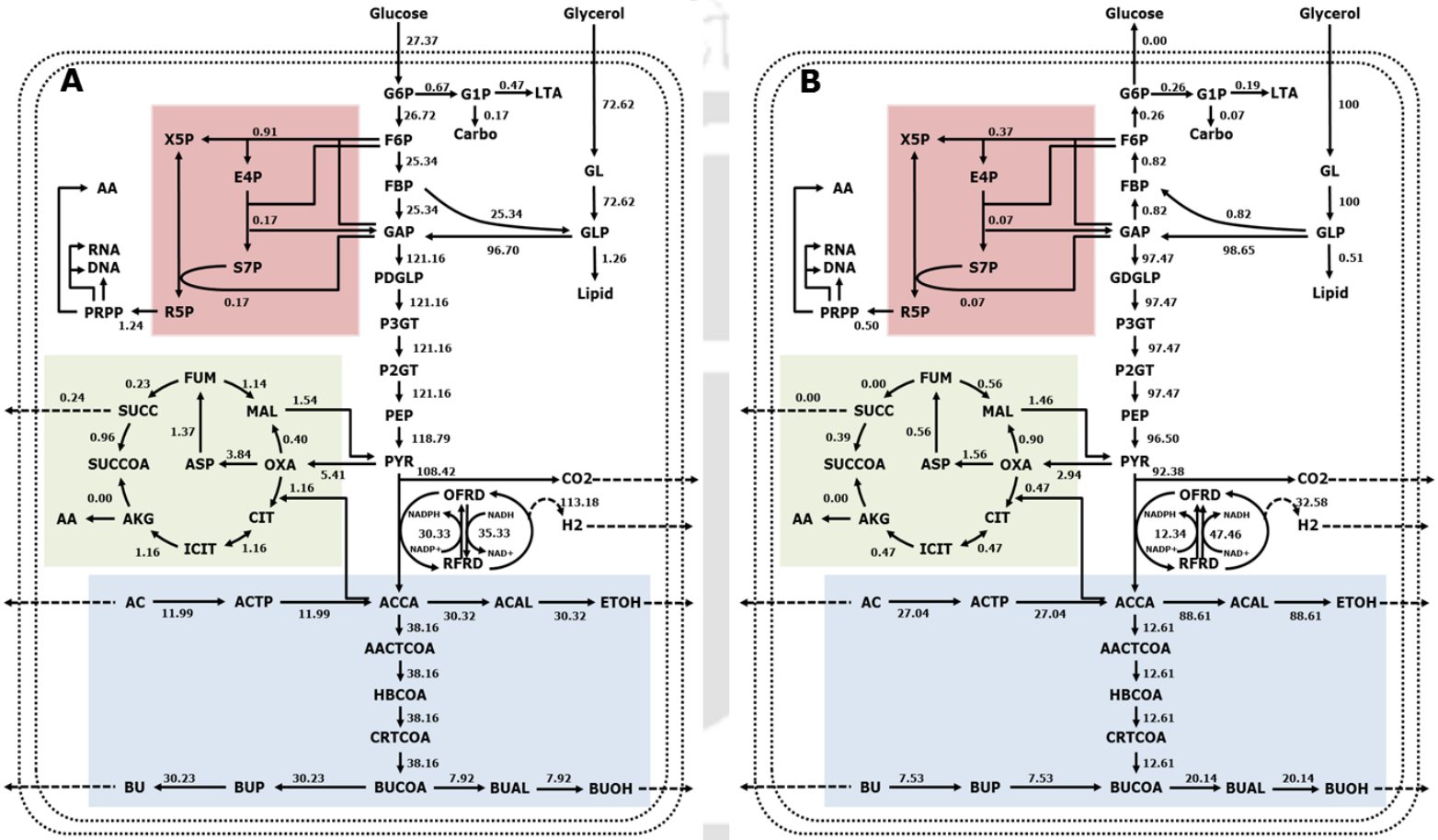


Fig. 6.9 Distribution of carbon fluxes under dual substrate batch fermentation using glucose- glycerol blend in a ratio of 20:80 (A) at 7 h with maximization of biomass as objective function and (B) at 18 h with maximization of biomass as objective function. All the flux values were normalized to 100 mmol total carbon substrate (glucose plus glycerol) assimilated and were measured in mmol g^{-1} DCW h^{-1}

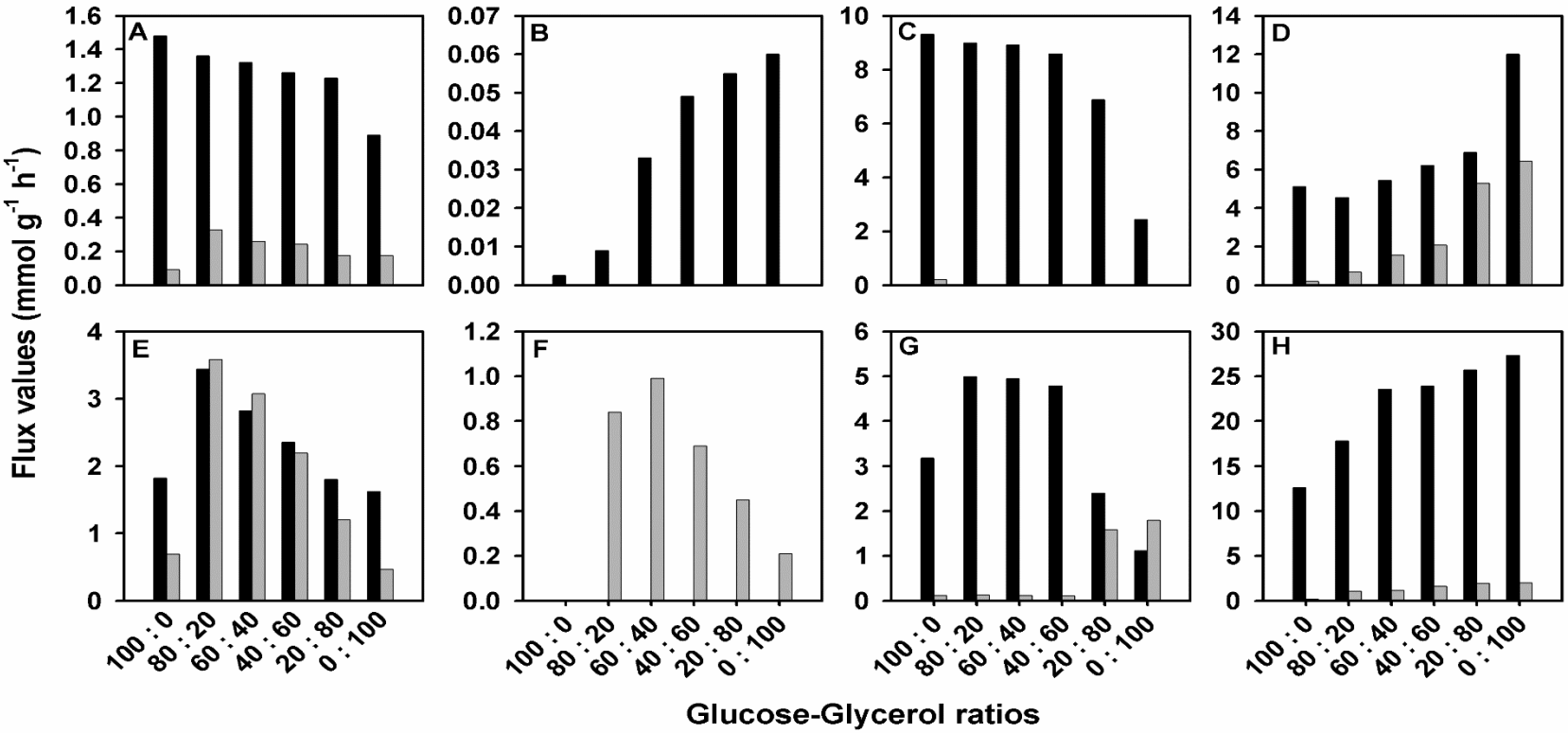


Fig. 6.10 Flux values at 7 h and 18 h of growth for various glucose-glycerol ratios diverted towards different metabolic pathways (A) pyruvate carboxylase (anaplerotic TCA), (B) succinic acid, (C) butyric acid, (D) ethanol (E) butanol, (F) butyric acid reutilization, (G) acetic acid reutilization and (H) hydrogen. Black and gray bars indicate flux estimates at 7 h with the objective function maximization of iomass and 18 h with maximization of butanol, respectively. All the fluxes were normalized with respect to C-mole basis and were measured in mmol g^{-1} DCW h^{-1}

At both 7 h and 18 h of cultivation, with increasing the glycerol contribution to the substrate blend, a linear increment in flux diverted to ethanol synthesis was observed along with a simultaneous decrease in the butanol flux (Fig. 6.10 D –6.10E). A reduction in the butanol flux at 18 h of cultivation was correlated to the decrease in flux towards butyric acid reutilization (Fig. 6.10 E – 6.10 F). Similar the reduction in the butanol flux at 7 h may be explained by the organism's preference to metabolize excess acetyl-CoA as a result of acetic acid utilization (Fig. 6.10 G) to a lesser reduced product such as ethanol (Fig. 6.10 D) and instead of butanol (detail in Section 3.3.2). This is in support of workby Wang et al. (2013) where supplementing butyric acid to the culture medium resulted in improved butanol formation by *C. beijerinckii* NCIMB 8052. On the other hand, Kumar et al. (2014) noted that ethanol producing elementary modes in *C. acetobutylicum* were associated with acetic acid utilization and were independent of butyric acid consumption. Therefore, a decreasing butanol flux can be directly correlated to reduced butyric acid reutilization flux while increasing the ethanol formation flux is a reflection of increased acetic acid consumption. This explains the selective upregulation of ethanol production over butanol during glycerol fermentation.

6.4 Conclusions

The FBA was performed using *C. sporogenes* NCIM 2918 to understand substrate dependent modulation of growth and alcohol biosynthesis while varying carbon source types and their concentrations in a mixture. The elevated ATP demand due to improved growth was satisfied by an upregulated carbon flux towards butyric acid synthesis during glucose and glucose-glycerol fermentations. Possible repression of pyruvate carboxylase resulting in lower carbon flux towards TCA cycle through anaplerotic reaction may be responsible for reduced growth in glycerol fermentation.

The ammonium acetate mediated induction of acetic acid utilization, during acidogenesis, led to excess acetyl-CoA generation and its subsequent metabolism to butyric acid or ethanol.

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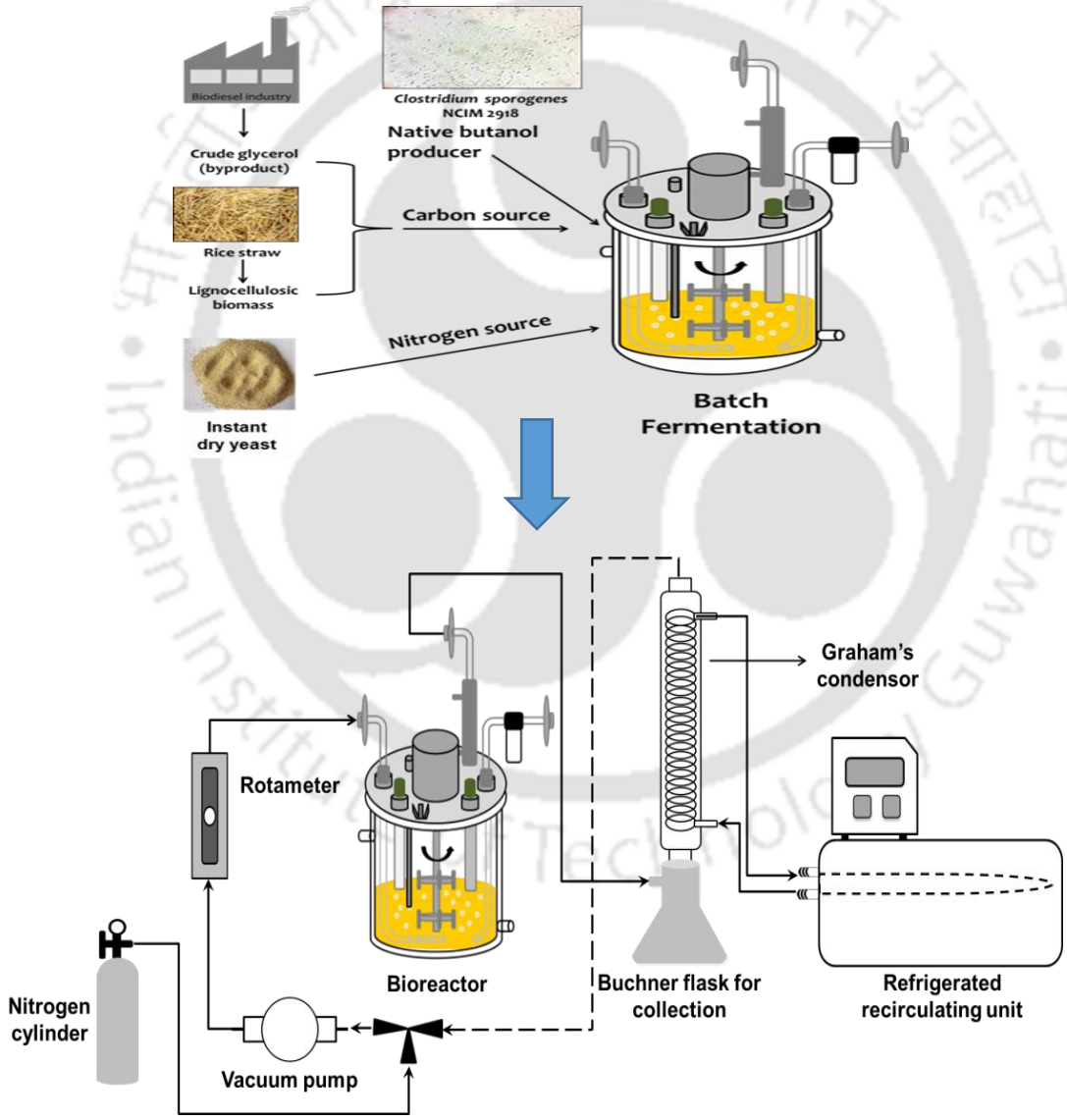
CHAPTER 7

Demonstrate process and scale up for production of sustainable biofuels via utilization of cheaper raw materials e.g. lignocellulosic biomass and glycerol



Schematic diagram for dual substrate fermentation coupled with gas stripping

7.1 Background and motivation

As a response to increasing gasoline price, fuel demand and environmental concerns research towards biological production of fuels such as ethanol and butanol has intensified (Qureshi et al., 2007; Rochon et al., 2017). ABE (Acetone, butanol and ethanol) production by *Clostridium* spp. was the first large scale industrial fermentation process developed during the first part of the previous century (Jones and Woods, 1986). However, due to various reasons such as (1) low butanol selectivity, (2) high cost of substrate, (3) low final product titer and productivity due to solvent toxicity and, (4) high product recovery cost, the bioilogical process was unable to compete with the fossil based process (Rochon et al., 2017; Lu et al., 2012). Conventional ABE fermentation results in production of acetone, butanol and ethanol in a ratio of 6:3:1, respectively (Qureshi et al., 2007; Rochon et al., 2017). Acetone cannot be used as an automotive fuel hence, its production may be considered as an unnecessary carbon sink during the solventogenic phase of clostridial fermentation (Ranganathan et al., 2010). Hence, attempts have been made to develop mutant strains bearing a non-functional acetone forming pathway (Jiang et al., 2009; Tummala et al., 2003) or converting acetone to isopropanol by genetic engineering (Lee et al., 2012). However, due to the instability of the mutant strains over long time periods, new clostridial strains such as *C. sporogenes* which show higher selectivity towards alcohol production are being isolated and characterized (Gottumukkala et al., 2013; Ranganathan et al., 2010).

Further, raw material cost is a key factor determining the process economic feasibility (Lu et al., 2012). Lignocellulosic biomass resources such as agricultural, forest, industrial and wood waste residues represent the largest renewable carbon source available for biofuel production without competing with crops for food supply

(Cai et al., 2016). A number of lignocellulosic materials such as sugarcane bagasse, corn stover, corn cob, rice straw, Napier grass, *Salix schwerinii*, Jerusalem artichoke stalk, eucalyptus and barley straw etc. have been investigated for ABE fermentation (Li et al., 2017; Rajagopalan et al., 2016; Xue et al., 2016; He et al., 2017; Yang et al., 2017; Xue et al., 2017; Zheng et al., 2015; Yang et al., 2015). To further increase economic feasibility of the bioprocess, adoption of bio-refinery concept is recommended (Clomburg et al., 2013; Tracy et al., 2012). One of the possibility for exploring various processes within a bio-refinery is the use of crude glycerol which is generated as a byproduct during both bioethanol and biodiesel production (Clomburg et al., 2013). Clostridial strains have shown the ability to perform multi substrate fermentation (Sabra et al., 2014) and hence, they can be examined by using a combination of cheaper raw materials or wastes. *C. sporogenes* has already shown versatility in its substrate utilization capabilities (Chapter 4, Section 4.3.1). For instance, *C. sporogenes* NCIM 2918 is able to utilize crude glycerol as sole carbon source and in combination with glucose in dual substrate fermentation however with low alcohol productivity (Chapter 5, Section 5.3.3). This increased fermentation duration can be attributed to the various impurities present in crude glycerol. Thus identification of impurities, critically affecting growth and alcohol production, is essential in order to develop targeted purification of crude glycerol (Venkataramanan et al., 2012). This partially purified crude glycerol could have better industrially feasible processing properties for biofuel production.

However, keeping aside raw material cost, another major factor which limits ABE fermentation is the low product titer and productivity, ultimately resulting in a high production and product recovery cost (Jones and Woods, 1986; Lu et al., 2012). One method to improve the ABE fermentation, is to change the fermentation mode

from batch and continuous to fed-batch which offers merits such as, use of concentrated substrates resulting in low water requirement and wastewater generation during fermentation (Lu et al., 2012; Ezeji et al., 2004a,2004b, 2005, 2007; Qureshi and Blaschek, 2001). Another important concern for growing clostridial strains in a chemostat is that this type of cultivation creates a selection pressure on the cells; hence non-sporulating cells which are fast growing are only able to survive. This causes cell degeneration or loss of cells which are solvent producing resulting in poor stability of the fermentation system (Ezeji et al., 2005; Setlhaku et al., 2012). However, due to the solvent toxicity faced by the organism it is not possible to operate a fed batch without coupling it to simultaneous product removal strategy (Ezeji et al., 2004b, 2005). Among the various product removal techniques, gas stripping has a number of advantages i.e. ease of operation, low energy requirement, easy to integrate with fermentation and selective removal of volatile components only without causing any harm to the cells in the fermentation broth (Rochon et al., 2017; Qureshi and Blaschek, 2001; Zheng et al., 2009; Dürre, 1998).

In the current study, three agricultural substrates, rice straw, areca nut husk and sugarcane bagasse were explored along with crude glycerol for biofuel production employing *C. sporogenes* NCIM 2918. Pretreatment and enzyme hydrolysis conditions were optimized for the selected biomass while inhibitor studies were undertaken to partially purify the crude glycerol. Finally, a dual substrate fed-batch reactor utilizing lignocellulosic hydrolysate and partially purified crude glycerol coupled with continuous gas stripping demonstrated a high level of liquid biofuel production. This is the first report of dual substrate waste utilization by *C. sporogenes*, demonstrating the highest alcohol titer in batch mode without product recovery and in fed batch mode with integrated product recovery.

7.2 Materials and methods

7.2.1 Organism, growth and maintenance conditions

C. sporogenes NCIM 2918 was procured from National Collection of Industrial Microorganisms (NCIM), India. Glycerol stocks were prepared and stored at -80°C. Revivals of glycerol stock, seed preparation and maintenance of anaerobic condition were carried out as mentioned in Chapter 3 (Section 3.2.1). For batch fermentation, 6 h old 20% (v/v) seed culture was used as inoculum. Experiments were performed in an optimized medium comprising of (in g L^{-1}) glucose 42, glycerol 28, instant dry yeast 56.6, K_2HPO_4 0.50, KH_2PO_4 0.50, CH_3COONH_4 2.2, para-amino-benzoic acid 0.01, biotin 0.001, $MgSO_4.7H_2O$ 0.22, $MnSO_4.H_2O$ 0.011, $FeSO_4.7H_2O$ 0.011, NaCl 0.011. Lignocellosic hydrolysate, pure and crude glycerol were autoclaved separately and added aseptically in the desired concentration. Batch fermentation was performed at 37°C in static condition and sampling was done at regular intervals for analysis of biomass, substrate utilization and product formation. The initial pH of the production media was kept between 6-6.5, which was left uncontrolled during the fermentation. All the experiments were conducted in triplicates and the values were measured as mean ± standard error. All the chemicals were purchased from Himedia, India unless otherwise mentioned.

7.2.2 Lignocellulosic biomass

The three lignocellulosic biomass, areca nut husk, rice straw and sugarcane bagasse used in this study were collected from a local farm (Amingaon and Karbi-Anglong district, Guwahati, Assam, India). Biomass was cut into small pieces of 5-8 cm, thoroughly washed with tap water and kept to dry overnight in hot air oven at 80 ºC. Further, the biomass samples were reduced to a powdered form using a mixer

grinder and sieved using a mesh of pore size 90 micron (Vinsyst Technologies, India) to obtain a uniform particle size.

7.2.3 Biomass pretreatment

7.2.3.1 Alkaline treatment

Five (5) g of biomass was mixed with 100 mL 2% (w/v) NaOH aqueous solution in a 250 mL conical flask (Saratale and Oh, 2015). The solid-to-liquid loading was maintained at 5% or 1:20 w/v for the initial pretreatment experiments (Fig. 7.1). Cotton plugged flasks were covered with aluminum foil and autoclaved at 121°C for 20 min (Fig. 7.1).

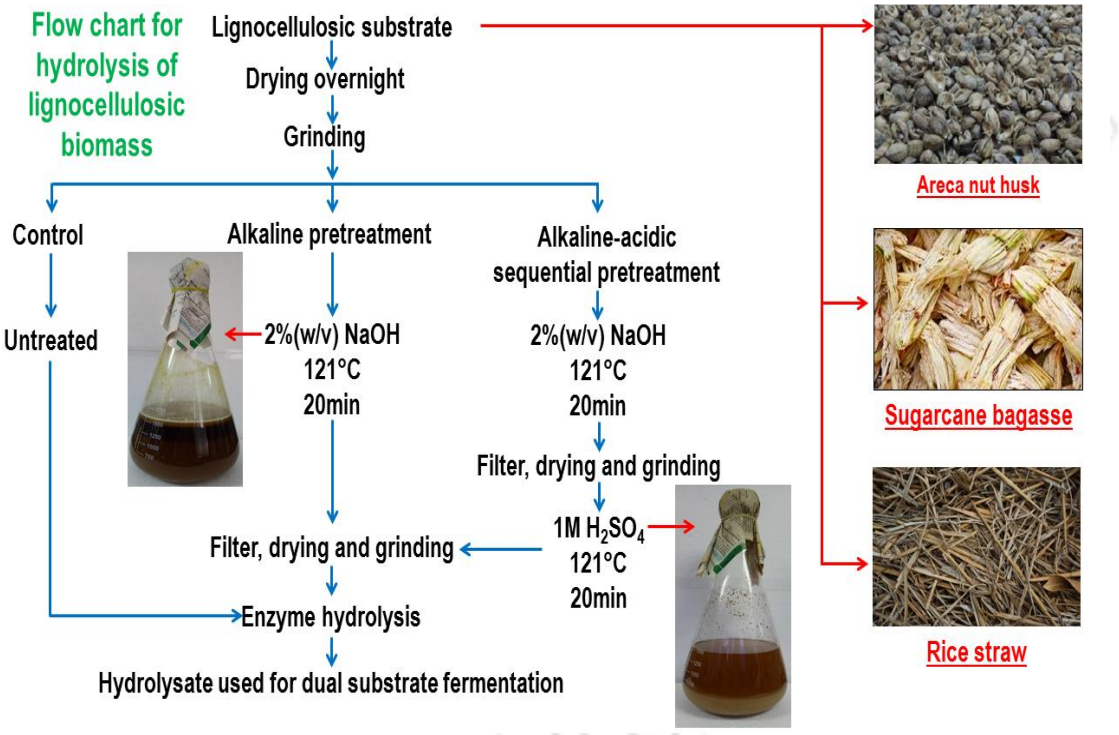


Fig. 7.1 Flowchart showing the various steps undertaken for hydrolysis of biomass

7.2.3.2 Alkaline and acid sequential treatment

Five (5) g of biomass was alkaline pretreated as previously described. After autoclaving, the flasks were cooled down and the biomass was filtered through a muslin cloth. Biomass obtained was dried overnight in a hot air oven at 80 ºC followed by grinding andsieving to achieve a fine powder. 5 g of alkaline pretreated

biomass powder was then mixed with 100 mL of 1M H_2SO_4 solution in 250 mL conical flask (Fig. 7.1). The flasks were plugged with cotton, covered with aluminum foil and autoclaved at 121°C for 20 min (Fig. 7.1).

The autoclaved flasks after both alkaline and alkaline-acidic pretreatment were cooled and the biomass was filtered through a muslin cloth. After drying overnight in a hot air oven at 80ºC, the biomass was further treated by grinding and subsequently sieving. The pretreated biomass powder and untreated biomass powder was used for enzymatic hydrolysis experiments. Untreated biomass was used as a control.

7.2.4 Biomass compositional analysis

7.2.4.1 Quantitative analysis

The determination of lignin, cellulose, and hemicellulose content of the biomass sample was undertaken using methods described by Jung et al. (2015). The biomass samples produced based on methods in Section 7.2.3 were converted to an extractive-free basis by the Soxhlet extraction method. The extractive free sample was then used to estimate the acid insoluble lignin (AIL) and acid soluble lignin (ASL) components according to the modified Klason lignin determination procedure and the UV spectroscopic method, respectively. Further, the extractive free sample was used to estimate the holocellulose content using a delignification procedure followed by estimating the cellulose content. Finally, the hemicellulose content was determined by subtracting the weight of holocellulose from the weight of cellulose.

7.2.4.2 Qualitative analysis

Field emission scanning electron microscope (FESEM)

Morphological characterization of the biomass was performed by employing field emission scanning electron microscopy (FESEM) using a SIGMA Zeiss instrument (Carl Zeiss SMT Ltd., UK). Overnight dried treated and untreated samples

were sprinkled evenly on the carbon tape placed over the surface of sample stub. In order to avoid any charging, the sample was coated with a thin layer of gold. Clear pictures were taken by testing different magnifications.

Thermogravimetric analysis (TGA)

Thermal decomposition of the biomass was carried out using a simultaneous thermal analyser (NETZSCH STA-449F3, Netzsch Ltd. Germany) with a heating rate of 10 ° C per min. Approximately 5–10 mg of treated and untreated sample was heated in an alumina crucible from room temperature to 900 ºC under a nitrogen atmosphere.

Fourier transform infrared spectroscopy (FTIR)

10 mg of treated and untreated samples were mixed with potassium bromide (KBr) in ratio of 1:100 (w/w) and grounded using a mortae and pestle. Pellets with a weight of 200 mg produced from biomass and KBr mixture were used to record an FT-IR spectra using a Fourier transform infrared (FT-IR) instrument (Perkin–Elmer, Spectrum Two) in the range of 400–4000 cm^{-1} at 4-cm^{-1} resolution. KBr pellet without biomass was used as the blank for each FTIR spectrum.

7.2.5 Enzymatic hydrolysis

7.2.5.1 Initial enzyme hydrolysis experiments

Two different cellulase enzymes were used to conduct the enzyme hydrolysis experiments. Cellulase 1 was a generous gift from AUM enzymes (Gujarat, India, 1500 EGU/g) while cellulase 2 was a purchased from Sigma (USA, Celluclast 1.5 L, ≥700 EGU/g). Five (5) g of raw and pretreated biomass powder were mixed with 100 mL of 50mM sodium acetate buffer with a pH of 4.8 in a 250 mL flask. The biomass to liquid loading was kept constant as 5% or 1:20 (w/v) for initial experiments. For each sample, three different experiments were conducted, where cellulase 1, cellulase

2 and cellulase 1 + cellulase 2 blend were added to 3 different flasks each containing buffer and biomass. This was done to screen the most efficient cellulose hydrolyzing enzyme or its blend. The enzymes were added such that total enzyme unit per unit gram of biomass was kept constant to 200 U/g and hydrolysis was performed at 50 ºC in a shaking incubator set at 120 rpm for 96 h. Samples were drawn every 12 h and the supernatant was analyzed for the reducing sugar content using high performance liquid chromatograph (HPLC) as described in Chapter 3 (Section 3.2.5). The pretreatment method and enzyme combination which resulted in maximum glucose release was selected for each of the three biomasses and used for further experiments.

7.2.5.2 Optimization of substrate loading for enzyme hydrolysis

Areca nut husk, rice straw and sugarcane bagasse were pretreated using the best pretreatment method obtained in Section 7.2.5.1, followed by enzyme hydrolysis using the most efficient enzyme combination (Section 7.2.5.1). The optimum biomass loading (g biomass per L of buffer) was obtained by performing enzyme hydrolysis experiments with varied biomass loading (5% to 12.5% w/v) while maintaining the conditions the same as outlined in Section 7.2.5.1. The optimum biomass loading ratio (w/v) obtained for each of the three biomasses were used for further experiments.

7.2.5.3 Dual substrate fermentation using lignocellulosic hydrolysate and pure glycerol

Rice straw, sugarcane bagasse and areca nut husk hydrolysate obtained using the optimized conditions, was concentrated in a rotary vacuum evaporator to produce concentrated sugar hydrolysate. This hydrolysate was used as a substitute for 42 g L^{-1} glucose in the production media discussed in Section 7.2.1. Dual substrate batch fermentation was performed in 100 mL air tight glass bottles as discussed in Section

7.2.1. Biomass samples which resulted in maximum glucose release was selected for further experiments.

7.2.5.4 Maximization of sugar release from hydrolysis via statistical optimization

A central composite design (CCD) based response surface methodology (RSM) was employed to optimize the hydrolysis conditions using three factors: enzyme units (required per unit gram of biomass) for cellulase 1 (X1), cellulase 2 (X2) and hydrolysis pH (X3) as depicted in Table 7.1.

Table 7.1 Actual levels of the selected enzyme hydrolysis parameter

Media components	**Parameter levels used in CCD for optimization**				
X_1 Cellulase 1 (Unit of enzyme/ g of biomass)	7.95	25	50	75	92
X_2 Cellulase 2 (Unit of enzyme/ g of biomass)	32.9	50	75	100	117
X_3 pH	3.89	4.3	4.9	5.5	5.9

A 3 factorial 5 level CCD was formulated (Table 7.3) using MINITAB (Version 16.1.1, Minitab Inc., USA) with maximization of glucose release considered as the objective function. All the experiments were performed in triplicates and mean value of glucose released after hydrolysis was considered as the response. The results were analyzed via considering the linear, quadratic and interaction effects between the selected hydrolysis parameters and glucose release which is mathematically expressed as in Eq. (7.1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (7.1)$$

Where, Y is the model predicted response (glucose release in g L^{-1}), X_i and X_j are the values for hydrolysis parameters (i and j varies from 1 to 3), k is the total number of parameters, β_0 is a constant, β_i, β_{ii} and β_{ij} are the regression coefficients for linear, quadratic and interaction functions respectively. Further, Analysis of variance

(ANOVA) and statistical regression analysis were used to predict the significance of the predicted model parameters. Validation of the optimized hydrolysis conditions was performed by comparing the experimental data with the predicted values.

7.2.6 Effect of impurities in crude glycerol on growth of C. sporogenes NCIM 2918

C. sporogenes has been shown to grow in the presence of crude glycerol as the sole carbon source or in combination with glucose in Chapter 5 (Section 5.3.3). However, the amount of time taken to utilize crude glycerol was much more compared to pure glycerol resulting in low alcohol productivity. This delay could be attributed to the presence of various impurities such as methanol, salts and free fatty acids. The effect of different concentrations of each impurity on the growth of *C. sporogenes* NCIM 2918 was investigated. This was performed by adding various concentrations of each impurity in the dual substrate production media (Section 7.2.1). The concentration of various impurities used for experimentation were taken from literature (Anand and Saxena, 2012; Venkataramanan et al., 2012). Methanol was added at 2.5 to 7.5 g L^{-1} while the salts (KCl and K_2SO_4) were added at 2.5 to 10% (weight of salt/weight of initial substrate concentration). The sodium salts of fatty acids (stearic acid, oleic acid, and linoleic acid) were added at 0.5-1.5 g L^{-1}. Dual substrate media containing pure glycerol without the addition of impurities was used as the control. Fatty acids have the most detrimental effect on the growth of the organism, therefore it was intended to establish if partially purifying the crude glycerol (Anand and Saxena, 2012) could result in similar growth as pure glycerol feed. This was performed by washing of crude glycerol in a 1:1 volume ratio with hexane at 200 rpm and 37ºC for 3 h. The upper phase of hexane and the lower aqueous phase were separated after centrifugation. The lower phase contained the

glycerol plus residual impurities which was subjected to washing again with hexane twice and finally hexane washed partially purified glycerol was obtained.

7.2.7 Dual substrate batch fermentation with and without continuous gas stripping

Rice straw was hydrolyzed using the optimized parameters as described in Section 7.2.5.4. The rice straw hydrolysate was concentrated in a rotary evaporator. The concentrated hydrolysate and hexane washed crude glycerol (Section 7.2.6) were used to replace the pure glucose and glycerol feed in the appropriate concentration in the production medium (Section 7.2.1). Batch fermentation was carried out in a 3.0 L automated bioreactor (Bio Console ADI 1025, Applikon Biotechnology, Holland) with 1.0 L media for 48 h at 37°C. Agitator speed was kept constant at 300 rpm while the initial pH of 6.5 was left uncontrolled post inoculation. Anaerobic condition was maintained by flushing N_2 (99.99%, Assam Air products, Guwahati, India) before and after inoculation. Sampling was done every 6 h and substrates, organic acids and alcohols were measured in high performance liquid chromatograph (HPLC) as mentioned in Chapter 3 (Section 3.2.5). The maximum alcohol producing capability of the organism was restricted to approximately 24 g L^{-1}. This might be attributed to the solvent toxicity faced by the organism, as observed earlier (Section 4.3.4) that the critical butanol tolerance for NCIM 2918 is between 10 to 15 g L^{-1}. Hence to overcome butanol toxicity, another experiment was performed where the batch process was coupled with *in situ* product recovery strategy via gas stripping. Batch fermentation was allowed to proceed as mentioned until the butanol concentration reached approximately 6 -7 g L^{-1}, from where continuous gas stripping was initiated. This was done since the minimum toxic butanol concentration which negatively effects the growth of NCIM 2918 was found to be 5 g L^{-1} (Chapter 4, Section 4.3.4)

and a minimum of 6-7 g L^{-1} butanol is also required for efficient gas stripping (Xue et al., 2012). As shown in the experimental set-up in graphical abstract at the starting of the chapter, reactor exhaust is connected to the inlet of the condenser while the outlet of the condenser was connected to the sparger of the reactor via a vaccum pump. This was done to maintain a continuous closed loop of nitrogen flow between the reactor and the condenser at a rate of 2.5 vvm. 100 % methanol (99.8%, AR grade) was continuously circulated through the condenser using a refrigerated circulatory water bath (WCL-P8, Daihan, Korea) maintained at -10°C. The condensate was collected in a 250 mL flask (kept immersed in ice) attached to the bottom of the condenser.

7.2.8 Dual substrate fed-batch fermentation with continuous gas stripping

Substrate limitation resulted in early termination of the gas stripping coupled batch fermentation process described in Section 7.2.7, hence to overcome this limitation, the fermentation mode was changed from batch to fed-batch. The fermentation was allowed to proceed until the butanol concentration reached approximately 6-7 g L^{-1}, after which continuous gas stripping was initiated. A minimum concentration of 10 g L^{-1} residual glucose and glycerol each was maintained in the fermentation broth by intermittent feeding of concentrated sugar hydrolysate and crude glycerol respectively. When the residual glucose concentration and glycerol in the fermentation broth declined below 10 g L^{-1}, concentrated sugar hydrolysate and crude glycerol were added to reach the initial glucose (42 g L^{-1}) and glycerol (28 g L^{-1}) concentration as used in the start of the fermentation. Gas stripping was continued until 72 h. Sampling and analysis was performed as described in Section 7.2.7.

7.3 Results and discussion

7.3.1 Characterization of the biomass before and after pretreatment

7.3.1.1 Compositional analysis and Field Emission Scanning Electron Microscopy (FESEM)

The composition of untreated and treated biomass is shown in Table 7.2. The first pretreatment method explored in this study is the alkaline method using sodium hydroxide (NaOH). This pretreatment method is well known for lignin removal from lignocellulosic biomass (Kim et al., 2016; Wang et al., 2016). As shown in the Table 7.2, for all the three biomass, alkaline treatment resulted in reduced lignin content in the pre-treated biomass compared with the untreated biomass.

Table 7.2 Cellulose, hemicellulose and lignin content of the untreated and pre-treated biomass

Type of Biomass		Control	NaOH	NaOH-H_2SO_4
Areca nut husk	**Cellulose**	34.2 ±2.6	42 ±4.3	68.8 ±3.4
	Hemicellulose	21.5 ±1.3	18.8 ±0.9	4.8 ±1.2
	Lignin	31.3 ±1.5	21.6 ±0.5	16.9 ±3.2
Sugarcane bagasse	**Cellulose**	34.0 ±2.2	59.2 ±3.9	84.2 ±5.6
	Hemicellulose	28.9 ±2.9	22.4 ±1.2	3.8 ±0.5
	Lignin	22.2 ±1.4	8.6 ±0.6	4.5 ±0.8
Rice straw	**Cellulose**	38.6 ±3.1	64.4 ±3.3	86.8 ±6.3
	Hemicellulose	27.8 ± 1.3	20.4 ±1.8	4.6 ±1.2
	Lignin	21.3 ±1.6	10.4 ±0.8	5.9 ±0.7

*The data in the table is represented as % based on dry weight

Black liquor (BL) which was separated from the pre-treated biomass was tested for the presence of any residual sugars such as glucose or xylose. However, there was no residual sugar present in the liquor showing that the efficiency of alkaline pre-treatment is mainly towards lignin removal (Girio et al., 2010). Similarly Zhu et al. (2010) observed that NaOH treatment of corn stover resulted in reduced

lignin content; however, the cellulose and hemi-cellulose were able to remain intact. Alkaline pretreatment functions by degrading the linkages between lignin monomers and between lignin and the cellulose/hemi-cellulose. Swelling of the biomass resulted in increasing the surface area; increased porosity of the lignocellulosic material and decreasing the cellulose crystallinity (Singh et al., 2015; Kim et al., 2016; Wang et al., 2016). Further, the increased porosity of the cell wall along with lignin removal lead to the formation of irregular pores that are shown in the FESEM images (Fig. 7.2 A-B and Fig. 7.2 D-E) of the treated and untreated biomass samples (Singh et al., 2014; Sant'Anna and de Souza, 2012). However, in case of the alkaline pretreated rice straw sample, the FESEM images showed a highly rough and granular surface; however the pores were not visible as observed in case of the areca nut husk and sugarcane bagasse samples. Lignin binds to the selected amino acids in the enzyme structure and subsequently, blocks the access of the enzymes to cellulose. This eventually leads to reduced enzyme hydrolysis efficiency (Kim et al., 2016; Wang et al., 2016). Hence, lignin removal is desirable for the development of an efficient enzyme hydrolysis process. Further, to remove the hemicellulose component which was unaffected by the alkaline treatment, acid pretreatment using sulphuric acid (H_2SO_4) was applied after the initial alkaline pretreatment. Acid pretreatment degradation of the hemicelluloses was evident from the presence of reducing sugar mainly xylose in the supernatant after acid pretreatment. As shown in Table 7.2, for all the three biomass samples, alkaline-acid pretreatment resulted in reduced hemicellulose content in the pre-treated biomass when compared with the untreated biomass sample. Acid pretreatment mostly causes dissolution of the hemicelluloses while the lignin is redistributed as discrete lignin droplets on the cellulose surface (Singh et al., 2015; Singh et al., 2014) as shown in FESEM pictures (Fig 7.2 C, 7.2 F and 7.2 I).

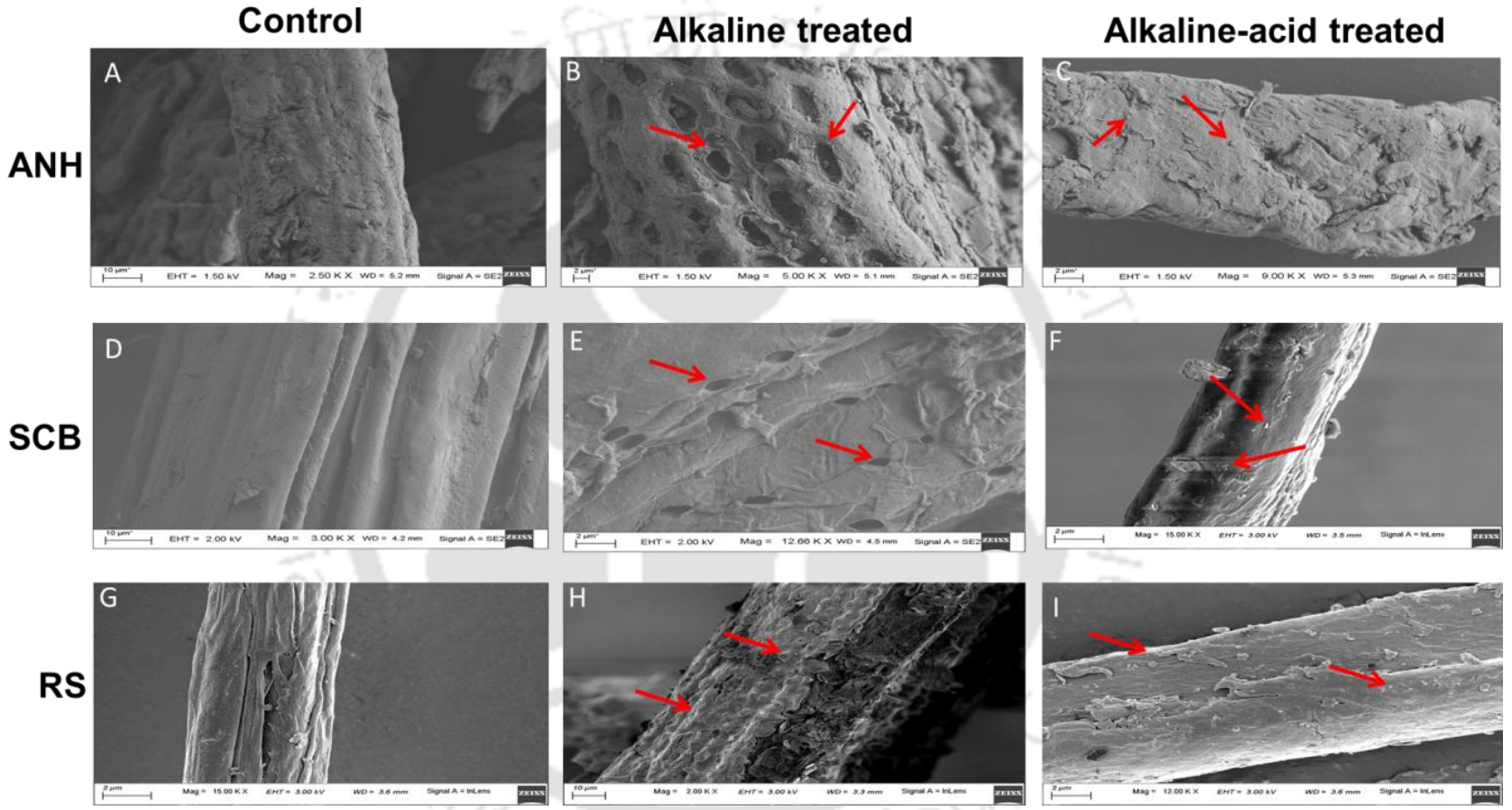


Fig. 7.2 Field Emission Scanning Electron Microscope (FESEM) image of raw and pretreated biomass (SCB- Sugarcane bagasse; ANH- Areca nut husk; RS- Rice straw). Red arrows point towards either the holes or lignin droplets left on the biomass surface after pretreatment

7.3.1.2 Thermo gravimetric analysis (TGA)

Thermogravimetric analysis profiles for the three lignocellulosic biomass samples before and after pre-treatment are shown in Fig. 7.3. Mass loss as function of temperature when the three biomass samples were heated at a constant temperature of 10ºC/min is shown in Fig. 7.3. The TGA curve can be divided into 3 major regions. The first region exists below 200ºC and it corresponds to the mass loss due to removal of moisture and some volatile components (Chen and Kuo, 2010; Varma and Mondal, 2016). As can be seen in Fig. 7.3, only a ~ 10% decrease in mass was observed in this zone for the treated and untreated biomass samples. In the second region, major mass loss occured from 200ºC to 450ºC. This region can further be divided into 2 subsections, 200ºC to 335ºC and 335ºC to 450ºC. The initial subsection (200ºC to 335ºC) refers to the degradation of hemicellulose and cellulose both while second subsection (335ºC to 450ºC) shows the degradation of cellulose (Varma and Mondal, 2016). As can be seen in Fig. 7.3 more than 50% mass loss is observed for all three biomass in the second region (200ºC-450ºC). However, in comparison to the pre-treated biomass, a higher percentage of mass loss is observed for the untreated biomass showing that the pre-treated biomass has a lower cellulose, hemicellulose and lignin content for thermal degradation to take place (Fig. 7.3). The third region beginning from 450ºC extends up to 800ºC corresponds to slow degradation of lignin and other heavy components (Chen and Kuo, 2010; Varma and Mondal, 2016). Mass loss is not significant in the third region and most of the sample left is in form of a biochar.

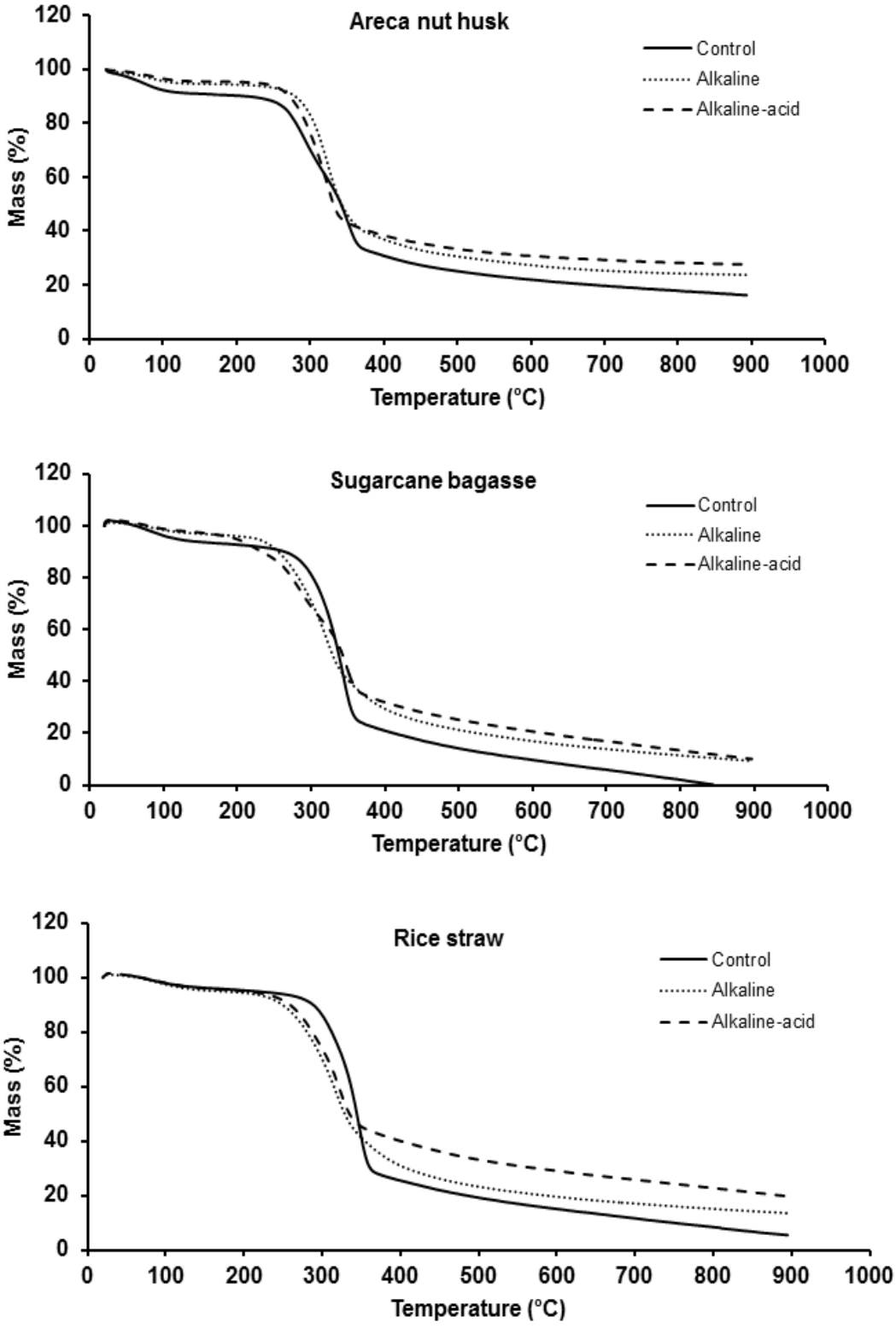


Fig. 7.3 Thermo gravimetric analysis (TGA) of treated and untreated areca nut husk, sugarcane bagasse and rice straw

7.3.1.3 Fourier Transform infrared spectrophotometry (FTIR)

FTIR data for untreated and pretreated biomass are shown in Fig. 7.4. A significant difference was observed in the transmittance and shape of FTIR curve between the untreated and pretreated biomass. These differences point towards the altered composition of the biomass after pretreatment. The transmittance of bands especially in range from 4000 to 3000 cm^{-1} and 1800 to 800 cm^{-1} is modulated by varied composition of biomass (cellulose, hemicellulose and lignin content) or by changes in the cellulose, hemicellulose and lignin structure (Swain and Krishnan, 2015). Different states of the lignocellulosic material such as crystalline and amorphous modulates the transmittance observed at different wavenumber such as at 3300 cm^{-1}, 2900 cm^{-1}, 1430 cm^{-1}, 1375 cm^{-1} and 900 cm^{-1} (Oh et al., 2005; Swain and Krishnan, 2015). The band ranging between 3550–3310 cm^{-1} depicts the O–H stretching caused by the vibration of hydroxyl group linked to cellulose and lignin structure (Kim et al., 2014) while bands near 2900 cm^{-1} show the C-H stretching vibration of the CH_2 and CH_3 groups from cellulose, lignin and hemicellulose (Varma and Mondal, 2016). Bands at near 3300 cm^{-1} and 2900 cm^{-1} are most intense in case of alkaline-acid pre-treated biomass followed by alkaline pre-treated biomass and untreated biomass (Fig. 7.4). The bands near 1059 cm^{-1} is assigned to interation between carbohydrate–lignin structures. These bands are more intense for the pre-treated biomass compared to the untreated biomass sample (Fig. 7.4). Hence, this qualitative analysis depicts substantial compositional and structural changes in the biomass after pre-treatment leads to improved enzymatic hydrolysis of complex lignocellulosic material into simple reducing sugar.

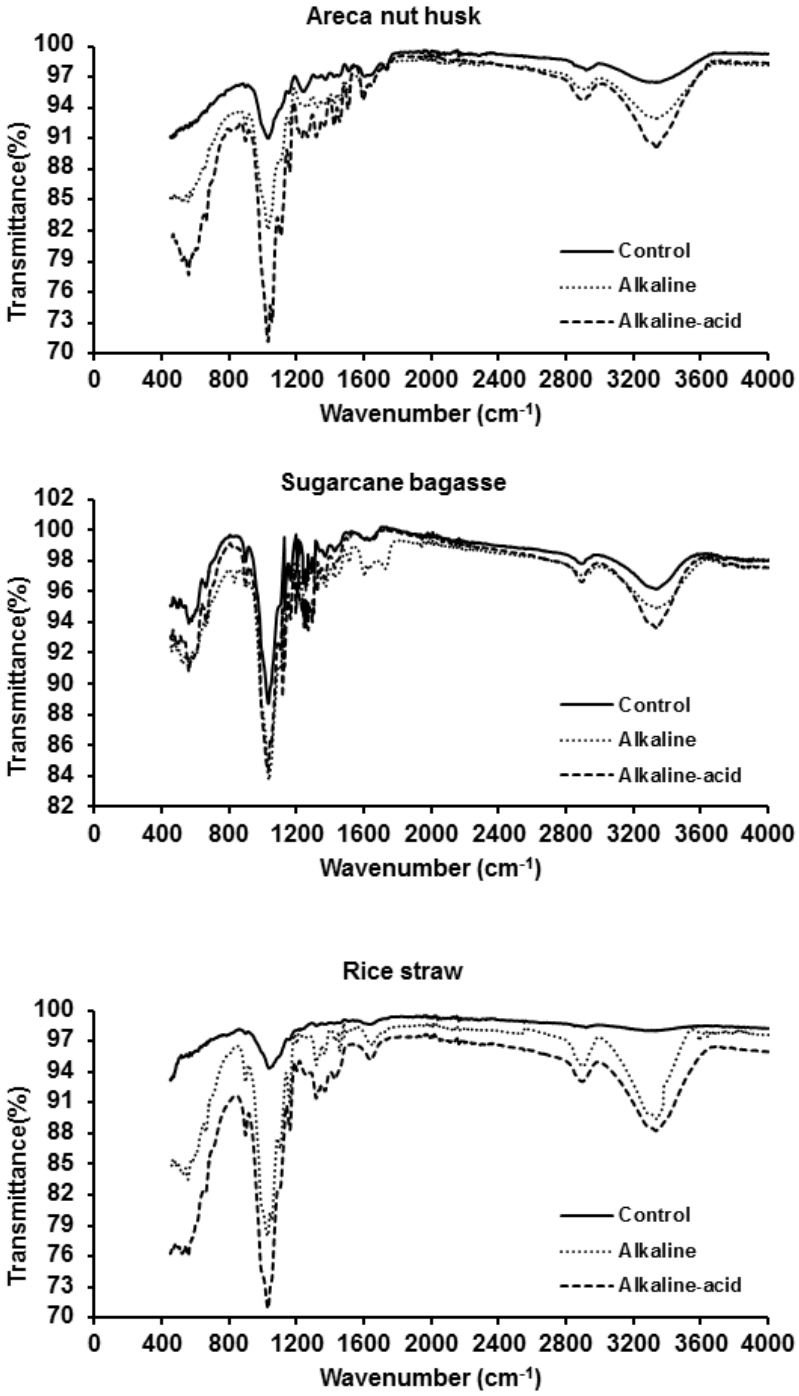


Fig. 7.4 Fourier Transform infrared spectrophotometry (FTIR) analysis of treated and untreated areca nut husk, sugarcane bagasse and rice straw

7.3.2 Enzymatic hydrolysis

7.3.2.1 Initial enzyme hydrolysis experiments

Untreated and pretreated (alkaline and alkaline-acid) areca nut husk, sugarcane bagasse and rice straw were used for the initial enzyme hydrolysis experiments. Each sample was hydrolyzed using 3 different enzyme formulations C1, C2 and C1+C2 using conditions as mentioned in Section 7.2.5.1. The reducing sugar concentrations in the hydrolysate are shown in Fig. 7.5. As evident from Fig. 7.5, untreated biomass resulted in minimum sugar hydrolysis stressing on the importance of biomass pretreatment. Sequential alkaline-acid pretreatment was more effective when compared to only alkaline pretreatment for the hydrolysis of maximum reducing sugar from all the three biomass samples. This could be attributed to the presence of hemicellulose in the alkaline pretreated biomass sample although lignin removal takes place through alkaline pretreatment. The hemicellulose fraction interferes in enzyme hydrolysis by forming a physical barrier around the cellulose; hence, the desire is to recover these sugars separately (Öhgren et al., 2007). Removing the two protective covering (lignin and hemicellulose) over cellulose through sequential alkaline-acid pretreatment results in an improved sugar yield. Alkaline-acid pretreated areca nut husk hydrolysis using the C1+C2 enzyme blend resulted in producing a mixture containing 14.3 g L^{-1} glucose, 1.1 g L^{-1} xylose and 0.98 g L^{-1} cellobiose (Fig. 7.5). Further alkaline-acid pretreated sugarcane bagasse hydrolysis using the C1+C2 enzyme blend resulted in 26.8 g L^{-1} glucose, 2.4 g L^{-1} xylose and 2.3 g L^{-1} cellobiose (Fig. 7.5). A hydrolysate containing maximum sugar levels of 33.8 g L^{-1} glucose, 1.5 g L^{-1} xylose and 1.7 g L^{-1} cellobiose was attained by hydrolyzing alkaline-acid pretreated rice straw using the C1+C2 enzyme blend (Fig. 7.5). For all the three biomass samples, a sequential alkaline-acidic pretreatment step followed by using

C1+C2 enzyme blend, was the most effective (Fig. 7.5). This treatment configuration was selected for further experiments.

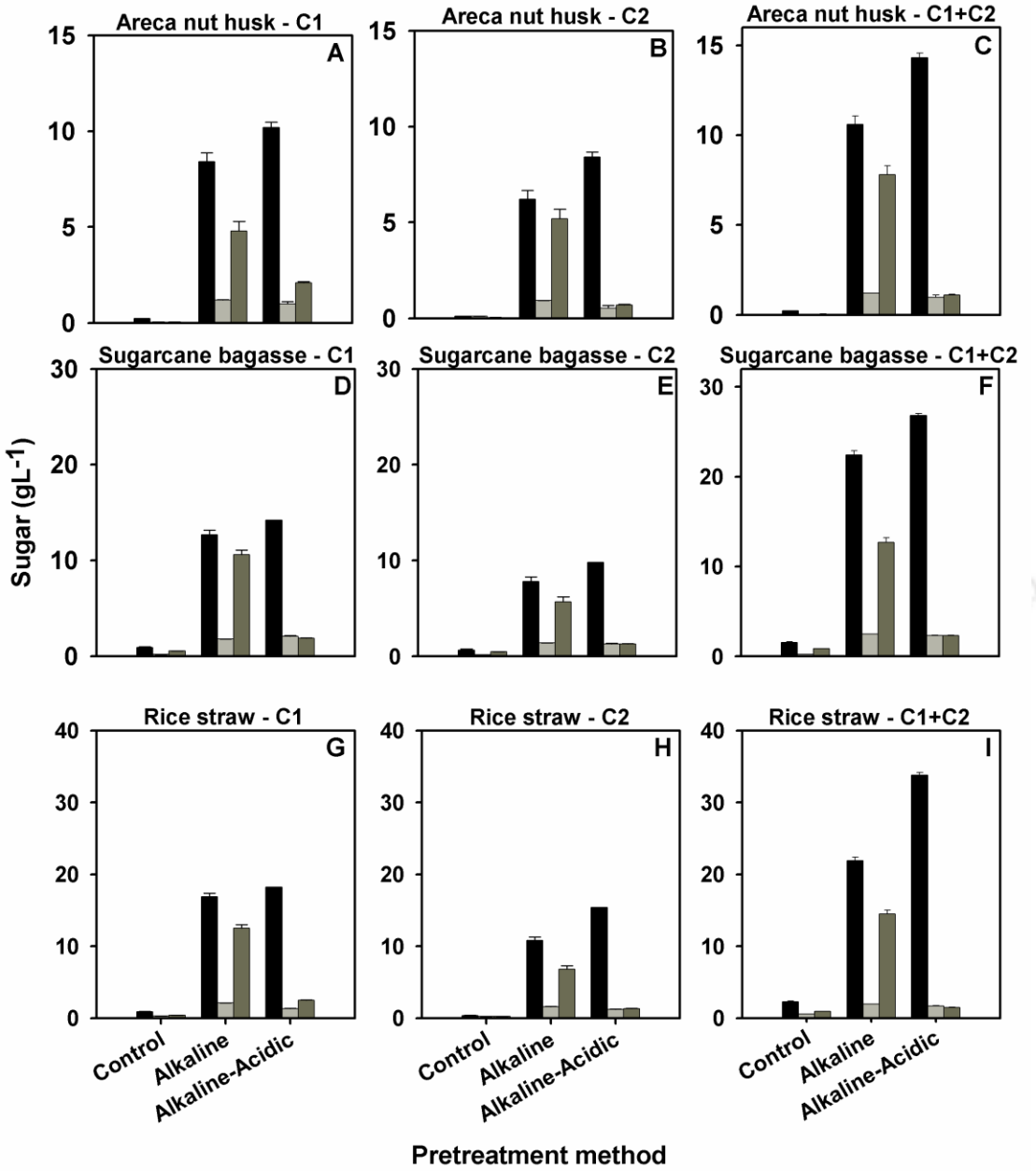


Fig. 7.5 Enzymatic hydrolysis of treated and untreated treated and untreated areca nut husk, sugarcane bagasse and rice straw. Glucose, cellobiose and xylose are represented by black, light grey and dark grey bars respectively

7.3.2.2 Optimization of substrate loading for enzymatic hydrolysis

Biomass to liquid loading was varied from 5% (w/v) to 12.5 % (w/v) to attain the most suitable loading ratio for maximum sugar hydrolysis. While biomass loading

of 10% (w/v) was optimum for areca nut husk and sugarcane bagasse, for rice straw a biomass loading of 7.5 % (w/v) was the best (Fig. 7.6). Higher amount of glucose (46.8 g L^{-1}) was released from rice straw when compared to sugarcane bagasse (36.8 g L^{-1}) and areca nut husk (22.8 g L^{-1}) using the optimized hydrolysis conditions (Fig. 7.6).

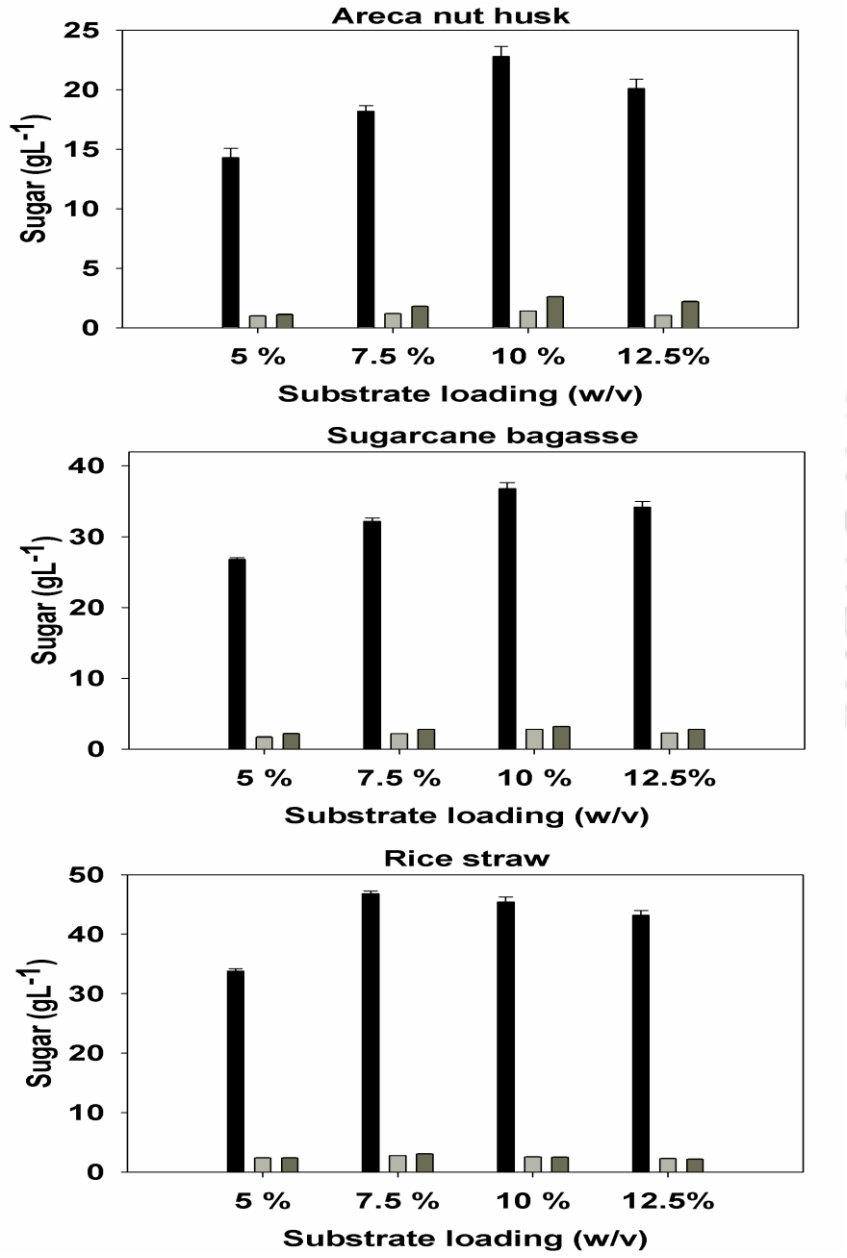


Fig. 7.6 Optimization of substrate loading for enzymatic hydrolysis. Glucose, cellobiose and xylose are represented by black, light grey and dark grey bars respectively

7.3.2.3 Dual substrate fermentation using lignocellulosic sugar hydrolysate and pure glycerol

Batch fermentations were performed by replacing pure glucose with the lignocellulosic sugar hydrolysate in the glucose-glycerol (60:40) dual substrate blend. However, for the areca nut husk sample, maintaining the desired glucose concentration at 42 g L^{-1} in the feed was not possible even after enzyme treatment and concentrating the hydrolysate. Hence, batch fermentation was performed with the sugar hydrolysate from the rice straw and sugarcane bagasse samples. The alcohol titer was similar for both the rice straw and sugarcane bagasse hydrolysates (Fig. 7.7). However, on the basis of higher reducing sugar release in case of rice straw sample (Fig. 7.6) compared to sugarcane bagasse, the rice straw sample was selected for further experiments.

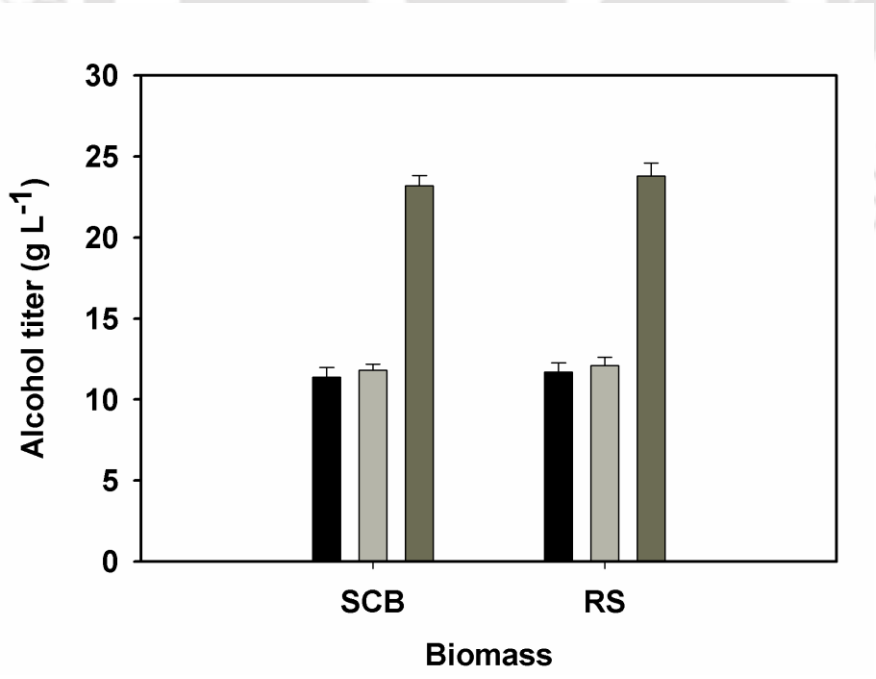


Fig. 7.7 Lignocellulosic hydrolysate-pure glycerol dual substrate fermentation. Butanol, ethanol and total alcohol titer is represented by black, light grey and dark grey bars respectively

7.3.2.4 Using statistical optimization to maximize the sugar release by enzyme hydrolysis of biomass samples

A CCD was constructed with 20 experiments to optimize the levels of Cellulase 1, Cellulase 2 and the optimum pH value required for efficient glucose hydrolysis. These studies showed that a wide range of glucose concentration from 4.50 ± 0.73 g L^{-1} to 47.2 ± 2.6 g L^{-1} for the conditions under consideration. Maximizing the glucose content in the hydrolysate was used as the objective function in the CCD-RSM based optimization. The experimental data obtained were tabulated along with the corresponding RSM predicted butanol titer in Table 7.3.

The RSM based model construction yielded a polynomial Eq. (7.2) which correlated the enzyme concentrations and pH with the predicted response of the glucose concentration (g L^{-1}).

$$Y = -1228.53 + 2.20X_1 + 2.21X_2 + 460.23X_3 - 0.01X_1^2 - 0.007X_2^2 - 44.63X_3^2 + 0.002X_1X_2 - 0.217X_1X_3 - 0.19X_2X_3 \quad (7.2)$$

Where, Y is the predicted glucose concentration (g L^{-1}) and X_i (i = 1-3) representing the concentration of Cellulase 1, Cellulase 2 and the optimum pH value respectively.

Table 7.3 Central composite design matrix of different enzyme units and pH used in RSM with the objective function of maximization of glucose (g L^{-1}) hydrolyzed from rice straw

Std. Order	Cellulase 1 (U g^{-1})	Cellulase 2 (U g^{-1})	pH	Experimental Glucose (g L^{-1})	Predicted Glucose (g L^{-1})
1	25.00	50.00	4.30	4.50	3.01
2	75.00	50.00	4.30	20.03	20.47
3	25.00	100.00	4.30	18.19	17.40
4	75.00	100.00	4.30	40.54	41.29
5	25.00	50.00	5.50	12.33	12.18
6	75.00	50.00	5.50	15.21	16.61
7	25.00	100.00	5.50	14.68	14.85
8	75.00	100.00	5.50	23.62	25.72
9	7.96	75.00	4.90	14.27	15.91
10	92.04	75.00	4.90	42.23	39.73
11	50.00	32.96	4.90	22.76	22.94
12	50.00	117.04	4.90	43.73	42.69
13	50.00	75.00	3.89	2.65	3.59
14	50.00	75.00	5.91	0.00	-1.80
15	50.00	75.00	4.90	44.50	46.34
16	50.00	75.00	4.90	45.90	46.34
17	50.00	75.00	4.90	46.80	46.34
18	50.00	75.00	4.90	47.22	46.34
19	50.00	75.00	4.90	47.10	46.34
20	50.00	75.00	4.90	46.40	46.34

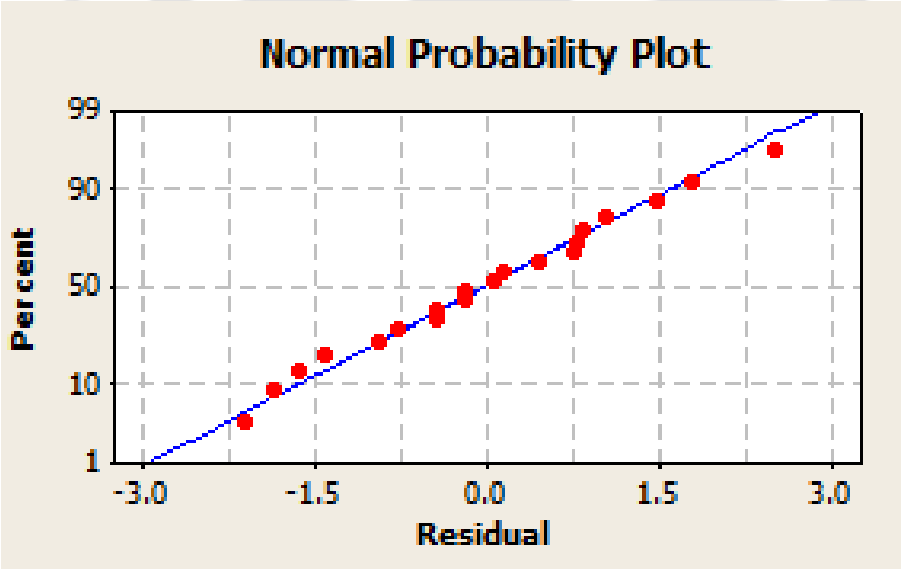


Fig. 7.8 Normality plot of residuals.

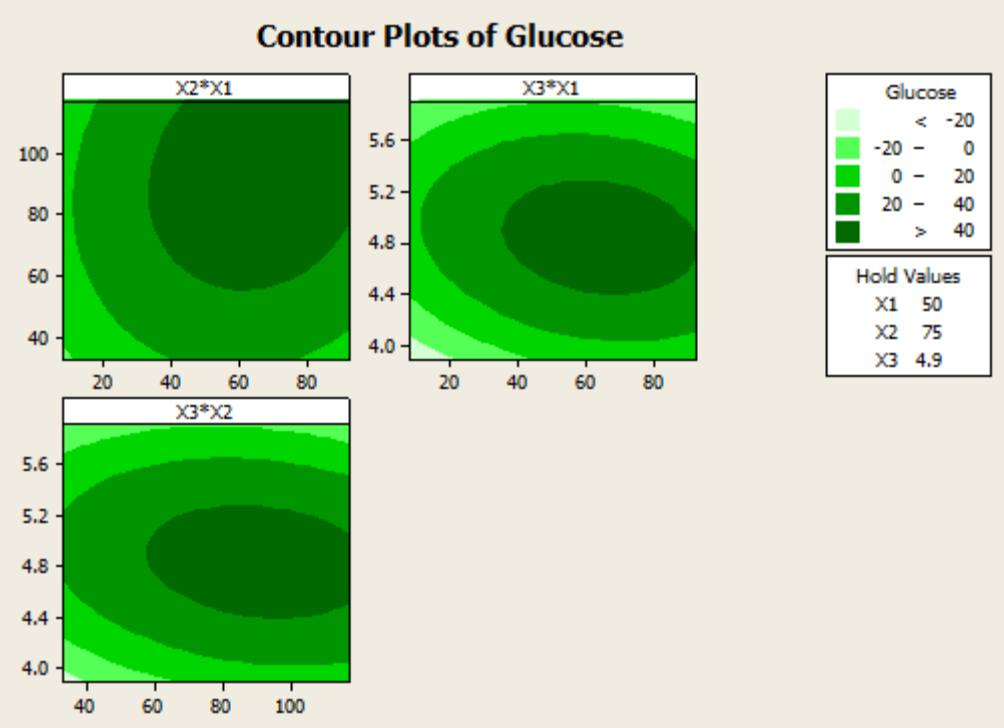


Fig. 7.9 Effect of design factors on the response variable.

An analysis of the data using ANOVA is shown in Table 7.4. The ANOVA indicates that the model is significant with *p*-value less than 0.05. The regression analysis showing a correlation coefficient (R^2) of 0.99 indicates that out of 20 experiments only 0.6 % could not fit the model.

Table 7.4 Analysis of variance for the quadratic regression model obtained from CCD-RSM employed in optimization of enzyme hydrolysis parameters

Source	F	P*	
Regression	29.23	0.001	Significant
Linear	28.21	0.001	Significant
X	5.7	0.001	Significant
C	74.87	0.001	Significant
pH	4.06	0.006	Significant
Square	58.64	0.001	Significant
X*X	128.26	0.001	Significant
C*C	39.13	0.001	Significant
pH*pH	37.68	0.002	Significant
Interaction	0.85	0.002	Significant
X*C	2.35	0.024	Significant
X*pH	0.01	0.002	Significant
C*pH	0.18	0.001	Significant
Residual Error			
Lack-of-Fit	281.42	0.056	Insignificant
Pure Error			
Total			

***Model parameters with *p*-value less than 0.05 was considered significant**

Validation of the optimized hydrolysis conditions was performed by comparing the experimental data with the predicted values. A maximum glucose concentration of 51.2 g L^{-1} was predicted by RSM when the biomass was hydrolysed with C1 unit of 67.4 U per gram of biomass, C2 unit of 94.9 U per gram of biomass and at a pH of 4.78 (Fig. 7.10). Validation of hydrolysis conditions predicted by RSM was performed in triplicates and the glucose concentration was found to be 48.8 ± 2.3 g L^{-1} which was similar to the predicted values. In comparison to the un-optimized hydrolytic conditions, RSM showed a 18.8% ± 0.9% decrease in the total unit of enzyme used to hydrolyse one gram of biomass. This reduction in enzyme use will aid in reducing the chemical cost and subsequently improve the process economics.

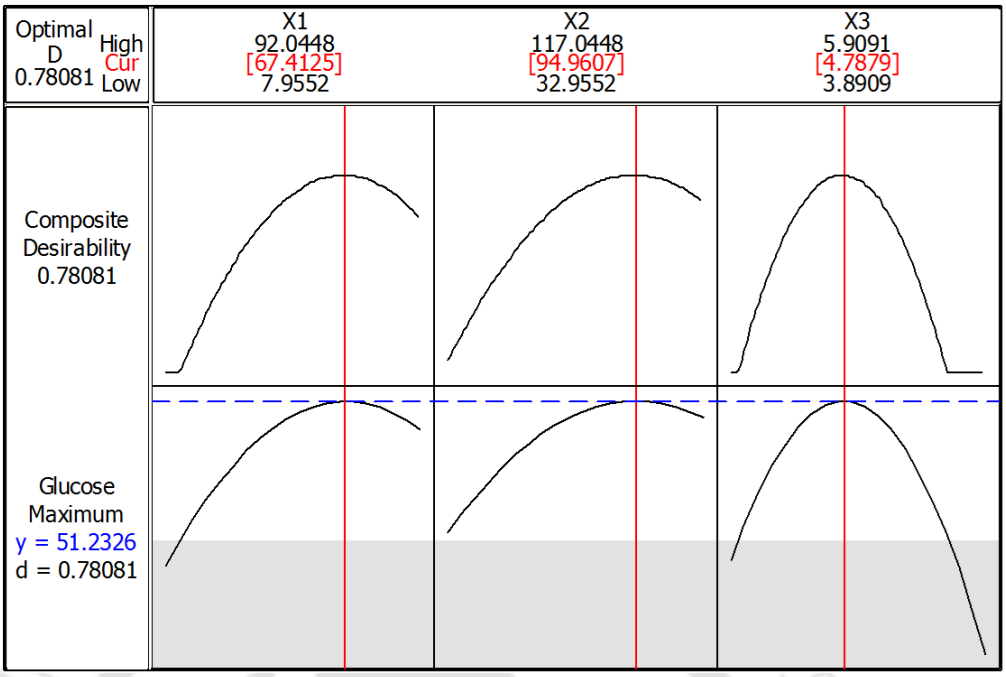


Fig. 7.10 Optimality plot to locate optimum factor levels for maximized response.

The rice straw hydrolysate which was produced using the optimized hydrolysis conditions was then used to replace pure glucose in dual substrate blend of glucose and glycerol (60:40) while pure glycerol was replaced with crude glycerol (Fig. 7.11). The clcohol titer (22.9 g L^{-1}) achieved under these conditions was comparable to that observed with pure sugars (24 g L^{-1}, chapter 5, section 5.3.2.2) but there was a significant reduction in alcohol productivity (0.21 g L^{-1} h^{-1}) compared to that observed for pure sugars (0.59 g L^{-1} h^{-1}, Chapter 5, Section 5.3.2.2). In order to explore possible reason behind the reduced alcohol productivity subsequent experiments were performed to examine the effect of various impurities present in crude glycerol on growth of *C. sporogenes*.

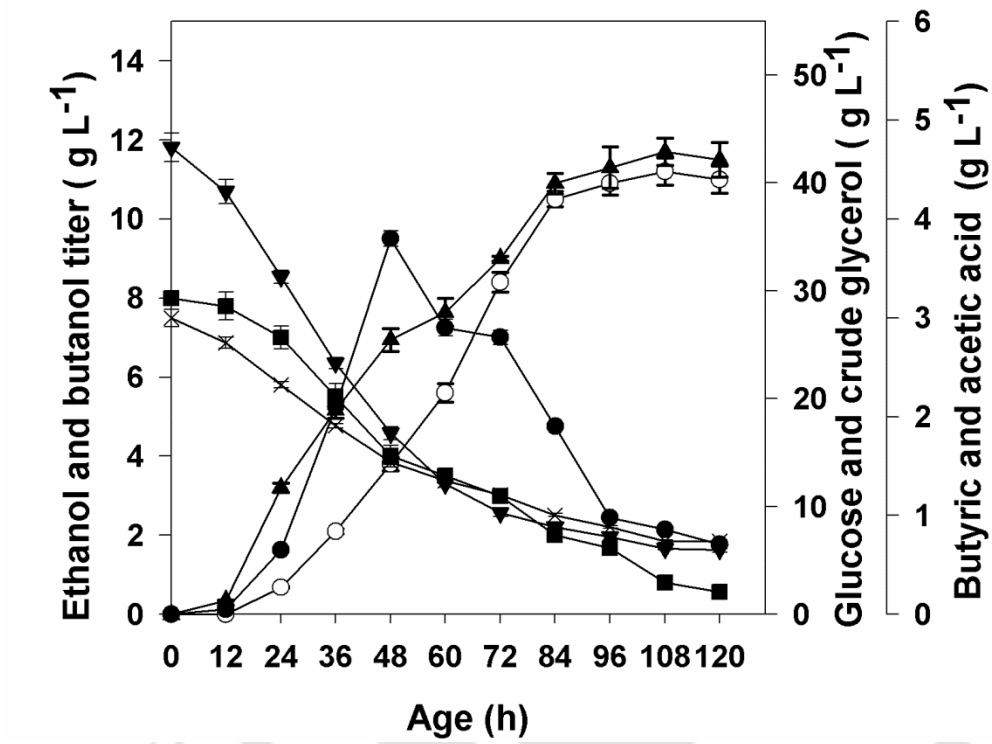


Fig. 7.11 Dynamic profiles for utilization of glucose (▼) utilization, crude glycerol (×) and production of butanol (○), ethanol (▲), acetic acid (■) and butyric acid (●) by *C. sporogenes* NCIM 2918 under dual substrate fermentation using glucose(rice straw hydrolysate) and crude glycerol blend in a ratio of 60:40

7.3.3 Effect of impurities present in crude glycerol on growth of C. sporogenes NCIM 2918

Effect of different concentrations of each impurity (methanol, salts and free fatty acids) on the growth of *C. sporogenes* NCIM 2918 was investigated. Under the conditions examined, methanol, potassium chloride, potassium sulfate, sodium stearate and sodium oleate did not affect microbial growth (Fig. 7.12). However, in the presence of sodium linoleate, which has a higher degree of unsaturation when compared to the other two C18 fatty acids, inhibited growthcaused a prolonged lag time (Fig. 7.12). Venkataramanan et al. (2012) observed similar detrimental effect on growth of *C. pasteurianum* ATCC 6013 by unsaturated fatty acids present in crude glycerol.

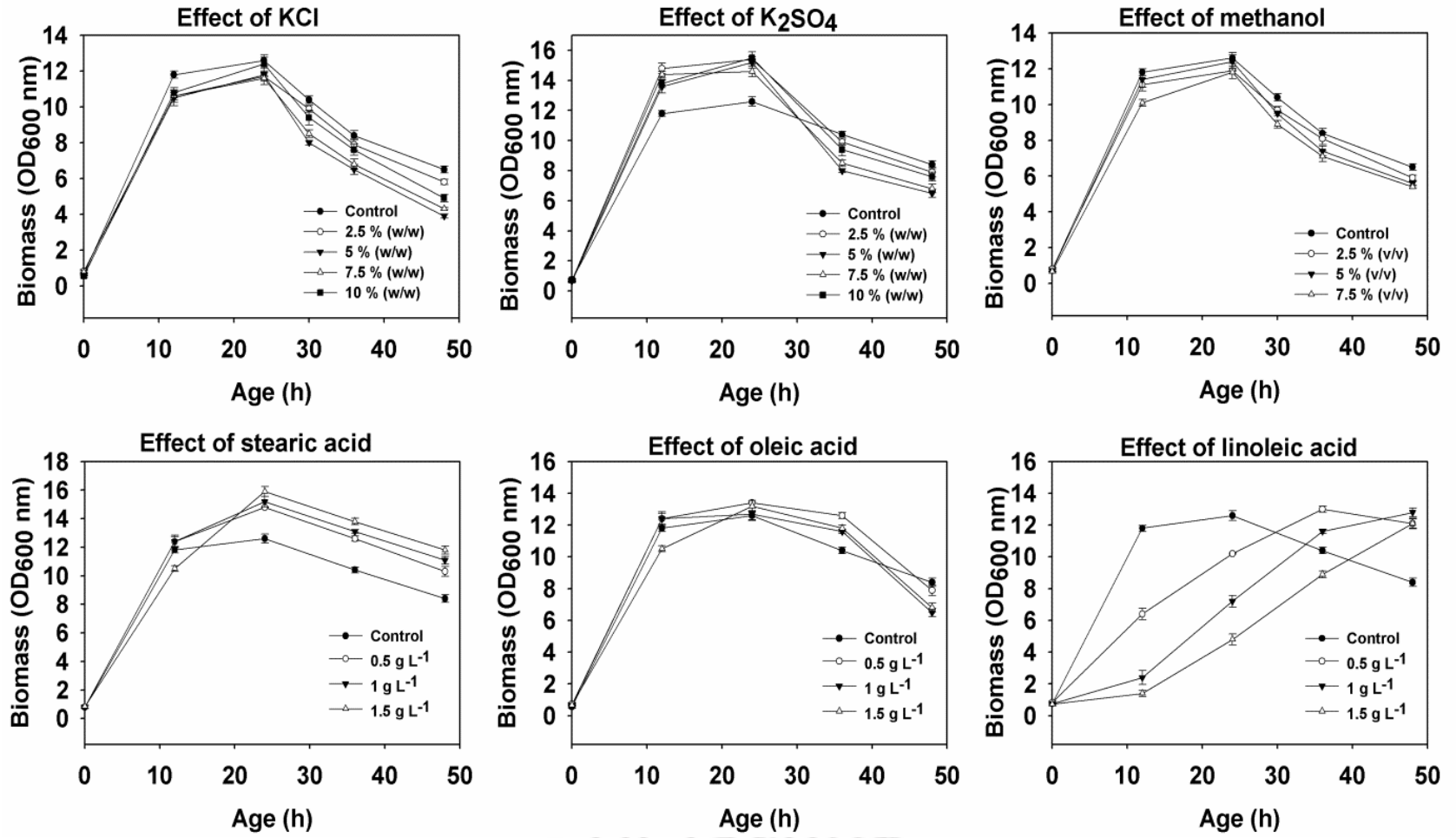


Fig. 7.12 Effect of different impurities present in crude glycerol on growth of *C. sporogenes* NCIM 2918

Unsaturated fatty acids consist of single or more double bonds resulting in bending of the molecule with cis double bond configuration in the oleic acid and linoleic acid structures. When this non-linear molecule interacts with the membrane of the organism it results in a hindrance to the diffusion of essential nutrients and metabolites across the membrane (Venkataramanan et al., 2012). Further Greenway and Dyke (1979) reported both free linoleic acid and the linoleic acid incorporated into the phospholipids resulting in altered membrane permeability contribute towards growth inhibition of *Staphylococcus aureus* cells. While Zheng at al. (2005) described for the first time that the inhibitory effect of unsaturated fatty acids, such as linoleic acid could be by blocking the fatty acid synthesis in *S. aureus*. On the other hand saturated fatty acids are incorporated into lipids of the bacterial membrane and do not hinder the diffusion process (Venkataramanan et al., 2012) or produce a membrane permeability increase (Greenway and Dyke, 1979). Hence, partially purifying the crude glycerol by removing the fatty acid impurities (Anand and Saxena, 2012) could result in a similar growth and product patterns as that observed for pure glycerol. Removing the fatty acid impurities was performed byhexane washing of crude glycerol in a 1:1 volume ratio at 200 rpm and at 37 ºC for 3 h. Fatty acids extraction using hexane washing (Fig.7.13 and 7.14) resulted in a partially purified crude glycerol which yielded an alcohol productivity of 0.51 g L^{-1} h^{-1} which was similar to that obtained from pure glycerol (0.59 g L^{-1} h^{-1}, Chapter 5, Section 5.3.3). Similar improvement in the crude glycerol utilization was reported by Venkataramanan et al. (2012) and Pyle et al. (2008). Therefore, the hexane washed crude glycerol was used for subsequent batch and fed-batch fermentation studies.

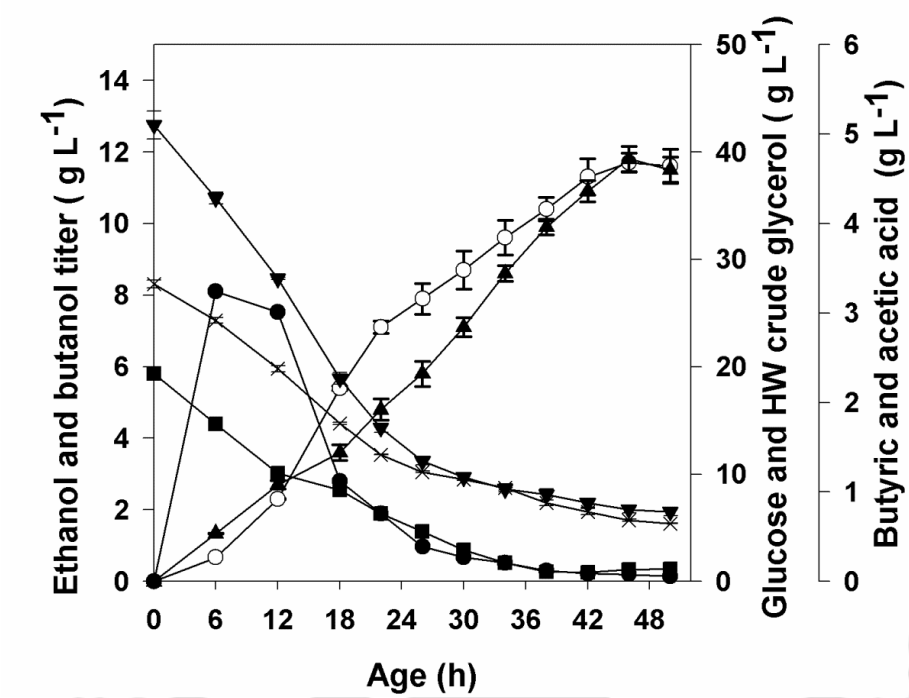


Fig. 7.13 Dynamic profiles for utilization of glucose (▼) utilization, crude glycerol (×) and production of butanol (○), ethanol (▲), acetic acid (■) and butyric acid (●) by *C. sporogenes* NCIM 2918 under dual substrate fermentation using glucose (rice straw hydrolysate) and hexane washed crude glycerol blend in a ratio of 60:40

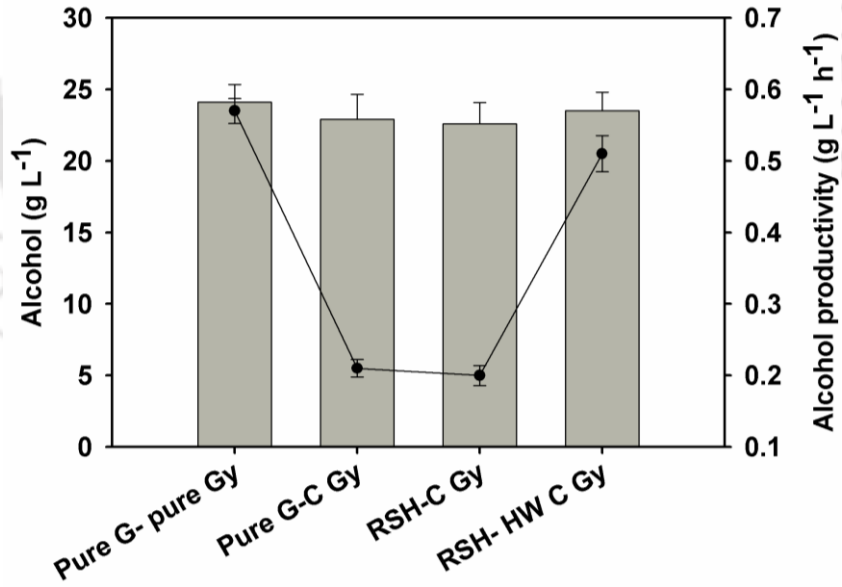


Fig. 7.14 Comparison of alcohol titer and productivity in dual substrate fermentation. Alcohol titer and productivity are represented by grey bar and black circle (●) respectively (G-Glucose; GY-Glycerol; RSH-Rice straw hydrolysate; C-Crude; HW-Hexane washed)

7.3.4 Dual substrate batch fermentation with and without continuous gas stripping in a 3 L bioreactor

Rice straw hydrolyzed under the optimized process conditions was concentrated in a rotary evaporator to produce a concentrate. This concentrateplus the hexane washed crude glycerol component were used at appropriate concentration to replace the pure glucose plus glycerol feed in the production medium. The total alcohol titer and productivity of 23.7 g L^{-1} and 0.52 g L^{-1} h^{-1} which was obtained was similar to that observed for batch fermentation in 500 ml bottles (Fig. 7.13). Butanol, ethanol and total alcohol titers produced by different *Clostridium* spp. during batch fermentation using different waste is compared with the titers obtained in the present study in Table 7.5. The total alcohol titer obtained is greater than most of the other *Clostridium* sp. stressing on the potential of *C. sporogenes* NCIM 2918 as a cell factory for biofuel production. The alcohol production from enzymatic hydrolysate was compared to the fermentation of a pure glucose and glycerol feed in a ratio of 60:40 (Chapter 5, Section 5.3.3.2) that produced 24 g L^{-1} alcohol with a composition of 11.9 g L^{-1} butanol and 12.1 g L^{-1} ethanol. A similar concentration of alcohol was obtained in the fermentation of rice straw hydrolysate-pure glycerol as compared in Fig. 7.14. Further, the maximum alcohol production capability of the organism was restricted due to the solvent toxicity. Dual substrate batch fermentation (using rice straw hydrolysate and hexane washed crude glycerol) coupled with gas stripping assisted in alleviating butanol toxicity. This resulted in the complete utilization of carbon sources (Fig. 7.15) with an improved alcohol titer of 26.4 g L^{-1} (butanol 13.6 g L^{-1} and ethanol titer 12.8 g L^{-1}). The total alcohol yields based on sugars consumption was 0.36 g g^{-1} (glucose plus glycerol) while alcohols were produced at a rate of 0.69 g L^{-1} h^{-1}. This yield and productivity are comparable to the results of previous studies

(Table 7.6). Although Wechgama et al (2017) reported a higher alcohol yield using sugarcane molasses feed, most of the *Clostridium* strains were unable to compete with the high alcohol productivity of 0.69 g L^{-1} h^{-1}reported in this study (Table 7.6).

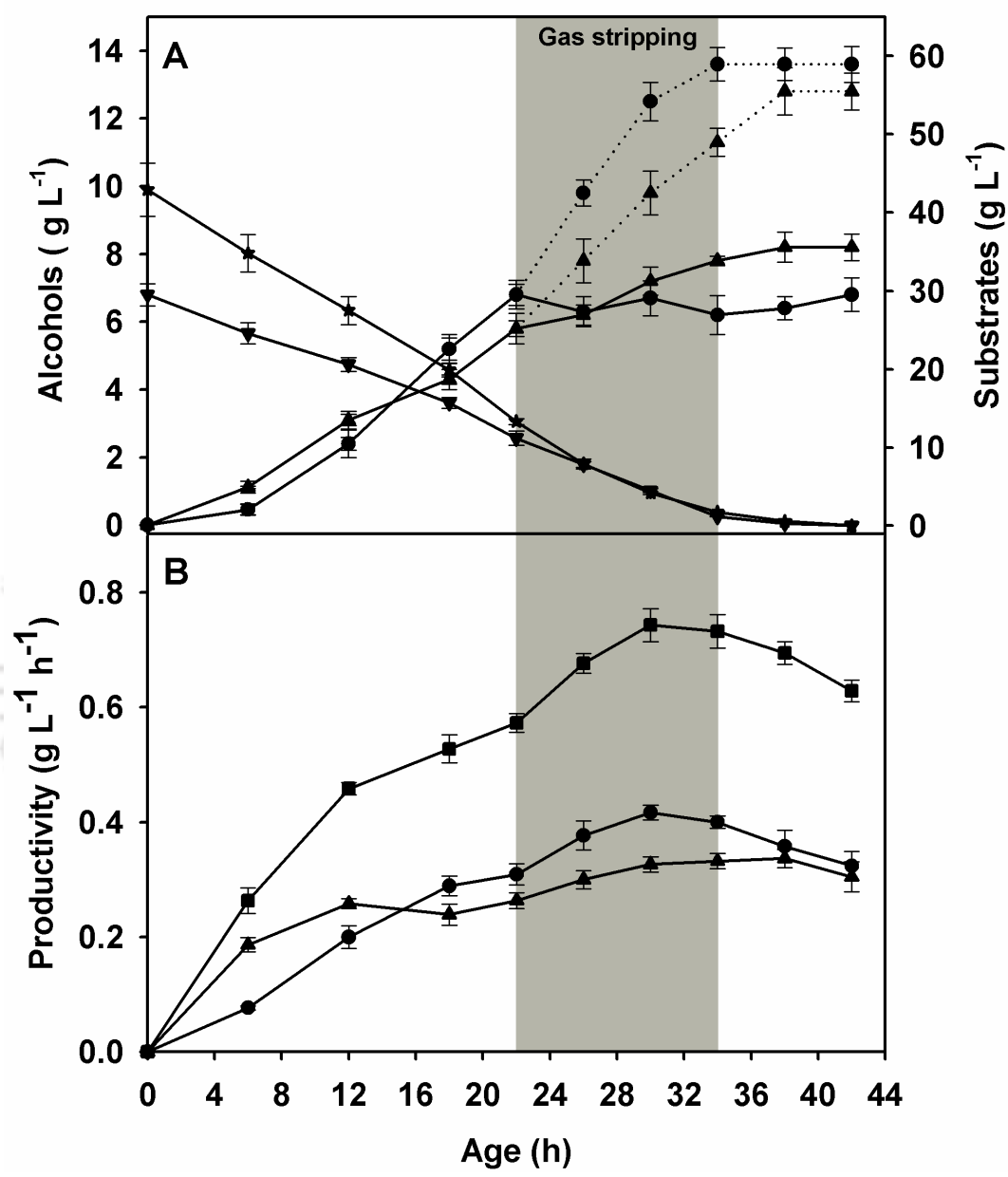


Fig. 7.15 Dynamic profiles for (A) butanol (●), ethanol (▲), cumulative butanol (--●--), cumulative ethanol (--▲--), glucose (*) and glycerol (▼) utilization (B) productivity for cumulative butanol (●), ethanol (▲) and total alcohol (■) during gas stripping coupled to batch fermentation of *C. sporogenes* NCIM 2918

Table 7.5 Comparison of butanol, ethanol and total alcohol titer produced by different *Clostridium* sp. under batch conditions using waste

Microorganism	Feed stock	Butanol titer ($g\ L^{-1}$)	Ethanol titer ($g\ L^{-1}$)	Total alcohol titer ($g\ L^{-1}$)	Reference
C. sporogenes NCIM 2918	**Rice straw hydrolysate + Hexane washed crude glycerol**	**11.7**	**11.8**	**23.5**	**This study**
C. beijerinckii P 260	Barley straw	18	1.7	19.7	Qureshi et al., 2010a
C. beijerinckii P 260	Corn stover	14.5	3.7	18.2	Qureshi et al., 2010b
C. pasteurianum DSMZ 525	Spruce Biomass hydrolysate + glycerol	17.4	ND	17.4	Sabra et al., 2014
C. acetobutylicum ABE 1401	Sweet sorghum bagasse	14.3	1.5-2.5	15.8-16.8	Cai et al., 2015
C. acetobutylicum IFP904	Jerusalem artichoke juice	14.8	0-0.3	14.8-15.1	Marchal et al., 1985
C. acetobutylicum ABE 1201	Sweet sorghum juice	13.22	1.25	14.5	Cai et al., 2016c
C. beijerinckii P 260	Sweet Sorghum Bagasse	13	1.4	14.4	Qureshi et al., 2016
C. beijerinckii BA101	Liquefied corn starch	13.4	0.7	14.1	Ezeji et al., 2007b
C. beijerinckii BA101	Saccharified liquefied cornstarch	13.4	0.8	14.2	Ezeji et al., 2007b
C. beijerinckii TISTR 1461	Sugarcane molasses	12.55	1.35	14	Wechgama et al., 2017
Clostridium strain BOH3	Rice bran + Sesame oilcake	13.5	-	13.5	Rajagopalan et al., 2016
C. beijerinckii P 260	Wheat straw	12	1.1	13.1	Qureshi et al., 2007
C. beijerinckii CC101	Corn stover	11.2	1.1	12.3	Xue et al., 2016
C. beijerinckii CC101	Jerusalem artichoke stalk	11.8	0.2	12	Xue et al., 2017

C. acetobutylicum ABE 1201	Corn cob bagasse	10.1	1.3	11.4	Cai et al., 2016b
C. acetobutylicum ATCC 824	Napier grass	9.5	1 to 2	10.5-11.5	He et al., 2017
C. acetobutylicum DSM 792	Sugarcane-sweet sorghum juices	10.5	1.2	11.7	Rochón et al., 2017
C. acetobutylicum JB 200	Cassava bagasse	9.71	1.37	11.08	Lu et al., 2012
C. acetobutylicum ABE 1301	Corn stalk	9.4	1.4	10.8	Cai et al., 2016a
C. acetobutylicum ATCC 824	Bamboo	10.4	-	10.4	Kolawole et al., 2016
C. acetobutylicum GX 01	Sugarcane	7.7	2	9.7	Pang et al., 2016
C. beijerinckii P 260	Corn stover	9	0.4	9.4	Qureshi et al., 2014
C. saccharoperbutylacetonicum N1-4	Eucalyptus	8.2	0.6	8.8	Zheng et al., 2015
C. acetobutylicum ATCC 824	Corn stover	7.1	1-1.5	8.1-8.6	Zhang et al., 2013
C. acetobutylicum DSM 1731	*Salix schwerinii*	8.1	0.6	8.7	Yang et al., 2017
C. acetobutylicum DSM 1731	Barley straw	7.9	0.5	8.4	Yang et al., 2015
C. acetobutylicum CH02	Sugarcane bagasse	7.7	0.6	8.3	Li et al., 2017
C. acetobutylicum NRRL B-591	Sorghum	7.1	1.1	8.2	Jafari et al., 2016
C. saccharobutylicum DSM 13864	Corn stover	7.9	0-0.5	7.9-8.4	Ding et al., 2016
C. beijerinckii NCIMB 8052	Pinusrigida	7.7	-	7.7	Kwon et al., 2016
C. acetobutylicum ATCC 824	Agricultural amorphophallus konjac waste	7.1	0.65	7.75	Shao et al., 2015
C. sporogenes BE01	Rice straw	3.4	1.9	5.3	Gottumukkala et al., 2013

Table 7.6 Comparison of butanol, ethanol and total alcohol titer produced by different *Clostridium* sp. under batch condition coupled to gas stripping using waste

Organism	Feed stock	Butanol titer ($g\ L^{-1}$)	Ethanol titer ($g\ L^{-1}$)	Total alcohol titer ($g\ L^{-1}$)	Alcohol Yield (g/g)	Alcohol productivity ($g\ L^{-1}\ h^{-1}$)	Reference
C. sporogenes NCIM 2918	**Rice straw + Hexane washed crude glycerol**	**13.6**	**12.8**	**26.4**	**0.36**	**0.69**	**This study**
C. acetobutylicum ABE 1201	Sweet sorghum juice	14	1.2	15.2	0.26	0.19	Cai et al., 2016c
C. beijerinckii TISTR 1461	Sugarcane molasses	14.13	0.83	15	0.41	0.31	Wechgama et al., 2017
C. beijerinckii CC 101	Wood pulping	13.5	0.1	13.6	0.34	0.19	Lu et al., 2013
C. beijerinckii P 260	Corn stover	11.6	1.1	12.7	-	0.21	Quresh et al., 2014
C. acetobutylicum ABE 1401	Sweet sorghum bagasse	17.2	1.5-2.5	18.7-19.7	0.26-0.28	0.23-0.25	Cai et al., 2015
C. beijerinckii BA101	Liquefied corn starch	15.1	1.1	16.2	0.29	0.21	Ezeji et al., 2007
C. beijerinckii BA101	Saccharified liquefied cornstarch (SLCS)	17.6	0.6	18.2	0.28	0.27	Ezeji et al., 2007

7.3.5 Dual substrate fed-batch fermentation with continuous gas stripping

Coupling fed batch fermentation to continuous gas stripping resulted in an extended cultivation period (Fig. 7.16). The organism was able to consume more sugar (70.4 g L^{-1} glucose and 47.6 g L^{-1} glycerol) when compared to the batch fermentation study. The fed batch system resulted in higher alcohol production of 44.4 g L^{-1} (butanol 21.5 g L^{-1} and ethanol 22.9 g L^{-1}) with an alcohol productivity of 0.62 g L^{-1} h^{-1} and yield of 0.37 alcohol per gram of sugar (glucose plus glycerol). Comparison of alcohol titer, yield and productivity observed during the study with various other fed batch studies is shown in Table 7.7. The results from this study clearly indicate that *C. sporogenes* NCIM 2918 is an improved biofuels producing microorganism when compared to any other wild type strains. However, higher alcohol production and tolerance capability with mutant strains have been observed in most of the fed batch studies. A comparison of alcohol titer and productivity data from various dual substrate fermentations performed showing stepwise improvement in the fermentation kinetics after utilizing different media and employing various process engineering strategiesis shown in Fig. 7.17. The current study showed that *C. sporogenes* NCIM 2918 produced the highest total alcohol titer amongst all the different wild type *Clostridium* spp. grown on waste materials in batch mode and without product recovery. This study also demonstrate the the highest individual (butanol or ethanol) as well as total alcohol titer and productivities for *C. sporogenes* batch fermentation using cheaper raw material without product recovery.

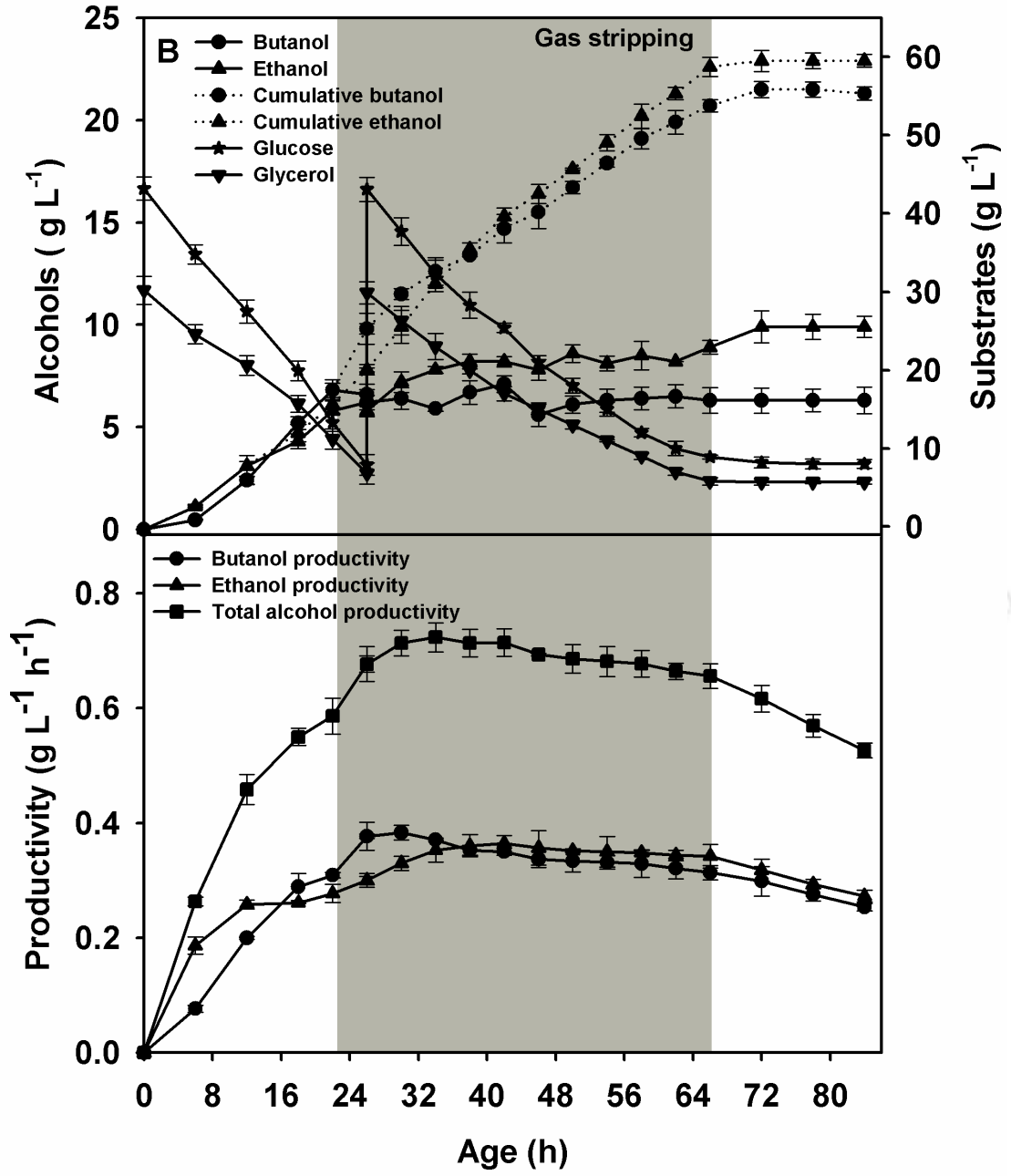


Fig. 7.16 Dynamic profiles for (A) butanol (●), ethanol (▲), cumulative butanol (--●--), cumulative ethanol (--▲--), glucose (*) and glycerol (▼) utilization (B) productivity for cumulative butanol (●), ethanol (▲) and total alcohol (■) during gas stripping coupled fed batch fermentation of *C. sporogenes* NCIM 2918

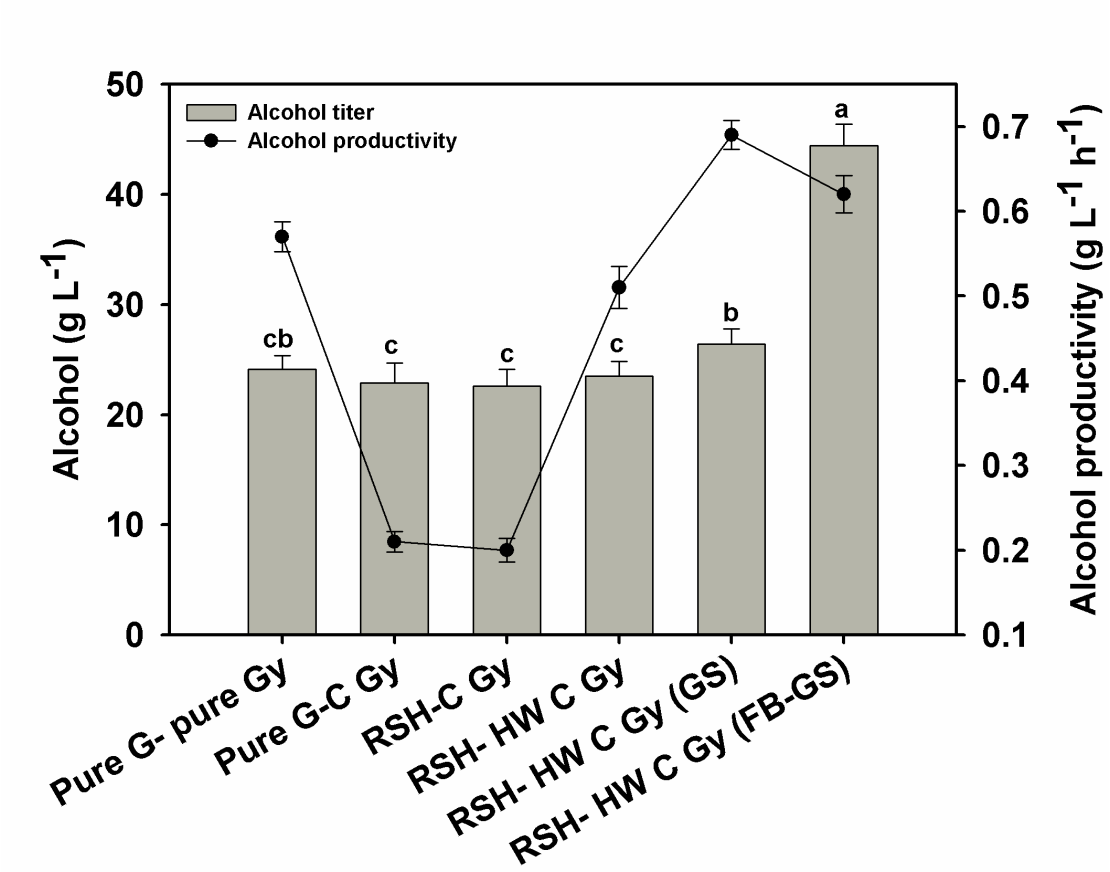


Fig. 7.17 Comparison of alcohol titer and productivity in dual substrate fermentation. Means that share the same letter do not differ significantly at the 95% confidence level based on the Tukey mean comparison method Alcohol titer and productivity are represented by grey bar and black circle (●) respectively (G-Glucose; GY-Glycerol; RSH-Rice straw hydrolysate; C-Crude; HW-Hexane washed; GS-Gas stripping; FB-Fedbatch)

Table 7.7 Comparison of butanol, ethanol and total alcohol titer produced by different *Clostridium* sp. under fed-batch mode of fermentation coupled to gas stripping using waste

Microorganism	Feedstock	Butanol titer (g L^{-1})	Ethanol titer (g L^{-1})	Total alcohol titer (g L^{-1})	Alcohol Yield (g g^{-1})	Alcohol productivity (g L^{-1} h^{-1})	Reference
C. sporogenes NCIM 2918	**Rice straw + Hexane washed crude glycerol**	**21.5**	**22.9**	**44.4**	**0.37**	**0.62**	**This study**
C. acetobutylicum ABE-P 1201 (Mutant strain)	Corn stover	18.6	2.4	21	0.21	0.1	Cai et al., 2017
C. acetobutylicum ABE 1201(Mutant strain)	Sweet sorghum juice	112.9	9.5	122.4	0.3	0.39	Cai et al., 2016c
C. acetobutylicum ABE 1401(Mutant strain)	Sweet sorghum bagasse	84.94	16.08	101.02	0.24	0.27	Cai et al., 2015
C. acetobutylicum DSM 792	Sugarcane-sweet sorghum juices	18.6	3.3	21.9	0.09	0.15	Rochón et al., 2017
C. acetobutylicum ABE 1401 (Mutant strain)	Sweet sorghum bagasse	101.3	15.1	116.4	0.25	0.6	Cai et al., 2015
C. acetobutylicum JB200 (Mutant strain)	Cassava bagasse	59.81	4.78	64.59	0.26	0.38	Lu et al., 2012
C. acetobutylicum strain JB201(Mutant strain)	Cassava bagasse	76.44	5.09	81.53	0.24	0.31	Lu et al., 2012
C. beijerinckii BA101	Saccharified liquefied cornstarch (SLCS)	56.2	1	57.2	0.25	0.42	Ezeji et al., 2007

This is the first instance where all the major nutrients used for *C. sporogenes* fermentation are replaced with cheaper raw material including glucose with rice straw hydrolysate, glycerol with crude glycerol and nitrogen source with instant dry yeast. This study is also the first to demonstrate the capability of a fed batch process for cultivation of *C. sporogenes* using a cheaper dual substrate combination coupled with *in situ* product recovery. Hence, the present study is a major step towards the development of sustainable bioprocess for producing of liquid biofuels from *C. sporogenes.*

7.4 Conclusions

The present study demonstrates a cost effective process towards the production of liquid biofuels (butanol and ethanol) using *C. sporogenes* NCIM 2918. The cost effectiveness was achieved by culturing the organism on a cheap raw materials sugar hydrolysate from a lignocellulosic biomass and crude glycerol, a waste from the biodiesel industry resulting in higher total alcohol titer. Batch fermentation of the organism resulted in a total alcohol titer of 23.7 g L^{-1} and a productivity of 0.52 g L^{-1} h^{-1}. Fed-batch fermentation with intermittent feeding of the sugar hydrolysate and crude glycerol feed coupled with an *in situ* product recovery process resulted in further improving the total alcohol titer to 44.4 g L^{-1} and productivity of 0.62 g L^{-1} h^{-1}.

7.5 References

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CHAPTER 8

Conclusions

Non-acetone producing *Clostridium acetobutylicum* strain NCIM 2918 was selected from six different strains screened for butanol production. This strain with the highest butanol titer of 3.7 g L^{-1} in the P2TY media was analyzed further as a potential biofuels producer. The absence of acetone production during the detailed characterization of NCIM 2918 actuated the need for its identification using phenotypic analysis through biochemical tests, followed by 16S rRNA gene sequencing and phylogenetic analysis. The results pointed towards the misclassification of the strain as *C. acetobutylicum* and subsequently, the organism was correctly classified as *C. sporogenes* NCIM 2918.

Initial screening of carbon and nitrogen sources resulted in the identification of sorbitol and instant dry yeast as effective sources. Carbon and nitrogen screening resulted in 157% increase in butanol titer which was further enhanced by 25% after media optimization. The final butanol titer of 12.1 g L^{-1} and 7.9 g L^{-1} of ethanol was attained which together as the total alcohol titer is outstanding when compared to many other *Clostridium* strains. Also the organism's inherent ability to utilize glycerol and xylose showed its potential in using cheap alternate substrates for butanol production offers a great potential towards developing a more economically viable fermentation process. Depending on the combination of carbon sources, the organism was able to manipulate its metabolism towards the synthesis of either ethanol or

butanol. This affected the total alcohol titer, which further motivated the assessment of substrate dependent product modulation. Different dual substrate combinations were screened among which a glucose-glycerol ratio of 60:40 resulted in a maximum butanol and ethanol titer of 11.9 and 12.1 g L^{-1} respectively. Further, evaluation of the strain with crude glycerol as a replacement to pure glycerol in the glucose-glycerol ratio resulted in butanol levels of 11.2 g L^{-1} and ethanol reaching 11.7 g L^{-1}. Thus the strain exhibits potential for utilization of cheaper substrates to produce biofuel.

Further, three different lignocellulosic biomass (rice straw, sugarcane bagasse and areca nut husk) were screened as a cheaper source for glucose replacement in the optimized 60:40 glucose-glycerol feed. The maximum glucose released from rice straw during the optimized enzyme hydrolysis conditions reached 48.81 ± 0.72 g L^{-1}. Finally, a process was demonstrated in 3 L bioreactor on dual substrate combination of rice straw hydrolysate and hexane washed crude glycerol. When grown on lignocellulosic biomass, the total alcohol titer of 23.5 g L^{-1} (butanol, 11.7 g L^{-1} and ethanol, 11.8 g L^{-1}) achieved was found to be the highest when compared to reported values. Maximum butanol producing capability was restricted due to the butanol toxicity, hence to overcome that the process was coupled with *in situ* product recovery strategy via gas stripping. This integrated process resulted in complete utilization of carbon sources and improved alcohol titer of 26.4 g L^{-1} (butanol 13.6 g L^{-1} and ethanol titer 12.8 g L^{-1}). To further extend the fermentation time, changing from batch to fed-batch the fermentation mode in a higher alcohol titer of 44.4 g $L^{-1}$and productivity of 0.62 g L^{-1} h^{-1}.

Strain characterization under different carbon and nitrogen sources revealed the existence of inherent metabolic versatility and adaptability of the organism in response to the change in substrate composition. In order to understand the underlying

regulation governing this metabolic flexibility, flux balance analysis (FBA) was conducted for different glucose-glycerol combinations. Glucose fermentation resulted in high butyric acid production and biomass flux while in case of glycerol a lower biomass and butyric acid along with high ethanol and hydrogen formation flux was noted. Key distinguishing feature among the two was lack of butyric acid reutilization flux during glucose fermentation. Growth limitation for high glycerol containing dual substrate mixture was attributed to low flux in anaplerotic pyruvate carboxylase reaction and butyric acid pathway. This coupled with high acetic acid reutilization resulted in higher ethanol production. A glucose-glycerol (60:40) ratio demonstrated a combination of features of sole carbon sources and maximum flux was diverted to alcohol production. This is the first instance of metabolic network reconstruction for *C. sporogenes* through which an attempt was made at understanding the modulation of growth and products in response to varied substrate composition. These results would aid in target identification for engineering of the organism towards high biofuel production.

Innovation in Science and Engineering Significance

Biofuel production by *Clostridium* spp. remains unachievable in terms of sustainability and economic feasibility. The work examined several aspects to improve the metabolic efficienceof biofuels production by integrating microbial approaches as well as engineering strategies are targeted to attain sustainability and economic feasibility. The following incremental strategies resulted in a significant increase in the alcohol titer and productivity.

- ✓ The physiochemical understanding of the organism designated as *Clostridium sporogenes* NCIM 2918 resulted in the identification of dual substrate combination of glucose-glycerol (60:40) feed and instant dry yeast as most suitable carbon and nitrogen sources respectively. Based on the studies, a dual substrate bioprocess strategy was developed in the present study with simultaneous utilization of glucose and glycerol for growth and alcohol production.
- ✓ A metabolic model was developed to understand the substrate dependent product modulation that was observed in *Clostridium sporogenes* NCIM 2918. The Model provided insights as to how the organism adapts its metabolism in response to varied environmental conditions. Metabolic flux balance analysis based modeling approach revealed the physiological significance of employing glucose-glycerol dual substrate fermentation when compared to the mono substrate (glucose or glycerol) fermentation strategy.
- ✓ Full-scale development of micorbial butanol production has several bottlenecks hindering its industrialization. These include high raw material cost, mixed product profile, solvent toxicity and low titer leading to high recovery cost. The

study describes a bioprocess that addresses all of the above limitations. The strain used has a unique property of producing only butanol and ethanol both of which qualify as biofuels.This eliminates the need for removing acetone which is a major component of ABE fermentation. Further, use of cheaper carbon and nitrogen source for fermentation reduces the process cost further aiding the commercialization potential of the developed strategy. Finally, use of fed batch strategy coupled with *in situ* product recovery process is a major development aiding in the alleviating of solvent toxicity and resulting in a higher biofuel titer. The present study is a major step towards the development of a sustainable bioprocess for biofuel production of s from *Clostridium sporogenes***.**

Future Prospects

- ✓ Evaluate the strains performance in pilot scale studies and demonstration of a large scale bioprocess for bio-alcohol production in continuous mode of operation using the wild type strain *Clostridium sporogenes* NCIM 2918.
- ✓ Perform adaptation studies or chemical mutagenesis to achieve a more tolerant strain of *Clostridium sporogenes* NCIM 2918, which will in turn aid in attaining higher alcohol titers. Further, metabolic engineering may be employed for overexpression of major stress protein genes, including *groES*, *dnaKJ*, *hsp18*, and *hsp90*.
- ✓ Conduct an economic feasibility analysis and sustainability analysis for the technology developed in this.
- ✓ Conduct a life cycle analysis to assess environmental impacts associated with all the stages of the process including raw materials use, manufacturing and end product use.

Appendix

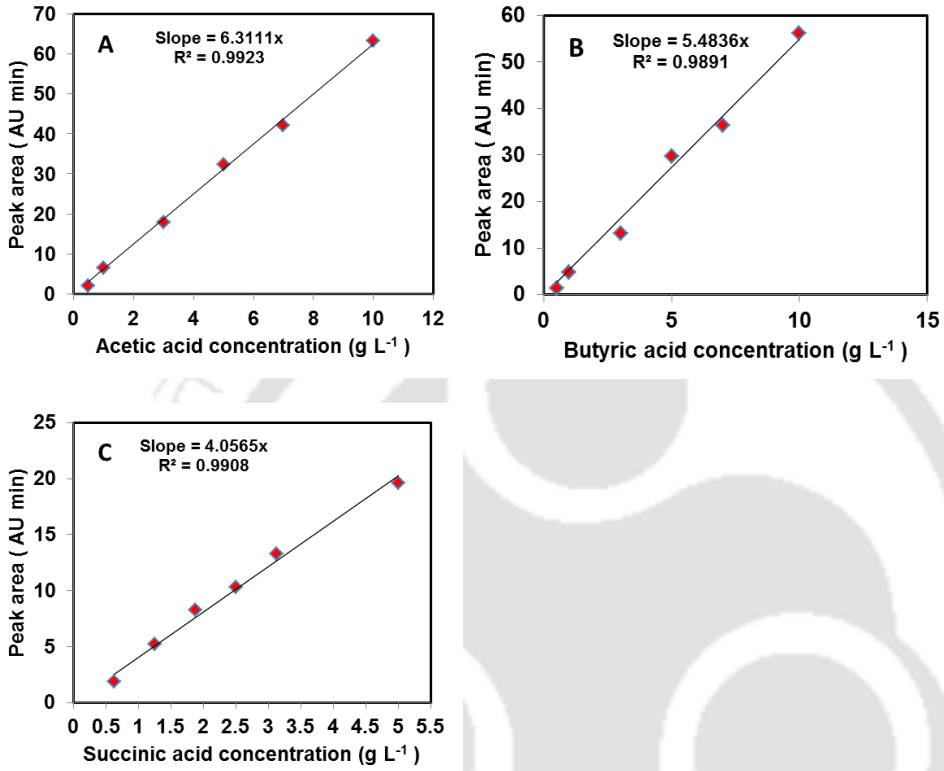

Fig. A1 Graphical representation of correlation between concentration of acids and their respective absorbance in HPLC

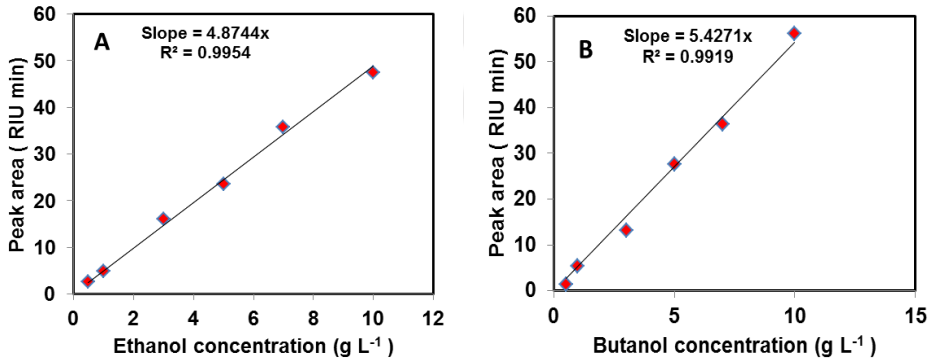

Fig. A2 Graphical representation of correlation between concentration of alcohols and their respective absorbance in HPLC

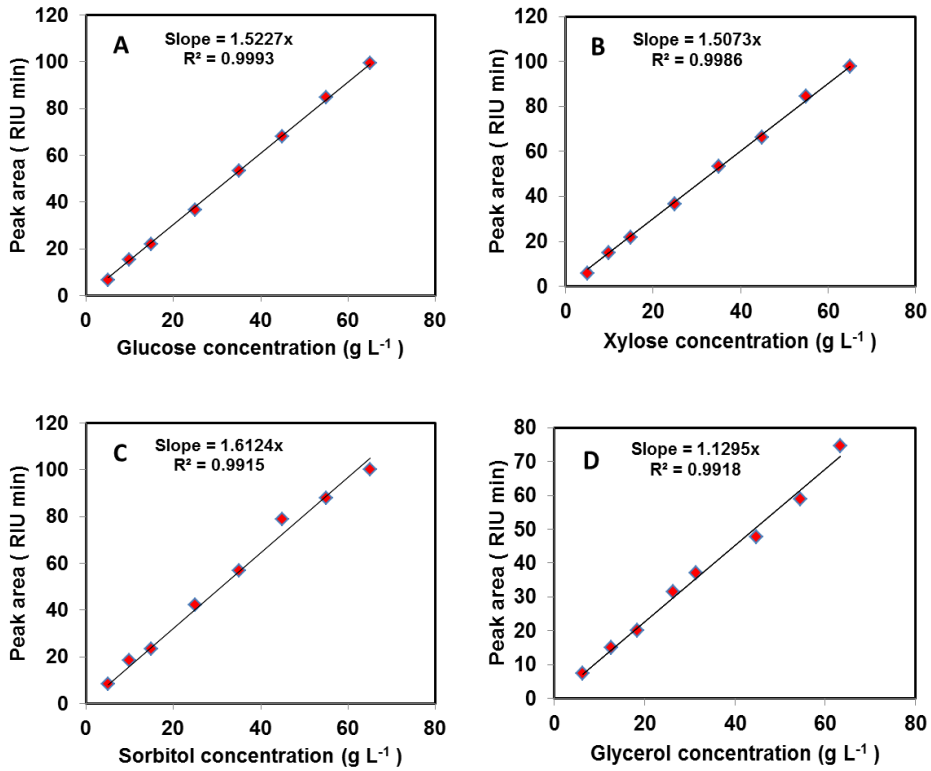

Fig. A3 Graphical representation of correlation between concentration of substrates and their respective absorbance in HPLC

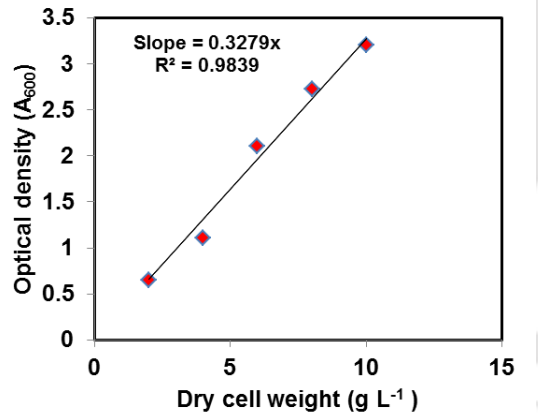

Fig. A4 Graphical representation of correlation between the dry cell weight and absorbance measured at 600 nm in a spectrophotometer

Table A1 Macromolecular composition of biomass

Macromolecular composition			
Component	**Proportion (% g per g)**		
Protein	50		
Carbohydrate	2		
RNA	7		
DNA	1		
Cell wall Component	21		
Peptidoglycan			
Lipids	12		
Lipoteichoic Acid	6		
Protein Composition			
Amino acids	**Mole Fraction**	**Mol. Weight (g per mol)**	**Avg Weight (g)**
Gly	0.115	57.1	6.5665
Ala	0.083	71.1	5.9013
Val	0.125	99.1	12.3875
Leu	0.046	113.2	5.2072
Ile	0.046	113.2	5.2072
Met	0.083	131.2	10.8896
Pro	0.049	97.1	4.7579
Phe	0.02	147.2	2.944
Tyr	0.085	163.2	13.872
Trp	0.005	186.2	0.931
Ser	0.045	87.1	3.9195
Thr	0.044	101.1	4.4484
Asn	0.017	114.1	1.9397
Asp	0.017	115.1	1.9567
Gln	0.014	129.1	1.8074
Glu	0.014	128.1	1.7934
Cys	0.13	103.2	13.416
Lys	0.036	128.2	4.6152
Arg	0.014	156.2	2.1868
His	0.016	137.2	2.1952
Total			106.9425
DNA Composition			
Bases	**Mole Fraction**	**Mol. Weight (g per mol)**	**Avg Weight (g)**
A	0.345	313.2	108.054
G	0.155	329.2	51.026
T	0.345	304.2	104.949
C	0.155	289.2	44.826
Total			308.855

RNA Composition			
Bases	**Mole Fraction**	**Mol. Weight (g per mol)**	**Avg Weight (g)**
A	0.271	329.2	89.2132
G	0.29	345.2	100.108
U	0.225	305.2	68.67
C	0.214	306.2	65.5268
Total			323.518

Lipid Composition			
Lipid Components	**Mole Fraction**	**Mol. Weight (g per mol)**	**Avg Weight(gms)**
Phosphatidylglycerol (dihexadecanoyl, n-C16:0)	0.497	721	358.337
Cardiolipin	0.0622	1441	89.6302
3-Phosphatidyl-1-(3-O-L-lysyl) glycerol	0.0623	867	54.0141
Diglucosyl-diacylglycerol	0.0747	1016	75.8952
3-D-Glucosyl-1,2-diacylglycerol	0.0125	836	10.45
DiacylGlycerol	0.207	656	135.792
Total			724.1185

Table A2 Reconstruction of the metabolic network of *C. sporogenes* NCIM 2918: List of reactions involved in the central metabolism.

S.No.	EC No.	Enzyme	Pathway	Reactions
1	2.7.1.2	glucokinase	**Glycolysis**	'Glc + ATP --> ADP + G6P'
2	5.3.1.9	glucose-6-phosphate isomerase		'G6P --> F6P'
3	5.3.1.9	glucose-6-phosphate isomerase		'F6P --> G6P'
4	2.7.1.11	phosphohexokinase		'F6P + ATP --> ADP + FBP'
5	4.1.2.13	fructose-bisphosphate aldolase		'FBP --> GLP + GAP'
6	4.1.2.13	fructose-bisphosphate aldolase		'GLP + GAP --> FBP'
7	3.1.3.11	fructose-bisphosphatase		'FBP --> F6P + Pi'
8	5.3.1.1	triose-phosphate isomerase		'GLP --> GAP'
9	5.3.1.1	triose-phosphate isomerase		'GAP --> GLP'
10	1.2.1.12	glyceraldehyde-3-phosphate dehydrogenase		'GAP + Pi + NAD --> PDGLP + NADH'
11	1.2.1.12	glyceraldehyde-3-phosphate dehydrogenase		'PDGLP + NADH --> GAP + Pi + NAD'
12	2.7.2.3	phosphoglycerate kinase		'PDGLP + ADP --> ATP + P3GT'
13	2.7.2.3	phosphoglycerate kinase		'ATP + P3GT --> PDGLP + ADP'
14	5.4.2.11	phosphoglycerate mutase		'P3GT --> P2GT'
15	5.4.2.11	phosphoglycerate mutase		'P2GT --> P3GT'
16	4.2.1.11	phosphoenolpyruvate hydratase		'P2GT --> PEP'
17	4.2.1.11	phosphoenolpyruvate hydratase		'PEP --> P2GT'
18	2.7.1.40	pyruvate kinase		'PEP + ADP --> Pyr + ATP'
19	1.1.1.6	glycerol dehydrogenase	**Glycerol metabolism**	'GLYC + NAD --> GL + NADH '
20	2.7.1.29	glycerone kinase		'GL + ATP --> GLP + ADP'
21	2.7.9.2	phosphoenolpyruvate synthase	**Pyruvate metabolism**	'Pyr + ATP --> PEP + AMP + Pi'

22	1.1.1.38	malate dehydrogenase		'Pyr + CO2 + NADH --> Mal + NAD'
23	1.1.1.38	malate dehydrogenase		'Mal + NAD --> Pyr + CO2 + NADH'
24	1.2.7.1	pyruvate:ferredoxin oxidoreductase		'Pyr + CoA + OFRD --> ACCA + CO2 + RFRD'
25	2.3.1.9	acetyl-CoA C-acetyltransferase	**Acidogenesis**	'2ACCA --> CoA + AACTLCoA'
26	2.3.1.9	acetyl-CoA C-acetyltransferase		'CoA + AACTLCoA --> 2 ACCA'
27	1.1.1.157	3-hydroxyacyl-CoA dehydrogenase		'AACTLCoA + NADH --> HBCoA + NAD'
28	4.2.1.17	enoyl-CoA hydratase		'HBCoA --> CRTCoA'
29	1.3.8.1	butyryl-CoA dehydrogenase		'CRTCoA + NADH --> BUCoA + NAD'
30	2.3.1.19	butyryl transferase		BUCoA + Pi --> BUP + CoA'
31	2.3.1.19	butyryl transferase		BUP + CoA--> Pi + BUCoA'
32	2.7.2.7	butyrate kinase		BUP + ADP --> BU + ATP'
33	2.7.2.7	butyrate kinase		BU + ATP --> BUP + ADP'
34	2.3.1.8	phosphate acetyltransferase		'ACCA + Pi --> ACTP + CoA'
35	2.3.1.8	phosphate acetyltransferase		'ACTP + CoA --> ACCA + Pi'
36	2.7.2.1	acetate kinase		'ACTP + ADP --> AC + ATP'
37	2.7.2.1	acetate kinase		'ATP + AC --> ACTP + ADP'
38	1.2.1.10	aldehyde dehydrogenase	**Solventogenesis**	'BUCoA + NADH --> NAD + BUAL + CoA'
39	1.1.1.1	alcohol dehydrogenase		'BUAL + NADH --> BUOL + NAD'
40	1.1.1.1	alcohol dehydrogenase		'BUOL + NAD --> BUAL + NADH'
41	1.2.1.10	acetaldehyde dehydrogenase		'ACCA + NADH --> ACAL + CoA + NAD'
42	1.2.1.10	acetaldehyde dehydrogenase		'ACAL + CoA + NAD --> ACCA + NADH'
43	1.1.1.1	alcohol dehydrogenase		'ACAL + NADH --> ETOH + NAD'
44	1.1.1.1	alcohol dehydrogenase		'ETOH + NAD --> ACAL + NADH'
45	6.4.1.1	pyruvate carboxylase	**TCA Cycle**	'Pyr + ATP + HCO3- --> ADP + Pi + OXA'

46	1.1.1.37	(S)-malate:NAD+ oxidoreductase		'OXA + NADH --> Mal + NAD'
47	4.2.1.2	fumarate hydratase		'Mal --> Fum'
48	4.2.1.2	fumarate hydratase		'Fum --> Mal'
49	1.3.5.4	fumarate reductase		'Fum + RFRD --> OFRD + Suc'
50	2.3.3.1	citric synthase		'OXA + ACCA --> Cit + CoA'
51	4.2.1.3	aconitate hydratase		'Cit --> iCit'
52	4.2.1.3	aconitate hydratase		'iCit --> Cit'
53	1.1.1.41	isocitrate dehydrogenase		'iCit + NAD --> AKG + NADH + CO2'
54	1.2.7.11	2-oxoacid oxidoreductase (ferredoxin)		'AKG + CoA + OFRD --> RFRD + SCOA + CO2'
55	6.2.1.5	succinyl-coenzyme A synthetase		'SCOA + ADP + Pi --> Suc + CoA + ATP'
56	6.2.1.5	succinyl-coenzyme A synthetase		'Suc + CoA + ATP --> SCOA + ADP + Pi'
57	5.1.3.1	Ribulose phosphate 3- epimerase	**Pentose phospate pathway**	'RL5P --> X5P'
58	5.1.3.1	Ribulose phosphate 3- epimerase		'X5P --> RL5P'
59	5.3.1.6	Ribose 5 phosphote isomerase		'R5P --> RL5P'
60	5.3.1.6	Ribose 5 phosphote isomerase		'RL5P --> R5P'
61	2.2.1.1	Transketolase		'R5P + X5P --> S7P + GAP'
62	2.2.1.1	Transketolase		'S7P + GAP --> R5P + X5P'
63	2.2.1.2	Transaldolase		'GAP + S7P --> F6P + E4P'
64	2.2.1.2	Transaldolase		'F6P + E4P --> GAP + S7P'
65	2.2.1.1	Transketolase		'F6P + GAP --> E4P + X5P'
66	2.2.1.1	Transketolase		'X5P + E4P --> F6P + GAP'
67	2.7.6.1	ribose-phosphate diphosphokinase		'ATP + R5P --> AMP + PRPP'
68	2.4.2.14,6.3.4.13 ,2 .1.2.2 ,6.3.5.3 ,6.3. 3.1,4.1.1.21 ,6.3.2. 6,	Lumped reaction	**Nucleicacid biosynthesis**	'2 Gln + PRPP + 4 ATP + Gly + fTHF + HCO3- + Asp --> AICAR + 2 Glu + 6 Pi + 4 ADP + THF + Fum'

	4.3.2.2			
69	2.1.2.3	AICAR transformylase		'AICAR + fTHF --> FPRICA + THF'
70	3.5.4.10	IMP cyclohydrolase		'FPRICA --> IMP'
71	3.5.4.10	IMP cyclohydrolase		'IMP --> FPRICA'
72	1.1.1.205	IMP dehydrogenase		'IMP + NAD --> XMP + NADH'
73	6.3.5.2	GMP synthase		'ATP + XMP + Gln --> AMP + 2 Pi + GMP + Glu'
74	2.7.4.8	guanylate kinase		'ATP + GMP --> ADP + GDP'
75	2.7.4.8	guanylate kinase		'ADP + GDP --> ATP + GMP'
76	2.7.1.40	pyruvate kinase		'GDP + PEP --> GTP + Pyr'
77	2.7.1.40	pyruvate kinase		'GTP + Pyr --> GDP + PEP'
78	1.17.4.2	ribonucleoside-triphosphate reductase		'GTP + RTRD --> dGTP + OTRD'
79	6.3.4.4	adenylosuccinate synthase		'IMP + GTP + Asp --> GDP + Pi + Fum + AMP'
80	2.7.4.3	adenylate kinase		'ATP + AMP --> 2 ADP'
81	2.7.4.3	adenylate kinase		'2ADP --> ATP + AMP'
82	1.17.4.2	ribonucleoside-triphosphate reductase		'ATP + RTRD --> dATP + OTRD'
83	6.3.5.5 ,2.1.3.2,3.5.2.3,1.3.1.14,2.4.2.10, 4.1.1.23	Lumped reaction		'CAP + Asp + NAD + PRPP --> UMP + CO2 + NADH + 3 Pi'
84	2.7.4.22	UMP kinase		'ATP + UMP --> ADP + UDP'
85	2.7.4.22	UMP kinase		'ADP + UDP --> ATP + UMP'
86	2.7.4.6	nucleoside-diphosphate kinase		'ATP + UDP --> ADP + UTP'
87	2.7.4.6	nucleoside-diphosphate kinase		'ADP + UTP --> ATP + UDP'
88	6.3.4.2	CTP synthase		'ATP + UTP + Gln --> ADP + Pi + CTP + Glu'
89	3.5.4.13	CTP deaminase		'CTP --> UTP + NH3'
90	2.7.4.14	CMP kinase		'ATP + CMP --> ADP + CDP'

91	2.7.4.14	CMP kinase		'ADP + CDP --> ATP + CMP'
92	2.7.4.6	nucleoside-diphosphate kinase		'CDP + ATP --> CTP + ADP'
93	2.7.4.6	nucleoside-diphosphate kinase		'CTP + ADP --> CDP + ATP'
94	3.5.4.13	dCTP deaminase		'dCTP --> dUTP + NH3'
95	3.6.1.23	dUTP diphosphatase		'dUTP --> dUMP + 2 Pi'
96	2.1.1.45	thymidylate synthase		'MLTHF + dUMP --> DHF + dTMP'
97	2.7.4.9	dTMP kinase		'ATP + dTMP --> ADP + dTDP'
98	2.7.4.6	nucleoside-diphosphate kinase		'dTDP + ATP --> dTTP + ADP'
99	1.17.4.2	ribonucleoside-triphosphate reductase		'CTP + RTRD --> dCTP + OTRD'
100	6.3.1.2	glutamine synthetase	**Aminoacid biosynthesis**	'Glu + NH3 + ATP --> Gln + ADP + Pi'
101	1.4.1.13	glutamate synthase		'Gln + AKG + NADPH --> 2 Glu + NADP'
102	2.6.1.1	aspartate transaminase		'OXA + Glu --> Asp + AKG'
103	2.6.1.1	aspartate transaminase		'AKG + Asp --> OXA + Glu'
104	6.3.5.5	carbamoyl-phosphate synthase		'Gln + HCO3- + 2 ATP --> Glu + CAP + 2 ADP + Pi'
105	2.7.2.8,1.2.1.38,2.6.1.11,2.3.1.35,2.1.3.3,6.3.4.5,4.3.2.1	Lumped reaction		2 ATP + NADPH + 2 Glu + CAP + Asp --> Arg + ADP + AKG + NADP + Fum + AMP + 4 Pi'
106	4.3.1.17	L-serine ammonia-lyase		'Pyr + NH3 --> Ser'
107	4.3.1.17	L-serine ammonia-lyase		'Ser --> Pyr + NH3'
108	2.1.2.1	glycine hydroxymethyltransferase		'Ser + THF --> Gly + MLTHF'
109	2.1.2.1	glycine hydroxymethyltransferase		'Gly + MLTHF --> Ser + THF'
110	2.3.1.30	serine O-acetyltransferase		'ACCA + Ser --> CoA + OAS'
111	2.5.1.47	cysteine synthase		'OAS + H2S --> Cys + AC'
112	2.7.7.4,1.8.99.2	Lumped reaction		'SO4 + ATP + RTRD --> SO3 + AMP + 2 Pi + OTRD'
113	1.8.1.2	hydrogen-sulfide:NADP+ oxidoreductase		'SO3 + 3 NADPH --> H2S + 3 NADP'

114	2.6.1.2	alanine transaminase		'Pyr + Glu --> Ala + AKG'
115	6.3.5.4	asparagine synthase		Gln + Asp + ATP --> Asn + AMP + 2 Pi + Glu'
116	2.7.2.4,1.2.1.11,4.3.3.7,1.17.1.8 ,2.3.1.117,2.6.1.17,3.5.1.18,5.1.1.7,4.1.1.20	Lumped reaction		'Pyr + ATP + Asp + NADPH + NADH + SCOA + Glu --> Lys + Suc + NADP + NAD + ADP + Pi + CoA + CO2'
117	2.7.2.4, 1.2.1.11, 1.1.1.3	Lumped reaction		'Asp + ATP + 2 NADPH --> HSer + ADP + 2 NADP + Pi'
118	2.3.1.46, 2.5.1.49, 4.4.1.8, 2.1.1.13	Lumped reaction		'HSer + SCOA + Cys + MTHF --> Met + CoA + Suc + Pyr + NH3 + THF'
119	2.7.1.39, 4.2.3.1	Homoserine kinase, threonine synthase		'HSer + ATP --> Thr + ADP + Pi'
120	4.1.2.48	low-specificity L-threonine aldolase		'Thr --> Gly + ACAL'
121	4.1.2.48	low-specificity L-threonine aldolase		'Gly + ACAL --> Thr'
122	2.7.2.11,1.2.1.41,1.5.1.2	Lumped reaction		'Glu + ATP + 2 NADPH --> Pro + ADP + 2 NADP + Pi'
123	4.3.1.19, 2.2.1.6, 1.1.1.86, 4.2.1.9, 2.6.1.42	Lumped reaction		'Pyr + Thr + NADPH + Glu --> Ile + AKG + NADP + NH3 + CO2'
124	2.2.1.6, 1.1.1.86, 4.2.1.9, 2.6.1.42	Lumped reaction		'2 Pyr + Glu + NADPH --> Val + AKG + NADP + CO2'
125	2.2.1.6, 1.1.1.86, 4.2.1.9, 2.3.3.13, 4.2.1.33, 1.1.1.85, 2.6.1.42	Lumped reaction		'2 Pyr + ACCA + Glu + NADPH + NAD --> Leu + AKG + NADP + NADH + CoA + 2 CO2'
126	2.5.1.54, 4.2.3.4, 4.2.1.10, 1.1.1.25, 2.7.1.71, 2.5.1.19, 4.2.3.5	Lumped reaction		'2 PEP + E4P + NADPH + ATP --> Chr + NADP + ADP + 4 Pi'
127	4.1.3.27, 2.4.2.18, 5.3.1.24, 4.1.1.48, 4.2.1.20	Lumped reaction		'Chr + Gln + PRPP + Ser --> Trp + Glu + GAP + Pyr + CO2 + 2 Pi'
128	5.4.99.5, 1.3.1.12 ,	Lumped reaction		'Chr + NAD + Glu --> Tyr + AKG + NADH + CO2'

	2.6.1.1			
129	5.4.99.5, 4.2.1.91,2.6.1.1	Lumped reaction		'Chr + Glu --> Phe + AKG + CO2'
130	2.4.2.17, 3.6.1.31, 3.5.4.19, 5.3.1.16, 2.4.2.-	Lumped reaction		'PRPP + ATP + Gln --> IGo3P + AICAR + Glu + 4 Pi'
131	4.2.1.19, 2.6.1.9, 3.1.3.15, 1.1.1.23	Lumped reaction		'IGo3P + Glu + 2 NAD --> His + 2 NADH + AKG + Pi'
132	2.6.1.16	glucosamine 6-phosphate synthase	**Aminosugar biosynthesis**	'Gln + F6P --> Glu + GS6P'
133	5.4.2.10	phosphoglucosamine mutase		'GS1P --> GS6P'
134	5.4.2.10	phosphoglucosamine mutase		'GS6P --> GS1P'
135	2.3.1.157	glucosamine-1-phosphate N-acetyltransferase		'ACCA + GS1P --> CoA + NAG1P'
136	2.7.7.23	UDP-N-acetylglucosamine diphosphorylase		'UTP + NAG1P --> UDPNAG + 2 Pi'
137	2.5.1.7	UDP-N-acetylglucosamine 1-carboxyvinyltransferase		'PEP + UDPNAG --> UDPNACG + Pi'
138	1.3.1.98	UDP-N-acetylmuramate dehydrogenase		'UDPNACG + NADPH --> UDPNAM + NADP'
139	6.3.2.8	UDP-N-acetylmuramate-L-alanine ligase		'ATP + UDPNAM + Ala --> ADP + Pi + UDPNAMA'
140	6.3.2.9	D-glutamate ligase		'UDPNAMA + Glu + ATP --> ADP + Pi + UDPNAMAG'
141	6.3.2.4	D-alanine-D- alanine Ligase		'ATP + 2 Ala --> ADP + Pi + DALDAL'
142	5.4.2.2	Phosphoglucomutase	**Carbohydrate biosynthesis**	'G6P --> G1P'
143	5.4.2.2	Phosphoglucomutase		'G1P --> G6P'
144	2.7.7.27, 2.4.1.21, 2.4.1.18	Lumped reaction		'G1P + ATP --> Carbo + ADP + 2 Pi'
145	6.4.1.2	acetyl-CoA carboxylase	**Fattyacid biosynthesis**	'ATP + HCO3- + ACCA --> ADP + Pi + MLCA'
146	2.3.1.38	[ACP]acetyltransferase		'ACCA + ACP --> CoA + AACP'
147	2.3.1.39	malonyl transacylase		'MLCA + ACP --> CoA + MLACP'
148	N/A	N/A		'AACP + 7 MLACP + 7 NADPH + 7 NADH --> HACP + 7

				ACP + 7 NAD + 7 NADP + 7 CO2'
149	2.3.1.85 / 3.1.2.14	fatty-acid synthase		'HACP --> ACP + HDA'
150	N/A	N/A		'HDA + CoA --> ACLCA'
151	1.1.1.94	glycerol-3-phosphate dehydrogenase	**Glycero-phospholipid biosynthesis**	'GLP + NADPH --> G3P + NADP'
152	1.1.1.94	glycerol-3-phosphate dehydrogenase		'G3P + NADP --> GLP + NADPH'
153	2.3.1.15,2.3.1.51	Lumped reaction		'2ACLCA + G3P --> PA + 2 CoA'
154	2.7.1.107	diacylglycerol kinase		'ATP + DAG --> PA + ADP'
155	2.7.7.41	phosphatidate cytidylyltransferase		'CTP + PA --> CDPDAGLYC + 2 Pi'
156	2.7.8.5,3.1.3.27	Lumped reaction		'CDPDAGLYC + G3P --> CMP + PG + Pi'
157	3.1.4.3	phospholipase C		'PG --> DAG + G3P'
158	N/A	N/A		'PG + Lys --> POG'
159	2.7.8.-	transferase for other substituted phosphate groups		'CDPDAGLYC + PG --> CDL + CMP'
160	2.7.7.9	UDPG phosphorylase	**Lipoteichoic acid Biosynthesis**	'G1P + UTP --> UDPGlu + 2 Pi'
161	2.7.7.9	UDPG phosphorylase		'UDPGlu + 2 Pi --> G1P + UTP'
162	2.4.1.157	1,2-diacylglycerol 3-glucosyltransferase		'UDPGlu + DAG --> UDP + 3D12DAG'
163	2.4.1.-	hexosyltransferase		'3D12DAG + UDPGlu --> DGDG + UDP'
164	2.7.8.20	phosphatidylglycerol-membrane-oligosaccharide		'PG + DGDG --> DAG + GPGL'
165	N/A	N/A		'PG + GPGL + Ala --> LTA'
166	1.18.1.3	NAD+-ferredoxin oxidoreductase	**ferredoxin-NADP+ reductase**	'RFRD + NAD --> OFRD + NADH'
167	1.18.1.3	NADH-ferredoxin oxidoreductase		'OFRD + NADH --> RFRD + NAD'
168	1.18.1.2	NADP+-ferredoxin oxidoreductase		'RFRD + NADP --> OFRD + NADPH'
169	1.8.1.9	thioredoxine reductase		'OTRD + NADPH --> RTRD + NADP'
170	1.12.7.2	hydrogenase		'RFRD --> OFRD + H2'
171	1.5.1.5	methylenetetrahydrofolate dehydrogenase	**One carbon pool by folate**	'MLTHF + NADP --> METHF + NADPH'

		(NADP)		
172	1.5.1.5	methylenetetrahydrofolate dehydrogenase (NADP)		'METHF + NADPH --> MLTHF + NADP'
173	1.5.1.20	5,10-methylenetetrahydrofolate reductase (NADPH)		'MLTHF + NADPH --> MTHF + NADP'
174	3.5.4.9	cyclohydrolase		'METHF --> fTHF'
175	3.5.4.9	cyclohydrolase		'fTHF --> METHF'
176	1.5.1.3	Dihydrofolate reductase		'DHF + NADP --> FOL + NADPH'
177	1.5.1.3	Dihydrofolate reductase		'FOL + NADPH --> DHF + NADP'
178	1.5.1.3	Dihydrofolate reductase		'THF + NADP --> DHF + NADPH'
179	1.5.1.3	Dihydrofolate reductase		'DHF + NADPH --> THF + NADP'
180	3.6.1.15	nucleoside-triphosphatase	**Maintenance Energy**	'ATP --> ADP + Pi'
181	4.2.1.1	carbonic anhydrase	**HCO3**	'CO2 --> HCO3-'
182	2.7.7.39	glycerol-3-phosphate cytidylyltransferase	**Wall Teichoicacid Biosynthesis**	'CTP + G3P --> CDPGLY + 2Pi'
183	2.7.7.39	glycerol-3-phosphate cytidylyltransferase		'CDPGLY + 2 Pi --> G3P + CTP'
184	N/A	N/A		'UDPNAG + CDPGLY --> WTA'
185	BIOMASS Precursors		**Cellwall biosynthesis**	'0.95 Peptido + 0.05 WTA --> Cellwall'
186			**Lipid Biosynthesis**	'0.497 PG + 0.0622 CDL + 0.0623 POG + 0.0747 DGDG + 0.0125 3D12DAG + 0.207 DAG --> LIPID'
187			**DNA biosynthesis**	'0.345 dATP + 0.155 dGTP + 0.155 dCTP + 0.345 dTTP + 0.0324 ATP --> DNA + 0.0324 ADP + 0.0324 Pi'
188			**RNA biosynthesis**	'0.77374 ATP + 0.29 GTP + 0.214 CTP + 0.225 UTP --> RNA + 0.50274 ADP + 0.50274 Pi'
189			**Protein biosynthesis**	'0.014 Glu + 0.115 Gly + 0.0635 Ala + 0.036 Lys + 0.017 Asp + 0.014 Arg + 0.014 Gln + 0.045 Ser + 0.083 Met + 0.005 Trp + 0.02 Phe + 0.085 Tyr + 0.13 Cys + 0.046 Leu + 0.016 His + 0.049 Pro + 0.017 Asn + 0.125 Val + 0.044 Thr + 0.046 Ile + 3.9776 ATP --> Protein + 3.9776 ADP + 3.9776 Pi'
190			**Peptidoglycan**	'UDPNAMAG + 3 ATP + NH3 + Lys + 5 Gly + DALDAL +

	BIOMASS Precursors	**biosynthesis**	UDPNAG --> Peptido'
191		**Biomass synthesis**	'4.675 Protein + 0.216 RNA + 0.032 DNA + 0.15 LTA + 0.17 LIPID + 0.108 Cellwall + 0.123 Carbo + 40 ATP --> Biomass + 40 ADP + 40 Pi'
192		**Exchange reactions**	'--> Glc'
193			'--> GLYC'
194			'--> NH3'
195			'--> SO4'
196			'--> Pi'
197			'Biomass -->'
198			'BUOL -->'
199			'ETOH -->'
200			'BU -->'
201			'AC -->'
202			'H2 -->'
203			'CO2 -->'
204			'Suc -->'
205			'--> AC'
206			'--> BU'

*- Abbreviations of the metabolites involved in the reactions are listed in Table A3

Table A3 Abbreviations and notations used in the reactions and model development

Abbreviations and meaning	
3D12DAG	3-D-Glucosyl-1,2-diacylglycerol
3PG	3-phospho glycerate
AACP	Acetyl-[acyl-carrier protein]
AACTLCoA	Acetoacetyl-CoA
AC	Acetate
ACP	Acyl-carrier protein
ACAL	Acetaldehyde
ACCA	Acetyl coenzyme A
ACTAC	Acetoacetate
ACTP	Acetyl phosphate
ACLCA	Acyl-CoA
ADP	Adenosine diphosphate
AICAR	5 Aminoimidazole 4 carboxamide ribonucleotide
AKG	α keto glutarate
Ala	Alanine
AMP	Adenosine monophosphate
Arg	Arginine
Asn	Aspargine
Asp	Asparate
ATP	Adenosine triphosphate
BUAL	Butyraldehyde
BUCoA	Butanoyl-CoA
BUP	Butanoyl phosphate
BU	Butyric acid
BUOL	Butanol
Carbo	Carbohydrate
CAP	Carbamoyl phosphate
CDP	Cytidine diphosphate
CDPDAGLYC	CDPdiacylglycerol
CDPGLY	CDP-Glycerol
CDL	Cardiolipin
Chr	Chorismate
Cit	Citric acid
CMP	Cytidine diphosphate
CO2	Carbon dioxide
CoA	Coenzyme A
CRTCoA	Crotonyl-CoA
CTP	Cytidine triphosphate
Cys	Cysteine
DAG	1,2-Diacylglycerol
DALDAL	D-Alanyl-D-Alanine

dATP	Deoxy adenosine triphosphate
dCDP	Deoxy cytidine diphosphate
dCMP	Deoxy cytidine 5' monophosphate
dCTP	Deoxy cytidine triphosphate
dUDP	Deoxy uridine diphosphate
DGDG	Digalactosyldiacylglycerol
dGTP	Deoxy guanosine triphosphate
dUMP	Deoxy uridine 5' monophosphate
dUTP	Deoxy uridine triphosphate
DHF	Dihydro folate
DNA	Deoxy ribose nucleic acid
dTMP	Deoxy thymidine 5' monophosphate
dTTP	Deoxy thymidine triphosphate
E4P	Erythrose 4 phosphate
ETOH	Ethanol
F6P	Fructose 6 phosphate
FBP	Fructose 1,6 biphosphate
FOL	Folate
FPRICA	5'-Phosphoribosyl-5-formamido-4-imidazolecarboxamide
Fum	Fumarate
HCO3	Bicarbonate
fTHF	10-Formyltetrahydrofolate
G3P	Glycerol 3-phosphate
G1P	Glucose 1 phosphate
G6P	Glucose 6 phosphate
GAP	Glyceraldehyde 3 phosphate
GL	Glycerone
Glc	Glucose
GLP	Glycerone phosphate/Dihydroxy acetone phosphate
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GLYC	Glycerol
GMP	Guanosine 5' monophosphate
GPGL	Glycerophophoglycoglycerolipid
GS1P	Glucosamine 1-phosphate
GS6P	Glucosamine 6-phosphate
GTP	Guanosine triphosphate
H2	Hydrogen
H2S	Hydrogen sulfide
HACP	Hexadecanoyl-[acp]
HBCoA	3-hydroxybutanoyl-CoA
HDA	Hexadecanoic acid

His	Histidine
HSer	Homo serine
iCit	Isocitrate
IGo3P	Imidazole glycerol 3 phosphate
IMP	Inosine monophosphate
Ile	Isoleucine
Leu	Leucine
LTA	Lipoteichoic Acid
Lys	Lysine
Mal	Malate
Met	Methionine
MTHF	5-Methyltetrahydrofolate
METHF	5,10-methenyltetrahydrofolate
MLTHF	5,10-Methylenetetrahydrofolate
MLCA	Malonyl-CoA
MLACP	Malonyl-[acyl-carrier protein]
MnTHF	N5,N10 Methylene tetra hydrofolate
MTHF	N5 Methyl tetrahydrofolate
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAG1P	N-Acetyl-D-glucosamine 1-phosphate
NH3	Ammonia
OAS	O-acetyl-L-serine
OFRD	Oxidized ferredoxin
OTRD	Oxidized thioredoxin
OXA	Oxaloacetate
P3GT	3-phospho-D-glycerate
P2GT	2-phospho-D-glycerate
PDGLP	3-phospho-D-glyceroyl phosphate
Peptido	Peptidoglycan
PEP	Phosphoenol pyruvate
PA	Phosphatidate
PG	Phosphatidylglycerol
POG	3-Phosphatidyl-1'-(3'-O-L-lysyl)glycerol
Phe	Phenyl alanine
Pi	Inorganic phosphate
PPP	Phytyl pyrophosphate
Pro	Proline
PRPP	5 phosphoribosyl 1 pyrophosphate
Pyr	Pyruvate

RL5P	Ribulose 5 phosphate
RFRD	Reduced ferredoxin
RNA	Ribose nucleic acid
R5P	Ribose 5 phosphate
RTRD	Reduced thioredoxin
SAHC	S- Adenosyl homocysteine
SAM	S Adenosyl methionine
SCOA	Succinyl coenzyme A
Ser	Serine
S7P	Sedoheptulose 7 phosphate
SO3	Sulfite
SO4	Sulfate
Suc	Succinate
THF	Tetra hydrofolate
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UDP	Uridine diphosphate
UDPGlu	UDP-glucose
UDPNAG	UDP-N-acetyl-D-glucosamine
UDPNACG	UDP-N-acetyl-3-(1-carboxyvinyl)-D-glucosamine
UDPNAM	UDP-N-acetylmuramate
UDPNAMA	UDP-N-acetylmuramoyl-L-alanine
UDPNAMAG	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate
UMP	Uridine 5' monophosphate
UTP	Uridine triphosphate
WTA	Wall Teichoic acid
XMP	Xanthosine 5'-phosphate
X5P	Xylulose 5 phosphate

Table A4 Flux distribution (mmole $gDCW^{-1}$ h^{-1}) in metabolic network of *C. sporogenes* NCIM 2918 for different dual substrate combinations when maximization of biomass (7 h) was used as the objective function

Reaction No	100:0	80:20	60:40	40:60	20:80	0:100
1	16.1	11.21	8.1	7.61995813	6.22	1.69E-08
2	6102.713768	4290.33044	2164.20221	177.853777	1080.95827	678.4782317
3	6086.781171	4279.27832	2156.25936	170.39225	1074.88577	678.5929913
4	15.71767554	10.8319885	7.68044905	7.12573843	5.86969143	1.12E-02
5	6085.238825	4144.12129	2042.15303	168.847301	1038.05024	679.1226986
6	6069.663047	4133.40569	2034.54514	161.721579	1032.29213	679.4820693
7	1.42E-01	1.16E-01	7.26E-02	1.37E-06	1.12E-01	3.71E-01
8	5829.169651	4128.70781	5501.82709	498.281929	7747.81418	7722.573134
9	5813.919854	4108.74965	5479.22523	475.766176	7725.84328	7698.645975
10	7001.926588	7180.44	7187.24664	646.243231	5930.49928	6061.491541
11	6971.32715	7149.97951	7157.24918	616.813882	5902.96952	6038.078777
12	7001.931115	7180.44155	7187.24697	646.243224	5930.50058	6061.492446
13	6971.331677	7149.98107	7157.24952	616.813883	5902.97082	6038.079683
14	7001.934668	7180.44291	7187.24743	646.243229	5930.50166	6061.493417
15	6971.33523	7149.98242	7157.24998	616.813898	5902.9719	6038.080653
16	7001.937288	7180.44398	7187.24792	646.243242	5930.50253	6061.494385
17	6971.33785	7149.98349	7157.25046	616.813922	5902.97277	6038.081621
18	30.14094094	30.0001491	29.504598	28.8548606	27.081123	23.00348209
19	2.96E-09	9.55E+00	1.53E+01	1.57E+01	1.65E+01	2.45E+01
20	3.57E-09	9.55E+00	1.53E+01	1.57E+01	1.65E+01	2.45E+01
21	1.54E-01	1.18E-01	8.26E-02	1.37E-06	9.14E-02	1.09E-02
22	1176.228461	1087.69303	1126.76877	101.974226	751.037848	679.2436478
23	1176.77607	1088.16308	1127.1811	102.30977	751.389587	679.4409441

24	27.3725707	27.4104488	26.9374568	26.3587582	24.6337126	21.14228842
25	3634.029028	4506.06458	3998.87669	333.46359	1715.46941	792.3494699
26	3622.899028	4493.63458	3987.15669	322.533394	1706.79941	788.3094699
27	11.13	12.43	11.72	10.9301954	8.67	4.04
28	11.13	12.43	11.72	10.9301955	8.67	4.04
29	11.13	12.43	11.72	10.9301955	8.67	4.04
30	2827.801615	2750.40018	2608.41504	216.794742	1214.41245	716.5777009
31	2818.491615	2741.41018	2599.51504	208.214627	1207.54245	714.1577009
32	2827.801628	2750.40021	2608.41509	216.794647	1214.41245	716.5776989
33	2818.491628	2741.41021	2599.51509	208.214558	1207.54245	714.1576989
34	1308.052057	1429.25866	1458.93261	126.232496	800.743443	689.2927749
35	1311.602061	1434.59761	1464.21996	131.368347	803.469449	690.6564241
36	1308.052057	1429.25866	1458.93261	126.232502	800.743444	689.2927756
37	1311.60206	1434.59761	1464.21996	131.368366	803.46945	690.6564248
38	1.819999999	3.44	2.82	2.35008012	1.8	1.62
39	1207.660323	1211.89364	1207.88245	108.012841	772.771936	695.3973336
40	1205.840323	1208.45364	1205.06245	105.662788	770.971936	693.7773336
41	1482.761636	1315.99543	1483.79445	147.414518	1209.97435	3554.732914
42	1477.701079	1311.50206	1478.39086	141.250834	1203.12792	3542.776809
43	1490.052607	1321.6166	1491.37516	148.274536	1218.29834	3563.616409
44	1484.942607	1317.0766	1485.92516	142.06451	1211.40834	3551.626409
45	1.485385283	1.36122252	1.323431	1.26168624	1.23088955	0.898526695
46	1.92E-01	1.42E-01	1.09E-01	1.35E-06	9.14E-02	1.11E-02
47	1175.108823	1087.4514	1127.67357	102.191911	753.326176	682.6213996
48	1175.464789	1087.77931	1127.97677	102.527467	753.586508	682.807605
49	1.24E-03	8.98E-03	3.21E-02	3.08E-07	5.44E-02	5.87E-02
50	0.301958674	0.28370383	0.28325383	0.33116485	0.26563251	0.207536569
51	1175.12441	1087.55014	1127.91071	102.527143	753.646697	682.8811796

52	1174.822451	1087.26644	1127.62746	102.196002	753.381065	682.673643
53	0.301958675	0.28370383	0.28325383	0.33111721	0.26563251	0.207536569
54	1.16E-03	2.04E-05	8.80E-04	4.91E-02	6.05E-04	1.33E-03
55	1174.983869	1087.68113	1128.31806	102.342005	755.157372	684.4207164
56	1175.231479	1087.91573	1128.55072	102.526031	755.375959	684.5899268
57	1173.881627	1085.98744	1124.91313	101.994563	750.270925	678.5940469
58	1174.160193	1086.25016	1125.17463	102.256352	750.516367	678.7850128
59	1173.881627	1085.98744	1124.91313	101.99457	750.270926	678.5940469
60	1174.160193	1086.25016	1125.17463	102.256344	750.516367	678.7850128
61	1173.245741	1085.44348	1124.3645	102.046898	749.96225	678.4395772
62	1173.289483	1085.48473	1124.40556	102.088414	750.000791	678.4695639
63	1173.245741	1085.44348	1124.3645	102.046887	749.96225	678.4395772
64	1173.289483	1085.48473	1124.40556	102.088425	750.000791	678.4695638
65	1173.914728	1086.03522	1124.95856	102.218434	750.374776	678.7002883
66	1173.679904	1085.81375	1124.73812	101.998147	750.167875	678.5393091
67	0.322308209	0.30397334	0.30257003	0.30327552	0.28398274	0.220952612
68	0.164865586	0.155487	0.15476918	0.1549921	0.14526151	0.11302063
69	0.192659273	0.1816996	0.18086077	0.18100526	0.16975026	0.132074088
70	1173.71423	1085.85941	1124.78185	102.185023	750.258047	678.6298787
71	1173.52157	1085.67771	1124.60099	102.004042	750.088297	678.4978047
72	0.024642736	0.02324091	0.02313361	0.02310944	0.02171248	0.016893387
73	0.024642737	0.02324091	0.02313361	0.0230855	0.02171248	0.016893387
74	1173.266941	1085.46443	1124.38523	102.078192	749.985267	678.4589634
75	1173.242298	1085.44119	1124.3621	102.055122	749.963555	678.44207
76	1173.71423	1085.85941	1124.78185	102.184984	750.258046	678.6298781
77	1173.52157	1085.67771	1124.60099	102.004078	750.088296	678.4978041
78	0.001367383	0.0012896	0.00128365	0.00128883	0.00120479	0.000937389
79	0.168016537	0.1584587	0.15772716	0.15784794	0.14803777	0.115180701

80	1188.047391	1095.36788	1134.49913	103.153389	757.194684	682.6947341
81	1186.954121	1094.36472	1133.53526	102.272958	756.276073	682.0402297
82	0.004102166	0.00386881	0.00385095	0.00384748	0.00361438	0.002812168
83	0.12096341	0.11408229	0.11355563	0.11411551	0.10657975	0.082924318
84	1173.454627	1085.63112	1124.55248	102.134424	750.104044	678.5355083
85	1173.333664	1085.51703	1124.43893	102.020316	749.997465	678.4525839
86	1173.955901	1086.07127	1124.9948	102.226077	750.398619	678.7146023
87	1173.713238	1085.84242	1124.767	101.996339	750.184812	678.5482496
88	0.16406303	0.13794755	0.10186275	0.02331155	0.102346	0.023982796
89	1.39E-01	1.15E-01	7.87E-02	6.84E-07	8.06E-02	7.08E-03
90	1174.406	1086.15861	1125.43781	102.221711	751.249492	680.9316323
91	1174.105672	1085.88613	1125.21712	102.056871	751.019677	680.8079344
92	1174.405999	1086.15861	1125.43781	102.221712	751.249492	680.931632
93	1174.105672	1085.88613	1125.21712	102.05687	751.019677	680.8079341
94	0.004102131	0.00386881	0.00385095	0.00388478	0.00361437	0.002812168
95	0.004102134	0.00386881	0.00385095	0.00388862	0.00361437	0.002812168
96	0.00410214	0.00386881	0.00385095	0.00389247	0.00361438	0.002812168
97	0.004102147	0.00386881	0.00385095	0.00387142	0.00361438	0.002812168
98	0.004102157	0.00386881	0.00385095	0.00385325	0.00361438	0.002812168
99	0.005469519	0.00515841	0.0051346	0.00516026	0.00481917	0.003749558
100	3.016754281	2.82835968	2.77985436	2.69938613	2.61582509	1.97959369
101	2.184451644	2.06018614	2.0506751	2.0477049	1.92469947	1.497511251
102	1183.596647	1094.47298	1133.44087	103.286437	755.790799	681.8526537
103	1182.604864	1093.53761	1132.50983	102.355927	754.916948	681.1727545
104	0.145282887	0.13701832	0.13638577	0.13683637	0.1280074	0.099596093
105	0.024319477	0.02293603	0.02283014	0.02272952	0.02142766	0.016671776
106	1183.008416	1093.96126	1132.92669	103.228079	755.464986	681.6647825
107	1182.04575	1093.05336	1132.02298	102.326169	754.616791	681.0048446

108	1176.050999	1087.90165	1126.83517	102.499117	751.588401	679.4167716
109	1175.545192	1087.42462	1126.36034	102.024383	751.14274	679.0700249
110	0.370003475	0.3489553	0.34734431	0.34593965	0.32600651	0.253649161
111	0.370003475	0.3489553	0.34734431	0.34590139	0.32600651	0.253649161
112	0.370003475	0.3489553	0.34734431	0.34590717	0.32600651	0.253649161
113	0.370003475	0.3489553	0.34734431	0.34591294	0.32600651	0.253649161
114	0.280412461	0.26446082	0.26323992	0.26340595	0.24706875	0.192231689
115	0.029530794	0.02785089	0.02772232	0.02760518	0.0260193	0.020244299
116	0.104594548	0.09864455	0.09818915	0.09827765	0.09215726	0.071702901
117	0.349962328	0.3300542	0.32853046	0.32732784	0.30834843	0.239910293
118	0.144179758	0.13597789	0.13535013	0.13480155	0.1270354	0.098839814
119	0.20578257	0.19407631	0.19318033	0.1925216	0.18131302	0.141070479
120	1173.297618	1085.49202	1124.41286	102.091237	750.006192	678.4731652
121	1173.248175	1085.44539	1124.36645	102.044881	749.962628	678.4392704
122	0.08511817	0.0802761	0.0799055	0.07958903	0.0749968	0.058351215
123	0.079906854	0.07536124	0.07501333	0.07471156	0.07040516	0.054778692
124	0.21713819	0.20478598	0.20384056	0.20301103	0.19131837	0.148855141
125	0.079906853	0.07536124	0.07501333	0.07473647	0.07040516	0.054778692
126	0.191081605	0.18021166	0.17937969	0.17873775	0.16836017	0.130992524
127	0.008685526	0.00819144	0.00815362	0.00814692	0.00765273	0.005954206
128	0.147653969	0.13925446	0.13861158	0.13805753	0.13009649	0.101221496
129	0.03474211	0.03276576	0.03261449	0.03250418	0.03061094	0.023816823
130	0.027793687	0.0262126	0.02609159	0.0260122	0.02448875	0.019053458
131	0.027793688	0.0262126	0.02609159	0.02599584	0.02448875	0.019053458
132	0.078253328	0.0738018	0.07346109	0.0739467	0.06894827	0.053645164
133	1173.270368	1085.46361	1124.38493	102.033906	749.969962	678.4408143
134	1173.348621	1085.53741	1124.4584	102.107838	750.03891	678.4944594
135	0.078253328	0.0738018	0.07346109	0.07391638	0.06894827	0.053645164

136	0.078253329	0.0738018	0.07346109	0.07388787	0.06894827	0.053645164
137	0.038123416	0.03595472	0.03578873	0.03606924	0.03359018	0.026134824
138	0.038123417	0.03595472	0.03578873	0.03602833	0.03359018	0.026134824
139	0.038123418	0.03595472	0.03578873	0.03598743	0.03359018	0.026134824
140	0.03812342	0.03595472	0.03578873	0.03593894	0.03359018	0.026134824
141	0.038123421	0.03595472	0.03578873	0.03589045	0.03359018	0.026134824
142	1173.611095	1085.76883	1124.69083	102.166818	750.197405	678.5929928
143	1173.443693	1085.61095	1124.53368	102.008404	750.049908	678.4782331
144	0.045703525	0.04310361	0.04290462	0.04274012	0.04026894	0.031331221
145	2.425408054	2.28743584	2.27687576	2.31420065	2.13700416	1.662694926
146	0.346486865	0.32677655	0.32526797	0.33059062	0.30528631	0.237527847
147	2.425408053	2.28743584	2.27687576	2.31421697	2.13700416	1.662694926
148	0.346486865	0.32677655	0.32526797	0.33060487	0.30528631	0.237527847
149	0.346486865	0.32677655	0.32526797	0.33054586	0.30528631	0.237527847
150	0.346486867	0.32677655	0.32526797	0.33045924	0.30528631	0.237527847
151	1174.469387	1086.52062	1125.44652	102.302464	750.693435	678.8902992
152	1174.143407	1086.21318	1125.1405	101.992534	750.406216	678.6668291
153	0.173243433	0.16338827	0.16263398	0.1651997	0.15264315	0.118763923
154	1.27E-01	1.09E-01	5.81E-02	4.71E-07	7.72E-02	4.93E-03
155	0.145668059	0.12661585	0.07550182	0.01799696	0.09354559	0.017673794
156	0.003935328	0.00371147	0.00369433	0.00390883	0.00346738	0.002697796
157	0.300327632	0.27247742	0.22069002	0.16501935	0.22981467	0.123697882
158	0.296398625	0.26877191	0.21700162	0.16080051	0.22635286	0.121004416
159	0.00392901	0.00370551	0.0036884	0.00404186	0.00346182	0.002693466
160	1173.456769	1085.63301	1124.55438	102.135347	750.105338	678.5363146
161	1173.33507	1085.51824	1124.44013	102.019691	749.99811	678.4528861
162	0.061244195	0.05776024	0.05749359	0.05828315	0.05396168	0.041984855
163	0.060454604	0.05701557	0.05675235	0.05735255	0.05326598	0.041443564

164	0.055735997	0.05256538	0.05232271	0.0527226	0.04910846	0.038208806
165	0.055736	0.05256538	0.05232271	0.05250965	0.04910846	0.038208806
166	1173.249622	1085.44854	1124.36936	102.067156	749.972011	678.4493323
167	1173.247616	1085.44665	1124.36748	102.065237	749.970244	678.4479567
168	0.002006491	0.00189235	0.00188362	0.00191578	0.0017679	0.001375517
169	1893.169239	1149.39837	1311.67212	123.451348	1567.64914	4854.492976
170	1886.254796	1147.26538	1315.64988	128.302599	1575.67703	4866.102903
171	7.822782136	7.3777728	7.3437127	7.37058355	6.89257878	5.362766827
172	0.380942543	0.35927213	0.35761352	0.35619795	0.33564485	0.261148276
173	12.63527394	17.8907247	23.5402687	23.8884764	25.7152428	27.33211345
174	1174.699998	1086.72221	1125.64923	102.331854	750.824828	678.9680715
175	1174.342474	1086.38503	1125.3136	101.995835	750.509816	678.7229768
176	0.144179758	0.13597789	0.13535013	0.13481222	0.1270354	0.098839814
177	1174.699998	1086.72221	1125.64923	102.331848	750.824828	678.9680714
178	1174.342474	1086.38503	1125.3136	101.99584	750.509816	678.7229766
179	1173.248579	1085.44756	1124.36838	102.066186	749.971106	678.448632
180	1173.248579	1085.44756	1124.36838	102.066201	749.971106	678.448632
181	1173.246695	1085.44577	1124.3666	102.064246	749.96939	678.4472782
182	1173.250797	1085.44964	1124.37045	102.068167	749.973004	678.4500903
183	1.49E-01	1.10E-01	9.87E-02	1.38E-06	9.31E-02	1.09E-02
184	4.22094181	3.94116368	3.89146172	3.86771536	3.64116262	2.773838346
185	0.040129921	0.03784708	0.03767235	0.03763211	0.03535809	0.027510341
186	0.063167471	0.0595741	0.05929907	0.05923135	0.05565625	0.043303314
187	0.011890342	0.01121395	0.01116218	0.01112455	0.01047647	0.008151212
188	0.080259848	0.07569415	0.0753447	0.07503269	0.07071618	0.055020681
189	1.73710552	1.6382878	1.63072448	1.62389766	1.530547	1.19084113
190	0.038123422	0.03595472	0.03578873	0.0358724	0.03359018	0.026134824
191	0.371573373	0.35043589	0.34881807	0.34735741	0.32738973	0.254725375

192	16.1	11.21	8.1	7.62	6.22	0
193	0	9.55	15.3	15.7	16.5	24.51
194	3.64994305	3.44231122	3.4264195	3.42376935	3.21593007	2.502152462
195	0.370003475	0.3489553	0.34734431	0.34590139	0.32600651	0.253649161
196	0.931886778	0.87887531	0.87481793	0.87840437	0.82107668	0.638838251
197	0.371573373	0.35043589	0.34881807	0.34735741	0.32738973	0.254725375
198	1.82	3.44	2.82	2.35	1.8	1.62
199	5.11	4.54	5.45	6.21	6.89	11.99
200	9.31	8.99	8.9	8.58	6.87	2.42
201	0	0	0	0	0	0
202	12.63527394	17.8907247	23.5402687	23.8884764	25.7152428	27.33211345
203	27.3012669	27.3343096	26.8393471	26.3392855	24.5171613	21.03612115
204	0.0024	0.009	0.033	0.049	0.055	0.06
205	3.18	4.99	4.94	4.79	2.4	1.11
206	0	0	0	0	0	0

Table A5 Flux distribution (mmole $gDCW^{-1}$ h^{-1}) in metabolic network of *C. sporogenes* NCIM 2918 for different dual substrate combinations when maximization of butanol (18 h) was used as the objective function

Reaction No	100:0	80:20	60:40	40:60	20:80	0:100
1	1.18	2.07	1.69	1.4	1.45E-08	8.27E-10
2	4025.070115	6.22827127	3786.763763	3861.5997	5.53032383	3643.9822
3	4023.900477	4.18079742	3785.096289	3860.22043	5.54609212	3643.998
4	1.16343339	2.10026803	1.68282286	1.39363939	0.05937607	5.71E-02
5	4023.422676	6.20388568	3781.742264	3857.72273	5.51352945	3644.0352
6	4022.275125	4.20442631	3780.122804	3856.38763	5.56290801	3644.0846
7	1.59E-02	1.01E-01	6.34E-02	5.85E-02	1.09E-01	1.06E-01
8	4021.946966	7.71484092	4586.692316	4529.64313	8.72029048	5122.1745
9	4020.819593	2.78924639	4581.806722	4525.18839	2.83037447	5116.3246
10	4148.225902	8.78653051	5562.402942	5175.27513	8.67722412	5075.2782
11	4145.964975	1.89190636	5555.928318	5169.51328	2.85798755	5069.499
12	4148.225899	8.78653051	5562.402933	5175.27512	8.67722412	5075.2782
13	4145.964973	1.89190636	5555.928309	5169.51327	2.85798755	5069.499
14	4148.225898	8.78653051	5562.402928	5175.27511	8.67722412	5075.2782
15	4145.964971	1.89190636	5555.928304	5169.51326	2.85798755	5069.499
16	4148.225896	8.78653051	5562.402924	5175.27511	8.67722412	5075.2782
17	4145.96497	1.89190636	5555.9283	5169.51325	2.85798755	5069.499
18	2.237603073	6.92700641	6.451134839	5.74369933	5.80027707	5.7584043
19	1.22E-08	2.97E+00	3.31E+00	3.16E+00	5.97E+00	5.93E+00
20	1.47E-08	2.97E+00	3.31E+00	3.16E+00	5.97E+00	5.93E+00
21	1.46E-02	1.15E-01	5.90E-02	5.77E-02	3.88E-02	3.69E-02
22	3979.546163	5.10022963	3699.476469	3799.67686	5.49578097	3643.0752
23	3979.58774	5.30060069	3699.610639	3799.80216	5.58349925	3643.161

24	2.061146383	6.46073076	6.04071828	5.36265963	5.51532164	5.4754968
25	3993.637165	6.58339017	3751.586498	3827.57219	5.91501194	3644.8196
26	3992.74008	3.83707425	3749.500189	3826.06778	5.16221145	3644.5567
27	0.897084732	2.74631592	2.086309016	1.50440368	0.75280049	0.2629132
28	0.897084732	2.74631592	2.086309016	1.50440368	0.75280049	0.2629132
29	0.897084732	2.74631592	2.086309016	1.50440368	0.75280049	0.2629132
30	3982.501938	4.77793664	3729.196198	3815.19961	5.31420276	3645.2827
31	3982.301938	5.61793664	3730.186198	3815.88961	5.76420276	3645.4927
32	3982.501938	4.77793664	3729.196198	3815.19961	5.31420276	3645.2827
33	3982.301938	5.61793664	3730.186198	3815.88961	5.76420276	3645.4927
34	3981.715498	5.10681368	3700.514531	3801.29744	4.74386947	3752.0462
35	3981.8584	5.28660242	3700.68432	3801.45324	6.35872159	3753.8711
36	3981.715498	5.10681368	3700.514531	3801.29744	4.74386947	3752.0462
37	3981.8584	5.28660242	3700.68432	3801.45324	6.35872159	3753.8711
38	0.697084732	3.58631592	3.076309016	2.19440368	1.20280049	0.4729132
39	3987.030525	7.01451586	3863.019074	3882.27291	6.14220179	3648.4645
40	3986.33344	3.42819994	3859.942765	3880.07851	4.9394013	3647.9916
41	3982.336663	5.52912369	3775.098118	3939.10237	8.3586251	5563.594
42	3982.149723	4.86577697	3773.544771	3937.03849	3.07328234	5557.1587
43	3982.37442	5.53246653	3775.75273	3939.94772	8.36135756	5567.5198
44	3982.18442	4.86246653	3774.19273	3937.87772	3.07135756	5561.0798
45	0.099665037	0.32623797	0.260049444	0.24111765	0.17601448	0.1739889
46	1.96E-02	1.52E-01	8.61E-02	8.11E-02	5.42E-02	5.22E-02
47	3.98E+03	5.17E+00	3.70E+03	3.80E+03	5.52E+00	3.64E+03
48	3981.04599	5.22089237	3700.022542	3800.67256	5.55498879	3643.9799
49	1.38E-04	1.20E-08	1.10E-05	1.13E-05	1.38E-04	1.36E-05
50	0.018670017	0.0404758	0.040476812	0.03723875	0.02838407	2.83E-02
51	3983.163959	5.21709067	3720.522305	3813.88018	5.55370519	3644.1296

52	3983.145289	5.17661488	3720.481829	3813.84294	5.52532112	3644.1012
53	0.018670017	0.0404758	0.040476812	0.03723875	0.02838407	2.83E-02
54	5.11E-05	2.86E-08	1.03E-06	1.03E-06	5.10E-05	1.03E-06
55	3.98E+03	5.18E+00	3.72E+03	3.81E+03	5.53E+00	3.64E+03
56	3.98E+03	5.21E+00	3.72E+03	3.81E+03	5.55E+00	3.64E+03
57	3981.146359	5.17790971	3699.748348	3800.63673	5.5250905	3643.991
58	3981.163602	5.21539423	3699.785832	3800.67121	5.55132977	3644.0173
59	3981.146359	5.17790971	3699.748348	3800.63673	5.5250905	3643.991
60	3981.163602	5.21539423	3699.785832	3800.67121	5.55132977	3644.0173
61	3981.14451	5.19370648	3699.722488	3800.61496	5.53614671	3643.9807
62	3981.147217	5.19959249	3699.728374	3800.62038	5.54026697	3643.9848
63	3981.14451	5.19370648	3699.722488	3800.61496	5.53614671	3643.9807
64	3981.147217	5.19959249	3699.728374	3800.62038	5.54026697	3643.9848
65	3981.15954	5.21245049	3699.770507	3800.65772	5.54926866	3644.0089
66	3981.145005	5.18085198	3699.738908	3800.62865	5.52714965	3643.9867
67	0.019950545	0.04337053	0.043370631	0.03990096	0.03035952	3.04E-02
68	0.010205008	0.02218474	0.022184768	0.02040998	0.01552936	1.55E-02
69	0.011925408	0.02592474	0.025924767	0.02385078	0.01814736	1.81E-02
70	3981.156066	5.20961301	3699.757759	3800.64647	5.54728205	3644.0018
71	3981.144141	5.18368828	3699.731834	3800.62262	5.52913469	3643.9836
72	0.00152536	0.00331598	0.003315987	0.00305071	0.0023212	2.32E-03
73	0.00152536	0.00331598	0.003315987	0.00305071	0.0023212	2.32E-03
74	3981.146468	5.19830743	3699.726366	3800.61857	5.53936738	3643.9835
75	3981.144943	5.19499145	3699.72305	3800.61552	5.53704618	3643.9812
76	3981.156066	5.20961301	3699.757757	3800.64647	5.54728205	3644.0018
77	3.98E+03	5.18E+00	3.70E+03	3.80E+03	5.53E+00	3.64E+03
78	8.46E-05	1.84E-04	1.84E-04	1.69E-04	1.29E-04	1.29E-04
79	0.010400048	0.02260876	0.02260878	0.02080007	0.01582616	1.58E-02

80	3981.359573	5.3171286	3700.828696	3801.53526	5.60190307	3644.3496
81	3981.286844	5.07593637	3700.643375	3801.36131	5.47469532	3644.2243
82	0.00025392	0.00055199	0.000551994	0.00050783	0.0003864	3.86E-04
83	0.007487512	0.01627705	0.016277115	0.01497493	0.01139404	1.14E-02
84	3981.151141	5.20478843	3699.740583	3800.63127	5.54390441	3643.992
85	3981.143653	5.18851138	3699.724306	3800.61629	5.53251037	3643.9806
86	3981.160237	5.21297795	3699.773125	3800.66002	5.54963799	3644.0103
87	3981.145217	5.18032476	3699.740472	3800.62998	5.52678065	3643.9874
88	1.80E-02	1.36E-01	6.47E-02	6.42E-02	4.23E-02	4.12E-02
89	1.65E-02	1.33E-01	6.14E-02	6.11E-02	3.99E-02	3.89E-02
90	3981.080071	5.27697368	3700.047898	3800.90515	5.56834797	3644.0841
91	3981.053004	5.11635279	3699.956872	3800.8171	5.50816807	3644.0239
92	3981.080071	5.27697368	3700.047898	3800.90515	5.56834797	3644.0841
93	3981.053003	5.11635279	3699.956871	3800.8171	5.50816807	3644.0239
94	2.54E-04	5.52E-04	5.52E-04	5.08E-04	3.86E-04	3.86E-04
95	2.54E-04	5.52E-04	5.52E-04	5.08E-04	3.86E-04	3.86E-04
96	0.00025392	0.00055193	0.000551957	0.00050779	0.0003864	3.86E-04
97	0.00025392	0.00055195	0.000551967	0.0005078	0.0003864	3.86E-04
98	0.00025392	0.00055197	0.000551981	0.00050782	0.0003864	3.86E-04
99	3.39E-04	7.36E-04	7.36E-04	6.77E-04	5.15E-04	5.15E-04
100	0.194604128	0.51978311	0.448569375	0.41730812	0.3109636	0.3098792
101	0.135215262	0.29394595	0.2939461	0.27043039	0.20576235	2.06E-01
102	3981.294763	5.26341027	3700.33213	3801.1496	5.58495967	3644.3071
103	3981.233372	5.12995316	3700.198673	3801.02682	5.49153956	3644.2137
104	0.008992862	0.01954955	0.019549615	0.01798563	0.01368479	1.37E-02
105	0.00150535	0.0032725	0.0032725	0.0030107	0.00229075	2.29E-03
106	3981.286991	5.26144929	3700.298829	3801.12046	5.58358582	3644.2896
107	3981.227403	5.13191041	3700.16929	3801.00129	5.49290845	3644.1989

108	3981.192099	5.23068914	3699.899187	3800.77073	5.5620399	3644.0784
109	3981.16079	5.1626265	3699.831124	3800.70811	5.5143959	3644.0307
110	0.022902825	0.04978875	0.04978875	0.04580565	0.03485212	3.49E-02
111	0.022902825	0.04978875	0.04978875	0.04580565	0.03485212	3.49E-02
112	0.022902825	0.04978875	0.04978875	0.04580565	0.03485212	3.49E-02
113	0.022902825	0.04978875	0.04978875	0.04580565	0.03485212	3.49E-02
114	0.017357237	0.03773309	0.037733107	0.03471446	0.02641319	2.64E-02
115	0.001827925	0.00397375	0.00397375	0.00365585	0.00278162	2.78E-03
116	0.006474293	0.01407454	0.014074542	0.01294858	0.00985218	9.85E-03
117	0.021662297	0.04709204	0.047092004	0.04332465	0.03296436	3.30E-02
118	0.008924575	0.01940125	0.01940125	0.01784915	0.01358087	1.36E-02
119	0.012737722	0.02769079	0.027690754	0.0254755	0.01938349	1.94E-02
120	3981.147457	5.19997615	3699.729039	3800.62098	5.54053548	3643.9852
121	3981.144396	5.19332286	3699.722385	3800.61486	5.53587824	3643.9806
122	0.005268725	0.01145375	0.01145375	0.01053745	0.00801762	8.02E-03
123	0.00494615	0.0107525	0.0107525	0.0098923	0.00752675	7.53E-03
124	0.013440625	0.02921875	0.02921875	0.02688125	0.02045312	2.05E-02
125	0.00494615	0.0107525	0.0107525	0.0098923	0.00752675	7.53E-03
126	0.01182775	0.0257125	0.025712499	0.0236555	0.01799875	1.80E-02
127	0.000537625	0.00116875	0.001168749	0.00107525	0.00081812	8.18E-04
128	0.009139625	0.01986875	0.01986875	0.01827925	0.01390812	1.39E-02
129	0.0021505	0.004675	0.004675	0.004301	0.0032725	3.27E-03
130	0.0017204	0.00374	0.003739999	0.0034408	0.002618	2.62E-03
131	0.0017204	0.00374	0.003739999	0.0034408	0.002618	2.62E-03
132	0.0048438	0.01052996	0.01052998	0.00968758	0.007371	7.37E-03
133	3981.143949	5.19138464	3699.722481	3800.61484	5.53452152	3643.9802
134	3981.148792	5.2019146	3699.733011	3800.62453	5.54189252	3643.9876
135	0.0048438	0.01052996	0.01052998	0.00968758	0.007371	7.37E-03

136	0.0048438	0.01052996	0.01052998	0.00968758	0.007371	7.37E-03
137	2.36E-03	5.13E-03	5.13E-03	4.72E-03	3.59E-03	3.59E-03
138	0.0023598	0.00512999	0.005129992	0.00471959	0.003591	3.59E-03
139	0.0023598	0.00512999	0.005129993	0.00471959	0.003591	3.59E-03
140	0.0023598	0.00512999	0.005129994	0.00471959	0.003591	3.59E-03
141	0.0023598	0.00512999	0.005129995	0.00471959	0.003591	3.59E-03
142	3981.154187	5.20791341	3699.75104	3800.64053	5.54609213	3643.998
143	3981.143824	5.18538727	3699.728514	3800.61981	5.53032382	3643.9822
144	0.002829	0.00615	0.00615	0.005658	0.004305	4.31E-03
145	0.150130245	0.32636973	0.326369934	0.30026031	0.22845906	2.28E-01
146	0.021447178	0.04662425	0.046624276	0.04289433	0.03263701	3.26E-02
147	0.150130245	0.32636973	0.326369934	0.30026031	0.22845906	2.28E-01
148	0.021447178	0.04662425	0.046624276	0.04289433	0.03263701	3.26E-02
149	0.021447178	0.04662425	0.046624276	0.04289433	0.03263701	3.26E-02
150	0.021447178	0.04662425	0.046624276	0.04289433	0.03263701	3.26E-02
151	3981.168513	5.21858533	3699.804731	3800.68784	5.5535641	3644.0276
152	3981.148335	5.17472049	3699.760866	3800.64748	5.52285867	3643.9969
153	0.010723589	0.02331213	0.023312138	0.02144716	0.0163185	1.63E-02
154	1.63E-02	1.37E-01	6.77E-02	6.66E-02	4.39E-02	4.38E-02
155	1.75E-02	1.40E-01	7.02E-02	6.89E-02	4.56E-02	4.56E-02
156	2.44E-04	5.30E-04	5.30E-04	4.87E-04	3.71E-04	3.71E-04
157	0.027067687	0.1606209	0.091026241	0.08804312	0.0601799	0.0601667
158	0.026824485	0.16009221	0.090497544	0.08755672	0.05980981	0.0597966
159	2.43E-04	5.29E-04	5.29E-04	4.86E-04	3.70E-04	3.70E-04
160	3981.151182	5.20483798	3699.740703	3800.63138	5.54393905	3643.9921
161	3981.143649	5.18846184	3699.724327	3800.61631	5.53247575	3643.9807
162	0.003790952	0.0082412	0.008241199	0.0075819	0.00576884	5.77E-03
163	0.003742077	0.00813495	0.008134949	0.00748415	0.00569446	5.69E-03

164	0.00345	0.0075	0.007499999	0.0069	0.00525	5.25E-03
165	0.00345	0.0075	0.007499999	0.0069	0.00525	5.25E-03
166	3981.145695	5.19678442	3699.724511	3800.61688	5.53830125	3643.9823
167	3981.145571	5.19651443	3699.724241	3800.61663	5.53811225	3643.9821
168	1.24E-04	2.70E-04	2.70E-04	2.48E-04	1.89E-04	1.89E-04
169	3982.36555	7.38905541	3744.369293	3840.68739	6.95725832	3826.4636
170	3980.971776	3.06086097	3740.521115	3837.89907	4.12391707	3823.6898
171	0.484222022	1.05265591	1.052656294	0.96844374	0.73685959	7.37E-01
172	0.023579945	0.05126063	0.051260674	0.04715981	0.03588253	3.59E-02
173	0.183062844	1.07988043	1.139874802	1.60588397	1.94503351	1.9649078
174	3981.172095	5.22070835	3699.818766	3800.70017	5.55505068	3644.0352
175	3981.149965	5.17259889	3699.770657	3800.65591	5.52137396	3644.0015
176	0.008924575	0.01940125	0.01940125	0.01784915	0.01358087	1.36E-02
177	3981.172095	5.22070835	3699.818766	3800.70017	5.55505068	3644.0352
178	3981.149965	5.17259889	3699.770657	3800.65591	5.52137396	3644.0015
179	3981.145633	5.19664942	3699.724374	3800.61675	5.53820675	3643.9822
180	3981.145633	5.19664942	3699.724374	3800.61675	5.53820675	3643.9822
181	3981.145508	5.19637346	3699.724107	3800.6165	5.53801355	3643.982
182	3.98E+03	5.20E+00	3.70E+03	3.80E+03	5.54E+00	3.64E+03
183	1.55E-02	1.12E-01	6.23E-02	5.60E-02	3.85E-02	3.69E-02
184	0.268993151	0.69434198	0.62815376	0.57977358	0.4336877	0.4316618
185	0.002484	0.0054	0.005399997	0.004968	0.00378	3.78E-03
186	0.00391	0.0085	0.008499999	0.00782	0.00595	5.95E-03
187	0.000736	0.00159999	0.001599991	0.00147199	0.00112	1.12E-03
188	0.004968	0.0108	0.0108	0.009936	0.00756	7.56E-03
189	0.107525	0.23374999	0.233750002	0.21505	0.163625	1.64E-01
190	0.0023598	0.00512999	0.005129995	0.00471959	0.003591	3.59E-03
191	0.023	0.05	0.05	0.046	0.035	3.50E-02

192	1.18	2.07	1.69	1.4	0	0
193	0	2.97	3.31	3.16	5.97	5.93
194	0.225927652	0.49114622	0.491146642	0.45185483	0.34380295	3.44E-01
195	0.022902825	0.04978875	0.04978875	0.04580565	0.03485212	3.49E-02
196	0.057682826	0.12539692	0.125397166	0.11536534	0.08777821	8.78E-02
197	0.023	0.05	0.05	0.046	0.035	0.035
198	0.697084732	3.58631592	3.076309016	2.19440368	1.20280049	0.4729132
199	0.19	0.67	1.56	2.07	5.29	6.44
200	0.2	0	0	0	0	0
201	0	0	0	0	0	0
202	0.183062844	1.07988043	1.139874802	1.60588397	1.94503351	1.9649078
203	2.05665012	6.45114571	6.031123152	5.35383101	5.50852471	5.4687746
204	1.89E-04	0.00E+00	1.20E-05	1.24E-05	1.89E-04	1.47E-05
205	0.12	0.13	0.12	0.11	1.58	1.79
206	0	0.84	0.99	0.69	0.45	0.21

List of Publications

Patent filed

1. **Mehak Kaushal,** Saumya Ahlawat, Gargi Goswami, Debasish Das. Method for production of biofuels by fermentation of a sugar bearing nutrient media. (Application no. 201831002144. Filed on 18th January 2018)

Published Manuscripts

1. **Mehak Kaushal**, K. Venkata Narayana Chary, Saumya Ahlawat, Basavaraj Palabhanvi, Gargi Goswami, Debasish Das* (2018). Understanding regulation in substrate dependent modulation of growth and production of alcohols in *Clostridium sporogenes* NCIM 2918 through metabolic network reconstruction and flux balance analysis. Bioresource Technology, 249, 767-779. (Impact factor: 5.6)
2. **Mehak Kaushal**, Saumya Ahlawat, Mayurketan Mukherjee, Muthusivaramapandian Muthuraj, Gargi Goswami, Debasish Das* (2017). Substrate dependent modulation of butanol to ethanol ratio in non-acetone forming *Clostridium sporogenes* NCIM 2918. Bioresource Technology, 225, 349-358. (Impact factor: 5.6)

Sequence submitted

1. **Mehak Kaushal**, Saumya Ahlawat, Muthusivaramapandian Muthuraj, Debasish Das (2015). *Clostridium sporogenes* strain NCIM 2918 16S ribosomal RNA gene, partial sequence (GenBank Accession no. KT441028.1).

Manuscripts submitted/ under preparation

1. **Mehak Kaushal**, Saumya Ahlawat, Muthusivaramapandian Muthuraj, Gargi Goswami, Debasish Das. Reclassification of non-type strain *Clostridium acetobutylicum* NCIM 2918 to *Clostridium sporogenes* NCIM 2918. (Under review- Annals of Microbiology).
2. **Mehak Kaushal**, Saumya Ahlawat, Bidhu Bhusan Makut, Gargi Goswami, Debasish Das. Co-utilization of crude glycerol and lignocellulosic hydrolysate by *Clostridium sporogenes* NCIM 2918 for biofuel production (under preparation).

Publications from collaborative work

Manuscript

1. Saumya Ahlawat, **Mehak Kaushal**, Mayurketan Mukherjee, Basavaraj Palabhanvi, Naveen Bedi, Gargi Goswami, Muthusivaramapandian Muthuraj, Debasish Das (2018). Process engineering strategy for development of a novel two stage fed-batch fermentation targeted towards improved butanol productivity. (Under review-Korean Journal of Chemical Engineering)

Patent filed

1. Mayurketan Mukherjee, Saumya Ahlawat, **Mehak Kaushal**, Gargi Goswami, Debasish Das. Improved culture media for butanol synthesis using *Clostridium acetobutylicum* ATCC 824. (Application no. 201731028507. Filed on 10th August 2017)

List of Conferences/Workshops/ Symposia

1. **Mehak Kaushal,** (2018) 1st International Conference on Sustainable Biofuel, February 26-27, 2018, Department of Biotechnology, Ministry of Science and Technology, Government of India and Mission Innovation-India Unit, New Delhi.

2. **Mehak Kaushal**, Saumya Ahlawat, Gargi Goswami, Debasish Das (2017). *Clostridium sporogenes* a cell factory for biofuel production: Process strategies and system biology approach. Bioprocessing INDIA 2017, held at Indian Institute of Technology, Guwahati, India.

3. **Mehak Kaushal**, Saumya Ahlawat, Gargi Goswami, Debasish Das (2017). Reclassification of *Clostridium acetobutylicum* NCIM 2918 to *Clostridium sporogenes* NCIM 2918: a step towards high energy liquid biofuel production. Reflux 2017, held at Indian Institute of Technology, Guwahati. **(Best poster presentation award)**.

4. **Mehak Kaushal**, Saumya Ahlawat, Mayurketan Mukherjee, Anwesha Purkayastha, Gargi Goswami, Debasish Das (2017). High energy liquid biofuel production utilizing crude glycerol by *Clostridium* sp. Research Conclave 2017, held Indian Institute of Technology, Guwahati.

5. **Mehak Kaushal**, Saumya Ahlawat, Mayurketan Mukherjee, Anwesha Purkayastha, Gargi Goswami, Debasish Das (2016). Dual substrate strategy for biofuel production using non-acetone producing *Clostridium* sp. 57th International Annual Conference of The Association of Microbiologists of India, held at Guwahati University, Guwahati, India.

6. **Mehak Kaushal**, Saumya Ahlawat, Mayurketan Mukherjee, Muthusivaramapandian Muthuraj, Debasish Das. (2016) Media engineering & process development for biobutanol production from non-acetone producing *Clostridium* sp. Using alternate carbon substrate and cheaper nitrogen source. Indo-US workshop on Cell factories, held at Indian Institute of Technology Bombay, Mumbai, India.

7. **Mehak Kaushal** (2015). Workshop on 'Frontier energy research with industry academia partnership', held at Indian Institute of Technology Guwahati.

8. **Mehak Kaushal** (2013). Training-cum-workshop on 'Application of Bioinformatics Tools in Agriculture' Organized by Bioinformatics Center, Unit of Simulation and Informatics, IARI, New Delhi.

9. **Mehak Kaushal** (2015). TEQIP Short term course on solid waste management, organized by Center for Environment, IIT Guwahati.

10. Saumya Ahlawat, **Mehak Kaushal**, Basavaraj palabhanvi, Vikram Kumar, Muthusivaramapandian Muthuraj, Debasish Das (2014). Screening and characterization of 12 *Clostridium* strains for butanol production: Effect of glucose and organic nitrate source. Bioprocessing INDIA 2014, held at Institute of Chemical Technology, Mumbai.

11. Naveen Bedi, Saumya Ahlawat, **Mehak Kaushal**, Basavaraj Palabhanvi, Vikram Kumar, Muthusivaramapandian Muthuraj, Debasish Das (2014). Development of an intermittent in situ butanol recovery strategy to overcome product toxicity and improve productivity. Bioprocessing INDIA 2014, held at Institute of Chemical Technology, Mumbai.

12. Saumya Ahlawat, **Mehak Kaushal**, Mayurketan Mukherjee, Muthusivaramapandian Muthuraj, Basavaraj Palabhanvi, Debasish Das (2016). Alleviation of end product toxicity by strain level improvement in *Clostridium acetobutylicum* via combinatorial strategy of mutagenesis and serial adaptation. International conference on 'Indo-US Workshop on Cell Factories', held at Indian Institute Of Technology Bombay, Mumbai.

13. Anwesha Purakayastha, Mayurketan Mukherjee, **Mehak Kaushal**, Saumya Ahlawat, Debasish Das. (2016) Unravelling the structure and interaction of SpoOA- A positive regulator in butanol biosynthesis from *Clostridium acetobutylicum* ATCC 824. Research Conclave 2016, held at Indian Institute of Technology Guwahati, Guwahati, India.

14. Mayurketan Mukherjee, Saumya Ahlawat, Basavaraj Palabhanvi, Anwesha Purakayastha, **Mehak Kaushal**, Debasish Das (2016). System biology approach to understand the regulation in metabolic shift from acidogenesis to solventogenesis in *Clostridium acetobutylicum* ATCC 824. International conference on 'Indo-US Workshop on Cell Factories', held at Indian Institute Of Technology Bombay, Mumbai.

15. Mayurketan Mukherjee, Anwesha Purkayastha, Saumya Ahlawat, **Mehak Kaushal**, Debasish Das (2016). Effect of metallic ions on butanol production from *Clostridium acetobutylicum* ATCC 824. 57th International Annual

Conference of the Association of Microbiologists of India, held at Guwahati University, Guwahati, India.

16. Mayurketan Mukherjee, Anwesha Purkayastha, Saumya Ahlawat, **Mehak Kaushal**, Gargi Goswami, Debasish Das (2017). Novel medium engineering strategy directed towards enhancing butanol production from *Clostridium acetobutylicum* ATCC 824. Bioprocessing INDIA 2017, held at Indian Institute of Technology, Guwahati, India.

17. KVN Chary, Saumya Ahlawat, Basavaraj Palabhanvi, Mayurketan Mukherjee, **Mehak Kaushal**, Debasish Das (2017). *In silico* analysis of carbon flux distribution *Clostridium acetobutylicum* through modelling approach. Research Conclave 2017, held at Indian Institute of Technology, Guwahati.

Underline represents presenting author

Vitae

The author was born on September 9th 1988 in New Delhi, India. She passed the Secondary School Examination conducted by the Central Board of Secondary Education, New Delhi, in 2004. She qualified the Higher Secondary School Examination conducted by Central Board of Secondary Education, New Delhi, in 2006. She completed Bachelor of Technology in Biotechnology from University Institute of Engineering and Technology, Kurukshetra, Haryana, in 2010.

Mehak Kaushal joined her Master of Technology Programme in July 2011 at Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India. She later converted to PhD Programme after completion of one year of course work in July 2012. She received Junior and Senior research fellowships under the scheme run by the Ministry of Human Resource and Development (MHRD), India. She successfully completed the course work with 9.3/10 Cumulative Point Index (CPI). She gave the Open (PhD Synopsis) Seminar on September 14th 2017 and presented her thesis work before the Doctoral Committee and her performance was satisfactory. She presented her final viva voce seminar on 25th May 2018.