# Development of a Bioprocess for Enhanced Butanol Production: Integrating Laboratory Experiments with *In-Silico* Predictions

*A Thesis*

*Submitted in partial fulfilment of the requirements*

*for the degree of*

## Doctor of Philosophy

*by*

**Saumya Ahlawat**


**Department of Biosciences & Bioengineering**
**Indian Institute of Technology Guwahati**
**Guwahati-781039, Assam, India**

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Dedicated

to

***My Family and Friends***

भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
Department of Biosciences & Bioengineering

# Statement

I do hereby declare that the content embodied in this thesis entitled **"Development of a Bioprocess for Enhanced Butanol Production: Integrating Laboratory Experiments with *In-Silico* Predictions"** is the result of investigations carried out by me in the Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India under the supervision of Professor Debasish Das.

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

Date : 27th September, 2019

**Saumya Ahlawat**
Roll No. 126106021

भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
Department of Biosciences & Bioengineering

# Certificate

It is certified that the work described in this thesis entitled **"Development of a Bioprocess for Enhanced Butanol Production: Integrating Laboratory Experiments with *In-Silico* Predictions"** by **Ms. Saumya Ahlawat** (Roll No. 126106021) 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 & Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

Debasish Das

Date : 27th September, 2019

**Dr. Debasish Das**
Professor
(Thesis Supervisor)
Department of Biosciences & Bioengineering
Indian Institute of Technology Guwahati
Guwahati - 781039, Assam, India

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# Abstract

Increasing global population and energy intensive lifestyle has resulted in increased global energy demand. As most of the demand is fulfilled by fossil fuels, it has become a cause of increased greenhouse gas emissions. Fuel crisis and climate change concerns have invigorated the need for sustainable environment friendly sources of energy. As a result, liquid biofuels have gained significant interest as replacements for gasoline and fossil based diesel. Butanol, produced by clostridial fermentation, is a promising transportation alternative as a drop-in fuel due to its physiochemical properties. Nevertheless, several limitations hindering commercialization of the process motivate the need for developing a holistic process involving interventions at the upstream and downstream processing levels.

The present study aims to address pressing bottlenecks and develop a suitable bioprocess for butanol production using *Clostridium* sp. Suitable strain for fermentation, *C. acetobutylicum* MTCC 11274, was selected from procured cultures based on a maximum butanol production capability of 11.5 g $L^{-1}$. Further, the strain was extensively characterized and suitable carbon and nitrogen sources were screened. The maximum butanol titer obtained under screening experiments was found to be restricted by butanol toxicity. Hence, for the purpose of media optimization, maximization of biomass productivity and maximization of butanol productivity were selected as objective functions. Two media compositions thus obtained were individually tested resulting in 103.5% improvement in biomass productivity and 63.6% enhancement in butanol productivity, compared to the unoptimized media.

Subsequently, chemical mutagenesis and serial adaptation experiments were conducted in order to obtain a strain exhibiting improved butanol tolerance and titer. Among the different strategies used i.e. UV mutagenesis, chemical mutagenesis using EMS and NTG, and serial adaptation, chemical mutagenesis yielded promising strains exhibiting higher butanol tolerance. However, none of the strains produced significantly increased butanol titer in comparison to the wild type strain. Therefore, the wild type strain was used for further experiments.

Based on batch experiments conducted using the media obtained for maximization of biomass productivity and maximization of butanol productivity, a two-stage fed-batch fermentation strategy was developed. The process involved sequential use of biomass productivity media followed by butanol productivity media, where butanol induction served as the transition point from one stage to the second stage. The strategy resulted in a 77 % improvement in the butanol productivity (0.55 g $L^{-1}$ $h^{-1}$) when compared to batch fermentation using the optimized medium for maximization of butanol productivity. Further, studies on limitation and starvation

of nutrients in the first stage yielded magnesium limitation as the most suitable factor and addition of 5 g $L^{-1}$ calcium carbonate in the second stage was found to be the best among supplementation experiments. Subsequently, magnesium limitation in stage one was coupled with calcium supplementation in stage two resulting in a combinatorial strategy. This combinatorial approach resulted in improved butanol titer and butanol productivity of 16.5 g $L^{-1}$ and 0.59 g $L^{-1}$ $h^{-1}$, respectively, which were among the highest values reported in literature. Further improvement in butanol titer was obtained by integrating the combinatorial strategy with *in situ* product recovery. Intermittent gas stripping served the dual purpose of alleviation of butanol toxicity and product recovery. To reduce the cost of nutrients used, glucose and peptone were replaced with a rice straw hydrolyzate and instant dry yeast, respectively. The final process resulted in a 54.3 g $L^{-1}$ butanol titer, 0.58 g $L^{-1}$ $h^{-1}$ butanol productivity, 40% replacement of glucose with rice straw hydrolyzate, and 100% replacement of peptone with instant dry yeast. Further reduction in substrate cost would be possible upon utilization of a highly concentrated hydrolyzate. Hence, the strategy demonstrated potential for sustainable butanol production.

Flux balance analysis was performed to understand the metabolic flexibility exhibited by the organism in response to nutritional modulation during batch and fed-batch fermentations. Early induction of solventogenesis during magnesium limited two-stage fed-batch fermentation was attributed as the secondary effect of improved growth. In order to fulfill the ATP demand, the elevated carbon flux was diverted toward acid synthesis pathways, which serve as an ancillary source of energy generation after glycolysis. Pathways related to biomass and butanol synthesis remained concomitantly active during acidogenesis and solventogenesis under combinatorial usage of magnesium limitation-calcium supplementation in two-stage fed-batch fermentation. This can be attributed to the presence of calcium in second stage which is reported to aid in upregulation of heat shock proteins, DNA synthesis, transcription, repair, and carbohydrate catabolic enzymes, and stabilize membrane proteins. Thus, calcium alleviated the negative effects of butanol and stalled the downregulation of solventogenic reactions.

# Contents

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| **AACTCOA** | acetoacetyl CoA |
| **ABE** | acetone-butanol-ethanol |
| **AC** | acetic acid |
| **ACCA** | acetyl CoA |
| **AHG** | 3, 6-anhydro-L-galactose |
| **ATCC** | American Type Culture Collection |
| **AR** | Analytical reagent |
| **ATP** | adenosine triphosphate |
| **BP** | British Petroleum |
| **BU** | butyric acid |
| **BUCOA** | butyryl CoA |
| **BUOH** | butanol |
| ***C.*** | *Clostridium* |
| **CCD** | central composite design |
| **CCS** | Carbon capture and storage |
| **CoA** | Coenzyme A |
| **DCW** | dry cell weight |
| **DEH** | 4-deoxy-L-erythro-5-hexoseulose |
| **EMA** | elementary mode analysis |
| **EMS** | ethyl methane sulfonate |
| **Eq.** | equation |
| **F6P** | fructose-6-phosphate |
| **FAME** | fatty acid methyl ester |
| **FBA** | flux balance analysis |
| **Fd** | ferredoxin |
| **Fig.** | figure |
| ***G.*** | *Granulobacter* |
| **G6P** | glucose-6-phosphate |
| **GA** | growth associated |
| **GAP** | glyceraldehyde-3-phosphate |
| **GHG** | greenhouse gas |
| **HPLC** | high performance liquid chromatograph |
| **IBE** | 1, 3-propandiol A-butanol-ethanol |
| **IDY** | instant dry yeast |
| **IMTECH** | Institute of Microbial Technology |
| **KBBE** | Knowledge-Based Bio-Economy |
| ***L.*** | *Lactobacillus* |
| **LLE** | liquid-liquid extraction |
| **LTA** | lipoteichoic acid |
| **Ltd.** | Limited |
| **MFA** | metabolic flux analysis |

| | |
|---|---|
| **MTCC** | Microbial Type Culture Collection |
| **Mtoe** | million tonnes of oil equivalent |
| **NA** | not available |
| **NAD(P)$^+$** | nicotinamide adenine dinucleotide phosphate (oxidized form) |
| **NAD(P)H** | nicotinamide adenine dinucleotide phosphate (reduced form) |
| **NCL** | National Chemical Laboratory |
| **ND** | not defined |
| **NGA** | non-growth associated |
| **NL** | no lag phase |
| **nm** | nanometer |
| **no.** | number |
| **NRRL** | Northern Regional Research Laboratory |
| **NTG** | N-methyl-N-nitro-N-nitrosoguanidine |
| **OD** | optical density |
| **PPP** | pentose phosphate pathway |
| **PTA-AK** | phosphotransacetylase-acetate kinase |
| **PTB-BK** | phosphotransbutyrylase-butyrate kinase |
| **PVA** | polyvinyl alcohol |
| **RES** | Renewable Energy Sources |
| **RID** | refractive index detector |
| **Rnf** | *Rhodobacter* nitrogen fixation |
| **rpm** | revolutions per minute |
| **RSM** | response surface methodology |
| **sp.** | specie |
| **spp.** | species |
| **TCA** | tricarboxylic acid |
| **TSFB** | two-stage fed-batch |
| **TY** | tryptone and yeast extract |
| **TYG** | tryptone-yeast extract-glucose |
| **UK** | United Kingdom |
| **US** | United States |
| **USA** | United States of America |
| **UV** | ultra-violet |
| **WL** | Wood-Ljungdahl |
| **WT** | wild type |
| **WW** | World War |

# Notations

| | |
|---|---|
| **%** | percentage |
| **MJ $kg^{-1}$** | megajoule per kilogram |
| **$** | United States (US) dollar |
| **1G** | first generation |
| **2G** | second generation |
| **g** | gram |
| **L** | liter |
| **h** | hour |
| **g $g^{-1}$** | gram per gram |
| **g $L^{-1}$** | gram per liter |
| **g $L^{-1}$ $h^{-1}$** | gram per liter per hour |
| **MJ $L^{-1}$** | megajoule per liter |
| **w/v** | weight/volume |
| **v/v** | volume/volume |
| **°C** | degree Celsius |
| ***g*** | gravitational acceleration |
| **M** | molar |
| **mL $L^{-1}$** | milliliter per liter |
| **w/w** | weight/weight |
| **mL $min^{-1}$** | milliliter per minute |
| **μL** | microliter |
| **vvm** | vessel volumes per minute |
| **mmol ATP $g^{-1}$ DCW** | millimoles of adenosine triphosphate per gram dry cell weight |
| **$P_{ow}$** | partition co-efficient between water and 1-octanol |

*"You cannot get through a single day without having an impact on the world around you. What you do makes a difference and you have to decide what kind of a difference you want to make."*

Jane Goodall (1934- )
English primatologist

# 1 Introduction

## 1.1 Background and motivation

Increased energy demand coupled with depleting resources and environmental concerns have led the researchers to search for alternative carbon neutral fuels. Initiatives to replace fossil fuels with renewable sources of energy have turned the attention towards biomass based fuels. Past experiences by many fossil fuel producers have considered ethanol as a fuel to replace gasoline. Even though it gained success in Brazil and the USA, operational difficulties such as being corrosive in nature and separation of gasoline-ethanol blends in the presence of water have limited its use only up to 20% blending with gasoline. In comparison, butanol, a four carbon primary alcohol, has many physiochemical properties (Table 1.1, Yusoff et al., 2015) similar to gasoline when compared to ethanol. These properties indicate butanol is a chemical which can replace gasoline (Branduardi and Porro, 2016) when compared to ethanol.

In comparison to ethanol, butanol is more hydrophobic and has a lower vapor pressure along with a higher energy density in comparison to gasoline. Less evaporative nature of butanol makes it safer to handle. A lower vapor pressure renders butanol less corrosive to materials like copper, brass, zinc, aluminum, steel, and lead which are major constituents of automotive engines and supply pipelines (AMF Fuel Information System). These properties make butanol a suitable drop-in fuel (Pfromm et al., 2010).

Butanol production by different *Clostridium* species through acetone-butanol-ethanol (ABE) fermentation is well known. *Clostridium* strains are believed to be the most efficient native butanol producers among bacteria, archea and eucarya (Qureshi et al., 2008). Few other microbes such as *Nesterenkonia* sp. strain F, *Butyribacterium*

**Table 1.1.** Comparison of fuel properties of butanol, gasoline and ethanol

| | Fuel properties | | |
|---|---|---|---|
| | Butanol | Gasoline | Ethanol |
| Chemical formula | $C_4H_9OH$ | $\sim C_8H_{15.6}$ | $C_2H_5OH$ |
| Air-fuel ratio | 11.1 | 14.8 | 9 |
| Heat of vaporization (MJ $kg^{-1}$) | 0.71 | 0.32 | 0.92 |
| Research octane number | 94 | 95 | 106-130 |
| Motor octane number | 80 | 85 | 89-103 |

*methylotrophicum*, and *Thermoanaerobacterium* sp. M5 produce butanol albeit in small amount (Amiri et al., 2016; Worden et al., 1991; Jiang et al., 2018). ABE fermentation was first reported by Louis Pasteur in 1861; however, gained popularity during the first and second World Wars. The first industrial process was developed by Chaim Weizmann using *Clostridium acetobutylicum*, also known as Weizmann organism. Decreasing demand, dwindling supply of raw materials, and competitive chemical processes resulted in almost demise of this process until the 1960s (Jones and Woods, 1986). However, interest in using renewable resources as a feedstock for the production of chemicals and recent developments in the field of biotechnology have resulted in a renewed interest in the fermentation route as a possible source of solvent production (Papoutsakis, 2008). Until 2005, butanol was primarily used as a solvent and its status as a future fuel was established after DuPont, a United States (US) based company and British Petroleum (BP) a United Kingdom (UK) registered company began a joint venture Butamax, to develop a commercial scale butanol production process (Kumar and Gayen, 2011). A memorandum of understanding has been signed between Dross Energy, an Indian renewable firm, and Celtic Renewables a Scottish company to use waste from whiskey industry for butanol production. This venture is intended to achieve dual purpose of biofuel generation and reducing pollution of the Ganges river (Kemp, 2018).

Success of any process at a commercial level depends on the economic feasibility which in turn will depend on raw material costs, cost of production and recovery, capital cost of plant infrastructure, and other operational and maintenance costs. The ABE fermentation suffers from major challenges such as: (i) high cost of starch and sugar substrates, (ii) end product toxicity, (iii) mixed end-products in undesirable solvent ratio, (iv) degeneration of strains, and (v) sporulation. All these factors contribute to low butanol yields making the production and recovery process uneconomical at the industrial level (Kumar and Gayen, 2011).

Researchers have attempted to solve these problems by employing various strain level improvements including identification of novel producer strains (Kaushal et al., 2017), microbial strain development for improved butanol titer and tolerance through adaptation, chemical mutagenesis, and metabolic engineering (Jiang et

al., 2009; Green, 2011; Jang et al., 2012; Zheng et al., 2015). System biology approaches such as static and kinetic modelling have been undertaken to understand the regulatory mechanisms involved in butanol biosynthesis (Kaushal et al., 2018). Process level improvements have been achieved through approaches including application of various alternate substrates (Gottumakkala et al., 2013; Kaushal et al., 2017), fermentation strategies to enhance butanol titer, yield and productivity, and coupling fermentation with *in situ* product recovery technologies to overcome the butanol toxicity (Qureshi et al., 2008; Lu et al., 2012). Another approach under investigation to achieve process level improvement in ABE fermentation is media engineering (Li et al., 2014) and employing environmental factors affecting the butanol synthesis potential of *Clostridium* spp. Several studies examining factors such as different pH strategies (Oshiro et al., 2010); supplementation of inducers such as butyric acid/sodium butyrate, zinc, calcium carbonate (Wang et al., 2013; Wu et al., 2013; Wu et al., 2016); and limitation of trace minerals: iron, magnesium, phosphate, sulphate (Bahl et al., 1986) have resulted in improved butanol process kinetics. Although progress has been made in addressing the bottlenecks hindering commercialization of butanol fermentation process, economic sustainability requires innovative process engineering strategies and detailed understanding of the fundamental regulation and controls involved in butanol biosynthetic pathway. Considering these issues, a series of objectives were proposed as a means to develop a promising and economically feasible process for butanol production.

## 1.2 Objectives of the present study

- Screening and characterization of *Clostridium* strains on the basis of high butanol production
- Enhancement of butanol tolerance of the selected strain via serial enrichment and mutagenesis
- Studies on effect of various factors governing growth and butanol production from *Clostridium* sp.
- Development of a mathematical model to understand the regulation of butanol synthesis in *Clostridium* sp.
- Development of a bioprocess coupled with *in situ* product recovery

## 1.3 Approach

In the first step, clostridial strains procured from different culture collection centers were screened for their butanol production capability. The strain with the ability to produce maximum butanol was selected for detailed characterization and process development via biochemical engineering and metabolic modelling approaches (Fig. 1.1). The biochemical engineering approach involved preliminary

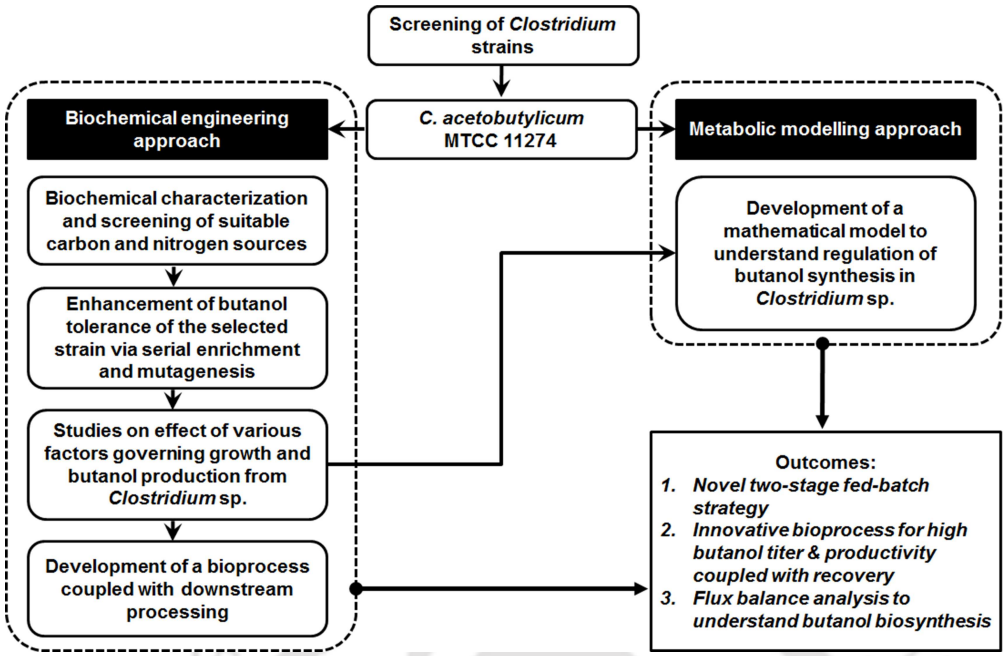


**Fig. 1.1.** Biochemical engineering and metabolic modeling approaches employed to achieve high butanol titer and butanol productivity and to understand the regulations involved in butanol biosynthesis for *Clostridium acetobutylicum* MTCC 11274.

characterization of the selected strain under different physico-chemical parameters according to Bergey's Manual of Determinative Bacteria (Buchanan and Gibbons, 1974). This was followed by a detailed characterization of the strain on wide range of carbon and nitrogen sources in order to screen the potential nutrients supporting growth and/or butanol production. Key nutrients were then optimized via a statistical optimization method to achieve maximization of biomass productivity and maximization of butanol productivity. Under biochemical engineering approach, strain improvement was also attempted via serial adaptation and chemical mutagenesis targeted towards enhanced butanol tolerance. However, a lack of substantial improvements in terms of butanol tolerance and butanol titer compelled further experiments to be conducted using the wild type strain only.

Further, a novel two-stage fed-batch fermentation strategy was developed to improve butanol productivity. Extensive experimentation was carried out to identify trace mineral nutrients triggering butanol production. This was then coupled with a two-stage process to design a strategic nutrient supplementation regime. Furthermore, the developed process was integrated with *in situ* gas stripping to achieve dual objectives to alleviate butanol toxicity and recover desired product. The feeding duration was modulated to minimize nutrient waste and finally, the process was demonstrated on cheaper nutrient sources which replaced 40% and 100% of the carbon and nitrogen source, respectively, with cost effective alternatives.

The metabolic modeling approach involved metabolic network reconstruction

and flux balance analysis which was carried out for two stage fermentation with and without butanol inducer supplemented condition to understand the complex carbon partitioning mechanism involved in the clostridial system. Carbon flux distribution towards butanol biosynthetic pathway, other metabolic pathways and non-growth associated maintenance energy was also captured for acidogenic and solventogenic phase. In the broader context, the current study was focused on development of an efficient strategy for butanol production via reduced fermentation time, enhanced butanol titer and productivity, and minimized nutrient waste and substrate cost.

## 1.4 Thesis organization

The thesis consists of eight chapters. **Chapter 1** deals with general background, motivation, objectives of present study, and approaches to resolve existing bottlenecks. **Chapter 2** comprises a detailed literature survey on ABE fermentation, clostridial metabolism research advancements towards butanol production and the existing bottlenecks associated with the current state of art technologies. **Chapter 3** describes selection of suitable butanol producer strain, biochemical characterization of strain followed by screening of suitable carbon and nitrogen sources as well as media optimization for high biomass and butanol productivity. **Chapter 4** focuses on improving butanol tolerance of selected strain via serial enrichment and chemical mutagenesis. **Chapter 5** details the study of various environmental cues that positively affect growth and butanol biosynthesis. **Chapter 6** describes the flux balance analysis (FBA) under different fermentation and butanol inducer supplemented condition to understand the regulations and controls involved in the butanol biosynthesis pathway. **Chapter 7** deals with strategic process development for high cell density-butanol productivity in fed-batch operation coupled with *in situ* product recovery. The strategy involves a combined approach of intermittent feeding of the limiting nutrients, butanol inducer supplementation, and alleviation of butanol toxicity by *in situ* product removal. Finally, the process is demonstrated by replacing key nutrients with cost effective alternatives. **Chapter 8** summarizes key research outputs from the present study with way forward for future prospects.

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*"If I have seen further it is by standing on the shoulders of Giants."*

Isaac Newton (1643-1727)
English physicist

# 2

# Literature Review

## 2.1 Energy demand, climate change, and renewable alternatives

Growing population and increasing economic development has contributed towards energy intensiveness in developed and under developed communities. This is related to the industrial as well as per-capita energy requirement, the former a product of industrialization while the latter depends upon the economic affluence of citizens. The global primary energy consumption increased by 2.2% with 135112.2 million tonnes of oil equivalent (Mtoe) consumed in 2017 (Fig. 2.1). China's consumption accounted for 23.2% of the global share. This was followed by US (16.5%) and India (5.6%) (BP statistical review of world energy 2018). At the global level oil (34%), coal (28%), and natural gas (23%) were the major feedstocks employed to fulfill the global energy demand. In the Indian context, coal (56%) and oil (30%) were the major source of energy. In case of India, only 3% of the total energy demand has been met by renewable energy, while globally it is only 4%. In addition to energy consumption, based on crude oil price of $44.67 per barrel in 2016, the price has increased by 21.1% to $54.10 per barrel in 2017 (value adjusted to 2017). Increasing energy demand is correlated to insufficient supply and a subsequent increase in cost. A large component of the current global energy demand is fulfilled by fossil fuel feedstock and hence, their depletion is also a concern.

Energy consumption using non-renewable sources is also linked to the environment, which relates the economical prowess and population of a country to its carbon dioxide and other greenhouse gas (GHG) emissions. Global $CO_2$ emission in 2017 was 33444 million tonnes of $CO_2$ (Fig. 2.2). China accounts for 27.6% of global $CO_2$ emissions in 2017 by releasing 9232.6 million tonnes of $CO_2$ (Fig. 2.2). China is followed by USA (15.2%) and India (7.0%) thus making the top three $CO_2$ emitters account for 49.8% of the global share (Fig. 2.2).

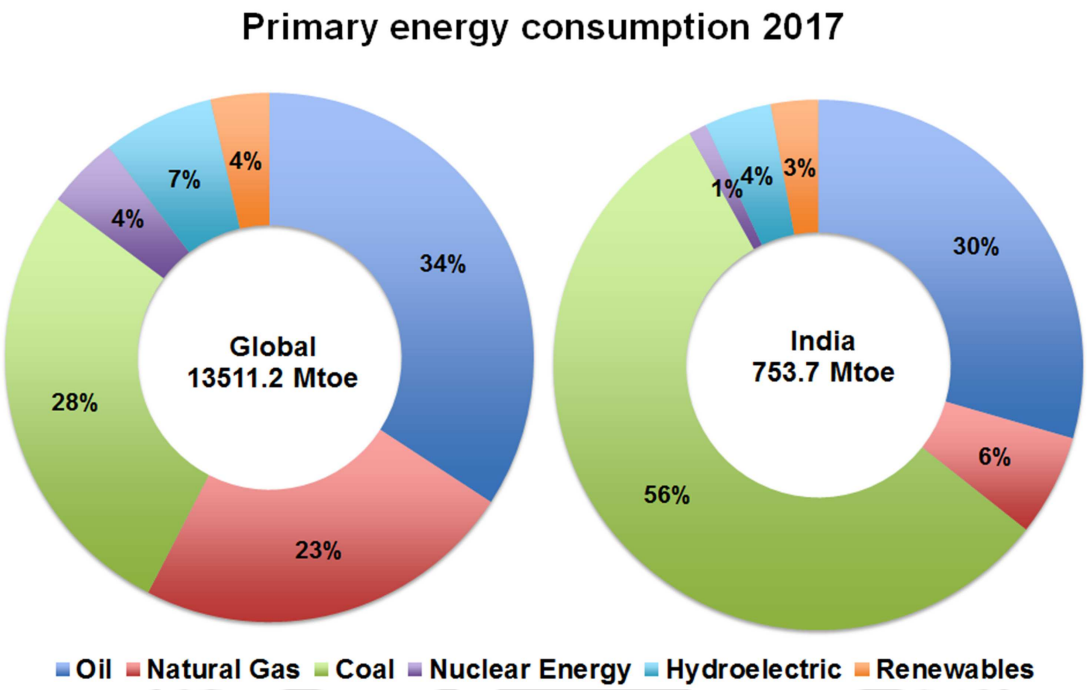


**Fig. 2.1.** Statistical distribution of the primary energy consumption by fuel in 2018: Global and Indian context (Source: BP statistical review of world energy 2018).

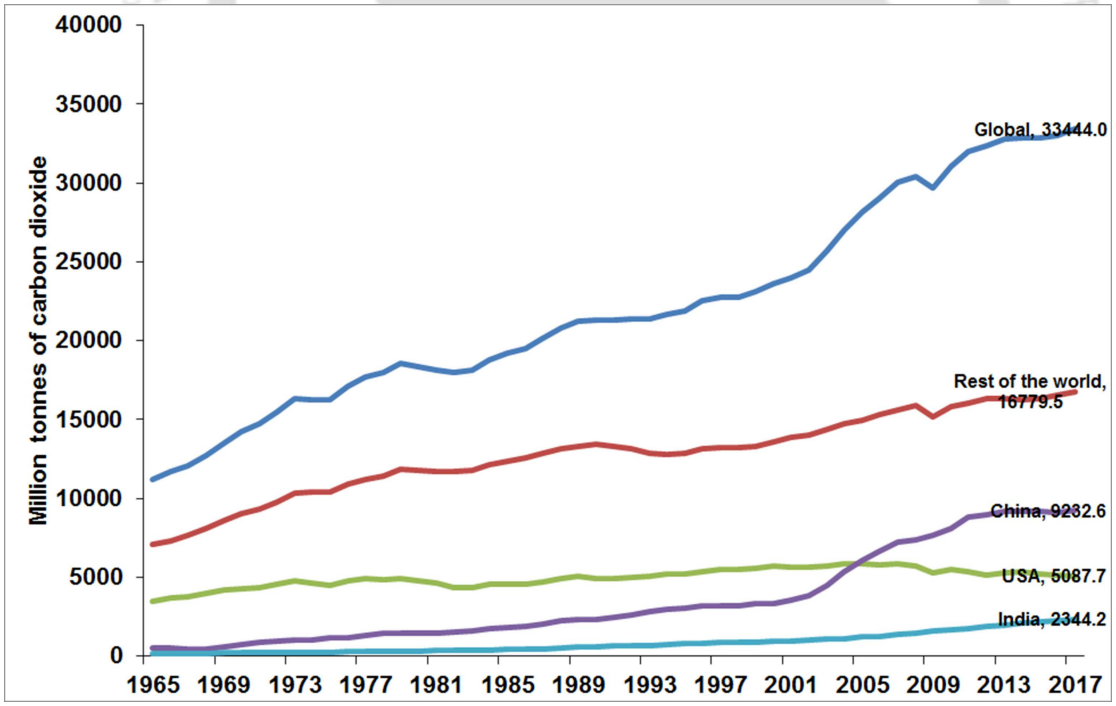


**Fig. 2.2.** $CO_2$ emission from the year 1965 to 2017 (Source: BP statistical review of world energy 2018).

Growth rate of global $CO_2$ emission was 1.6% which was higher than the averaged 1.3% for the years 2006-2016. Growth rate for China and India was 1.6% and 4.4%, respectively, while USA had negative growth rate of 0.5% depicting its decreased emissions in comparison to 2016. Increased GHG concentration in the atmosphere causes rise in the atmospheric temperature which further leads to

severe environmental concerns affecting terrestrial and aquatic life. According to the decarbonisation scenarios of the Energy Roadmap 2050 (Scarlat et al., 2015), significant reductions in greenhouse gas emissions are needed by 2030 (57-65%) and near-complete decarbonisation by 2050 (96-99%). In order to fulfil the energy demand while keeping the climate change in check, economical, carbon neutral energy sources are needed. Energy sources which do not have greenhouse gas emissions or carbon footprint are termed carbon neutral. Carbon footprint of a process is the amount of greenhouse gases primarily carbon dioxide released into the atmosphere by direct and in-direct emission. This involves estimation of carbon dioxide emission at different stages of the process such as raw material procurement, pretreatment, fermentation, transportation and combustion and electricity requirement of the total process etc. Renewable sources which can reduce GHG emissions and provide nonperishable energy include: solar, wind, hydro, geothermal, and biomass. In terms of transportation fuels, electric vehicles are under research and have technical issues that hinder widespread usage. Most economical would be the use of biofuels through blending or complete replacement of gasoline/biodiesel. The European Commission (2005) developed the concept of the Knowledge-Based Bio-Economy (KBBE) which entails (i) replacement of fossil fuel feedstocks by renewable raw materials, (ii) replacement of chemical processes by biological ones for the production of biological resources, and (iii) conversion of bioresources into food, feed, bio-based products, and bioenergy (Scarlat et al., 2015). NER300 is an EU funding programme for innovative low-carbon energy demonstration projects, in particular for carbon capture and storage (CCS) and innovative Renewable Energy Sources (RES) technologies (Scarlat et al., 2015). CCS technologies include pre-combustion, post-combustion, oxyfuel, and industrial applications. RES technologies include carbon-neutral fuels such as bioenergy, concentrated solar power, photovoltaics, geothermal, wind, ocean, hydropower, and smart grids. According to European Commission, bioenergy is expected to have an important role within the long-term goal to become a competitive low carbon economy (Scarlat et al., 2015).

Fig. 2.3 depicts the biofuel production from the year 1990 to 2017. Global production increased by 3.5% to 84.121 Mtoe in 2017. USA led the world in biofuel production with 36.9 Mtoe (43.9% of the global share), while China (2.147 Mtoe) and India (0.435 Mtoe) ranked very low amounting to 2.6% and 0.5%, respectively, of the global share (Fig. 2.3). However, in case of India and China, the production of biofuels has only started from 2000 and 2001, respectively. India ranks second in the global population, third in primary energy consumption and $CO_2$ emission, and is one of the fastest growing economies of the world (BP statistical review of world energy 2018). Hence, India must focus on building its biofuel production capacity and to increase its global share from 0.5% to a number corresponding to

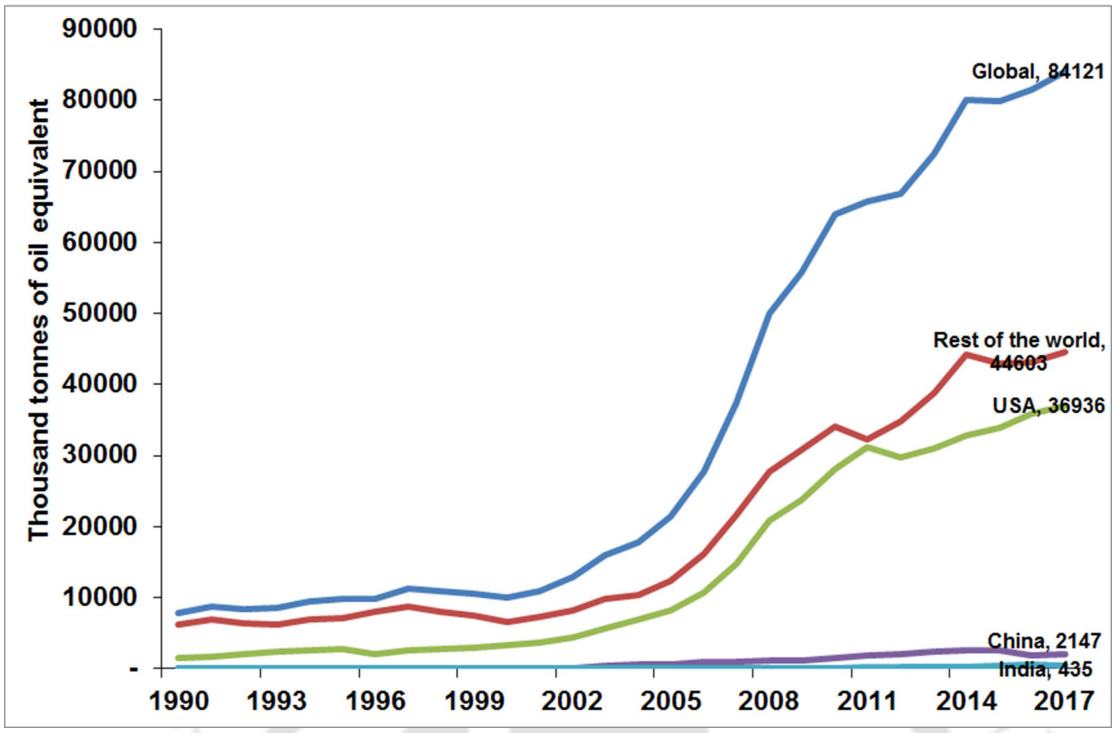


**Fig. 2.3.** Biofuel production from the year 1990 to 2017 (Source: BP statistical review of world energy 2018).

its consumption. The biofuel data shown in Fig. 2.3 comprises only ethanol and biodiesel because biobutanol industrial production is low and still in the research phase. According to Renewables 2018: Global status report (REN21), the global biofuel production existing at the end of 2017 comprised bioethanol (106 billion liters), FAME biodiesel (31 billion liters), and hydrotreated vegetable oil (6.5 billion liters); however, no report on biobutanol was available.

Biofuels classification is dependent on the biomass used as substrate during production (Fig. 2.4) (Nigam and Singh, 2011; Dutta et al., 2014). Biofuels produced from soybean, sunflower, corn, rapeseed, barley, wheat, palm, sugarcane, etc. are designated as first generation (1G) biofuels. 1G biodiesel production involves esterification and transesterification of oils, whereas, fermentation and thermochemical processes are used for 1G bioethanol and biobutanol production. Apart from reducing GHG emissions, 1G biofuels utilize simple and well established technology. However, they are land intensive and give rise to food versus fuel debate. To that end, research has been directed towards developing second generation (2G) biofuels. 2G biofuels are biodiesel, bioethanol, and biobutanol produced from non-edible oil seeds, agricultural and plant residue, grass, weeds, energy crops, municipal waste, etc. They serve the binary advantage of mitigating food versus fuel crisis and utilizing waste for energy and chemicals production. A major hindrance in cost effectiveness of 2G biofuels is the costly pretreatment process involved in the conversion of biomass into readily utilizable sugar.

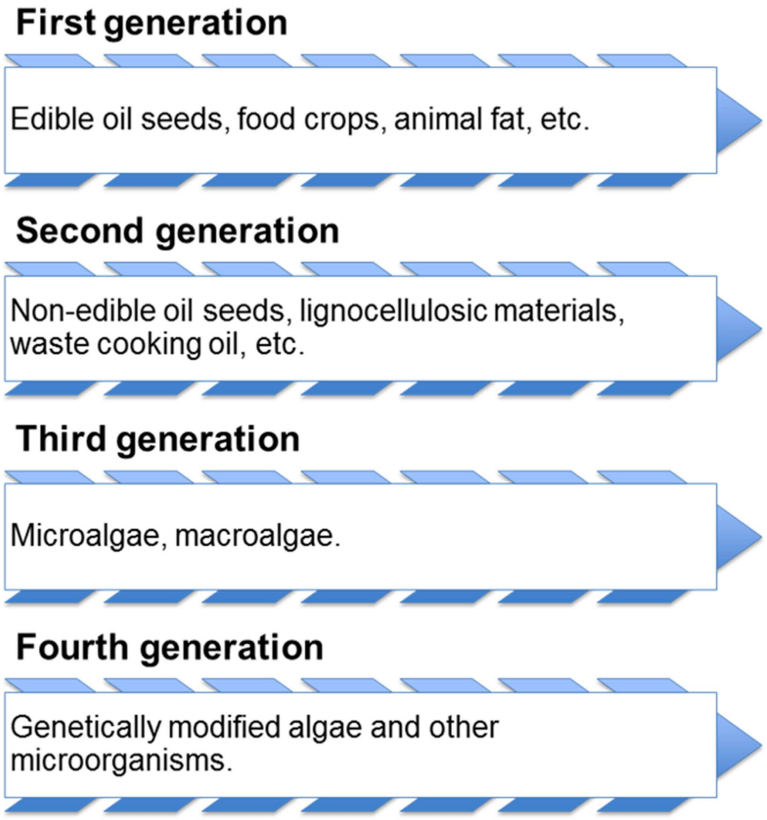


**Fig. 2.4.** Classification of different biofuel generations according to feed stocks (Source: Dutta et al., 2014).

Third and fourth generation of biofuels involve use of algae and genetically modified algae/microbes, respectively. Algae are an exceptional feedstock for biorefinery in terms of easy cultivation, no competition as food, and can use wastewater for growth. Another advantage of using algae is that after lipid extraction for transesterification, the remaining biomass can be used for fermentation. However, use of algae also pose problems in terms of high energy requirement for algal cultivation (for mixing, filtration, centrifugation, etc.), low lipid content, chances of biomass contamination in open cultivation systems, and high cost of photo-bioreactor operation. In order to address the above mentioned bottlenecks, research is required to develop robust genetically modified algae with high lipid content and to design economical unit operations.

***Biofuel policies***

As of 2015, out of the 3.1% contribution of biofuels towards transportation fuel such as bioethanol and biodiesel contributed only to 2.8% of this amount. Despite the drivers such as energy security, demand and climate change, biofuels have not been the forerunner in the transportation sector due to a lack of policy support related to the uncertainty of sustainable feedstocks supply. A major step in this direction was taken in 2017 with the establishment of the BioFuture Platform with 19 participating countries including India (BioFuture Platform Declaration 2017). This

organization mutually agreed to escalate their efforts towards the implementation of biofuel production and blending targets. To that end, the Mission Innovation Sustainable Biofuels Challenge was established with the objective of encouraging and facilitating commercialization of sustainable biofuels.

The number of countries developing a regulatory framework for renewable energy in transport increased from approximately 10 in 2004 to 70 in 2017. Forty-two countries have developed national targets (Renewables 2018: Global status report, REN21). Several countries have biofuel blending mandates such as Argentina (E12), Romania (E8), Zimbabwe (E10), Mexico (E10), New Zealand (Biodiesel 7%), and Slovania (Biodiesel 20%). USA and Australia do not have national mandates and implement only sub-national biofuel blending targets. A national policy on biofuels implemented by India in 2018, provides funding, tax incentives, and concessions towards developing the bioethanol and biodiesel production sectors, as well as implementing higher purchase prices for 2G biofuels, and mandating biodiesel blending. The policies are framed towards implementing bioethanol and biodiesel usage together with no policy assisted timeline existing for usage of biobutanol either mandatory or voluntary (global or national). Biobutanol is a superior fuel in comparison to ethanol and it is true that several technological roadblocks have hindered commercialization of biobutanol; however, policy support is imperative to achieve the desired results.

## 2.2 Acetone-Butanol-Ethanol (ABE) fermentation

### 2.2.1 History

Greek physician Hippocrates (about 460-370 BC) , probably the first report on *Clostridium* species, described a disease in Epidemics III which in retrospect may have been gas gangrene caused by *Clostridium histolyticum* (Durre, 2001). The first instance of biological formation of butanol was reported by Louis Pasteur in 1861 (Pasteur, 1861). Interestingly this was also the first report on microbial cultivation in the absence of air and led him to coin the term ‘anaerobic’. His designated microbe, *Vibrion butyrique*, was probably a mixed culture with *Clostridium* as the dominating species because of butyric acid and butanol production (Durre, 2005).

The term *Clostridium* was first used by A. Trecul in 1867, while describing one of the three morphological forms of *Amylobacter*, a particle he observed growing on rotten plant material (Trecul, 1867). *Clostridium* is the latinized form of Greek word ‘klostridion’meaning ‘ small spindle’ which in turn stems from ‘kloster’ (spindle) (Durre, 2001). van Tieghem (1877) and Fitz (1878) worked on *Bacillus amylobacter* and *B. butylicus*, respectively. In 1880, Prazmowski isolated a bacterium similar to the above-mentioned strains and recommended that *Vibrion butyrique*, *Amylobacter*, and *B. amylobacter* were identical and should be referred as *C. butyricum* (Prazmowski,

1880). Despite not coining the term *Clostridium*, Prazmowski is credited with introducing the nomenclature *C. butyricum*. This may be due to the fact that Trecul proposed and maintained that these particles were spontaneously generated from organic material and were not bacterium (Durre, 2001). The next solventogenic bacteria were isolated by Beijerinck (1893), termed as *Granulobacter butylicum*and *G. saccharobutyricum*. *G. butylicum* was designated as *C. butylicum* in 1930 and later re-designated as *C. beijerinckii* (George et al., 1983). These anaerobic bacteria were able to produce butanol, hydrogen, carbon dioxide, and butyric acid forming and used maltose and glucose as carbon sources (Durre, 2001). In 1902, Winogradsky described in detail another butanol forming bacteria, *C. pasteurianum*, with the unique property of fixing molecular nitrogen (Winogradsky, 1902). The first report on microbial production of acetone was published by Schardinger (1905).

Research towards the commmercialization of ABE fermentation began at the start of the 20$^{th}$ century when increased demand led to the inflated pricing of natural rubber (Durre, 1998). In 1910, Strange and Graham Ltd. (England) began research on microbial production of acetone and butanol which were precursors for producing monomers of synthetic rubber i.e. dimethyl butadiene and butadiene (Jones and Woods, 1986; Durre, 2005). In 1912-1914, Chaim Weizmann isolated a strain, termed as BY and later identified as *C. acetobutylicum*, capable of utilizing maize starch and producing higher acetone and butanol in comparison to other existing strains (Jones and Woods, 1986). This organism, also known as the Weizmann organism, became the model organism for ABE fermentation and the first taxonomically acceptable description for *C. acetobutylicum* was published in 1926 (McCoy et al., 1926). Weizmann process was granted a British patent in 1915 (Weizmann, 1915) and played critical role in World War I (WWI) as well as the establishment of Israel. WWI escalated the demand for acetone which was required for manufacturing cordite (smokeless ammunition). As the war affected the supply of raw material (calcium acetate) for acetone production through chemical route, attention was directed towards the fermentative route leading the establishment of industrial ABE fermentation units in Britain, Canada, and the United States (Durre, 1998). At the end of WWI in 1918, acetone demand decreased and hence, lessened the interest in ABE fermentation process. However, the process revived in 1920, with renewed interest in butanol production. Ethanol prohibition in the United States (1920) created a shortage of amyl alcohol, a raw material for amyl acetate, which was a by-product of ethanol fermentation. The automobile industry, newly revolutionized by Henry Ford, created a massive demand for amyl acetate which was used as lacquer solvent. Butanol presented an alternative by serving as a raw material for butyl acetate which was also a competent solvent for lacquers (Ezeji et al., 2005). Hence ABE fermentation industries grew in parallel to the automotive industry.

WWII (1939-1945) created a huge demand for acetone and by 1945, two-third and one-tenth of United States's butanol and acetone, respectively, were produced by ABE fermentation (Rose, 1951). At that time, ABE fermentation ranked second after ethanol fermentation by yeast; however, increasing cost of molasses and the availability of cheaper solvent from petrochemical industry caused a decline and eventual closing of ABE production facilities in the 1960s (Durre, 2001; Jones, 2001). The global oil crisis in 1973, caused after the Israeli war against Egypt and Syria, redirected focus towards butanol as a source of renewable energy. Since then the demand for bioenergy and mitigation of negative effects of climate change have fueled the research interest towards strain development for desired characteristics, improvement in upstream and downstream processing, and understanding regulations governing metabolic process.

### 2.2.2 Microorganisms

Traditionally, butanol production has been achieved through ABE fermentation where strains of *Clostridium* genus produce butanol along with acetone and ethanol in a ratio of 6:3:1 (Xin et al., 2018). *C. acetobutylicum* and *C. beijerinckii* are the two species most commonly used for butanol production followed by *C. pasteurianum* and *C. saccharoperbutylacetonicum* (Xin et al., 2018). New clostridial strains have been isolated for butanol production and many existing strains have been explored for butanol formation (Kaushal et al., 2017). In recent years, few strains belonging to genus *Nesterenkonia* and *Thermoanaerobacterium* have also been reported to natively produce butanol (Amiri et al., 2016; Jiang et al., 2018). Native producers of butanol differ in terms of their substrate utilization pattern and product profile as shown in Table 2.1. The strain to be used is selected depending upon the availability of raw material, type of solvent mix required, requirement of supplementary nutrients, etc. (Jones and Woods, 1986). Among all the butanol producers, *C. acetobutylicum*, particularly type strain ATCC 824, is the most widely explored in terms of genetic engineering, process development, and mathematical modelling (Xin et al., 2018). *C. acetobutylicum* is a gram-positive, rod shaped, motile, and sporulating obligate anaerobic bacteria capable of producing acetone, butanol, and ethanol using a variety of monosaccharides and polysaccharides such as glucose, fructose, xylose, starch, pectin, etc. (Jones and Woods, 1986).

The typical fermentation profile shows a biphasic pattern wherein the first phase depicts rapid growth and the second phase exhibits low to no growth. The first phase is termed as the acidogenic phase because the main metabolic products are acids, namely acetic acid and butyric acid. The acids in un-dissociated form diffuse passively through the cytoplasmic membrane, dissociate, and accumulate intracellularly as acetate and butyrate. During acidogenesis, the accumulation of ionized form of acids causes acid stress. Hence, as a regulatory measure,

accumulation of acids beyond a threshold concentration causes switch in activity of enzymes resulting in reutilization of acids to produce solvents. In *C. acetobutylicum*, this switch has been reported to be genetically regulated as acetoacetyl-CoA-acetate/butyrate-CoA-transferase, catalyzing acetate and butyrate uptake, is not expressed in acidogenic phase (Gheslaghi et al., 2009). This enzyme catalyzes the reduction of dissociated acetic acid and butyric acid, and therefore this reaction is thermodynamically feasible only when the intracellular substrate concentrations

**Table 2.1.** Microbial species capable of natively producing butanol

| Microorganisms | Main substrate | Product profile | Reference |
|---|---|---|---|
| *C. acetobutylicum* | Glucose | Butanol, acetone, and ethanol | Huese-mann et al., 2012 |
| *C. beijerinckii* | Glucose | Butanol, acetone, and ethanol | Qureshi et al., 2010 |
| *C. saccharoperbutylacetonicum* | Glucose | Butanol, acetone, and ethanol | Thang et al., 2010 |
| *C. saccharoperbutylicum* | Glucose | Butanol, acetone, and ethanol | Berezina et al., 2009 |
| *C. pasteurianum* | Glucose | Butanol | Sabra et al., 2014 |
| *C. pasteurianum* | Glycerol | Butanol and 1,3-propanediol | Sabra et al., 2014 |
| *C. sporogenes* | Glucose | Butanol and ethanol | Kaushal et al., 2017 |
| *C. tetanomorphum* | Glucose | Butanol and ethanol | Gong et al., 2016 |
| *C. carboxidivorus* | Carbon monoxide | Butanol and ethanol | Fernández-Naveira et al., 2016 |
| *C. aurantibutyricum* | Glucose | Butanol, acetone, and isopropanol | George et al., 1983 |
| *Clostridium drakei* | $H_2/CO_2$ or CO | ethanol and butanol | Gobner et al., 2008 |
| *Clostridium ragsdale* | Syngas (CO: $CO_2$: $H_2$) | acetic acid, ethanol, and minimal butanol | Kundiyana et al., 2010 |
| *Clostridium ragsdale* | Fatty acids | propanol, butanol, pentanol, and hexanol | Isom et al., 2015 |
| *Nesterenkonia* sp. strain F | Glucose | Butanol, acetone, and ethanol | Amiri et al., 2016 |
| *Butyribacterium methylotrophicum* | Carbon monoxide | Butanol and ethanol | Worden et al., 1991 |
| *Thermoanaerobacterium* sp. M5 | Xylan | Butanol and ethanol | Jiang et al., 2018 |

(acetate and butyrate) are high and the product concentrations are low. This uptake takes place during the second phase, designated as the solventogenic phase, is due to predominant production of solvents (Patakova et al., 2011).

Reactions involved in the central metabolic pathway of *C. acetobutylicum* when glucose is used as a substrate are listed in Fig. 2.5. Glucose is converted to pyruvate through Embden-Meyerhof pathway which also results in the production of ATP and NADH. Pyruvate undergoes phosphoclastic cleavage catalyzed by pyruvate-ferredoxin oxidoreductase along with acetylation of coenzyme A (CoA). The ensuing products are acetyl-CoA, carbon dioxide, and reduced ferredoxin. Acetyl-CoA is the central metabolite that branches into acids and solvents through a diverged metabolic pathway. Ferredoxin along with hydrogenase, NADH ferredoxin oxidoreductase, and NADPH ferredoxin oxidoreductase plays an important role in electron distribution. Ferredoxin transfers electrons to the hydrogenase enzyme which catalyzes hydrogen production via the reduction of protons.

NADH ferredoxin oxidoreductase mediates the transfer of electron between NAD± and ferredoxin thus, catalyzes the oxidation and reduction of NAD±, respectively (Gheslaghi et al., 2009). Unlike NADH ferredoxin oxidoreductase, the main function of NADPH ferredoxin oxidoreductase is the generation of NADPH during microbial growth as the oxidative pentose phosphate route is absent in *C. acetobutylicum* (Gheslaghi et al., 2009; Amador-Noguez et al., 2010; Crown et al., 2011). During acidogenesis, the acetic acid is produced via acetyl phosphate from acetyl-CoA while butyryl-CoA is converted to butyric acid via butyryl phosphate (Fig. 2.5). Carbon flow from acetyl-CoA to butyryl-CoA involves three intermediate metabolites: acetoacetyl-CoA, 3-hydroxybutyryl-CoA, and crotonyl-CoA (Fig. 2.5).

Acetic and butyric acid production results in ATP generation while the net yield is double during acetic acid production in comparison to butyric acid (Jones and Woods, 1986). According to Jones and Wood (1986), lactate pathway is not operational in *C. acetobutylicum* under normal cultivation conditions. The lactate production was observed under the conditions of carbon monoxide (CO) sparged fermentations (Kubowitz, 1934; Simon, 1947; Datta and Zeikus, 1985). This was reported to occur due to hydrogenase inhibition by CO and as a result the pathway was directed towards lactate production to facilitate oxidation of NADH. Kim et al. (1984) also observed pyruvate decarboxylase inhibition during CO sparging. It was also observed that CO sparging altered solvent production in favor of butanol while reducing acetone production. Datta and Zeikus (1985) observed that under such conditions early induction of solventogenesis was observed which occurred at peak concentration of lactate instead of butyrate peak concentration.During solventogenesis lactate was reutilized, similar to acetate and butyrate, to form solvents (mainly acetate and butyrate).

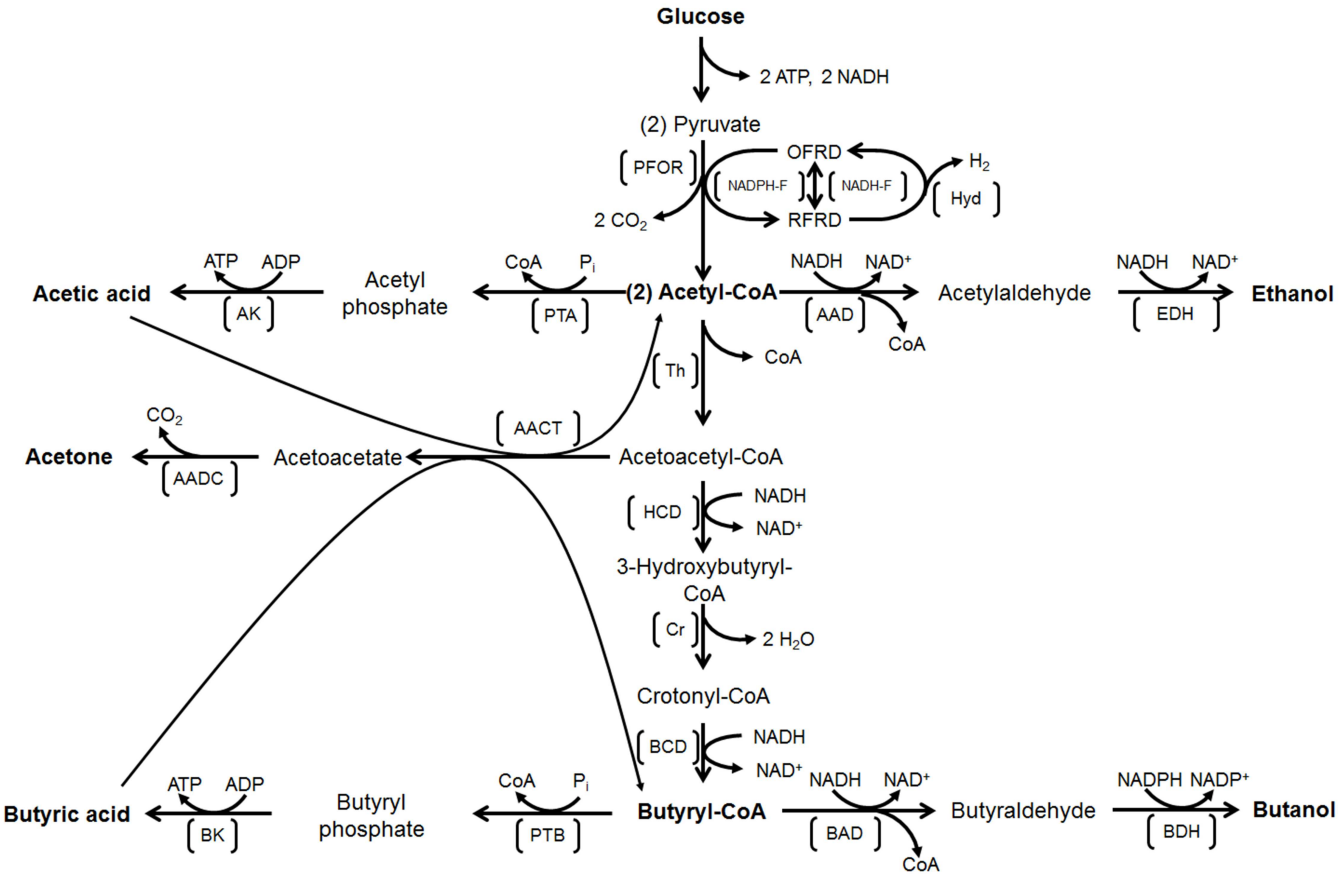


**Fig. 2.5.** Metabolic pathways in *C. acetobutylicum*. Enzymes catalyzing the reactions are abbreviated in the parenthesis as follows: (PFOR)-pyruvate-ferredoxin oxidoreductase; (NADH-F)-NADH-ferredoxin oxidoreductase; (NADPH-F)-NADPH-ferredoxin oxidoreductase; (Hyd)-hydrogenase; (AK)-acetate kinase; PTA- phosphate acetyltransferase (phosphotransacetylase); AAD- acetaldehyde dehydrogenase; EDH-ethanol dehydrogenase; Th- thiolase; AACT- acetoacetyl-CoA:acetate/butyrate:CoA transferase; AADC- acetoacetate decarboxylase; HCD- 3-hydroxybutyryl-CoA dehydrogenase; Cr- crotonase; BCD- butyryl-CoA dehydrogenase; PTB- phosphate butyltransferase (phosphotransbutyrylase); BK- butyrate kinase; BAD- butyraldehyde dehydrogenase; BDH- butanol dehydrogenase.

In contrast, CO sparged fermentation using *C. acetobutylicum* ATCC 4259 did not report lactate production (Kim et al., 1984). Solventogenesis involves the following three key branching points: acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA. Through the reaction catalyzed by CoA transferase enzyme, acetic and butyric acid are re-converted to acetyl-CoA and butyryl-CoA, respectively. Acetyl-CoA and butyryl-CoA further get converted to ethanol and butanol via acetylaldehyde and butyraldehyde, respectively, the catalyzing enzyme being bifunctional aldehyde dehydrogenases. During the acid re-assimilation reaction by acetoacetyl-CoA:acetate/butyrate:CoA transferase, another key metabolite acetoacetyl-CoA is converted to acetoacetate. Acetoacetate decarboxylase catalyzes the irreversible decarboxylation of acetoacetate to acetone. In addition, acid and sugar uptake also occur simultaneously during solventogenesis (Jones and Woods, 1986). The genes encoding for solvent synthesis are arranged as *adhE-ctfA-ctfB* comprising the sol operon. *adhE* encodes the bifunctional enzyme butyraldehyde/butanol dehydrogenase and *ctfA-ctfB* encode for the two subunits of CoA transferase enzyme. The sol operon along with the *adc* operon (encoding acetoacetate decarboxylase) reside on the 0.192 Mbp plasmid designated as pSOL1. Hence, the loss of megaplasmid upon repeated subculturing renders the strain incapable of producing solvents (Poehlein et al., 2017; Patakova et al., 2011).

*C. beijerinckii*, especially NCIMB 8052, is the second most widely explored and studied strain after *C. acetobutylicum* (Xin et al., 2018). While this strain undergoes biphasic ABE fermentation, similar to *C. acetobutylicum*, there exist many strain of *C. beijerinckii* which also produce isopropanol (Chen and Hiu, 1986; Hiu et al., 1987; Milne et al., 2011). Unlike *C. acetobutylicum*, *C. beijerinckii* does not contain the pSOL1 plasmid and the solventogenic genes are present on its 6.7-Mbp chromosome (Wilkinson and Young, 1995; Milne et al., 2011).

*C. saccharoperbutylacetonicum* and *C. saccharobutylicum* have sol operon arrangement similar to *C. beijerinckii*; *C. saccharoperbutylacetonicum* also contains a megaplasmid (Poehlein et al., 2013; Poehlein et al., 2017). Similar to *C. acetobutylicum*, the above mentioned strains produce acetone, butanol, and ethanol using monosaccharides and preferably glucose (Keis et al., 2001).

*C. pasteurianum* differs from *C. acetobutylicum* both in terms of products and choice of substrates. Instead of the typical ABE fermentation, it follows IBE (1, 3-propandiol -butanol-ethanol) pattern (Biebl, 2001). *C. pasteurianum* ATCC 6013 reported to use pure or crude glycerol as sole carbon source for IBE fermentation (Taconi et al., 2009). Interestingly, *C. pasteurianum* prefers glycerol over glucose as it produced more butanol (13.9 g $L^{-1}$) during glycerol fermentation as opposed to 6.9 g $L^{-1}$ during glucose fermentation (Sabra et al., 2014). A similar phenomenon was observed for *C. sporogenes* NCIM 2918, where a higher total alcohol level (8.8 g $L^{-1}$)

was produced when using glycerol as sole carbon source in comparison to glucose (Kaushal et al., 2017). *C. sporogenes* is also unique in terms of its product mixture and produces only alcohols (butanol and ethanol) without acetone production (Kaushal et al., 2017). Like *C. sporogenes*, *C. tetanomorphum* also produces butanol and ethanol (Gottwald et al., 1984; Gong et al., 2016).

The absence of the acetone synthesis pathway in *C. tetanomorphum* DSM665 and *C. sporogenes* DSM 795 was reported by Gong et al. (2016) and Poehlein et al. (2015a), respectively. Clostridial species capable of using gaseous substrates for butanol synthesis include *C. carboxidivorans*, *C. drakei*, and *C. ragsdale* (Fernández-Naveira et al., 2017; Gößner et al., 2008; Kundiyana et al., 2010). While *C. carboxidivorans* and *C. drakei* exclusively use gases like CO, $CO_2$, and $H_2$ or syngas (Liou et al., 2005); *C. ragsdale* has been reported to utilize both syngas and fatty acids resulting in butanol formation (Kundiyana et al., 2010; Isom et al., 2015).

### 2.2.3 Substrates

In the total production cost of a bioprocess as high as approximately 50% is allocated towards the raw material expense (Yadav et al., 2014; Sabra et al., 2014). Further, food versus fuel issue raises concerns over use of edible feedstock for production (Algayyim et al., 2018). Novel clostridial cell factories are under investigation for using cheaper raw material while others are genetically modified to serve the same purpose. A number of cost-effective feedstocks are being used as a replacement to expensive raw material for developing an industrially viable process.

***Lignocellulosic raw material***

Lignocellulosic biomass consisting of forest residues, energy crops, agriculture residues, industrial waste, municipal solid waste and liquid waste etc. (Table 2.2) constitute one of the most abundant sources of carbon (Zhang et al., 2007). Lignocellulosic biomass is mainly composed of the following (Rajendran et al., 2017): (1) cellulose (glucose polymer), (2) hemicellulose (polymer of mostly five carbon sugar such as xylose and arabinose), and (3) lignin (consisting of phenylpropanoid units). Since the composition of these three polymers vary in different type of biomass, developing biomass specific pretreatment methods become imperative (Seidl and Goulart, 2016). Numerous pretreatment methods employed to produce monomeric sugars from lignocellulosics (Rajendran et al., 2017). These methods can be grouped into four categories: (1) Physical i.e. microwave and sonication (Kumar et al., 2009; Rajendran et al., 2017), (2) Physicochemical i.e. steam explosion and ammonia fiber explosion, (3) Thermochemical i.e. alkali treatment, acidic treatment, organosolvent treatment etc. (Kumar et al., 2009; Putro et al., 2016), and (4) Biological i.e. involving the use of microorganisms such as brown, white or soft rot fungi (Galbe and Zacchi, 2007). Since the microbial method is time

consuming, its usage is economically unviable for biofuel production (Rajendran et al., 2017). A more economical approach is using enzymes from microbes in bulk quantity for hydrolysis of lignocellulosic biomass. Hence, pretreatment methods such as physical, physiochemical, or thermochemical are used in combination with enzymatic hydrolysis for efficient release of simple sugars (Kaushal et al., 2019). No universal pretreatment method can be applied for all biomass feedstocks. A combination of different methods such as steam explosion and acid pretreatment has resulted in improved sugar hydrolysis (Ranjan et al., 2013). Other pretreatment method combination includes supercritical $CO_2$ and steam explosion (Alinia et al., 2010) and organosolv and biological (Monrroy et al., 2010). Thus, an efficient and cost-effective pretreatment method has to be developed such that the utilization of lignocellulosic biomass for biofuel production becomes industrially feasible.

Microbial contamination from the feedstock can be a major problem during enzyme hydrolysis. It can affect fermentation by reducing sugar release, formation of inhibitory compounds, competing with producer organisms, and altering product mixture. Several methods exist for controlling contamination from feedstock or other sources during hydrolysis and fermentation. These include: (i) sterilization of pretreatment biomass by autoclave before enzymatic hydrolysis, (ii) addition of antibiotics to the hydrolyzate or fermentation media, (iii) sterilization of hydrolysis and fermentation equipment and pipelines, (iv) optimization of pretreatment products in such a way that they curb contamination without affect producer organism, and (v) engineering microorganisms to produce endolysins (Serate et al., 2015). Among the above mentioned, autoclave and antibiotic addition have been reported to be suitable for controlling microbial contamination during enzymatic hydrolysis (Serate et al., 2015).

***Crude glycerol***

Crude glycerol, a byproduct from biodiesel production, has become an attractive alternative substrate for biofuel production (Da Silva et al., 2009). *Clostridium* spp. such as *C. pasteurianum* and *C. sporogenes* have an inherent capability to utilize glycerol efficiently, while *C. acetobutylicum* utilizes glycerol only in mixed substrate fermentation along with glucose (Vasconcelos et al., 1994). Butanol production reaching levels of 17 g $L^{-1}$ has been reported by Biebl (2001) during glycerol fermentation by *C. pasteurianum*, while Taconi et al. (2009) demonstrated a butanol yield of 0.30 g $g^{-1}$ of crude glycerol. Impurities such as methanol, salts, and fatty acids present in crude glycerol were studied. Fatty acids were found to impair clostridial growth resulting in long batch reaction times and low productivity (Venkataramanan et al., 2012). Therefore, the removal of these impurities becomes an unavoidable step during glycerol fermentation.

**Table 2.2.** Biofuel production from various lignocellulosic materials

| Microorganism | Substrate | Pretreatment and hydrolysis methods | Reference |
|---|---|---|---|
| *C.saccharoperbutyl-acetonicum* N1-4 | Switchgrass | Acetic acid+ Enzyme | Wang et al., 2019 |
| *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. beijerinckii* TISTR 1461 | Rice straw | Ionic liquid+Enzyme | Boonsombuti et al., 2019 |
| *C.acetobutylicum* YM1 | Palm kernel cake | Acid | Al-Tabib et al., 2018 |
| *C. beijerinckii* P260 | Apple pomace | Hot water+Acid/Alkali+Enzyme | Jin et al., 2019 |
| *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. beijerinckii* YVU1 | Sugarcane field residue | Alkali+Acid +Enzyme | Reddy et al., 2017 |
| *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 |

***Marine macroalgae (seaweeds)***

Marine macroalgae usage as a substrate for biofuel production has many key advantages such as high carbohydrate composition, low lignin content, and no need for arable land for cultivation (Ohgren et al., 2007; Wargacki et al., 2012). The key hindrance in efficient utilization of marine macroalgae is the presence of sugars such as L-rhamnose, 3, 6-anhydro-L-galactose (AHG), and 4-deoxy-L-erythro-5-hexoseulose (DEH). These sugars are non-fermentable by clostridia (van der Wal et al., 2013; Yun et al., 2015) and thus wild type strains have to be genetically engineered to render them capable for bioprocess development utilizing macroalgae.

***Syngas***

Mixture of CO, $CO_2$, and $H_2$ gases is designated as syngas. Syngas is mainly produced from carbon based materials such as natural gas, coal, biomass, and waste by a process known as gasification (Basu, 2018). Utilization of syngas as a carbon source for biofuel production (Weissermel and Arpe, 2008) is not only a cost-effective alternative but also makes way for environmental benefits. Clostridial spp. such as *C. glycolicum*, *C. ljungdahli*, *C. autoethanogenum*, and *C. carboxidivorans* ( Köpke et al., 2010; Bruant et al., 2010; Guo et al., 2010; Fernández-Naveira et al., 2017; Küsel et al., 2001) are able to ferment syngas. However, only *C. carboxidivorans* has been reported to produce butanol (Fernández-Naveira et al., 2017) since others lack the butanol synthesis genes required to produce butanol using syngas (Guo et al., 2010; Köpke et al., 2010). The Wood-Ljungdahl (WL) pathway is functional in species utilizing syngas with the first step in WL pathway involving the conversion of CO or $CO_2$ plus $H_2$ to produce acetyl-CoA. Acetyl-CoA serves as the common branching point for both the acid and alcohol synthesis pathways (Drake et al., 2008).

### 2.2.4 Factors affecting growth and solvent production

A complete understanding of factors responsible for product titer, yield, and productivity of a particular process is required before conclusions can be made in favor of desired product formation. Several studies have been carried out as an attempt to gain an insight into the physiological factors controlling growth and solventogenic phase of *Clostridium* sp. in terms of initiation and duration (Table 2.3). Typical feature of ABE fermentation is the decrease of culture pH during acid formation followed by increase in pH during solvent production. The low pH value when this transition takes place, also known as 'breakpoint pH', is associated with the onset of solventogenesis (Millat et al., 2013). A threshold value of undissociated acids (acetic and butyric) concentrations (Bahland Gottschalk, 1985) and internal pH have been identified as a trigger for the solventogenic phase (Gottwald and Gottschalk, 1985). Several studies involving pH control (Tashiro et al., 2004; Oshiro et al., 2010) and the addition of acids (Tashiro et al., 2004; Wang et al., 2013) have

**Table 2.3.** Different environmental cues affecting growth and solvent production in *Clostridium* sp.

| Factors | Organism | Mode of cultivation | Remarks | Reference |
|---|---|---|---|---|
| pH control | *Clostridium beijerinckii* IB4 | Batch | Improved butanol titer | Jiang et al., 2014 |
| pH control | *C. acetobutylicum* ATCC 824 | Batch | Improved butanol titer | Monot et al., 1984 |
| pH control | *C. acetobutylicum* XY16 | Batch | improved solvent production | Guo et al., 2012 |
| pH control | *C. acetobutylicum* ATCC 824 | Batch | Improved butanol titer | Yang et al., 2013 |
| Sodium butyrate supplementation | *Clostridium beijerinckii* NCIMB 8052 | Batch | Improved butanol titer and butanol selectivity | Wang et al., 2013 |
| Sodium butyrate | *Clostridium beijerinckii* NCIMB 8052 | Batch | Improved butanol yield | Lee et al., 2008b |
| Butyric acid addition | *C. saccharoperbutylacetonicum* N1-4 | Fed-batch | Improved yield and butanol titer | Tashiro et al., 2004 |
| Butyric acid addition | *C. acetobutylicum* ATCC 824 | Batch | Improved butanol selectivity and 2% increase in butanol titer | Martin et al., 1983 |
| Butyric acid addition | *C. acetobutylicum* ATCC 39236 | Batch | Improved butanol titer | Datta and Zeikus, 1985 |
| Glucose concentration | *C. acetobutylicum* ATCC 824 | Batch | Improved butanol titer | Monot et al., 1982 |
| Phosphate limitation | *C. acetobutylicum* | Chemostat | Solventogenesis at low pH | Bahl and Gottschalk, 1985 |
| Sulphate limitation | *C. acetobutylicum* | Chemostat | Solventogenesis at low pH | Bahl and Gottschalk, 1985 |
| Magnesium limitation | *C. acetobutylicum* | Chemostat | Not appropriate for continuous | Bahl and Gottschalk, 1985 |
| Iron limitation | *C. beijerinckii* NCIB 8052 | Batch | Decrease in butanol titer but improved butanol selectivity | Junelles et al., 1988 |
| Iron limitation | *C. pasteurianum* DSM 525 | Phosphate limited chemostat | Improved butanol titer | Dabrock et al., 1992 |
| Iron limitation | *C. acetobutylicum* | Chemostat | B:A= 8 (at low pH) | Bahl et al., 1986 |
| CO pressure | *C. acetobutylicum* ATCC 39236 | Batch | Improved butanol titer | Datta and Zeikus, 1985 |
| CO pressure | *C. pasteurianum* DSM 525 | Phosphate limited chemostat | Improved butanol titer | Dabrock et al., 1992 |
| Methyl Viologen supplementation | *C. acetobutylicum* | Batch | Improved butanol selectivity and butanol titer at controlled pH of 5 | Peguin et al., 1994 |

| | | | | |
|---|---|---|---|---|
| 1 mM methyl viologen | *C. acetobutylicum* ATCC 824 | Batch | Improved butanol selectivity and butanol titer | Honicke et al., 2012 |
| MV+Fe limitation | *C. acetobutylicum* | Batch | Improved butanol titer at controlled pH of 5.5 | Peguin and Soucaille, 1995 |
| Whey fermentation | *C. acetobutylicum* | Continuous culture | Decrease in butanol titer but improved butanol selectivity | Bahl et al., 1986 |
| Redox control (—290 mV, air) | *C. acetobutylicum* | Batch | Improved solvent production | Wang et al., 2012 |
| Glycerol supplementation | *C. beijerinckii* | Batch | Improved furfural detoxification | Ujor et al., 2014 |
| Tiron supplementation | *C. acetobutylicum* YM1 | Batch | Improved solvent production | Al-Shorgani et al., 2015 |
| Furfural or HMF supplementation | *C. beijerinckii* BA101 | Batch | Improved growth (13%) and solvent production (19%) | Ezeji et al., 2007 |
| Furfural + HMF supplementation | *C. beijerinckii* P260 | Batch | Improved ABE productivity | Qureshi et al., 2012 |
| $ZnSO_4$ supplementation | *C. acetobutylicum* L7 | Batch | Improved growth and butanol titer | Wu et al., 2013 |
| $CaCO_3$ supplementation | *C. acetobutylicum* | Batch | Improved growth, sugar uptake, solvents | Li et al., 2013b |
| $CaCO_3$ supplementation | *C. beijerinckii* NCIMB 8052 | Batch | Ameliorated LDMIC inhibition | Zhang and Ezeji, 2014 |
| $CaCO_3$ supplementation | *C. acetobutylicum* | Batch | Relieves carbon catabolite repression | El Kanouni et al., 1998 |
| $CaCO_3$ supplementation | *C. sporogenes* BE01 | Batch | Improved hydrogen, volatile fatty acids and solvents | Gottumukkala et al., 2015 |

shown improvement in the butanol titer and yield. Variations in growth media are also known to alter the dynamic solvent production profile. Long et al. (1984) observed that excess glucose was required for initiation of butanol production and limitations of phosphate improved the yield (Bahl and Gottschalk, 1985). Limitation of other media nutrients such as iron, magnesium, sulphate, and nitrogen have been shown to affect growth and solvent production in *Clostridium* sp. (Bahl et al., 1986; Junelles et al., 1988; Peguin and Soucaille, 1995). In some cases of *C. beijerinckii* cultivation, addition of furfural and/or 5-hydroxymethyl furfural was reported to improve growth and solvent production (Ezeji et al., 2007; Qureshi et al., 2012), while *C. acetobutylicum* reportedly exhibited no change in fermentation profile (Zhang et al., 2012). Wu et al. (2013) reported that zinc supplementation to fermentation media improved clostridial growth and butanol production. Changes in incubation temperature, increased hydrogen partial pressure, and intermittent oxidative stress are also shown to affect butanol production (Jones and Woods, 1986).

Supplementation of electron carriers such as methyl/benzyl viologen or carbon monooxide gassing is shown to improve butanol selectivity (Kim and Kim, 1988; Dabrock et al., 1992; Peguin et al., 1994; Honicke et al., 2012). Even though required in small amounts, electron carriers such as methyl/benzyl viologen are costly and would affect the process economics on large scale operation. On the other hand, carbon monooxide being a toxic gas poses environmental threat and would require additional precautionary measures when used in industry. Another important aspect is related to the maximum achievable butanol concentration which is restricted by butanol toxicity (Dunlop, 2011). Therefore, factors providing tolerance to the strain can also be targeted for improvement in the production capacity. On such inducer is calcium carbonate, which has been reported to improve butanol tolerance along with buffering capacity, increased substrate utilization, and efficient acid re-assimilation (Richmond et al., 2011; Han et al., 2013; Gottumukkala et al., 2015).

### 2.2.5 Solvent toxicity

During ABE fermentation clostridial cells produce a mixture of solvents i.e. acetone, butanol, and ethanol (Jones and Woods, 1986). Among the three solvents, acetone and ethanol are not produced at inhibitory levels (Jones and Woods, 1986). Butanol which is the major product in the mixture is also the most toxic (Izard et al., 1989). Adverse effect of butanol on growth and solvent production is varied in different *Clostridium* strains, starting with as low as 5 g $L^{-1}$ and causing complete inhibition between 12-15 g $L^{-1}$ (Izard et al., 1989; Kaushal et al., 2017). *C. acetobutylicum* is the most studied organism for butanol toxicity (Nicolaou et al., 2010). The exact mechanism of inhibition is unknown; however, the impact is reported to be a linear function of the butanol concentration (Izard et al., 1989). Toxicity of hydrocarbons is also shown to be a function of carbon number, functional

group (carboxyl or hydroxyl), chain structure (straight or branched), and partition coefficient between octanol and water (Wilbanks and Trinh, 2017). Hydrocarbons with more carbon atoms, straight chained structure, hydroxyl group, and high partition coefficient are considered more toxic (Wilbanks and Trinh, 2017). Hence, butanol with its four carbon straight chained structure ending with a hydroxyl group, and partition coefficient of 0.8 (log $P_{ow}$) is highly toxic toward microorganisms (Nicolaou et al., 2010; Wilbanks and Trinh, 2017).

***Mechanism of butanol toxicity***

Butanol toxicity is attributed to disrupted membrane functions, which is further related to hydrophobicity of the butanol molecule (Izard et al., 1989; Weber and de Bont, 1996). Unsaturated and saturated fatty acids and fatty acid-protein interaction cause gaps in the cell membrane to allow movement of acyl chains and thereby provide fluidity to the membrane (Ingram 1976). The hydrophobic nature of butanol allows it to dissolve into the outer cell membrane; however unlike ethanol, it is bigger than gaps in the membrane and creates further gaps in the cytoplasmic membrane, thereby increasing the membrane fluidity (Ingram 1976). Butanol also increases disorder in the lipid bilayer of the membrane, particularly the glycerol backbone (Weber and de Bont, 1996). This destabilizes the bilayer and affects the lipid-lipid and lipid-water interactions and thus, disrupts the membrane function (Weber and de Bont, 1996).

Apart from providing structural integrity to the cell, a membrane also controls the cellular influx/outfluxes of ions and metabolites through its selective permeability. The increase in membrane permeability in *C. acetobutylicum* by butanol accumulation results in leakage of protons, potassium ions (Bowles and Ellefson 1985), and phophoenolpyruvate (Hutkins and Kashket, 1986). Dissipation of proton motive force was also observed in solventogenic *C. acetobutylicum* by Terracciano and Kashket, (1986). Hviid et al. (2017) postulated that the increase in membrane permeability of *L. lactis* in the presence of high butanol concentration might be due to butanol-induced stabile pores in the membrane.

In addition to above mentioned functions, cell membrane is also a matrix for enzymes which are important for solute transport, electron transport chain, etc. (Weber and de Bont, 1996). Enzymatic activity might be affected by membrane disorder caused by solvents, for example, butanol induced inhibition of membrane-bound ATPase was observed in *C. acetobutylicum* ATCC 824 cells by Moreira et al. (1981). Other cellular functions adversely affected by butanol are maintenance of internal pH (Gottwald and Gottschalk 1985; Bowles and Ellefson 1985); membrane pH gradient (Bowles and Ellefson 1985; Terracciano and Kashket, 1986); intracellular ATP levels (Bowles and Ellefson 1985); and $Na^+/H^+$ exchanger (Wang et al., 2005).

Another membrane related function affected by presence of butanol above a threshold concentration is inhibition of sugar and amino acid uptake (Moreira et al., 1981; Bowles and Ellefson, 1985). Inhibition is amplified during xylose fermentation where complete inhibition of *C. acetobutylicum* ATCC 824 growth was reported at 75% lower butanol concentration than required to achieve similar effect during glucose fermentation. Butanol levels at 7 g $L^{-1}$ and 10.5 g $L^{-1}$ have been reported to reduce the incorporation of xylose and glucose into cell material by 50%, respectively (Ounine et al., 1985). Butanol affects sugar transport system; however, the reason behind differential degree of inhibition between xylose and glucose grown cells is unknown. It is also hypothesized that butanol may exert its toxicity by affecting some other cellular functions (Wang et al., 2005); however, a plethora of literature linking inhibition of microbial growth with hydrophobicity mediated perturbation of membrane structure and function advocates that the cell membrane is the primary location of inhibitory interactions (Linden and Moreira, 1982).

***Mechanism of butanol tolerance***

Under non-inhibitory butanol concentrations, microbial cells exhibit stress responses such as changes in saturated to unsaturated fatty acid ratio, lipid tail length, content of plasmalogens, cyclopropane ring in fatty acids of the cell membrane, and expression of heat shock proteins, which have been explored to improve butanol tolerance (Patakova et al., 2018). Addition of elaidic and oleic acids to cultivation medium of *C. acetobutylicum* has resulted in their incorporation into the cell membrane; however, butanol tolerance was not improved (Baer et al., 1987). Although cyclopropane fatty acid synthase over-expression yielded a tolerant *C. acetobutylicum* ATCC 824 strain, the resulting mutant lost butanol production capability (Zhao et al., 2003). Overexpression of groESL has shown to improve butanol tolerance and titer of *C. acetobutylicum* (Tomas et al., 2003). Improved butanol tolerance and production duration in *C. acetobutylicum* were also observed through overexpression of spo0A (Alsaker et al., 2004) and CAC1869 (Borden and Papoutsakis, 2007), although data regarding overall butanol titer was not available.

In addition, strain improvement strategies such as chemical mutagenesis and adaptive evolution have also been undertaken to improve butanol tolerance. Since chemical mutagenesis and adaptive evolution are non-targeted strategies, they are not always successful in achieving a butanol tolerant strain. Further, not all reported instances of improved tolerance have reflected improvements in butanol titer (Jones and Woods, 1986). Table 2.4 shows a few examples of improvement in butanol tolerance through adaptive enrichment, chemical mutagenesis, or genetic engineering which can lead to enhanced butanol titer. However, absence of fully elucidated pathways involving clostridial metabolism and stress responses has hindered development of butanol tolerant high producer strain.

**Table 2.4.** Different strain improvement strategies for enhanced butanol tolerance

| Strain | Type[a] | Parental strain | Method | Improved titer (g $L^{-1}$) | Control titer (g $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| JB200 | A | *C. acetobutylicum* ATCC 55025 | Serial enrichment in fibrous bed bioreactor | 21 | 12.5 | Yang and Zhao, 2013[b] |
| ATCC 55025 | M | *C. acetobutylicum* ATCC 4259 | EMS treatment | 13 | 10.6 | Jain et al., 1993[b] |
| GS4-3 | M | *C. acetobutylicum* GX01 | NTG treatment plus genome shuffling | 18.1 | 14 | Li et al., 2016 |
| BKM19 | M | *C. acetobutylicum* PJC4BK | NTG treatment | 17.6 | 15.9 | Jang et al., 2013 |
| SA-1 | A | *C. acetobutylicum* ATCC 824 | Serial enrichment | 8.6 | 7.6 | Lin and Blaschek, 1983 |
| T64 | A | *C. acetobutylicum* D64 | ASBE[c] (serial enrichment) | 15.3 | 12.2 | Liu et al., 2013 |
| BT14 | M | *C. beijerinckii* NCIMB 8052 | ARTPd treatment | 16.7 | 12.2 | Kong et al., 2016 |
| p(GROE1) | E | *C. acetobutylicum* ATCC 824 | groESL overexpression | 17.1 | 13 | Tomas et al., 2003 |

[a]A-Adapted, M-Mutant, E-Engineered; [b]Strain protected by US patent; [c]Artificial simulation of bio-evolution; [d]Atmospheric and room-temperature plasmas

### 2.2.6 Different fermentation strategies

Low product titer, yield, and productivity are the key limitations of the ABE fermentation process. Both strain and process level improvements have been examined to overcome the above challenges. Even after achieving strain level improvement, further improvement at the process level by applying various process strategies are imperative to achieve the desired yield.

***Batch, fed-batch, and continuous mode of fermentation***

The three key fermentation modes of reactor operation can be differentiated on the basis of type of substrate feeding performed during the fermentation process. In batch fermentation, media used to initiate fermentation is filled in the reactor and the process parameters are set for a selected reaction time. Fermentation is continued until the metabolic activity of the organism ceases and no more substrate utilization and product formation takes place. On the other hand, in case of fed-batch fermentation, intermittent feeding of the media components takes place. This is advantageous especially in cases when high initial substrate concentration is inhibitory to the microbial growth. Continuous fermentation is the term given to the mode of fermentation where the feeding of media components is accompanied by continuous product removal. This mode of fermentation results in faster attainment of the exponential phase which is not possible in case of the batch and fed-batch processes.

***Co-cultivation and multistage fermentation strategy***

Fermentation in which the characteristics of two different microorganisms are explored to achieve improved process yield is designated as co-culture fermentation. In some cases an organism capable of desired product formation may not be able to utilize cheaper substrates and hence has to be co-cultured with another organism which can efficiently utilize the desired substrates. For instance, Nakayama et al. (2011) co-cultivated cellulose-utilizing *C. thermocellum* strain with a butanol-producing *C. saccharoperbutylacetonicum* strain for efficient low cost lignocellulosic biomass utilization. Li et al. (2013a) co-cultivated *C. tyrobutyricum* with *C. beijerinckii* with the aim that butyric acid produced by the first organism will be utilized by the latter organism resulting in high butanol levels reaching 12.8 g $L^{-1}$. Further, with the aim of exploring the distinct metabolic stages showcased by *Clostridium* spp. multistage fermentation comes into existence. Two-stage continuous fermentation was reported by Mutschlechner et al. (2000) where *C. beijerinckii* was grown to produce a maximum acid yield in the first stage with subsequent transfer to the solventogenesis second stage.

***Immobilization and cell recycling***

In order to overcome the bottlenecks observed with using suspended cells, such as product inhibition, loss of cells viability, difficulty in product separation from cells, inability in using high cell concentration during fermentation, cell washout, and low productivity (Jones and Woods, 1986), cell immobilization systems was employed to address these issues . Immobilization supports such as calcium alginate beads, ceramic beads, chitosan, PVA, sugarcane bagasse, coke, bonechar, sponge etc. have been reported in many studies (Moon et al., 2016; Sarchami et al., 2016; Gottumukkala et al., 2017). Kong et al. (2015) reported an ABE productivity of 11.3 g $L^{-1}$ $h^{-1}$ using chemically modified sugarcane bagasse as a support for immobilization of *C. acetobutylicum* cells. Similarly, Qureshi et al. (2000) used a clay brick packed bed reactor for *C. beijerinckii* BA101 culture under continuous fermentation, resulting in total solvent productivity of 15.8 g $L^{-1}$ $h^{-1}$. In order to achieve high cell concentration, researchers have used a porous membrane as parts of the cell recycle strategy. The fermentation broth is filtered through the membrane to retain the cells which can then be recycled back to the reactor to initiate next fermentation process. This results in high cell density and ultimately improved the productivity as evident in studies by Malaviya et al. (2012) and Zheng et al. (2013). Malaviya et al. (2012) reported a butanol productivity of 7.8 g $L^{-1}$ $h^{-1}$ during continuous glycerol fermentation, while Zheng et al. (2013) achieved 1.2 g $L^{-1}$ $h^{-1}$ during continuous xylose fermentation.

***Multi-substrate fermentation***

Most of cheap raw material such as lignocellulosic biomass, industrial, and

municipal waste contains a mixture of different carbon or nitrogen sources. This necessitates the use of industrial strains that are able to utilize a broad range of carbon/nitrogen sources in multi-substrate fermentation. For instance, Xin et al. (2014) reported fermentation of horticultural waste cellulosic hydrolysate containing glucose and xylose using wild type *Clostridium* sp. strain BOH3 to produce a butanol level of 11.7 g $L^{-1}$. Most of the wild type strains suffer from carbon catabolite repression because they are unable to utilize other sugars in presence of glucose. In order to improve xylose utilization in a sugar mixture, Gu et al. (2009) overexpressed the transaldolase enzyme in *C. acetobutylicum*, while Ren et al. (2010) silenced the CcpA gene resulting in simultaneous utilization of glucose and xylose. Further, dual substrate fermentation (glucose and glycerol) has been reported for both *C. pasteurianum* (Sabra et al., 2014) and *C. sporogenes* (Kaushal et al., 2017).

### 2.2.7 Downstream processing

Downstream processing involves separation and purification of a product from fermentation broth. In order for a process to be economical, the cost of product should be higher than the cost incurred during upstream and downstream processes. In case of butanol, which is expected to serve as an energy source, the energy generated from butanol should be more than the energy consumed during fermentation and recovery processes (Jiménez-Bonilla and Wang, 2018). Butanol contains more energy in comparison to ethanol; net heat of consumption of butanol is 29.2 MJ $L^{-1}$, while that of ethanol is 21.1 MJ $L^{-1}$ (Abdehagh et al., 2014). However, distillation of butanol is expensive in comparison to ethanol due to lower butanol concentration in the fermentation broth (approx. 20 g $L^{-1}$ for butanol; approx. 150 g $L^{-1}$ for ethanol), closer boiling point of butanol/water azeotrope (93°C) to water (100°C) (ethanol/water azeotrope 78.2°C), and lower final distilled concentration for butanol (55.5% for butanol; 95.5% for ethanol). Hence, it is necessary that other efficient technologies are used for economically recovery of butanol (Jiménez-Bonilla and Wang, 2018). Product inhibition is one of the main reasons that limit butanol titer beyond a certain limit and is detrimental to recovery process economics. Although strain improvement and media engineering efforts have improved butanol tolerance, the degree of success is not up to the desired level. In order to mitigate butanol toxicity and efficiently recover butanol, several *in situ* product recovery strategies have been explored (Ezeji et al., 2007; Xue et al., 2014b; Lee et al., 2016). These separation techniques can be categorized into extraction and evaporation based techniques. Extraction based techniques include liquid-liquid extraction, reactive extraction, perstraction, and adsorption-based techniques, while evaporation based techniques include pervaporation, vacuum and flash fermentation, and gas stripping (Jiménez-Bonilla and Wang, 2018).

Many *in situ* product recovery techniques have proven to improve fermentation

titer, productivity, and yield at laboratory level but establishment of an optimum strategy for industrial use has not been reported. Advantages and disadvantages of different *in situ* product recovery techniques are listed in Table 2.5 (Xue et al., 2013; Abdehagh et al., 2014; Lee et al., 2016; Jiménez-Bonilla and Wang, 2018).

Liquid-liquid extraction (LLE) is a butanol recovery technique where an organic extractant is added to the fermentation broth to selectivity extract butanol (Abdehagh et al., 2014). A hydrophobic extractant is used to facilitate separation of butanol-laden organic phase. LLE is used during fermentation of different *C. acetobutylicum* and *C. beijerinckii* strains (Evans and Wang, 1988; Groot et al., 1990; Cascon et al., 2011; Chavez et al., 2012); however, with the exception of oleyl alcohol many of the extractants with high selectivity have been reported to be toxic to the culture (Abdehagh et al., 2014). Perstraction is a membrane assisted separation process where one side of membrane is in contact with fermentation broth while the other has the extracting solvent. Process efficiency depends on the selectivity of the membrane and the selected extractant. Separation between culture and extractant allows the use of highly selective (toxic) extractants without affecting fermentation and provides improvement over LLE; however, high cost of membrane, slow diffusion, and clogging of membrane has prevented implementation in large-scale facilities (Table 2.5).

Use of adsorption-based techniques involve sequential adsorption of butanol

**Table 2.5.** Advantages and disadvantages of different *in situ* product recovery techniques

| Techniques | Energy requirement (MJ $Kg^{-1}$ butanol) | Advantage | Disadvantage |
|---|---|---|---|
| Liquid-Liquid extraction | 8-9 | High selectivity; energy efficient; ease of operation | High cost; toxic to culture; extractant and nutrient loss; emulsion formation |
| Perstraction | 7.7 - 9 | Non-toxic to culture; high selectivity | High cost; membrane fouling; slow diffusion |
| Adsorption | upto 33 | Low energy requirement; high biocompatibility; ease of operation | High cost; low selectivity; instability of adsorbant |
| Pervaporation | upto 145 | Non-toxic to culture; high selectivity; energy efficient; no nutrient loss; low operating temperature | High cost; membrane fouling; membrane leakage |
| Vacuum fermentation | NA[a] | Non-toxic to culture; no fouling | Low selectivity |
| Gas stripping | 14 - 31 | Non-toxic to culture; no fouling; low water requirement; no nutrient loss | Low selectivity |

[a]NA- not available

on an adsorbent and desorption from a suitable adsorbent. The adsorbent should be highly selective to butanol, non-toxic to the cells, and have a high adsorption capacity. Also, the development of an efficient desorption process is equally important, which further adds to the operating cost of the process (Abdehagh et al., 2014). Pervaporation is another membrane based technique where a hydrophobic membrane allows selective permeation of butanol from the fermentation broth. Butanol after permeation is carried as a vapor to the cold trap for condensation (Abdehagh et al., 2014). This is achieved either by creating vacuum or by employing a carrier gas. Although pervaporation is non-toxic to the cells, energy efficient, and highly selective, the technology suffers from the limitations of high cost, membrane leakage, and membrane fouling (Lee et al., 2016).

During vacuum fermentation, butanol is removed under vacuum conditions in a fermenter operated at normal temperature, while in flash fermentation, the fermenter is operated at normal pressure and butanol separation occurs by passing the filtered broth through a vacuum chamber where distillation takes place. Vacuum fermentation of *C. beijerinckii* showed improvement in butanol titer from 80.6 g $L^{-1}$ to 106 g $L^{-1}$. During flash fermentation, concentrated substrate (10-30%, w/v) could be used but a decrease in yield was observed (Jiménez-Bonilla and Wang, 2018).

Gas stripping is a simple butanol recovery technique which was first demonstrated by Ennis et al. (1986). The stripping gas, which can be pure nitrogen or a mixture of carbon dioxide and hydrogen, is bubbled into the fermentation broth from sparger and carries volatile compounds such as butanol along with it through fermenter exhaust. The exhaust is connected to a condenser where the solvents from the solvent-laden gas condense and are collected at the bottom, while the gas is recycled into the fermenter (Ennis et al., 1986). Important factors affecting efficiency of butanol recovery are gas flow rate, concentration of butanol and microbial cells in the broth, and condensation temperature (Xue et al., 2014a). Major advantages of gas stripping include simple operation, low operating cost, ease of integration with other techniques, no detrimental effect to cell growth and fermentation, no loss of nutritional compounds or other metabolites, no requirement of expensive extractants, and no dependence on membrane technology (Ezeji et al., 2005; Zheng et al., 2009; Xue et al., 2014a). In addition, gas stripping also facilitates the use of concentrated substrates when integrated with fed-batch fermentation, thus reducing the water requirement.

Improvements in butanol titer, productivity, and yield were reported when gas stripping was used intermittently or continuously coupled with fermentation (Ezeji et al., 2004; Xue et al., 2012; Vrije et al., 2013). Considering all the separation techniques in the current context of technological expertise, evaporation based strategies such as gas stripping, vacuum fermentation, and pervaporation are the

most suitable for large scale use (Zheng et al., 2009; Jiménez-Bonilla and Wang, 2018). Integration of these techniques with different mode of cultivation will improve the fermentation performance as well as provide a concentrated solution for the distillation process.

### 2.2.8 Metabolic modeling

ABE fermentation carried out by various *Clostridium* spp. is observed to be a biphasic phenomenon. The switch from one phase (acidogenesis) to another phase (solventogenesis) is governed by complex metabolic regulation at the both transcriptional and translational levels. In order to understand this shift in metabolic machinery between two metabolic states, use of mathematical models mimicking the clostridial metabolic network has been reported by many researchers. To that end, possible hypotheses are tested by using available experimental data and then validating the theoretical results obtained through the simulation studies.

The first clostridial model dates back to 1984, when Papoutsakis (1984) wrote equations for butyric acid production by *Clostridium* sp. while later in 1999, Desai et al. (1999) developed the first clostridial stoichiometric model. Two years thereafter, Nolling et al. (2001) proposed the complete genome sequence of *C. acetobutylicum* ATCC 824. This laid the foundation for developing the genome-scale metabolic models for *C. acetobutylicum* by two different research groups in 2008 (Lee et al., 2008a; Senger and Papoutsakis, 2008). The two models differed in the number of reactions and metabolites. The model by Senger and Papoutsakis (2008) consisted of 422 intracellular metabolites, 552 reactions, and 80 membrane transport reactions, while the model reported by Lee et al. (2008a) included 502 reactions and 479 metabolites. Both groups attempted to resolve the TCA cycle by different approaches. In case of Senger and Papoutsakis (2008), they incorporated a urea cycle whereas Lee et al. (2008a) proposed an active reductive pathway between pyruvate and -ketoglutarate.

In 2010 and 2011, two different groups (Amador-Noguez et al., 2010; Crown et al., 2011) made attempts to elucidate the inconsistencies existing in the prevailing notion of TCA cycle. Both suggested a complete but broken TCA cycle along with disconnected oxidative and reductive branches. On the basis of the above hypothesis, McAnulty et al. (2012) developed another extended model for *C. acetobutylicum*. Au et al. (2014) possible another possible route with the TCA cycle configured in the oxidative direction, with no prominent flux between $\alpha$-ketoglutarate and succinyl-CoA or succinate and fumarate. Dash et al. (2014) used the insights reported by Au et al. (2014) to develop a model three-fold larger in size compared to earlier models (Senger and Papoutsakis., 2008; Lee et al., 2008a), which was further extended in size (20%) by Yoo et al. (2015). Although the model developed by Yoo et al. (2015) was a highly refined version with incorporation of transcriptomic, proteomic, and

fluxomic data, still key questions that would unfold the intricacies of the TCA cycle remained unanswered.

Meanwhile, Ehsaan et al. (2016) re-sequenced the genome of *C. aceto-butylicum* ATCC 824 to find some discrepancies in the earlier sequencing results (Nölling et al., 2001); however, it is yet to be ascertained how it will reflect on the metabolic network. Apart from *C. acetobutylicum*, genome scale metabolic models have been developed for other *Clostridium* spp. namely *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* (Serrano-Bermúdez et al., 2017).

Apart from GSM, methods for small scale metabolic network analysis such as elementary mode analysis (EMA), flux balance analysis (FBA), and metabolic flux analysis (MFA) are widely employed (Millat et al., 2017). These techniques assist researchers in simulating fermentation kinetics under varied environmental conditions even when limited experimental data is available. This is able to assist in elucidating and predicting versatile cellular phenotypes and their interactions with the metabolism (Kumar et al., 2014; Li et al., 2014; Gallardo et al., 2016; Sharma et al., 2017). For instance, Kumar et al. (2014) used elementary mode analysis as a tool to quantify metabolic fluxes in *C. acetobutylicum* under different external pH and in response to the addition of exogenous acids. Similarly FBA was used by Kaushal et al. (2018) to study the effect of substrate modulation on alcohol production by *C. sporogenes*, while Gallardo et al. (2016) used it to estimate the altered flux distribution of *C. acetobutylicum* in response to external electron supply. Further, $^{13}$C-MFA was used to constrain the model for *C. acetobutylicum* and internal flux distribution was studied in the different modelled conditions using different optimization objectives such as growth rate maximization, ATP maintenance, and the formation of NADH and NADPH (Wallenius et al., 2016).

## 2.3 Current challenges

ABE fermentation for economic fuel production is hindered by limitations such as low product titer and productivity, solvent toxicity, cost of substrate, high recovery cost, and lack of comprehensive knowledge regarding clostridial metabolism (Kaushal et al., 2018; Kumar et al., 2018; Menchavez and Ha, 2019; Li et al., 2019). Many clostridial strains have low inherent butanol production capacity, while in most cases the butanol titer and productivity are limited due to solvent toxicity. Butanol concentration upon reaching a strain specific level commences adverse effect on growth and production, and at a threshold limit completely inhibits the fermentation. Cessation of fermentation restricts the maximum attainable butanol titer, while sluggish fermentation affects the overall productivity. Simple sugars

or sugar alcohols selected substrates for high butanol producing microbial strains and hydrolysis of lignocellulosic material is cost intensive due to involvement of enzymes. These factors cause the substrate cost amount to approximately 50% of the operational cost. Adverse effect of low solvent concentration is also reflected in high energy requirements during product recovery thus increasing the downstream processing cost. Lack of understanding of complex clostridial metabolic regulation has hindered the progress in genetic engineering of robust strains and process development. Therefore, the present study was designed to address the above bottlenecks and to develop a bioprocess for enhanced butanol production.

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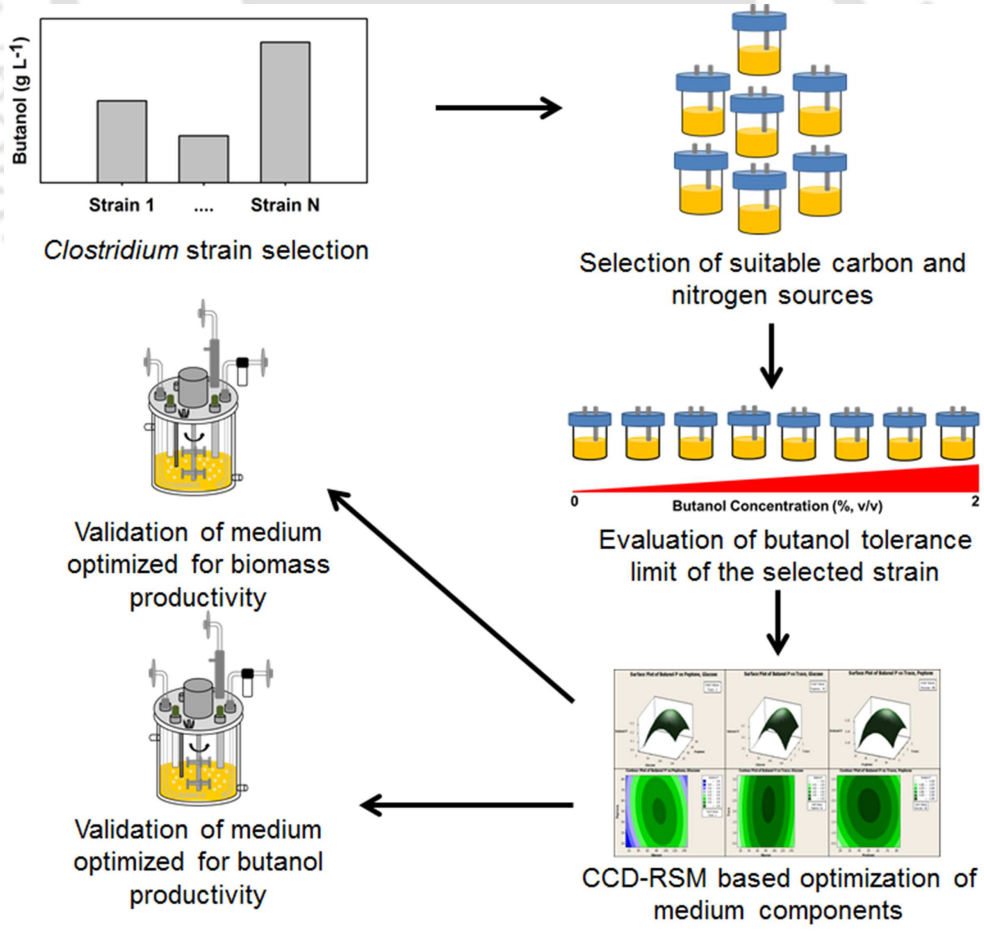
*"Gentlemen, it is the microbes that will have the last word."*

Louis Pasteur (1822-1895)
French chemist and microbiologist

# 3

# Screening and characterization of *Clostridium* strains on the basis of high butanol production




Selection of potential *Clostridium* strain followed by screening of suitable carbon and nitrogen sources and media optimization

## 3.1 Background and motivation

Renewed interest in butanol fermentation can be attributed to increased energy demand coupled with depleting resources and rising environmental concerns (Papoutsakis, 2008). Butanol is considered as an alternative to gasoline due to its physiochemical properties such as high energy density, high air-fuel ratio, low heat of vaporization, etc. In terms of fuel properties, butanol has greater resemblance to gasoline compared to ethanol and hence is more suitable as an alternative to gasoline (Jang et al., 2012; Tracy, 2012). Synthesis of butanol takes place through conventional acetone-butanol-ethanol (ABE) fermentation by *Clostridium* sp. which suffers from major bottlenecks such as high cost of substrates, end product toxicity, mixed products, degeneration of strains, and sporulation (Kumar and Gayen, 2011). All the above limitations along with low butanol titer and productivity make the production and recovery process commercially infeasible (Zheng et al., 2015).

Genus *Clostridium* displays microbial diversity in terms of habitat, genetic material, metabolism, and nutritional and cultivation requirements (Tracy, 2012; Jones and Woods, 1986). Among the butanol producing clostridial strains, diversity exist in terms of substrate requirements, growth dynamics, and product profiles (Xin et al., 2018). For instance, *C. acetobutylicum* and *C. beijerinckii* are known to utilize hexoses and pentoses to produce ABE, whereas *C. pasteurianum* uses glycerol to produce butanol-propanediol (Xin et al., 2018). While most *C. acetobutylicum* strains are unable to consume glycerol as a sole carbon source, Yadav et al. (2014) identified *C. acetobutylicum* KF158795 as a glycerol consumer. Similarly a previously reported *C. acetobutylicum* strain was identified as *C. sporogenes* based on absence of acetone production, biochemical characterization, and phylogenetic analysis (Kaushal et al., 2017). Hence, it is imperative to screen new strains for selection of suitable strain and to undertake extensive characterization prior to process development (Zheng et al, 2015). Optimization is used to improve the performance of a system without affecting the cost and one of the most commonly used optimization tool in chemical and biochemical processes is response surface methodology (RSM) (Ba and Boyac, 2007). RSM based optimization of media components is advantageous over one-variable-at-a-time optimization as it provides information regarding effect of individual components as well as their interaction, is less time and labor intensive, cost effective as less experiments are required, and generates a mathematical model for the process analysis (Wani et al., 2012; Ba and Boyac, 2007). Major steps in optimization involve designing and conducting statistical experiments, generation and validation of mathematical model to describe the response surface, and use of the mathematical model to predict optimum concentrations of the variables for achieving desired response i.e. maximization or minimization of the defined objective function (Maddox and Richert, 1977).

The energy sector involves low value high volume products. Therefore, productivity must be considered as one of the critical process parameter while designing a production strategy. This is even more relevant for synthesis of butanol, which follows a complex metabolic network resulting in multiple end products. Another major strain-level bottleneck is the butanol toxicity towards wild-type strains at a concentration of 13-16 g $L^{-1}$ (Gao et al., 2012; Jones and Woods, 1986). This results in limited scope of improving the butanol titer beyond a particular limit. Therefore, researchers are presently focusing on improving the butanol productivity or productive fermentation time. To that end, different clostridial strains were procured and characterized under different media to screen the most potential butanol producer. Further, in-depth characterization of the selected strain was carried out, followed by screening of suitable carbon and nitrogen sources, and statistical optimization of the media components for maximization of biomass productivity and maximization of butanol productivity.

## 3.2 Materials and methods

### 3.2.1 Microorganisms, maintenance, and inoculum preparation

Six *C. acetobutylicum* strains were procured from various culture repositories (Table 3.1) and screened on the basis of their butanol producing ability. The lyophilized cultures were revived using Cooked Meat Media (Himedia M149) and stored as glycerol stocks at 80°C. One mL glycerol stock, of each of the cultures, was inoculated into culture tubes containing 14 mL tryptone-yeast extract-glucose (TYG) medium comprising (g $L^{-1}$) tryptone: 30.0, glucose: 20.0, yeast extract: 10.0, and cysteine hydrochloride: 0.05 (Annous and Blaschek, 1990). The inoculated cultures were incubated at 37°C under static anaerobic conditions. Anaerobic condition was maintained by intermittent purging with pure nitrogen (99.99%, Assam Air products, Guwahati, India), while the anaerobicity was confirmed by addition of a redox indicator dye, resazurin at 1 g $L^{-1}$. 10 mL of each of the actively growing cultures was transferred to 500 mL airtight glass bottles containing 100 mL TGY medium and incubated under similar conditions for the development of seed culture. When biomass with optical density ($OD_{600}$) of 3.0 was obtained, 10% (v/v) seed

**Table 3.1.** List of *Clostridium* strains procured from various culture repositories

| S. No. | Strain name | Strain No. | Source |
|---|---|---|---|
| 1 | *Clostridium acetobutylicum* | NCIM 2842 | NCL, Pune |
| 2 | *Clostridium acetobutylicum* | NCIM 2878 | NCL, Pune |
| 3 | *Clostridium acetobutylicum* | NCIM 2879 | NCL, Pune |
| 4 | *Clostridium acetobutylicum* | B-591 | NRRL, USA |
| 5 | *Clostridium acetobutylicum* | B-596 | NRRL, USA |
| 6 | *Clostridium acetobutylicum* | MTCC 11274 | IMTECH, Chandigarh |

culture was used as inoculum for the production media in all experiments, unless otherwise mentioned. All the chemicals (AR grade) were purchased from Himedia, India unless otherwise mentioned.

### 3.2.2 Screening of potential butanol producing strain and selection of suitable production medium

Since bacterial strains have different nutritional requirements, the procured strains were characterized for their butanol production potential in two different media compositions: (i) P2 medium, and (ii) P2+TY medium. P2 medium comprises (in g $L^{-1}$) of glucose: 80.0, $K_2HPO_4$: 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, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001 (Monot et al., 1982). The P2+TY medium 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). All media were autoclaved at 121°C for 20 min, while the vitamin stocks were sterilized through 0.2 μm syringe filter. The batch fermentation was carried out in 500 mL airtight glass bottles containing 100 mL of the respective medium for 120 h at 37°C under static conditions. Samples were drawn every 12 h for analysis of growth, medium pH, substrate utilization, and product formation. The medium and the strain exhibiting highest butanol titer were selected for process development.

Biochemical characterization of the selected strain was carried out according to Bergey's Manual of Determinative Bacteria (Buchanan and Gibbons, 1974) and the tests conducted are as follows: 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, lecithinase test, and growth on twenty four different carbon sources. Biochemical analysis data for *C. acetobutylicum* strains were taken from various available literature reports for comparative purpose (Buchanan and Gibbons, 1974; Lemmel et al., 1986; Forsberg et al., 1986; Keis et al., 2001; Servinsky et al., 2014). For the growth experiments in different carbon sources, glucose in TYG media was replaced with respective carbon sources, while maintaining equimolar concentration of 0.6 M carbon. In order to ensure that no residual glucose was transferred from seed media to test media containing different carbon sources, the culture was centrifuged at 5,000×*g* for 10 min at 4°C prior to inoculation and re-suspended in the respective media. Samples were drawn for analysis of biomass, acid, and solvent production for all the carbon sources.

### 3.2.3 Characterization of the selected strain under different carbon and nitrogen sources

Further, the selected strain was characterized under different carbon and nitrogen sources to study their effect on growth and butanol production. Seventeen

carbon sources including 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) were used to screen and classify the growth promoting and butanol inducing carbon source. Glucose, in the selected medium, was replaced with different carbon sources in the equivalence of 2.66 M of total carbon, while all other media components were kept same. The carbon source that supported maximum butanol production was selected and used for all subsequent characterization and optimization experiments. Similarly, ten different organic and inorganic nitrogen sources (tryptone, yeast extract, peptone, proteose peptone, beef extract, ammonium acetate, ammonium sulphate, ammonium chloride, glycine, and urea) were used to select the suitable nitrogen source. To that end, tryptone and yeast extract in the medium were replaced with the above mentioned organic nitrogen sources at an equimolar concentration of 0.342 M of total nitrogen. Inorganic nitrogen sources were used at 0.065 M concentration to replace organic nitrogen source in the medium. The nitrogen source resulting in maximum butanol production was selected and used in further experiments. It is important to note that absence of residual carbon and nitrogen sources carried forwarded from seed media was ensured as described in section 3.2.2. The fermentation conditions and sampling time points were kept same as detailed in section 3.2.2.

### 3.2.4 Evaluation of the strain for solvent tolerance

The threshold limit of butanol tolerance for the selected strain was determined by exposing the cells to different concentrations of butanol. Batch fermentation was carried till biomass OD of 3.0 was attained. The exponentially growing culture was equally distributed in fermentation bottles containing different butanol concentrations (%, w/v) of 0.25, 0.5, 0.75, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, and 2.0. The organism when grown in the media without supplementation of any exogenous butanol was termed as control culture. The growth was monitored up to 120 h of incubation at 37°C. Samples were taken out every 24 h to estimate growth and butanol production.

### 3.2.5 Statistical medium optimization for maximization of biomass and maximization of butanol productivity

Central composite design (CCD) based RSM was employed to optimize the medium composition for maximizing the biomass and butanol productivity. The actual and coded levels of the above selected parameters used in CCD-RSM experimental design are shown in Table 3.2.

The CCD was formulated using statistical software Minitab statv.16.1.1 (Minitab Inc., Pennsylvania, USA) with three factors and five levels (Table 3.2). The

CCD required 18 experiments which included eight factorial points, six axial points, and four replicates of the center point to search linear, quadratic, and interaction effects of parameters on biomass and butanol productivity. The interactive effects are represented by the regression Eq. (3.1).

$$Y = \beta_0 + \Sigma_{i=1}^{k} \beta_i X_i + \Sigma_{i=1}^{k} \beta_{ii} X_i^2 + \Sigma_{i=1,i<j}^{k-1} \Sigma_{j=2}^{k} \beta_{ij} X_i X_j \quad (3.1)$$

where, Y is the biomass productivity or butanol productivity (g $L^{-1}$ $h^{-1}$) as model response, Xi is the $i^{th}$ parameter, $k$ is the total number of parameters and $\beta_0$, $\beta_{ii}$, $\beta_{ii}$ and $\beta_{ij}$ are the regression coefficients. The experiments were carried out as described in section 3.2.2. The medium compositions optimized for both biomass productivity and butanol productivity were validated under similar cultivation conditions.

**Table 3.2.** Parameters, their codes and level values used in CCD-RSM based optimization

| Factors | Levels code and corresponding values | | | | |
|---|---|---|---|---|---|
| | **-1.68** | **-1** | **0** | **1** | **1.68** |
| Initial glucose concentration (g $L^{-1}$) | 12.72 | 40 | 80 | 120 | 147.27 |
| Initial peptone concentration (g $L^{-1}$) | 16.36 | 30 | 50 | 70 | 83.63 |
| Initial trace elements concentration (mL $L^{-1}$) | 3.18 | 10 | 20 | 30 | 36.81 |

### 3.2.6 Characterization of the strain under batch mode in bioreactor

Two separate batch fermentations were carried out using the medium compositions obtained for biomass productivity and butanol productivity, respectively. Each experiment was carried out in a 3.0 L automated bioreactor (Bio Console ADI 1025, Applikon Biotechnology, Holland) with 1.0 L optimized medium. The reactor was operated at 37°C, initial pH 6.1-6.3 (uncontrolled), and agitator speed of 300 rpm. $N_2$ was purged through the optimized media prior to inoculation to ensure anaerobic conditions and was monitored by the use of redox indicator dye, resazurin (1 g $L^{-1}$). Samples were drawn at regular intervals for the analysis of growth, substrate utilization, and product formation.

### 3.2.7 Analytical methods

Five mL of fermentation broth was collected and centrifuged at 10,000×*g* for 10 min at 4°C. The pellet was washed and re-suspended in saline (0.85%, w/w of NaCl) to measure growth in terms of OD at 600 nm using UV-visible spectrophotometer (Cary 50, Varian, Australia). The OD was converted in to dry cell weight (DCW) using the calibration equation 1 OD=0.328 g $L^{-1}$ DCW . The supernatant was used to obtain dynamic profiles for pH, substrate, organic acids (acetate and butyrate), and solvents (acetone, ethanol, and butanol). The concentrations of substrates, organic acids, and solvents were measured in high

performance liquid chromatograph (HPLC, LC-20AD, Shimadzu, Japan) equipped with a Rezex ROA column (300 x 7.8 mm, Phenomenex, USA). Organic acids were detected in ultra-violet (UV) detector at 210 nm, while substrates and solvents were measured in refractive index detector (RID) at 37°C. 0.005 N $H_2SO_4$ (98%, Merck, Germany) used as mobile phase was flown through the column at a flow rate of 0.5 mL $min^{-1}$. The minimum detection limits estimated for substrates, acids, and solvents were found to be 0.1g $L^{-1}$, 0.04 g $L^{-1}$, and 0.06 g $L^{-1}$, respectively. 10 µL of sample was used for analysis and filtered with 0.2 µm filters prior to injection. Correlation standard curves obtained are mentioned in the appendix. All experiments were conducted in triplicates and the values have been represented as mean ± standard error.

## 3.3 Results and discussion

### 3.3.1 Screening of *Clostridium* strains as a potential cell factory for butanol production

Six *Clostridium* strains were screened in order to select potential strain as a cell factory for butanol production. The characterization of these strains, in terms of growth and butanol production, was performed in two different media compositions as reported in the literature works (Annous and Blaschek, 1990; Monot et al., 1982). This was done owing to the fact that the maximum production potential of a strain is specific to particular medium composition (Ezeji et al., 2005). The growth and butanol titer of six *Clostridium* strains on two different media are shown in Fig. 3.1. Among the six strains characterized, only two strains exhibited butanol production

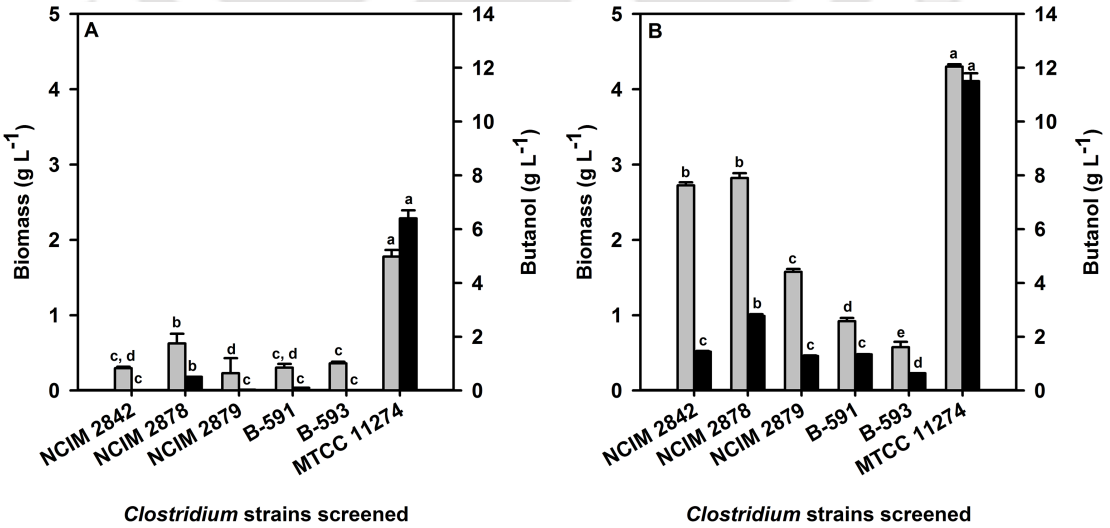



**Fig. 3.1.** The comparison of six *C. acetobutylicum* strains in terms of maximum growth (grey bar) and butanol titer (black bar) when grown in (A) P2 medium (P2) and (B) P2 medium supplemented with tryptone and yeast extract (P2+TY). Means that share the same letter do not differ significantly at the 95% confidence level based on the Tukey method.

in P2 medium with a maximum titer of 6.4 g $L^{-1}$ obtained from MTCC 11274 (Fig. 3.1A). Interestingly, all the strains except MTCC 11274 showed less growth in P2 medium when compared to the growth obtained from the other media (P2+TY). It is important to note that P2 is a synthetic medium devoid of any organic source of nitrogen (Monot et al., 1982) and hence does not support high growth of such fastidious organisms (Welsh et al., 1987) which in turn resulted in minimal or no butanol production.

In order to validate this hypothesis, in the present study, the organisms were further characterized on P2+TY medium where P2 medium was supplemented with organic nitrogen source in the form of tryptone and yeast extract in the concentrations used by Annous and Blaschek (1990). Remarkably, all the strains exhibited growth and butanol production in P2 + TY medium with a maximum butanol titer of 11.5 g $L^{-1}$ in case of MTCC 11274 (Fig. 3.1B). The rest of the strains produced butanol in the range of 1 g $L^{-1}$ to 3.8 g $L^{-1}$. Therefore, MTCC 11274 was selected as the best producer strain on P2+TY medium and considered for subsequent process development. Biochemical characterization of MTCC 11274 and its comparison to features associated with *C. acetobutylicum* strains revealed several phenotypical traits typical of the strain (Table 3.3). The cells were rod shaped, motile, and tested negative for lipase, $H_2S$ production, oxidase test, catalase test, urease production, indole production, Voges Proskauer test, proteolytic test, nitrate reduction test, and Nagler test (lecithinase) which coincides with the results for *C. acetobutylicum* strains. The strain was unable to utilize glycerol and sorbitol but it could ferment mannitol and starch as a sole carbon sources which is one of the typical phenotypic behavior for *C. acetobutylicum* strains (Buchanan and Gibbons, 1974; Keis et al., 2001).

### 3.3.2 Characterization of the strain under different carbon and nitrogen sources

Variation in carbon and nitrogen sources alters the growth and butanol production in *Clostridium* spp. (Al-Shorgani et al., 2011; Welsh et al., 1987). In the present set of experiments, seventeen different carbon sources were screened in order to determine the most potential carbon source yielding maximum butanol titer by MTCC 11274. Amongst all the carbon sources considered, glucose was found to be best growth promoting (4.5 g $L^{-1}$) and butanol-supporting (11.6 g $L^{-1}$) carbon source (Fig. 3.2A). The other carbon sources such as arabinose, starch, mannose, maltose, fructose, and dextrin were found to produce butanol in the range of 7.8 g $L^{-1}$ to 8.7 g $L^{-1}$. Interestingly, the organism was able to utilize xylose, thereby producing 4 g $L^{-1}$ of butanol (Fig. 3.2A). Similarly other *Clostridium* species such as *C. sporogenes*, *C. saccharoperbutylicum*, and *C. beijerinckii* have been reported to utilize xylose as the sole carbon source to produce butanol titer of 3.2, 1.2, and 5.8 g $L^{-1}$, respectively (Kaushal et al., 2017, Xiao et al., 2012; Al Shorgani et al., 2011).

**Table 3.3.** Characteristics of strain *C. acetobutylicum* MTCC 11274 and *C. acetobutylicum* strains

| Phenotypic trait | Strain MTCC 11274 | *C. acetobutylicum* strains |
|---|---|---|
| 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[a] | - | + |
| Temperature | 37 | 30-37 |
| pH range | 5.0 -7 | 5.0 -7 |
| Fermentation end products[b] | A,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 | - | NT |
| Xylan | + | + |
| Pectin | + | + |
| Inositol | - | - |
| Citrate | - | NT |
| Glucuronic acid | - | -/+ |

+ represents positive; - represents negative; -/+ represents mixed results on different strains; a-represents that the proteolytic test and gelatin liquefaction were 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* ATCC 824 was taken from literature; NT-not tested

Butanol production was found to be minimal on lactose, sucrose, galactose, and gluconate, whereas no production was detected on sugar alcohols such as glycerol or sorbitol and also with ribose and carboxy methyl cellulose as the carbon source. This is in accordance to the studies conducted for various strains of *C. acetobutylicum*, where the strains failed to utilize glycerol as the sole carbon source and glycerol could only be used in a mixture with glucose (Gheshlaghi et al., 2009; Andrade and Vasconcelos, 2003). An interesting finding from the current research is the ability of the organism to produce significant amount of butanol when grown on pentose sugars, arabinose, and xylose, which opens up scope for development of a sustainable bioprocess via utilization of lignocellulosic biomass.

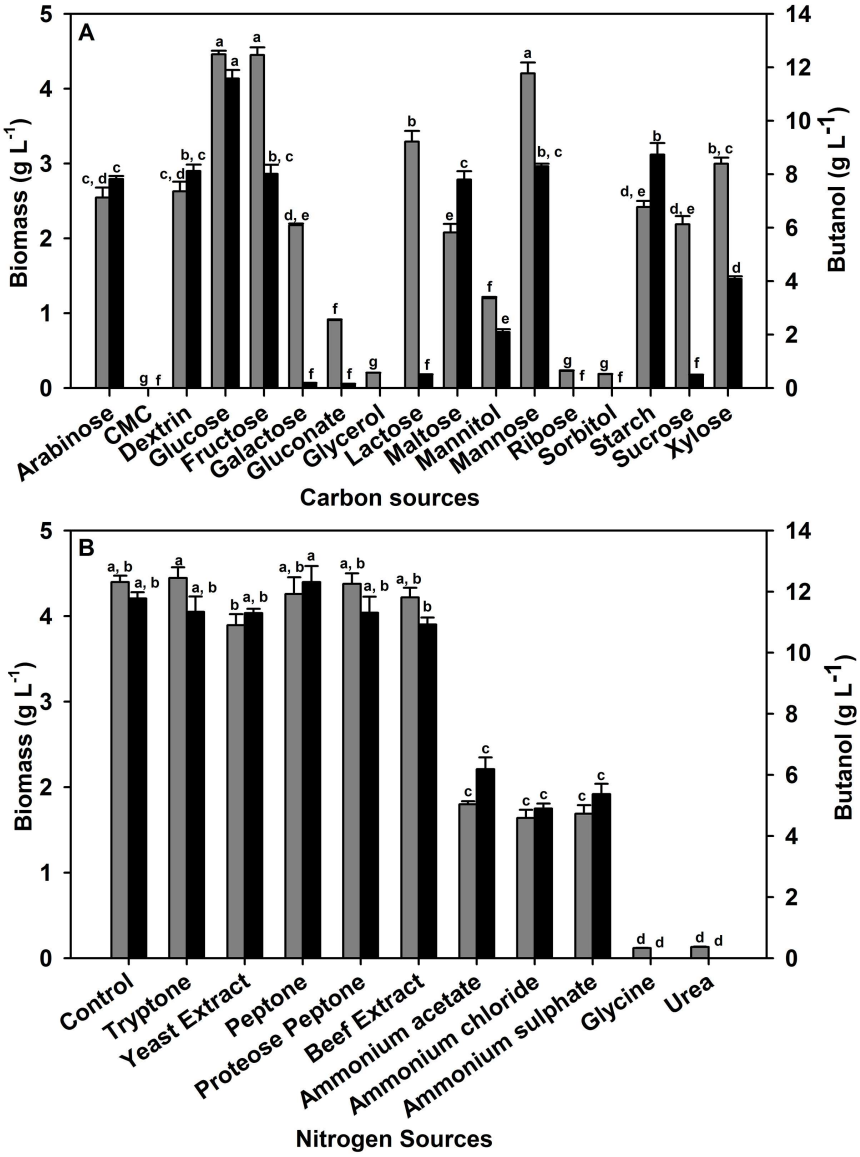



**Fig. 3.2.** Characterization of *C. acetobutylicum* MTCC 11274 in terms of growth (grey bar) and butanol titer (black bar) under different (A) carbon sources and (B) nitrogen sources. Means that share the same letter do not differ significantly at the 95% confidence level based on the Tukey method.

Wang et al. (2014) characterized *C. acetobutylicum* ATCC 824 on glucose, xylose, and glucose-xylose blends simulating microalgae based carbohydrates and demonstrated its potential in using the same for butanol production of 13.03, 8.89 and 11.5 g $L^{-1}$, respectively. On the basis of maximum growth and highest butanol titer, glucose was selected as the carbon source for the subsequent screening experiments conducted in order to determine the most significant nitrogen source influencing growth and butanol production by MTCC 11274.

Among the eight organic nitrogen sources screened, similar biomass in the range of 3.9 to 4.5 g $L^{-1}$ was obtained for all except glycine and urea (Fig. 3.2B). Butanol production was observed in the range of 10.9 g $L^{-1}$ to 12.3 g $L^{-1}$ with the highest being achieved when peptone was used as the nitrogen source. Ranjan et al. (2012) observed that yeast extract was a significant factor in solvent production by *C. acetobutylicum* MTCC 481. The organic nitrogen substrates act as a source of essential amino acids and growth factors and, thereby increase the growth rate of the microorganisms (Ezeji et al., 2005). Among the inorganic nitrogen sources screened, ammonium acetate showed maximum accumulation of butanol (6.2 g $L^{-1}$) followed by ammonium chloride and ammonium sulphate. Kanchantawee et al. (1990) observed maximum butanol production (6 g $L^{-1}$) by using ammonium sulphate, which was further increased by supplementing with yeast extract. Peptone, which showed maximum butanol titer, was selected as the nitrogen source for further experiments.

### 3.3.3 Evaluation of the strain for solvent tolerance

Meager increase in butanol titer after nitrogen screening and similar titer obtained under different organic nitrogen sources indicated possible product inhibition, a known bottleneck in ABE fermentation (Jones and Woods, 1986). This led us to assess butanol tolerance limit of the strain in order to ascertain the cause of restricted butanol titer obtained during batch fermentation. Exponentially growing cells were exposed to different concentrations of butanol to study the effect of exogenous butanol on the growth of the organism (Fig. 3.3).

In the presence of 0.25% and 0.5% butanol, no difference in growth was observed till 24 h of cultivation when compared with the control culture. However, in terms of final biomass titer, a reduction in 11.75% was observed upon exposure to 0.5% butanol as compared to the control culture (Fig. 3.3). The growth was found to reduce significantly when the cells were exposed to further elevated butanol concentrations in the range of 0.75% to 1.2% (Fig. 3.3). Cells were unable to survive when exposed to a butanol concentration of 1.4% and above (Fig. 3.3). An interesting observation was made when 1.3% butanol was added to the media; cells were able to survive although there was no increase in growth in terms of biomass. In order to evaluate the viability, the cells exposed to 1.3% butanol were harvested

and re-suspended in the fresh medium. It was observed that if transferred to the fresh medium within 24 h of fermentation, cells could resume their growth albeit with a longer lag time. In a similar study, Gao et al. (2012) reported that wild

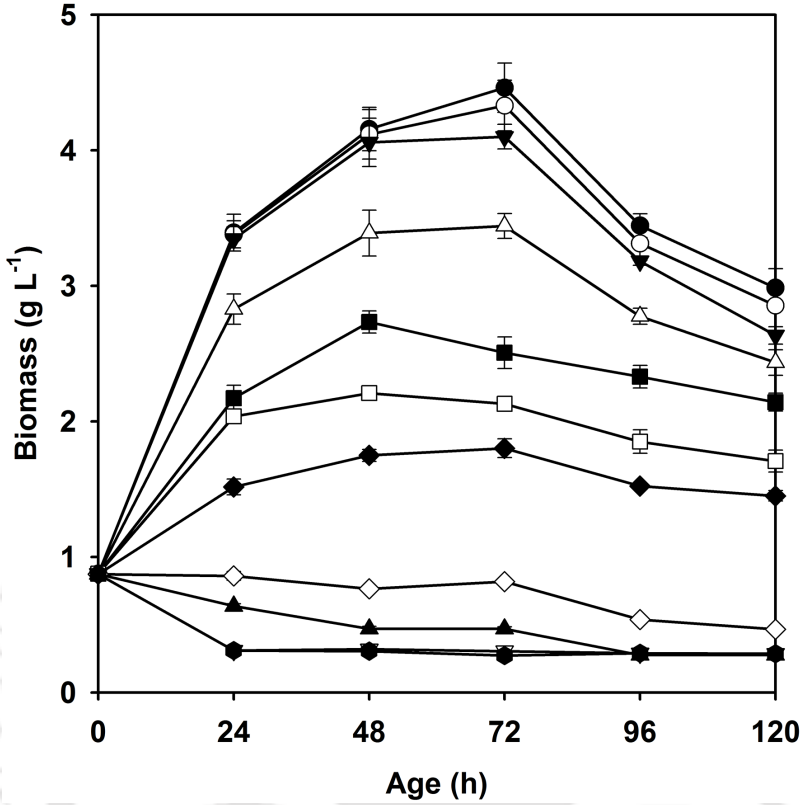



**Fig. 3.3.** Dynamic profiles for growth of *C. acetobutylicum* MTCC 11274 under supplementation with various concentrations of extraneous butanol (%, w/v): 0.25 (○), 0.5 (▼), 0.75 (▽), 1.0 (■), 1.1 (□), 1.2(⧫), 1.3 (◊), 1.4 (▲), 1.5 (▽) and 2.0 (✠). Control (●) symbolizes culture not exposed to exogenous butanol stress.

type *C. acetobutylicum* ATCC 824 failed to grow when exposed to 14 g $L^{-1}$ butanol. This led to a conclusion that improvement of butanol titer beyond a certain limit was not achievable due to limited butanol tolerance of the strain. Hence, further medium optimization experiments were carried out to improve butanol productivity in order to make the process industrially feasible. This study was also particularly significant in ascertaining the threshold butanol concentration at which the recovery process needs to be initiated in order to avoid butanol toxicity from inhibiting the fermentation process.

### 3.3.4 Statistical optimization of media for maximization of biomass productivity and maximization of butanol productivity

Phenomenon of product inhibition exerts a limit on the maximum attainable titer in any process; therefore, further improvement in such a process can be achieved by improving the productivity rather than the product titer. Taking into account the product toxicity experienced by the strain, as observed from the butanol tolerance

experiments, optimization of media composition was carried out separately with the aim of maximizing the butanol and biomass productivity. A CCD was constructed to optimize the initial concentration of three media components *viz.*, glucose, peptone, and trace elements (Table 3.4) and to study the effect of their interaction on model response.

The experimental results were analyzed through RSM for two objective functions: (i) maximization of biomass productivity and (ii) maximization of butanol productivity. As biomass formation and butanol production are two mutually exclusive parameters, the nutritional conditions required for growth and butanol production were optimized separately. The experiments resulted in wide range of biomass productivity from 0.08 g $L^{-1}$ $h^{-1}$ to 0.21 g $L^{-1}$ $h^{-1}$, while the butanol productivity varied from 0.12 g $L^{-1}$ $h^{-1}$ to 0.36 g $L^{-1}$ $h^{-1}$. The experimental data along with the corresponding RSM predicted values, for both the objective functions, are tabulated in Table 3.4.

**Table 3.4.** Tabular representation of CCD design of media parameters containing experimental and predicted values for maximum biomass productivity and maximum butanol productivity as two separate responses

| Std. order | Glucose (g $L^{-1}$) | Peptone (g $L^{-1}$) | Trace (mL $L^{-1}$) | Max. biomass productivity | | Max. butanol productivity | |
|---|---|---|---|---|---|---|---|
| | | | | Exp. | Pred. | Exp. | Pred. |
| 1 | 40 | 30 | 10 | 0.2 | 0.202573 | 0.22 | 0.209913 |
| 2 | 120 | 30 | 10 | 0.16 | 0.147769 | 0.27 | 0.271022 |
| 3 | 40 | 70 | 10 | 0.17 | 0.172489 | 0.27 | 0.255798 |
| 4 | 120 | 70 | 10 | 0.1 | 0.098824 | 0.25 | 0.249146 |
| 5 | 40 | 30 | 30 | 0.2 | 0.195831 | 0.23 | 0.222809 |
| 6 | 120 | 30 | 30 | 0.13 | 0.133647 | 0.3 | 0.302557 |
| 7 | 40 | 70 | 30 | 0.16 | 0.166567 | 0.26 | 0.254255 |
| 8 | 120 | 70 | 30 | 0.09 | 0.085522 | 0.26 | 0.266242 |
| 9 | 12.73 | 50 | 20 | 0.21 | 0.200183 | 0.12 | 0.139161 |
| 10 | 147.27 | 50 | 20 | 0.08 | 0.085947 | 0.21 | 0.200627 |
| 11 | 80 | 16.36 | 20 | 0.19 | 0.193988 | 0.28 | 0.28587 |
| 12 | 80 | 83.64 | 20 | 0.13 | 0.128222 | 0.29 | 0.293917 |
| 13 | 80 | 50 | 3.18 | 0.15 | 0.159692 | 0.3 | 0.307283 |
| 14 | 80 | 50 | 36.82 | 0.15 | 0.142838 | 0.33 | 0.332504 |
| 15 | 80 | 50 | 20 | 0.17 | 0.175037 | 0.36 | 0.35758 |
| 16 | 80 | 50 | 20 | 0.18 | 0.175037 | 0.36 | 0.35758 |
| 17 | 80 | 50 | 20 | 0.17 | 0.175037 | 0.36 | 0.35758 |
| 18 | 80 | 50 | 20 | 0.18 | 0.175037 | 0.36 | 0.35758 |

Max.- Maximum; Exp.- Experimental; Pred.- Predicted

RSM based model construction yielded two polynomial equations (Eq. 3.2

and 3.3), which correlated the medium parameters with the predicted response of biomass productivity and butanol productivity.

$$Y = 0.49 + 0.0020X_1 + 0.0021X_2 + 0.1X_3 - 2.16x10^{-5}X_1^2 - 3.75x10^{-5}X_2^2 - 0.026X_3^2 - 1.79x10^{-5}X_1X_2 - 1.40 \times 10^{-4}X_1X_3 - 3.12x10^{-5}X_2X_3 \quad (3.2)$$

$$Y = 0.25 + 0.008X_1 + 0.008X_2 - 0.061X_3 - 4.17x10^{-5}X_1^2 - 5.98x10^{-5}X_2^2 - 0.013X_3^2 - 2.12x10^{-5}X_1X_2 - 1.16 \times 10^{-4}X_1X_3 - 1.8x10^{-4}X_2X_3 \quad (3.3)$$

where, $Y$ is the predicted biomass and butanol productivity (g $L^{-1}$ $h^{-1}$), respectively, while $X_i$ ($i$ = 1-3) represents the medium components; glucose, peptone, and trace elements, respectively. Analysis of variance showed a high degree of significance for both biomass and butanol productivity as evident from the $p$-value less than 0.05 for both the objective functions (Tables 3.5 and 3.6). The regression analysis showed a correlation coefficient ($R^2$) of 0.98 for both the objective functions, indicating that only 2% of the 18 experiments performed could not be fitted by the model. A normal distribution of residuals ensures an adequate fit of the model with the experimental data (Fig 3.4 and 3.5).

**Table 3.5.** Analysis of variance for the quadratic regression model obtained from CCD-RSM employed in optimization of media components for the biomass productivity from *Clostridium acetobutylicum* MTCC 11274

| Source | DF | Seq SS | Adj SS | Adj MS | F | p value |
|---|---|---|---|---|---|---|
| Regression | 9 | 0.21647 | 0.21647 | 0.024052 | 47.81 | 0 |
| Linear | 3 | 0.19615 | 0.19615 | 0.065383 | 129.96 | 0 |
| Glucose | 1 | 0.146419 | 0.146419 | 0.146419 | 291.03 | 0 |
| Peptone | 1 | 0.046543 | 0.046543 | 0.046543 | 92.51 | 0 |
| Trace | 1 | 0.003187 | 0.003187 | 0.003187 | 6.34 | 0.036 |
| Square | 3 | 0.01841 | 0.01841 | 0.006137 | 12.2 | 0.002 |
| Glucose*Glucose | 1 | 0.009443 | 0.013967 | 0.013967 | 27.76 | 0.001 |
| Peptone*Peptone | 1 | 0.001443 | 0.003059 | 0.003059 | 6.08 | 0.039 |
| Trace*Trace | 1 | 0.007525 | 0.007525 | 0.007525 | 14.96 | 0.005 |
| Interaction | 3 | 0.001909 | 0.001909 | 0.000636 | 1.27 | 0.35 |
| Glucose*Peptone | 1 | 0.001653 | 0.001653 | 0.001653 | 3.29 | 0.107 |
| Glucose*Trace | 1 | 0.000253 | 0.000253 | 0.000253 | 0.5 | 0.498 |
| Peptone*Trace | 1 | 0.000003 | 0.000003 | 0.000003 | 0.01 | 0.939 |
| Residual Error | 8 | 0.004025 | 0.004025 | 0.000503 | | |
| Lack-of-Fit | 5 | 0.003956 | 0.003956 | 0.000791 | 34.53 | 0.007 |
| Pure Error | 3 | 0.000069 | 0.000069 | 0.000023 | | |
| **Total** | 17 | 0.220494 | | | | |

* p-value higher than 0.05 shows insignificance

# $X_1$, $X_2$, $X_3$ represents the media components glucose, peptone, and trace elements, respectively

**Table 3.6.** Analysis of variance for the quadratic regression model obtained from CCD-RSM employed in optimization of media components for the butanol productivity from *Clostridium acetobutylicum* MTCC 11274

| Source | DF | Seq SS | Adj SS | Adj MS | F | p value |
|---|---|---|---|---|---|---|
| Regression | 9 | 0.065083 | 0.065083 | 0.007231 | 59.21 | 0 |
| Linear | 3 | 0.005407 | 0.005407 | 0.001802 | 14.76 | 0.001 |
| Glucose | 1 | 0.004561 | 0.004561 | 0.004561 | 37.34 | 0 |
| Peptone | 1 | 0.000078 | 0.000078 | 0.000078 | 0.64 | 0.447 |
| Trace | 1 | 0.000078 | 0.000078 | 0.000078 | 0.64 | 0.447 |
| Square | 3 | 0.057103 | 0.057103 | 0.019034 | 155.86 | 0 |
| Glucose*Glucose | 1 | 0.057103 | 0.057103 | 0.019034 | 155.86 | 0 |
| Peptone*Peptone | 1 | 0.057103 | 0.057103 | 0.019034 | 155.86 | 0 |
| Trace*Trace | 1 | 0.002246 | 0.002246 | 0.002246 | 18.39 | 0.003 |
| Interaction | 3 | 0.002574 | 0.002574 | 0.000858 | 7.02 | 0.012 |
| Glucose*Peptone | 1 | 0.002296 | 0.002296 | 0.002296 | 18.8 | 0.002 |
| Glucose*Trace | 1 | 0.000174 | 0.000174 | 0.000174 | 1.42 | 0.267 |
| Peptone*Trace | 1 | 0.000104 | 0.000104 | 0.000104 | 0.85 | 0.383 |
| Residual Error | 8 | 0.000977 | 0.000977 | 0.000122 | | |
| Lack-of-Fit | 5 | 0.000963 | 0.000963 | 0.000193 | 41.27 | 0.006 |
| Pure Error | 3 | 0.000014 | 0.000014 | 0.000005 | | |
| **Total** | 17 | 0.06606 | | | | |

* p-value higher than 0.05 shows insignificance

# $X_1$, $X_2$, $X_3$ represents the media components glucose, peptone, and trace elements, respectively

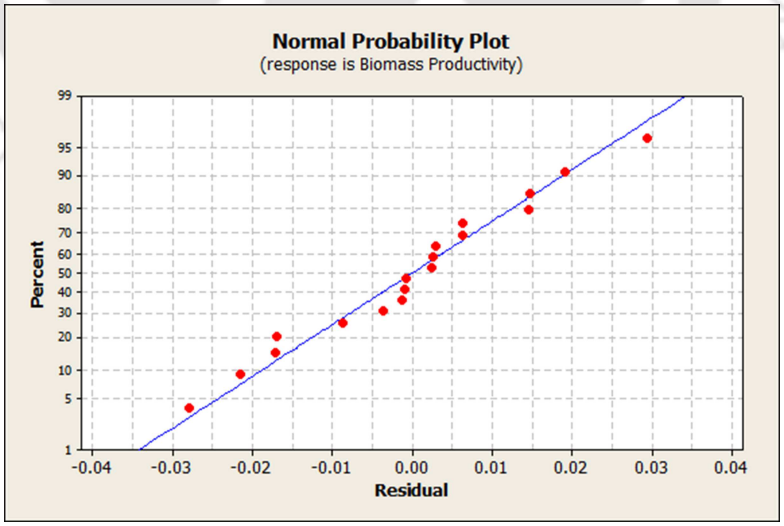



**Fig. 3.4.** Normality plot of residuals where the response was biomass productivity.

The linear and quadratic effects of all the three parameters were found to be significant on the growth of the strain. No significant interaction among the media components was found for biomass productivity within the selected concentration range. In case of butanol productivity, both linear (except peptone) and quadratic

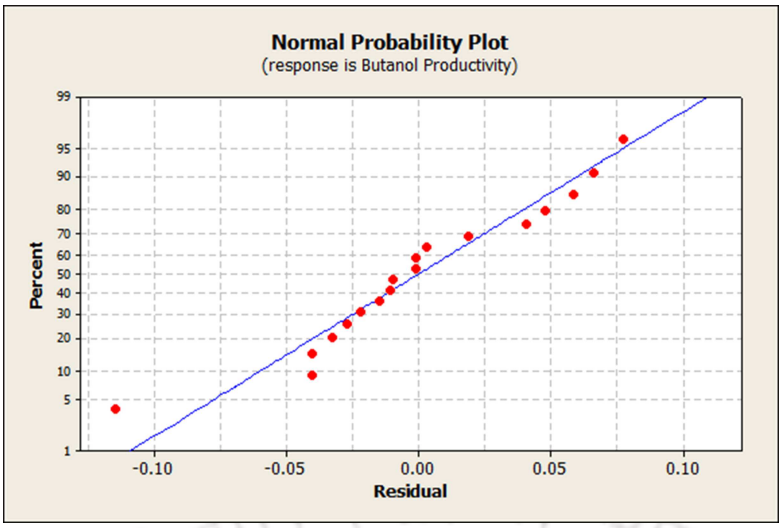



**Fig. 3.5.** Normality plot of residuals where the response was butanol productivity.

**Table 3.7.** Comparison of biomass and butanol productivity from C. acetobutylicum MTCC 11274 grown in un-optimized media and CCD-RSM based optimized media composition

| **Media component (g $L^{-1}$)** | **Un-optimized media** | **Media optimized for biomass productivity** | **Media optimized for butanol productivity** |
|---|---|---|---|
| Glucose | 80 | 33.11 | 82.04 |
| Peptone | 40 | 21.12 | 49.66 |
| $K_2HPO_4$ | 0.5 | 0.5 | 0.5 |
| $KH_2PO_4$ | 0.5 | 0.5 | 0.5 |
| $MgSO_4.7H_2O$ | 0.2 | 0.36 | 0.46 |
| $MnSO_4.H_2O$ | 0.01 | 0.018 | 0.023 |
| $FeSO_4.7H_2O$ | 0.01 | 0.018 | 0.023 |
| NaCl | 0.01 | 0.018 | 0.023 |
| PABA | 0.01 | 0.01 | 0.01 |
| Biotin | 0.01 | 0.01 | 0.01 |
| Ammonium acetate | 2.2 | 2.2 | 2.2 |
| Biomass productivity (g $L^{-1}$ $h^{-1}$) | 0.135 | 0.28 | ND |
| Butanol productivity (g $L^{-1}$ $h^{-1}$) | 0.22 | ND | 0.36 |
| % Increase in biomass productivity[a] | ND | 103.5 | ND |
| % Increase in butanol productivity[b] | ND | ND | 63.6 |

a & b - represents the percentage increase of the biomass and butanol productivity in the optimized medium as compared to the un-optimized medium under flask conditions; ND - Not determined

effects of all the parameters along with interaction between glucose and peptone were found to be significant. Maximum biomass productivity of 0.28 g $L^{-1}$ $h^{-1}$ was predicted by the RSM for concentrations of glucose, peptone, and trace elements of 33.12 g $L^{-1}$, 21.12 g $L^{-1}$ and 18.1 mL $L^{-1}$, respectively. Similarly, maximum butanol productivity of 0.36 g $L^{-1}$ $h^{-1}$ was predicted for 82.04 g $L^{-1}$ of glucose, 49.66 g $L^{-1}$ of peptone and 23.2 mL $L^{-1}$ of trace elements. Concentrations of glucose, peptone, and trace elements were found to be significantly different in the medium optimized for butanol and biomass productivity implying mutually exclusive nature of these two parameters. The RSM predicted media composition was validated by corresponding experimental values. CCD-RSM based media optimization resulted in 103.5% increment in biomass productivity and 63.6% increment in butanol productivity as compared to the biomass (0.135 g $L^{-1}$ $h^{-1}$) and butanol productivity (0.22 g $L^{-1}$ $h^{-1}$) obtained from the un-optimized media composition (Table 3.7).

Various studies have been targeted towards increasing butanol titer (Kaushal et al., 2017; Kao et al., 2013; Yadav et al., 2014). However, limited reports are available with respect to improved butanol productivity for *Clostridium* spp. via media optimization. One such study was carried out by Moon et al. (2011), where maximization of butanol and 1, 3-PDO was achieved during active growth phase of *C. pasteurianum* DSM 525 through medium optimization.

### 3.3.5 Characterization of the strain in optimized media in bioreactor

Dynamic profiles of growth, substrate utilization, and product formation were obtained by growing the organism in media optimized for biomass productivity (Fig. 3.6A) and butanol productivity (Fig. 3.6B). Cultivation on both the media was marked by two distinct metabolic phases, a typical characteristic of ABE fermentation (Jones and Woods, 1986; Duerre, 2007) in clostridial strains. The first phase, termed as acidogenic phase, was marked by the formation of acetic acid and butyric acid, while the second phase, termed as solventogenic phase, was marked by the formation of solvents namely acetone, butanol, and ethanol (Fig. 3.6A and 3.6B). In the initial phase of the fermentation, fall in extracellular medium pH from 6.1 to 4.5 was attributed to the accumulation of organic acids in the broth (Fig. 3.6A and 3.6B). Higher concentration of acids results in disruption of pH gradient across the cell membrane resulting in hindrance of normal metabolic functions of the cells (Jones and Woods, 1986). In order to combat this acid stress, cells start a process of detoxification via reassimilation of acids to various solvents (Ezeji et al., 2005) and hence a phase transition from acidogenesis to solventogenesis was observed at around 10 h and 18 h of fermentation for growth supporting and butanol supporting medium, respectively (Figs. 3.6A and 3.6B). This transition between the two metabolic phases has been shown to be associated with the shift in the activity of the corresponding enzymes in acid and solvent forming pathways (Lee et al., 2008).

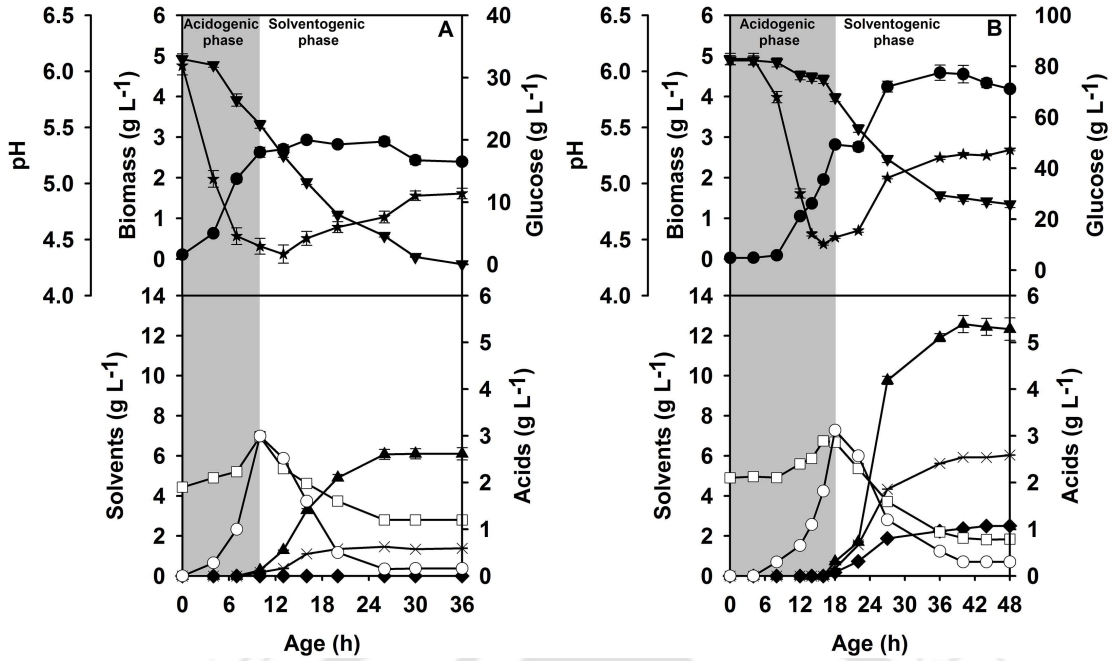



**Fig. 3.6.** Dynamic profile for growth (●), pH (★, glucose (▼), butanol (▲), acetone (×), ethanol (⧫), acetic acid (○) and butyric acid (□) when *C. acetobutylicum* MTCC 11274 was grown in optimized medium for (A) maximum biomass productivity and (B) maximum butanol productivity.

When grown on the medium optimized for biomass productivity, cells entered into the exponential phase of growth without any distinct lag phase (Fig. 3.6A). This dynamics of growth was concomitant with the rapid utilization of glucose from extracellular medium. However, in case of the medium optimized for butanol productivity, a distinct lag phase of 8 h was observed characterized by insignificant change in the extracellular glucose concentration (Fig. 3.6B). This distinct lag phase may be attributed to higher initial concentration of glucose in the medium resulting in substrate inhibition for growth (Qureshi and Blaschek, 2001). From the Fig. 3.6B, it is also evident that the rate of glucose utilization increased with the onset of the exponential phase of growth. Significantly higher specific growth rate (111%) and maximum volumetric biomass productivity (75%) was achieved in case of growth supporting medium as compared to the medium optimized for butanol productivity albeit with a lower biomass titer (Table 3.7). In both the media, butanol production was triggered as soon as the extracellular medium pH reached a critical value of 4.5 though the time required for initiation of butanol synthesis differed significantly depending on the type of medium used. In case of growth supporting medium, butanol production was initiated at 10 h, whereas the medium supporting butanol productivity required an additional 8 h for butanol induction. However, a two-fold higher maximum butanol titer of 12.56 g $L^{-1}$ with a productivity of 0.31 g $L^{-1}$ $h^{-1}$ was achieved in case of butanol supporting medium as compared to the medium favoring growth (Table 3.8).

In case of growth supporting medium, cessation of butanol synthesis at 26 h

**Table 3.8.** Comparison of ABE fermentation in batch fermentation using optimized media for biomass productivity and butanol productivity by *C. aceto-butylicum* MTCC 11274

| Parameters | Medium optimized for biomass productivity | Medium optimized for butanol Productivity |
|---|---|---|
| Biomass (g $L^{-1}$) | 2.92 | 4.48 |
| Butanol (g $L^{-1}$) | 6.07 | 12.56 |
| Acetone (g $L^{-1}$) | 1.4 | 5.9 |
| Ethanol (g $L^{-1}$) | 0 | 2.5 |
| ABE (g $L^{-1}$) | 7.47 | 20.96 |
| Lag time (h) | NL | 8 |
| Max specific growth rate ($h^{-1}$) | 0.38 | 0.18 |
| Maximum biomass productivity (g $L^{-1}$ $h^{-1}$) | 0.28 | 0.16 |
| Overall butanol productivity (g $L^{-1}$ $h^{-1}$) | 0.23 | 0.31 |
| Butanol yield (g $g^{-1}$ glucose) | 0.18 | 0.23 |
| Batch time (h) | 26 | 40 |
| Residual acetic acid (g $L^{-1}$) | 1.2 | 1.8 |
| Residual butyric acid (g $L^{-1}$) | 0.2 | 0.3 |
| Residual glucose (g $L^{-1}$) | 0 | 26.9 |

NL-No lag phase

of fermentation may be attributed to lower concentration of glucose below a critical value of 5 g $L^{-1}$ (Long et al., 1984). It is important to note that when grown on the optimized medium for butanol productivity, the growth and butanol production was terminated followed by a decrease in the biomass concentration at around 40 h, in spite of presence of sufficient amount of residual glucose in the extracellular medium (Fig. 3.6B). This may be attributed to the possible butanol toxicity on the cells which corroborates with the findings of butanol tolerance experiments (Fig. 3.3). Similar results were reported for *C. acetobutylicum* ATCC 824, where accumulation of butanol around 12-14 g $L^{-1}$ resulted in onset of sporulation and cessation of fermentation (Maddox et al., 1989).

The organism exhibited a marked difference with reference to total solvent production in two different media. When the organism was cultivated in the medium optimized for butanol productivity approximately three-fold higher total ABE titer (20.96 g $L^{-1}$) was obtained as compared to the growth supporting medium (7.47 g $L^{-1}$). This lower titer of total solvent in case of the medium optimized for biomass productivity was attributed to non-production of ethanol coupled with lower production of acetone and butanol. ABE titer together with the distribution of individual solvent was reported to vary significantly over a range of 5.76 g $L^{-1}$ to 19.9 g $L^{-1}$ depending on the medium composition and the strain used (Chen et

al., 2014, Wu et al., 2013, Ezeji et al., 2004; Monot et al., 1982). Termination of fermentation due to glucose depletion in batch fermentation using medium optimized for biomass productivity gave the lead for development of a fed-batch strategy for improvement of butanol titer coupled with high biomass productivity. On the other hand, Termination of fermentation due to butanol toxicity in batch fermentation using medium optimized for maximization of butanol productivity gave the lead for development of fermentation strategy coupled with continuous product recovery.

## 3.4 Conclusion

Six strains were screened for maximum butanol production in two media compositions. *Clostridium acetobutylicum* MTCC 11274 was identified as the best butanol producer strain among the strains screened and selected for further studies. The tryptone-yeast extract-glucose media (TYG) with P2 salt solution was found to be the best growth promoting and butanol producing media. Glucose and peptone were selected as the carbon and nitrogen source, respectively, on the basis of maximum butanol production. Butanol tolerance limit of *C. acetobutylicum* MTCC 11274 was found to be 14 g $L^{-1}$. Statistical optimization resulted in two different media composition each for biomass productivity and butanol productivity. In case of media optimized with biomass productivity objective function an increase of 103.5% was observed, whereas 63.6% increase in butanol productivity in case of butanol productivity as objective function. Glucose depletion in batch fermentation using medium optimized for biomass productivity gave the lead for development of a fed-batch strategy for improved butanol titer, while butanol toxicity in batch fermentation using medium optimized for maximization of butanol productivity actuated the need for integration of fermentation with *in situ* product recovery.

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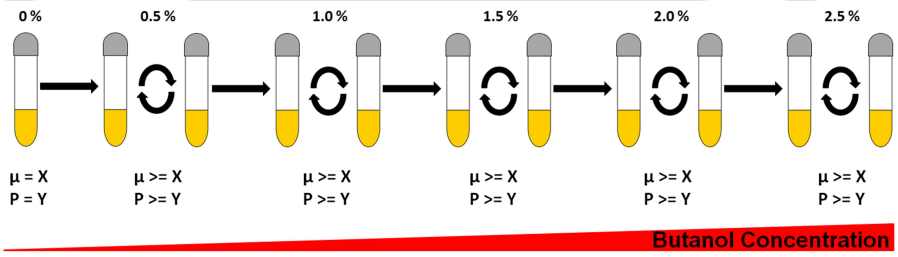
*"But still try, for who knows what is possible...."*

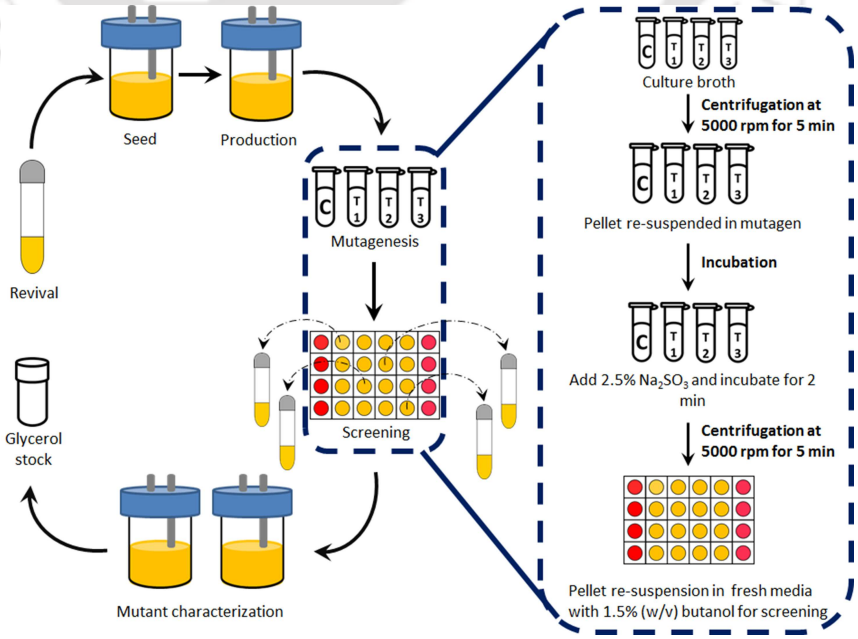
Michael faraday (1791-1867)
English chemist and physicist

# 4

# Enhancement of butanol tolerance of the selected strain via serial enrichment and mutagenesis




Enhancement of butanol tolerance via serial enrichment




Enhancement of butanol tolerance via mutagenesis

## 4.1 Background and motivation

Upsurge in fuel prices, threat of depleting traditional energy sources, and climate change concerns have diverted attention towards re-commercialization of butanol fermentation (Grassi et al., 2018; Patakova et al., 2018). Advent of petrochemical route of butanol synthesis and decreased supply of raw material led to virtual demise of acetone-butanol-ethanol (ABE) fermentation at industrial scale during 1950s and 60s (Jones and Woods, 1986). Since last few decades the demand for clean fuels and inclination to reduce reliance on petrochemical products have revived interest in butanol synthesis through ABE fermentation (Sauer, 2016). Clostridial cultivation for ABE fermentation has been around for 100 years, yet industrial production is hampered by high cost of substrate, mixed product profile, low butanol titer, yield and productivity, solvent toxicity, and lack of understanding of metabolism (Kumar and Gayen, 2011; Patakova et al., 2018). Butanol toxicity is one of the critical factors as it restricts the maximum attainable butanol titer and contributes towards high recovery costs (Jones and Woods, 1986).

Mechanism of butanol toxicity is a complex phenomenon affecting different cellular functions including membrane related processes (Jones and Woods, 1986; Peabody and Kao, 2016). Butanol is a hydrophobic molecule and has the ability to partition into the cell membrane (Weber and de Bont, 1996). This incorporation into the lipid bilayer results in disordering of glycerol backbone of the lipid, affects lipid-lipid and lipid-water interactions, and thus disrupts the membrane function (Weber and de Bont, 1996). Butanol partitioning may also lead to increased permeability causing dissipation of proton motive force, affecting internal pH, or causing loss of metabolites (Weber and de Bont, 1996). These hypotheses corroborate with the findings of Bowles and Ellefson, (1985), where high butanol concentration induced decrease of internal pH, intracellular ATP levels, and glucose uptake during batch fermentation of *C. acetobutylicum* ATCC 4259. Butanol accumulation also affects the activity of membrane bound enzymes exemplified by 50% inhibition of membrane ATPase in *C. acetobutylicum* ATCC 824 when exposed to 3% (w/w) butanol (Moreira et al., 1981). Ounine et al. (1985) showed that effect of butanol toxicity was amplified when xylose was used as carbon source. Complete inhibition of *C. acetobutylicum* ATCC 824 was observed at 14 g $L^{-1}$ butanol concentration in case of glucose, whereas similar level of inhibition occurred at 8 g $L^{-1}$ butanol concentration in case of xylose (Ounine et al., 1985).

Although there are overwhelming examples of butanol toxicity, several adaptive changes have been observed in microorganisms as a response to solvent accumulation (Weber and de Bont, 1996). Organic solvent tolerance is a strain specific property depending upon genetic as well as environmental factors (Sardessai and Bhosle, 2002) and organisms adopt several stress responses in combating such

a scenario by altering the membrane composition, use of efflux pumps, inducing general stress responses, and certain other unknown transcriptional and translational changes (Dunlop, 2011). Researchers have tried to use these stress responses in improving tolerance of *Clostridium* species towards butanol. Tomas et al. (2003) overexpressed *groESL* in *C. acetobutylicum* ATCC 824 and observed 85% increase in butanol tolerance and 54% improvement in butanol titer of the engineered strain in comparison to the wild type. Several analyses have shown that the saturated to unsaturated fatty acid ratio of cell membrane increases as a response to butanol stress conditions (Jones and Woods, 1986). In some cases as observed by Astumi et al. (2009) change in membrane compositions increased solvent tolerance but the production remained unchanged. Gram negative bacteria are known to use efflux pumps as a primary mechanism of tolerance by pumping out excess solvents from cytoplasm. However, no such mechanism is established in gram positive bacterium such as *Clostridium* (Dunlop et al., 2011).

Microbial solvent tolerance is a complex trait involving multiple genes; hence, genetic engineering has not been successful in achieving the desired tolerance levels. In comparison, adaptation and random mutagenesis approaches have proven to be more successful. *Clostridium beijerinckii* BA 101, a mutant strain developed by NTG treatment of *Clostridium beijerinckii* NCIMB 8052, was reported to produce 18.6 g $L^{-1}$ butanol which was further improved to 20.9 g $L^{-1}$ upon acetate addition (Formanek et al., 1997; Chen and Blaschek, 1999). Another NTG mutant strain *C. pasteurianum* MBEL_GLY2 produced 17.8 g $L^{-1}$ butanol when grown on glycerol under optimized batch conditions (Malaviya et al., 2013). Serial adaptation to increasing butanol concentration has also shown promising results. *Clostridium acetobutylicum* T64 with tolerance up to 4% (v/v butanol) produced 15.5 g $L^{-1}$ butanol (Liu et al., 2013). A different adaptive engineered strain, *Clostridium acetobutylicum* JB200, was shown to produce 21 g $L^{-1}$ butanol when grown in stirred tank bioreactor (Yang and Zhao, 2013). To that end, serial enrichment and random mutagenesis were undertaken to improve butanol tolerance limit of the selected strain and investigate its effect on butanol production. Three types of mutagens were used in mutagenesis experiments ultraviolet irradiation (UV), N-methyl-N-nitro-N-nitrosoguanidine (NTG), and ethyl methane sulfonate (EMS).

## 4.2 Materials and methods

### 4.2.1 Microorganism, inoculum, and media

The lyophilized culture of best butanol producer *Clostridium acetobutylicum* MTCC 11274 was revived and stored as glycerol stocks at 80°C. Inoculum preparation was carried out in tryptone-yeast extract-glucose (TYG) medium as detailed in section 3.2.1. Adaption and mutagenesis experiments were carried out in the optimized

medium for maximization of butanol productivity comprising (in g $L^{-1}$) glucose: 82.0, peptone: 49.0, $K_2HPO_4$: 0.50, $KH_2PO_4$: 0.50, $MgSO_4.7H_2O$: 0.46, $MnSO_4.H_2O$: 0.023, $FeSO_4.7H_2O$: 0.023, NaCl: 0.023, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001. Experimental conditions and sampling were kept same as in section 3.2.2, unless otherwise mentioned. All the chemicals (AR grade) were purchased from Himedia, India unless otherwise mentioned.

### 4.2.2 UV irradiation of cultures and induction of mutants

The procedure followed for UV mutagenesis is schematically depicted in Fig 4.1. Batch fermentation was carried out till the cells reached exponential phase. 5 mL of fermentation broth was taken in a petri plate and placed below a UV germicidal light fixed in a chamber. The distance between UV lamp and culture plate was kept 25 cm. The UV irradiation was carried out at varying exposure times from 50 to 350 sec. A control experiment was carried out parallel to the irradiation experiments.

The experiments were carried out in triplicate and about 0.1 mL each from these exposed plates was inoculated in fresh medium and biomass ($OD_{600}$ nm) was estimated after incubation made in dark at 37°C for 24 h. Killing curve was constructed by plotting the maximum absorbance ($OD_{600}$ nm) against the time of exposure. The exposure time with 90% killing was selected and used for development of solvent tolerant strain. The killing percentage was calculated by the following formulae:

$$\text{Killing percentage (\%)} = \frac{(OD_{600_{Control}} - OD_{600_{Treated}})}{OD_{600_{Control}}} \times 100 \qquad (4.1)$$

The mutagenic experiments were carried out in triplicate and 0.1 mL from each of the three aliquots was inoculated in fresh medium for subsequent screening experiments.

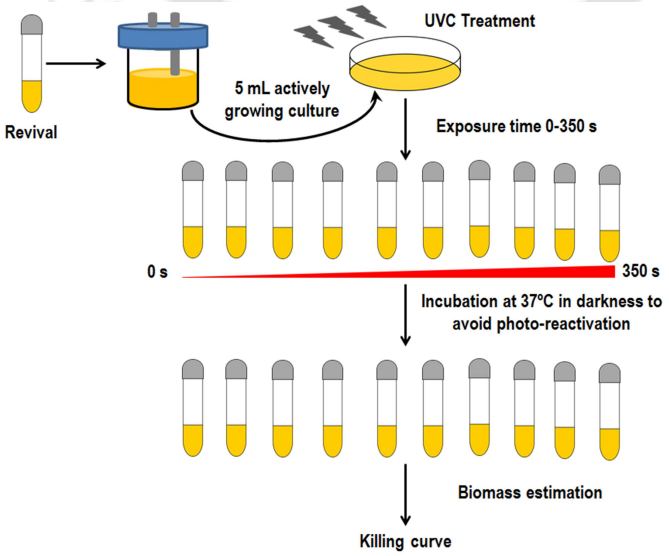



**Fig. 4.1.** Schematic representation of UV mutagenesis protocol.

### 4.2.3 Chemical mutagenesis using N-methyl-N-nitro-N-nitrosoguanidine (NTG)

Schematic representation of mutagenesis protocol is shown in Fig. 4.2. 2 mL exponential phase cells ($OD_{600}$ nm 3.0) were centrifuged at 5,000 rpm for 5 min. The pellet was re-suspended in fresh media containing different concentration of NTG solution (0.1 - 1.2 g $L^{-1}$). After 15 min of incubation at 37°C, 1 mL of 2.5% (w/v) sodium thiosulphate was added to terminate the reaction. Cells were washed twice with fresh medium. 200 µL from each eppendorf tube were inoculated in fresh media and incubated at 37°C. A control experiment was performed parallel to mutagenesis. After 24 h incubation, the biomass ($OD_{600}$ nm) was calculated and killing curve was constructed by plotting the maximum absorbance ($OD_{600}$ nm) against the mutagen concentration (Fig. 4.2A). The concentration with 90% killing, calculated according

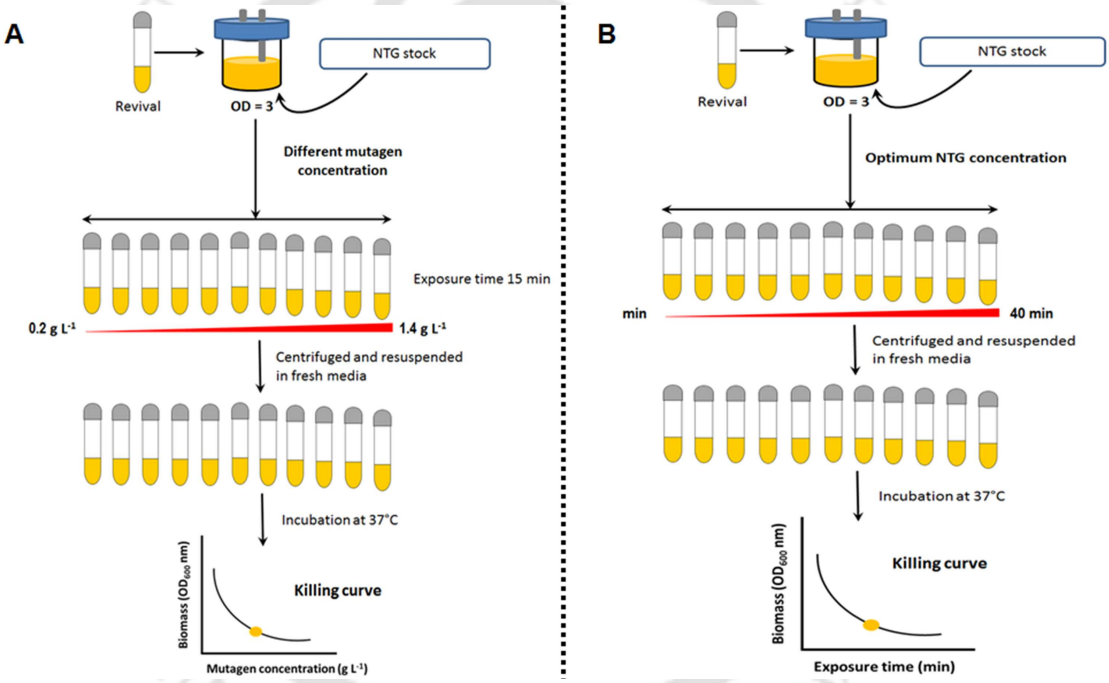



**Fig. 4.2.** Schematic representation of chemical mutagenesis protocol.

to Eq. 4.1, was selected for further optimization of incubation time (5-40 min) of mutation treatment (Fig. 4.2B). Similarly, killing curve was constructed to select the duration for mutagen exposure. The optimized mutagen concentration and treatment time were used for development of the solvent tolerant strain.

### 4.2.4 Chemical mutagenesis using ethyl methane sulfonate (EMS)

The mutagenesis experiments were carried out similar to as described in section 4.2.3. The EMS concentration used were varied from 0.1-30 g $L^{-1}$ and initial exposure time was kept at 30 min. A control experiment was performed parallel to mutagenesis. The concentration chosen on the basis of 90% killing (calculated according to Eq. 4.1) was selected for further optimization of mutagen exposure

time (10-60 min) of mutation treatment. The optimized mutagen concentration and treatment time were used for development of the solvent tolerant strain.

### 4.2.5 Rational screening of solvent tolerant mutant(s)

All the mutagenesis experiments (UV, NTG, and EMS) described in sections 4.2.2-4.2.4 were performed in triplicate along with control. After mutagenic treatment, the cells were re-suspended in fresh media and cultured in micro titer plates containing different concentration of selection pressure (butanol). The wells exhibiting growth after incubation were further inoculated in tubes with medium containing selection pressure for second round of screening. The tubes showing growth were used for glycerol stock preparation and stored at -80°C for further characterization in terms of butanol tolerance and butanol production.

### 4.2.6 Characterization of mutants for butanol tolerance and butanol production

The stored glycerol stocks were revived and inoculum preparation was carried out as explained in section 4.2.1. All the characterization experiments were conducted in 500 mL customized cultivation bottles under static condition in an incubator at 37°C. Media preparation and experimental procedure were kept same as in section 3.2.2. The cultures were characterized on the basis of growth, pH, glucose utilization, and butanol production. A control experiment was carried out to compare performance of tolerant strains with the wild type strain. Mutants were also characterized in terms of their butanol tolerance and butanol challenge experiments were carried out as detailed in section 3.2.4.

### 4.2.7 Adaptive evolution to enhance butanol tolerance

While growing in nature bacterial cells encounters various environmental stresses which negatively alter their growth. One such stress is accumulation of large amount of toxic product, which is termed as product inhibition. However, the cells are also known to exhibit a process of adaptation whereby they gradually get accustomed to survive in harsh conditions also known as adaptive evolution. Keeping this in mind, a set of experiments were carried out to adapt the wild type *C. acetobutylicum* MTCC 11274 to higher concentration of butanol. Inoculum preparation was carried out as mentioned earlier.

Exponentially growing cells were inoculated in two culture bottles containing butanol concentration of 0 (control) and 0.25 g $L^{-1}$. The batch was monitored for 72 h for dynamic profiling of growth and butanol production. In addition, after reaching biomass of 1.0 g $L^{-1}$ ($OD_{600}$ nm 3.0) 10% (v/v) of the cells were subcultured in fresh media having same butanol concentration as before and similar analysis was carried out. This process constituted one phase of adaptation and individual subcultures in each phase were termed as adaptation cycles. Several cycles were

performed in one adaptation phase till the specific growth rate of the stressed cells matched that of control (unstressed cells). Once this condition was fulfilled, the stress concentration was increased by exposing the cells to higher concentration of butanol, thereby initiating second phase of adaptation, and similar adaptation cycles were carried out.

### 4.2.8 Analytical methods

The collected sample was centrifuged at 10,000×*g* for 10 min at 4°C. The pellet was used to measure growth using UV-visible spectrophotometer (Cary 50, Varian, Australia) as detailed in section 3.2.7. The substrates, organic acids, and solvents concentrations present in the supernatant were measured in high performance liquid chromatograph (HPLC, LC-20AD, Shimadzu, Japan) equipped with a Rezex ROA column (300×7.8 mm, Phenomenex, USA), detailed in section 3.2.7. All experiments were conducted in triplicates and the values have been represented as mean ± standard error.

## 4.3 Results and discussion

### 4.3.1 Optimization of mutagenic conditions

Conditions for mutagenesis experiments were optimized for the following three mutagenic conditions: UV, NTG, and EMS. Killing curves were constructed when the cells were exposed to different exposure times in case of UV irradiation. For chemical mutagenesis (NTG and EMS), the cells were first exposed to different concentrations of mutagens keeping the exposure time constant. The mutagen concentration exhibiting around 90% killing was selected. The selected concentration was used to expose the cells for different duration of mutagenic treatment. Similar to mutagen concentration, a killing curve was constructed to estimate the time required to kill 90% of the cells. The selected mutagen concentration and exposure time were further used for mutagenesis experiments.

UV irradiation of cell suspension from MTCC 11274 was performed and the percentage of survival against time was plotted to estimate the UV exposure time required to kill 90% of cells. Therefore, an exposure time of 230s was chosen for mutagenesis experiments (Fig. 4.3). MTCC 11274 cells were exposed to different NTG concentration from 0.1 to 1.2 (g $L^{-1}$) while keeping the exposure time constant to 15 min. At concentration of 0.1 g $L^{-1}$ no killing was observed and beyond this concentration a decline in OD was observed. Almost 100% killing was observed at a concentration of 0.6 g $L^{-1}$ and above. The desired killing of 90% cells was observed at 0.4 g $L^{-1}$ that was selected as the optimized concentration and used for experiments at different exposure time (Fig. 4.4A). The exposure time was varied from 5-40 min while keeping the NTG concentration constant at 0.4 g $L^{-1}$. The

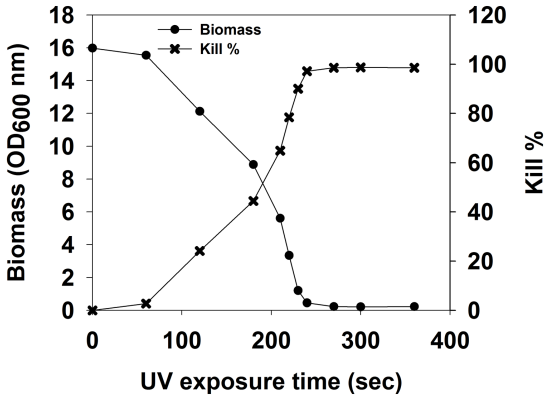



**Fig. 4.3.** Killing curve of *C. acetobutylicum* MTCC 11274 when exposed to different duration of UV irradiation from a distance of 25 cm.

selected exposure time was 15 min, which resulted in killing of 90% of the cells (Fig. 4.4B). Similar to chemical mutagenesis using NTG, the concentrations and exposure time of EMS treatment were optimized. 90% killing of cells was observed at 16 g $L^{-1}$ (Fig. 4.5A), which was selected for further optimization of exposure duration of cell to the mutagen. Optimum time required to kill 90% of the cells was found to be 30 min (Fig. 4.5B). The optimized conditions were then used to perform mutagenesis experiments followed by rational screening for butanol tolerant mutants.

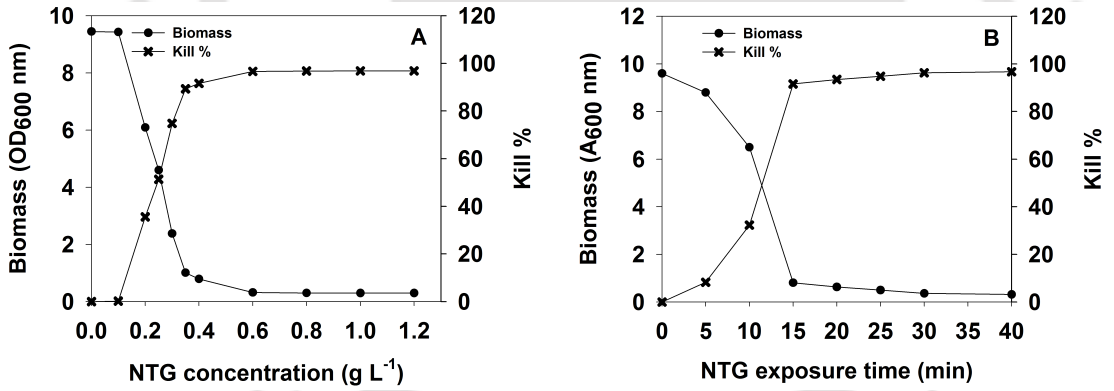



**Fig. 4.4.** Killing curve *C. acetobutylicum* MTCC 11274 when exposed to different (A) NTG concentration (g L-1) and (B) exposure time (min).

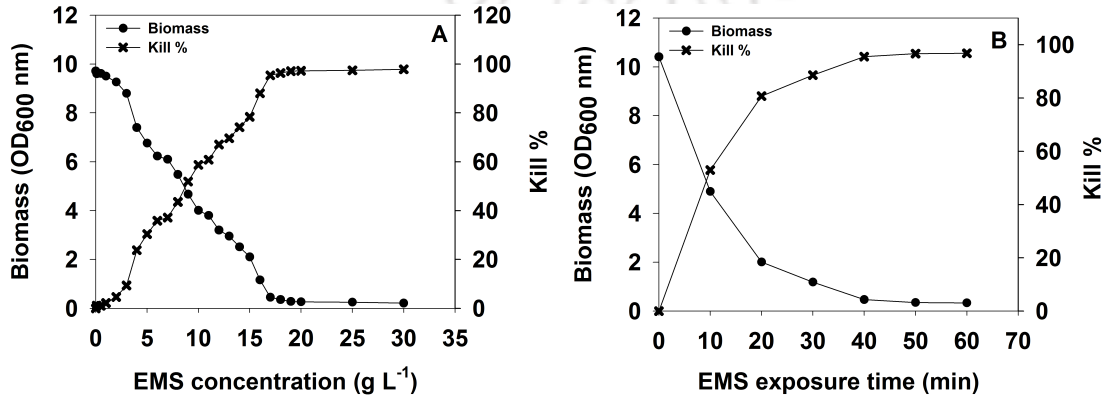



**Fig. 4.5.** Killing curve *C. acetobutylicum* MTCC 11274 when exposed to different (A) EMS concentration (g L-1) and (B) exposure time (min).

### 4.3.2 Rational screening of solvent tolerant mutant(s)

Evolutionary engineering comprising mutagenesis followed by selection has been widely applied to improve microbial tolerance to environmental stresses (Zhu et al., 2015). Keeping this view in mind, three mutagenesis experiments were carried out namely, UV irradiation, NTG treatment and mutagenesis using EMS, according to the optimum conditions obtained in section 4.3.1.

After the mutagenesis treatment, the untreated and treated cells were exposed to the selection pressure, which was 1.5% (w/v) butanol in the present study. A control experiment parallel to screening was also carried out where the untreated and treated cells were grown in absence of selection pressure. In case of UV irradiation, even after repeated trials the mutagenesis failed to generate any probable improvement in terms of solvent tolerance. Biomass growth was observed in the control experiments showing that the cells were able to survive the mutagenesis experiment; however, they were not mutated to tolerate high butanol concentration. After a number of trials, NTG and EMS mutagenesis resulted in one and eight putatively improved strains, respectively. As reported in previous literature works, among the mutagens, EMS and NTG are the most effective ones and UV irradiation was not found to be an effective way to mutagenize solvent-producing *Clostridia* (Lemmel, 1984; Bowring and Morris, 1985). The results obtained in the present study corroborates with the findings of other researchers.

### 4.3.3 Characterization of putatively improved strain(s) for butanol tolerance and butanol production

The eight strains (E1-E8) selected after mutagenesis using EMS on the basis of improved butanol tolerance and the strain (M1) selected after mutagenesis using NTG, were characterized in batch fermentation to compare their growth, butanol titer, and butanol productivity in comparison to the wild type (WT) strain MTCC 11274. It was observed that despite having improved tolerance, none of the strains obtained after mutagenic treatment showed significant improvement in any of the desirable attributes such as biomass titer, butanol titer or butanol productivity (Fig. 4.6). A maximum butanol titer of 12.85 g $L^{-1}$ was obtained by M1 which was similar to WT (12.3 g $L^{-1}$). EMS and NTG treatments were repeated to get improved strains; however, repeated trials did not yield any desirable strains. Therefore, further experiments were carried out using the wild type strain MTCC 11274.

### 4.3.4 Adaptive evolution to enhance butanol tolerance

Evolutionary engineering of cells via adaptation towards high butanol tolerance was carried out. Phase one of serial adaptation was started with exposure of the cells to 0.25% (w/v) butanol and incubating at 37°C for 24 h. This constituted one adaptation cycle and was carried out in the similar way till the stressed cells

showed growth similar to control (unstressed cells). After four-adaptation cycles at

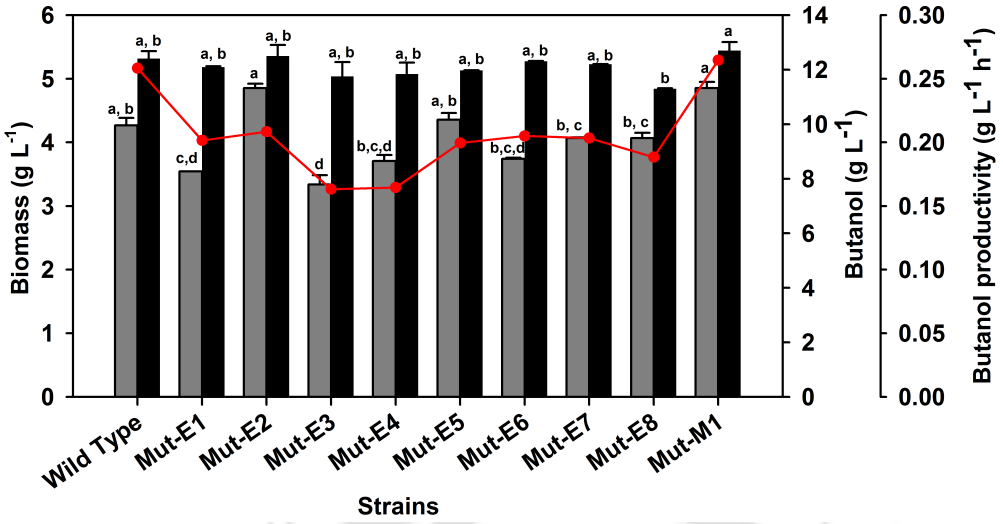



**Fig. 4.6.** Bar chart depicting comparison of putatively improved strains after mutagenic treatment with wild type (WT) strain in terms of biomass (grey bar), butanol titer (back bar) and butanol productivity (red circle).

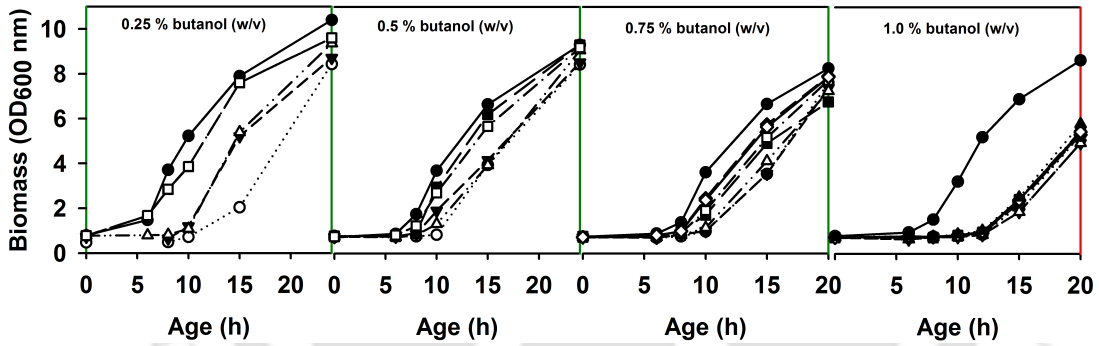



**Fig. 4.7.** Dynamic profile of biomass growth for cells exposed to different butanol concentration in comparison to unstressed cells. Control (●); cycle 1 (○); cycle 2 (▼); cycle 3 (△); cycle 4 (■); cycle 5 (□); cycle 6 (♦); cycle 7 (◊); cycle 8 (▲).

0.25% (w/v) butanol, the cells exhibited similar biomass, specific growth rate, and lag time as the control and were considered to be adapted (Fig. 4.7). Further, second phase of adaptation was started with 0.5% (w/v) butanol and after five adaptation cycles, the strain was found to be adapted (Fig. 4.7). Similarly, the strain found to be adapted in the third phase of adaptation (exposure to 0.75% butanol) after seven cycles (Fig. 4.7). Adaptation to 1.0% was carried out for 18 cycles; however, no further improvement was achieved in terms of specific growth rate.

## 4.4 Conclusion

Chemical mutagenesis was successfully applied to the solvent-producing *C. acetobutylicum* strain. After NTG treatment, an improved strain M1 was obtained which was found to be tolerant to 1.6% butanol i.e. 23% higher than wild type. M1

cells were viable for a longer period of time when compared to wild type; however, its butanol productivity and titer were similar to the wild type. EMS treatment yielded eight putatively improved strains, none of which showed improved butanol titer in comparison to wild type. Additionally, UV mutagenesis and serial adaptation did not result in butanol tolerant strains. Hence, further experiments were carried out using the wild type strain MTCC 11274.

## 4.5 References

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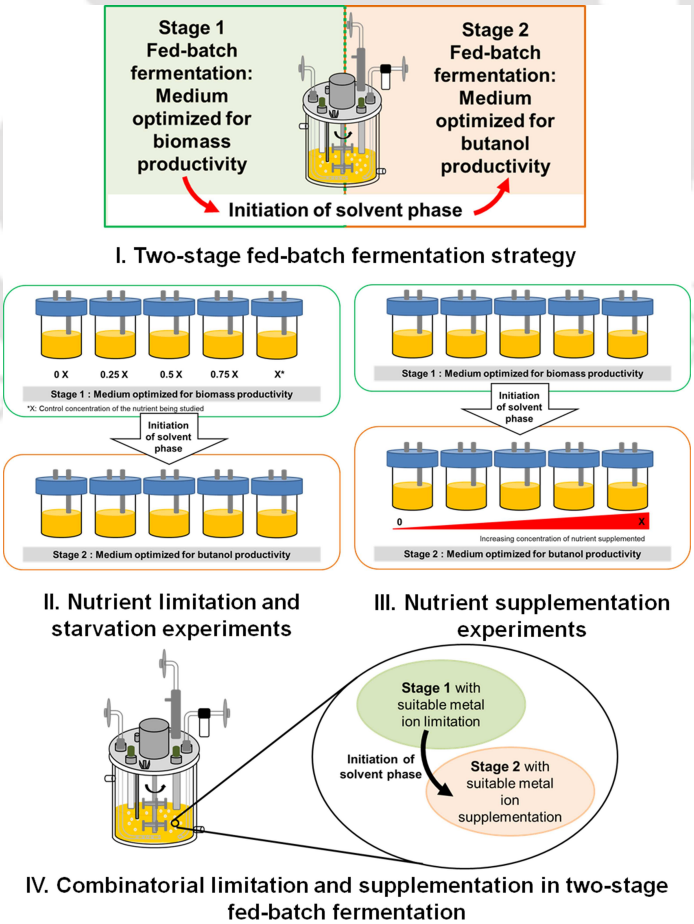
*"Somewhere, something incredible is waiting to be known."*

Carl Sagan (1934-1996)
American astrophysicist and astrobiologist

# 5

# Studies on effect of various factors governing growth and butanol production from *Clostridium* sp.




Schematic diagram of studies of various factors affecting growth and butanol production

## 5.1 Background and motivation

Looming threats of energy scarcity and depleting reserves of conventional fuels along with concerns over climate change issues have led to increased interest in bioenergy and biofuels (Papoutsakis, 2008; Kaushal et al., 2018). Butanol is considered as a promising next generation biofuel due to its resemblance to gasoline and superior physiological properties compared to ethanol; however, its production has not reached a commercially viable stage (Zheng et al., 2015; Wu et al., 2016). Fermentative production of butanol, typically by *Clostridium* spp., suffers from several limitations such as solvent toxicity, low titer, degeneration of strain, complex metabolic pathway resulting in mixed product profile, and high cost of substrate and product recovery (Wu et al., 2016; Kaushal et al., 2017). To address the above issues, diverse studies have been carried out in terms of mode of cultivation (Ezeji et al., 2004; Lu et al., 2012; Sethalku et al., 2012), strain improvement (Ezeji et al., 2004; Jang et al., 2012; Xue et al., 2012), dual substrate fermentation (Sabra et al., 2014; Kaushal et al., 2017), lignocellulosic substrates (Gottumakkala et al., 2013; He et al., 2017), recovery strategies (Xue et al., 2012), and use of novel strains (Kaushal et al., 2017). Another avenue being explored for achieving improvement in ABE fermentation in terms of product titer, yield, and productivity is the use of environmental cues affecting *Clostridium* spp. in terms of growth, transition from acidogenesis to solventogenesis, modulating the metabolic pathway towards desired product, imparting solvent tolerance etc.

Several studies have been carried out employing environmental cues such as different pH strategies (Tashiro et al., 2004; Oshiro et al., 2010); supplementation of butyric acid/sodium buyrate (Tashiro et al., 2004; Wang et al., 2013), lactic acid (Oshiro et al., 2010), electron carriers (Kim and Kim, 1988; Peguin et al., 1994; Honicke et al., 2012), zinc (Wu et al., 2013; Wu et al., 2016), calcium carbonate (Richmond et al., 2011; Yang et al., 2013; Wu et al., 2016), furfural (Ezeji et al., 2007; Zhang et al., 2012; Qureshi et al., 2012), glycerol (Ujor et al., 2014; Kaushal et al., 2017); carbon monoxide (CO) gassing (Datta and Zeikus, 1985; Dabrock et al., 1992); limitation of trace minerals: iron, magnesium, phosphate, sulphate, nitrogen (Bahl et al., 1986; McNeil and Kristiansen, 1985; Junelles et al., 1988; Peguin and Soucaille, 1995); and manipulation of hydrogen partial pressure (Doremus et al., 1985). Use of electron carriers such as methyl/benzyl viologen and carbon monooxide gassing is reported to alter the solvent ratio in favor of butanol (Kim and Kim, 1988; Dabrock et al., 1992; Peguin et al., 1994; Honicke et al., 2012). However, methyl/benzyl viologen are cost intensive while use of carbon monooxide poses environmental threat. Zinc supplementation of 0.001 g $L^{-1}$ is shown to increase butanol titer from 11.7 g $L^{-1}$ to 12.6 g $L^{-1}$ and further to 16.1 g $L^{-1}$ in association with calcium carbonate addition (Wu et al., 2013; Wu et al., 2016). While zinc is known

to support butanol titer and growth, calcium carbonate has a number of advantages such as buffering capacity, increased substrate utilization, acid re-assimilation, and improved solvent tolerance (Richmond et al., 2011; Han et al., 2013; Gottumakkala et al., 2015).

To that end, a two-stage fed-batch process engineering strategy was developed exhibiting enhanced butanol productivity by exploiting metabolic shift from growth supporting medium to the butanol promoting medium. Further, effect of different concentrations of $MgSO_4$, $MnSO_4$, $FeSO_4$, $PO_4^{2-}$, $CaCO_3$, and $ZnSO_4$ were studied on butanol production during the two-stage fed-batch fermentation. The metal ions supporting butanol production were used in combination to achieve higher butanol titer and productivity.

## 5.2 Materials and methods

### 5.2.1 Microorganisms, maintenance, and inoculum preparation

The lyophilized culture of best butanol producer *Clostridium acetobutylicum* MTCC 11274 was revived and stored as glycerol stocks at 80°C. Inoculum preparation was carried out in tryptone-yeast extract-glucose (TYG) medium as detailed in section 3.2.1. Experiments were carried out in the optimized medium for maximization of biomass productivity and butanol productivity. The biomass productivity medium comprised of (in g $L^{-1}$) glucose: 33, peptone: 21.12, K2$HPO_4$: 0.50, $KH_2PO_4$: 0.50, $MgSO_4.7H_2O$: 0.36, $MnSO_4.H_2O$: 0.018, $FeSO_4.7H_2O$: 0.018, NaCl: 0.018, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001. The butanol productivity medium consisted of (in g $L^{-1}$) glucose: 82, peptone: 49, $K_2HPO_4$: 0.50, $KH_2PO_4$: 0.50, $MgSO_4.7H_2O$: 0.46, $MnSO_4.H_2O$: 0.023, $FeSO_4.7H_2O$: 0.023, NaCl: 0.023, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001. Experimental conditions and sampling were kept same as in section 3.2.2, unless otherwise mentioned. All the chemicals (AR grade) were purchased from Himedia, India unless otherwise mentioned.

### 5.2.2 Two-stage fed-batch process engineering strategy

A two stage fed-batch process engineering strategy was employed to increase the volumetric productivity of butanol. In the first stage, the organism was grown in the medium supporting maximum biomass productivity. The concentrations of glucose and peptone were maintained at their respective optimal values by intermittent feeding throughout the entire first-stage of the fermentation. With the onset of butanol production, the concentrations of carbon and nitrogen sources were adjusted to the corresponding values optimized for maximum butanol productivity, thereby initiating the second stage of fed-batch fermentation process. Similar to the first stage, the respective optimal concentrations of glucose and peptone were also

maintained in the second stage by intermittent feeding till the end of the fermentation. The cultivation conditions were kept same as detailed in section 3.2.6. Sampling was carried out at intervals of 2 h and the concentrations of glucose and peptone were monitored in order to determine the frequency of feeding.

### 5.2.3 Effect of limitation and starvation of metal ions in two-stage fed-batch process

The effect of starvation or limitation of ions ($Mg^{2+}$, $Mn^{2+}$, $Fe^{2+}$ and $PO_4^{3-}$) on butanol production was evaluated by varying the concentration of the individual ion in the first stage of the two-stage fermentation process, while keeping the concentration of the rest of the ions at their respective optimal value. Concentration of the ion of interest was varied as follows: X, 0.5X, 0.25X, 0.125X, 0X, where X denotes its concentration in the optimized medium for biomass productivity and was considered as control. Upon induction of solventogenic phase, the concentrations of glucose and peptone were adjusted to mimic the medium optimized for maximum butanol productivity, thereby initiating the second stage of the process. The cultivation conditions were kept same as described in section 3.2.5.

### 5.2.4 Effect of supplementation of metal ions in two-stage fed-batch process

With the aim of increasing the butanol titer and productivity, the two-stage fermentation process was emended with the supplementation of potential inducers viz., calcium carbonate ($CaCO_3$) and zinc sulphate ($ZnSO_4$), in the second stage of the fermentation process. As described in section 5.2.3, the organism was initially grown in the medium supporting maximum biomass productivity during the first stage of the two-stage process engineering strategy. With the onset of butanol production, the concentrations of glucose and peptone were adjusted (as described in section 5.2.3) and the medium was supplemented with different individual concentrations of the selected inducers. The concentrations of $CaCO_3$ and $ZnSO_4$ were varied through 2-10 g $L^{-1}$ and 0.0005-0.5 g $L^{-1}$, respectively. The batch without supplementation of any inducer was considered as control. The cultivation and sampling conditions were kept same as described in section 3.2.5.

### 5.2.5 Two-stage fed-batch process combined with metal ions limitation and supplementation

In order to evaluate the combinatorial effect of limitation of a metal ion and supplementation of an inducer on butanol titer and productivity, the metal ion limited first stage was coupled with the inducer supplemented second stage in a two-stage fed-batch fermentation process. In the first stage, the biomass productivity medium was limited with half of the initial concentration of $MgSO_4.7H_2O$ and upon initiation of the second stage, optimum concentration of $CaCO_3$ was complemented together with glucose and peptone in concentrations mimicking the optimal butanol productivity

medium. During the entire course of fermentation, the respective concentrations of glucose and peptone were maintained by intermittent feeding. The experiment was carried out as detailed in section 5.2.2.

### 5.2.6 Effect of calcium carbonate on butanol tolerance

To study the effect of supplementation of the inducer on tolerance limit, MTCC 11274 was grown, under batch mode, in the medium promoting maximum butanol productivity supplemented with optimum concentration of $CaCO_3$. On attaining biomass of 1 g $L^{-1}$, the exponentially growing cells were equally distributed in 250 mL bottles. Each bottle was supplemented with different butanol concentration in the range of 0-2% (w/v) and the growth was monitored up to 72 h. In addition, the effect of $CaCO_3$ supplementation on growth, glucose utilization, and butanol production of the strain was also evaluated by carrying out batch fermentation with and without calcium addition. Medium optimized for maximum butanol productivity was used for fermentation and the experimental conditions were kept same as described in section 3.2.5.

### 5.2.7 Analytical methods

Estimation of biomass and concentrations of glucose, organic acids (acetate and butyrate), and solvents (acetone, ethanol, and butanol) were performed as described in section 3.2.7. The OD was converted in to dry cell weight (DCW) using the calibration equation 1 OD = 0.328 g $L^{-1}$ DCW . The amount of nitrogenous compounds in form of free amino acids and protein/peptide were estimated separately and were used to determine the concentration of peptone. As the nitrogen percentage in amino acid and protein/peptide is same, the summed amount of these components was used in preparation of calibration curve with peptone which depicts that 1 g peptone contained 0.707 g of protein and free amino acids. The protein concentration was estimated by Lowrys method using Bovine Serum Albumin (BSA) as the standard (Lowry et al., 1951). Concentration of total amino acids was quantified by ninhydrin reaction method using glycine as the standard (Yemm et al., 1955). All experiments were conducted in triplicates and the values have been represented as mean±standard error.

## 5.3 Results and discussion

### 5.3.1 Two-stage fed-batch process engineering strategy

Based on the results obtained from the batch fermentation experiments, a two stage fed-batch process engineering strategy was developed with the aim of improving the volumetric rate of butanol production. In the first stage of fermentation, the cells were grown in the medium optimized for biomass productivity followed by the second stage of fermentation in the medium optimized for butanol productivity.

Similar to batch fermentation on growth supporting medium, the cell growth pattern was characterized by the absence of a lag phase. A significant increase in biomass titer was observed with the transition of fermentation medium from growth supporting to butanol promoting (Fig. 5.1). This process engineering strategy resulted in an overall biomass productivity of 0.37 g $L^{-1}$ $h^{-1}$ which was substantially higher than that observed for individual batch fermentation in two different media (Table 5.1). A maximum butanol titer of 13.1 g $L^{-1}$ was achieved within a shorter fermentation time (24 h) when compared to the batch fermentation on the medium optimized for both biomass and butanol productivity (Fig. 5.1).

It is important to note that by using this process engineering strategy, we were able to significantly reduce the time required to achieve maximum butanol titer by (i) eliminating the lag phase for growth in turn, reducing the time required for butanol induction and (ii) increasing the butanol production rate. Upon switch from growth supporting medium to butanol inducing medium, cells already under solventogenesis, were exposed to higher concentration of glucose. This higher concentration of glucose favors butanol synthesis as excess of sugar is essential for onset and maintenance of

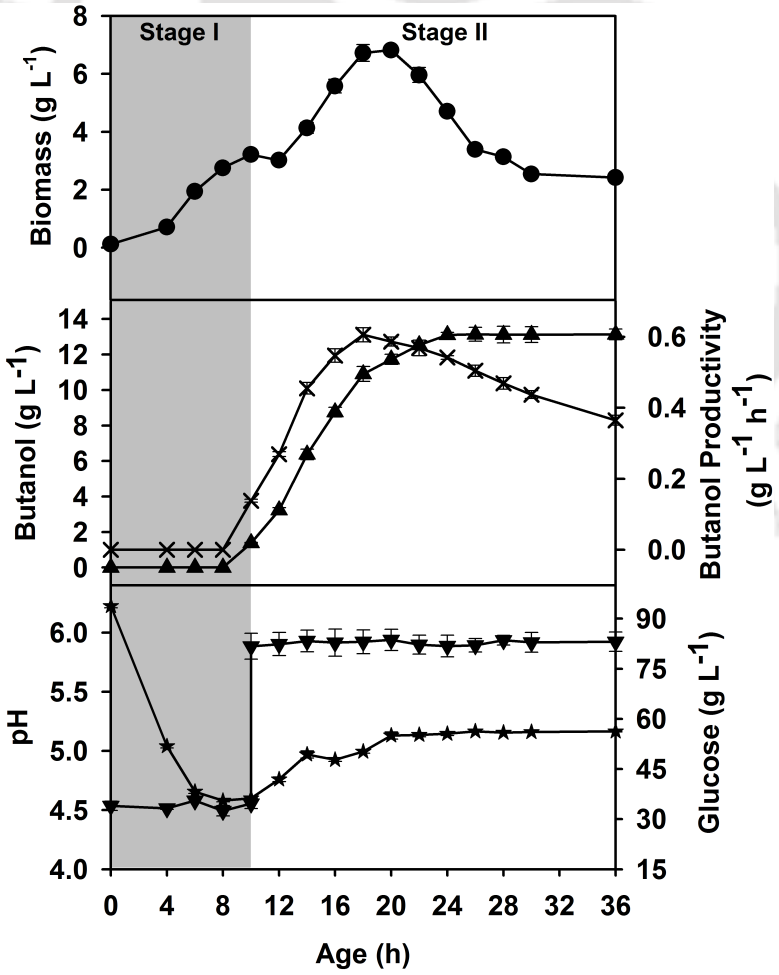



**Fig. 5.1.** Dynamic profile for growth (●), butanol (▲), butanol productivity (×), pH (★) and glucose (▼) when *C. acetobutylicum* MTCC 11274 was grown in two-stage fed-batch mode of fermentation.

**Table 5.1.** Comparison of ABE fermentation during individual batch fermentations using optimized media for biomass productivity and butanol productivity by *C. acetobutylicum* MTCC 11274 with two-stage fed-batch fermentation

| Parameters | Batch fermentation | | Fed-batch fermentation |
|---|---|---|---|
| | Medium optimized for biomass productivity | Medium optimized for butanol Productivity | Two-stage |
| Biomass (g $L^{-1}$) | 2.92 | 4.58 | 6.82 |
| Butanol (g $L^{-1}$) | 6.07 | 12.56 | 13.1 |
| Acetone (g $L^{-1}$) | 1.4 | 5.9 | 6.3 |
| Ethanol (g $L^{-1}$) | 0 | 2.5 | 2.84 |
| ABE (g $L^{-1}$) | 7.47 | 20.96 | 22.44 |
| Lag time (h) | No Lag phase | 8 | No Lag phase |
| Maximum specific growth rate ($h^{-1}$) | 0.38 | 0.18 | 0.5 |
| Maximum biomass productivity (g $L^{-1}$ $h^{-1}$) | 0.28 | 0.16 | 0.37 |
| Overall butanol productivity (g $L^{-1}$ $h^{-1}$) | 0.23 | 0.31 | 0.55 |
| Butanol yield (g $g^{-1}$ glucose) | 0.18 | 0.23 | 0.22 |
| Batch time (h) | 26 | 40 | 24 |
| Residual acetic acid (g $L^{-1}$) | 1.2 | 1.8 | 2.69 |
| Residual butyric acid (g $L^{-1}$) | 0.2 | 0.3 | 1.84 |
| Residual glucose (g $L^{-1}$) | 0 | 26.9 | 82.9 |

solventogenesis (Jones and Woods, 1986). It was reported earlier that even under higher concentration of sugar, the solvent producing enzymes are not catabolically repressed during solventogenesis (Jones and Woods, 1986). This resulted in butanol productivity of 0.55 g $L^{-1}$ $h^{-1}$, an improvement of 77% as compared to the batch fermentation on butanol supporting medium. The improvement was achieved without compromising the butanol yield (0.22 g $g^{-1}$ glucose). This is the first report on use of a novel two-stage fed-batch fermentation strategy demonstrating significantly higher butanol productivity as compared to the others reported in the literature works (Table 5.2).

**Table 5.2.** Comparison of butanol productivity by different *Clostridium* sp. under fed batch conditions without product recovery

| Organism | Type | Substrate | Productivity (g $L^{-1}$ $h^{-1}$) | Butanol titer (g $L^{-1}$) | Reference |
|---|---|---|---|---|---|
| ***C. acetobutylicum* MTCC 11274** | **WT** | **Glucose** | **0.55** | **13.1** | **This study** |
| *C. acetobutylicum* ATCC 824 | WT | Glucose | 0.28 | 13.25 | Ventura et al., 2013 |
| *C. acetobutylicum* ATCC 824 | E | Glucose | 0.22 | 19.12 | Ventura et al., 2013 |
| *C. pasterianum* NRRL B-598 | WT | Glucose | 0.17 | 8.3 | Lipovsky et al., 2016 |
| *C. acetobutylicum* ATCC 824 | WT | Glucose | 0.08 | 13.2 | Menchavez and Ha, 2019 |

Novelty of the process lies in the ease of its operational strategy where the organism was cultivated in two different media compositions (two stages) in a single bioreactor. These cultivation stages were separated temporally and not spatially and hence, eliminated the need of using multiple bioreactors and additional downstream processing which require higher capital and operational costs (Setlhaku et al., 2013). Further, the process engineering strategy can be applied to wide spectrum of clostridial strains depicting its universality.

### 5.3.2 Effect of limitation and starvation of metal ions in two-stage fed-batch process

A set of five concentrations (including control) for each ion were screened, to select the most potential contributor towards butanol production. Fig. 5.2A-D shows the maximum butanol titer and productivity obtained for different concentration of $MgSO_4$, $MnSO_4$, $FeSO_4$, and $PO_4^{3-}$. Among all the concentration of $MgSO_4$ screened, 0.5X exhibited highest butanol titer (13.41 g $L^{-1}$) when supplemented in biomass productivity medium (Fig. 5.2A). Magnesium is an important divalent ion, playing a crucial role in biological system such as preventing ribosome degradation during cell division, cofactor of various enzymes, and part of the gram complex, thereby providing structural stability to cell wall (Webb, 1949; Wacker, 1969; Thomas and

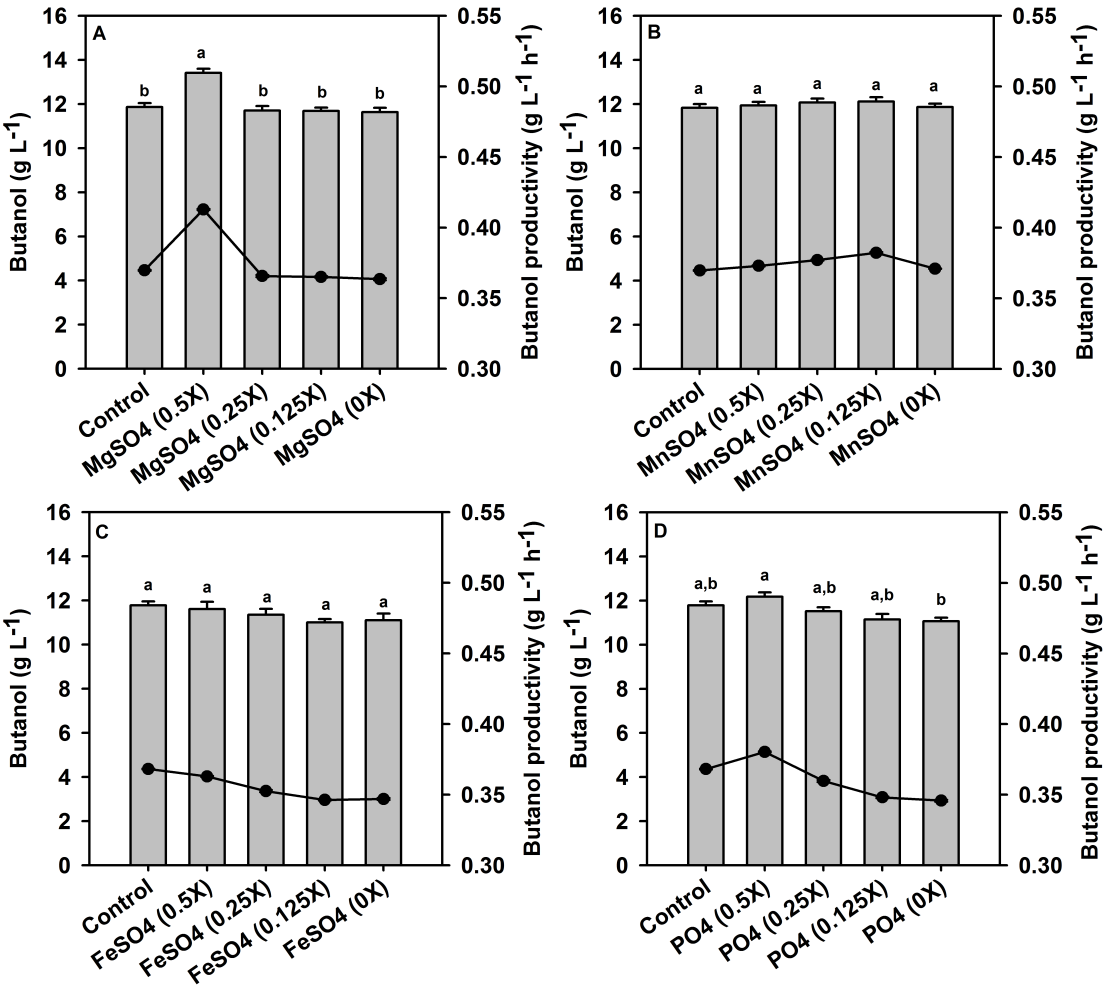



**Fig. 5.2.** Comparison of butanol titer (grey bar) and butanol productivity (•) under different limitation and starvation conditions of (A) $MgSO_4$, (B) $MnSO_4$, (C) $FeSO_4$, and (D) $PO_4^{3-}$. Means that share the same letter do not differ significantly at the 95% confidence level based on the Tukey method.

Rice, 2014). It is reported that presence of magnesium in optimum concentration is essential, as excess or deficiency may result in inhibition of cell division and activity of enzymes which use the metallic ion as cofactor (Webb, 1949). For instance, maximum growth and butanol production was reported for the strain *C. acetobutylicum* ATCC 824 when culture medium was supplemented with an optimal $MgSO_4$ concentration in the range of 0.05 g $L^{-1}$ - 0.2 g $L^{-1}$ (Monot et al., 1982). Complete elimination of $MgSO_4$ from the medium resulted in 78% reduction in growth, while butanol titer was reduced by 57.9% (Monot et al., 1982). On the other hand, presence of $MgSO_4$ in higher concentration (0.35 g $L^{-1}$ - 0.5 g $L^{-1}$) resulted in lowered growth and butanol titer by 11.7% and 15%, respectively, in comparison to that obtained in case of optimal concentration range (Monot et al., 1982).

Monot et al. (1982) showed that manganese did not have much effect on butanol production from *C. acetobutylicum* ATCC 824. Similar results were obtained in the present study, where $MnSO_4$ concentration of 0.125X resulted in the highest butanol titer of 12.1 g $L^{-1}$, which was found to be similar to the control (Fig. 5.2B).

Phosphate and iron limited chemostat have shown positive regulation towards solventogenesis (Bahl et al., 1986). However, in the present study phosphate and iron limitation/starvation during two-stage fed-batch fermentation did not show any effect as butanol titer similar to control (Fig. 5.2C and 5.2D) was obtained in all the cases.

### 5.3.3 Effect of supplementation of metal ions in two-stage fed-batch process

Two inducers in different concentrations were screened for their potential role in enhancing butanol titer. The inducers were supplemented in the second stage of the two-stage fed-batch strategy. While $CaCO_3$ at 2 g $L^{-1}$ exhibited a lower titer than control (no inducer addition), further increase in concentration upto 5 g $L^{-1}$ resulted in gradual increase in butanol titer up to a maximum of 14.66 g $L^{-1}$ (Fig. 5.3A). The titer thus obtained was 18.1% higher when compared to control (12 g $L^{-1}$) and surpassed the butanol inhibitory concentration for MTCC 11274 (1.4%, w/v in preliminary batch fermentation experiments in section 3.3.3). $CaCO_3$ has been reported to contribute towards butanol tolerance of the strains via various mechanisms e.g. upregulating heat shock proteins, DNA repair, and stabilizing membrane proteins (Gottumukkala et al., 2015; Ujor et al., 2016).

To investigate the potential role of $CaCO_3$ with respect to alleviation of solvent toxicity, butanol challenge experiments were conducted; the organism was grown in presence of different concentrations of butanol along with supplementation of $CaCO_3$ in the production medium. Interestingly, with supplementation of $CaCO_3$ the organism was able to grow even in the presence of butanol concentration of 1.5% (w/v) (Fig. 5.3C). While no visible increase in biomass was observed when exposed to 1.75% (w/v) butanol, the supplemented cells were able to resume growth when sub-cultured in fresh media devoid of butanol ascertaining that the cells retained viability. The results indicate that $CaCO_3$ possibly imparts improved solvent tolerance to the strain. This observation of improved tolerance and hence improved titer corroborates with other studies reporting benefits of $CaCO_3$ supplementation (Han et al., 2013; Gottumukkala et al., 2015; Ujor et al., 2016).

Han et al. (2013) reported that supplementation of 4 g $L^{-1}$ $CaCO_3$ to batch cultures of *C. beijerinckii* NCIMB 8052 resulted in butanol titer of 16.3 g $L^{-1}$ (control 11.4 g $L^{-1}$), 6.2 g $L^{-1}$ biomass (control 3.5 g $L^{-1}$), and no residual glucose (control 17.8 g $L^{-1}$) in a shorter span of time compared to control (Han et al., 2013). Proteomic analysis attributed this enhanced performance to the upregulation of genes involved in DNA synthesis, transcription, repair, carbohydrate catabolic enzymes, and heat shock proteins (Hsp20, GrpE, molecular chaperone DnaK, chaperone protein DnaJ, and HPr). In addition, supplementation of 4 g $L^{-1}$ $CaCO_3$ to degenerate strain of *C. beijerinckii* NCIMB 8052 (DG-8052) restored butanol production capability of the strain along with improved growth (Jiao et al., 2016). This was hypothesized to be

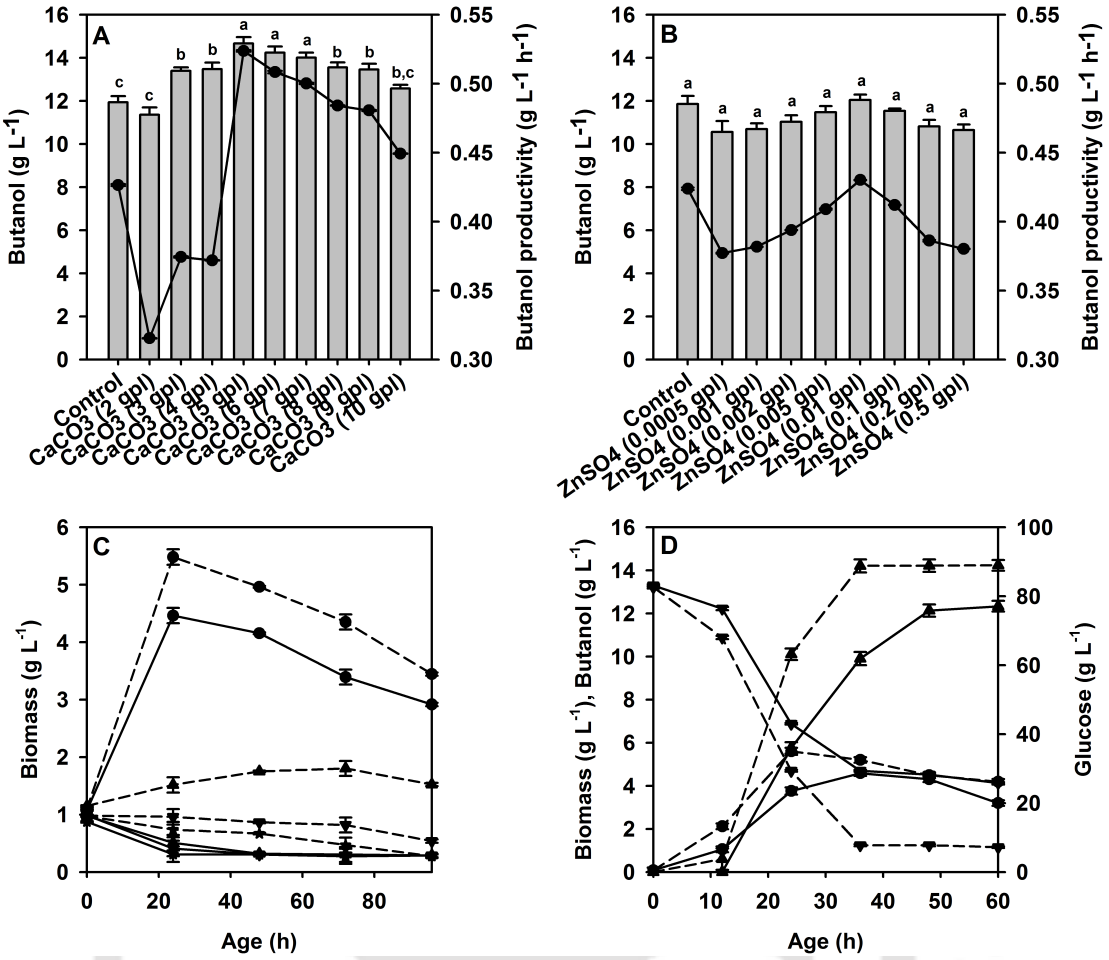



**Fig. 5.3.** Comparison of butanol titer (grey bar) and butanol productivity (•) under supplementation conditions of (A) $CaCO_3$ and (B) $ZnSO_4$. Means that share the same letter do not differ significantly at the 95% confidence level based on the Tukey method. (C) Dynamic profiles for growth of *C. acetobutylicum* MTCC 11274 under $CaCO_3$ supplemented (- -) and un-supplemented (-) batch fermentation when exposed to extraneous butanol concentrations of (%, w/v): 0 (•), 1.5 (▲), 1.75 (▼) and 2.0 (★). (D) Dynamic profile for growth (•), butanol (▲) and glucose (▼) when batch fermentation of C. acetobutylicum MTCC 11274 was carried out in optimized medium for maximum butanol productivity with (- -) and without (-) $CaCO_3$ supplementation.

due to role of $CaCO_3$ as a buffering agent and of $Ca^{2+}$ as an effector of metabolic pathway (Jiao et al., 2016). Transcriptional analysis revealed up-regulation of genes involved in glycolysis, solventogenesis, cell motility, and molecular chaperones in presence of $CaCO_3$ (Jiao et al., 2016). $Ca^{2+}$ is also hypothesized to act as a quorum sensing molecule for inducing growth and solventogenesis; however, confirmatory experimental evidence is still required (Ujor et al., 2016).

Zinc supplementation to media during batch fermentation increased butanol titer and growth along with reduced fermentation time (Wu et al., 2013). This feat is attributed to positive affect on glycolytic enzymes and butanol dehydrogenase enzyme (Ujor et al., 2016). However, in the present study zinc addition did not

result in any of the aforementioned outcomes (Fig. 5.3B). During batch fermentation with and without $CaCO_3$ supplementation biomass titer remained similar; however, marked difference in terms of butanol titer, productivity, and residual substrate were observed (Fig. 5.3D). While 20 g $L^{-1}$ glucose remained in the end of fermentation for un-supplemented set, only 7 g $L^{-1}$ residual glucose was observed in $CaCO_3$ supplemented bottle (Fig. 5.3D). $CaCO_3$ supplementation resulted in maximum butanol titer of 14.2 g $L^{-1}$ in 36 h (Fig. 5.3D), while in un-supplemented bottle 12.21 g $L^{-1}$ was achieved in 48 h. Overall butanol productivity of 0.39 g $L^{-1}$ $h^{-1}$ was achieved, which even though higher than the un-supplemented set, was still lower than achieved for $CaCO_3$ supplemented two-stage fed-batch strategy (0.52 g $L^{-1}$ $h^{-1}$), hence emphasizing the efficacy of the strategy (Fig. 5.3D).

### 5.3.4 Combinatorial use of limitation and supplementation of metal ions in two-stage process engineering strategy

Metal ions, especially cations, play an important role in growth and development of any living being. While the list of essential cations include zinc, magnesium, calcium, manganese, sodium, etc., the first three are considered especially important. The effect they have on the metabolism of an organism depends on their individual influence and on their interaction with other nutrients. Wu et al. (2016) reported the synergistic role of zinc and calcium towards improved butanol production and tolerance of *C. acetobutylicum* L7.

Among the metal ions screened via limitation and starvation experiments, $MgSO_4$ when used in half of the optimized concentration in media (0.18 g $L^{-1}$) gave positive results, while in supplementation studies 5 g $L^{-1}$ $CaCO_3$ showed superior results (Fig. 5.2A and 5.3A). In view of these, a combinatorial strategy was implemented to evaluate their synergistic effect on butanol titer and productivity. Two-stage fed-batch process combined with $MgSO_4$ limitation and $CaCO_3$ supplementation resulted in induction of butanol at 8 h (Fig. 5.4) which was 2 h earlier than the two-stage fed-batch process without metal ions limitations/supplementations (control experiment).

Mechanism of early induction of butanol synthesis can be described as a broader effect of $Mg^{2+}$ at the cellular level, improving growth which in turn resulted in early attainment of threshold level of acids required for metabolic switch from acidogenesis to solventogenesis. Further, butanol titer was elevated to a concentration of 16.5 g $L^{-1}$, an improvement of 26% when compared with the control experiment. However, the overall productivity was found to be marginally increased to a value of 0.59 g $L^{-1}$ $h^{-1}$ (Fig. 5.4). This combinatorial strategy resulted in one of the highest butanol titers and productivity amongst the various other studies conducted using calcium carbonate (Table 5.3).

Magnesium and calcium though being one of the most important cations for organism have antagonistic role, competing for the same receptors. Maintaining appropriate ratio of magnesium and calcium is reported to aid yeast fermentation (Trofimova et al., 2010). However, no study is reported with regard to combinatorial effect of magnesium and calcium ions on butanol fermentation by *Clostridium* spp.

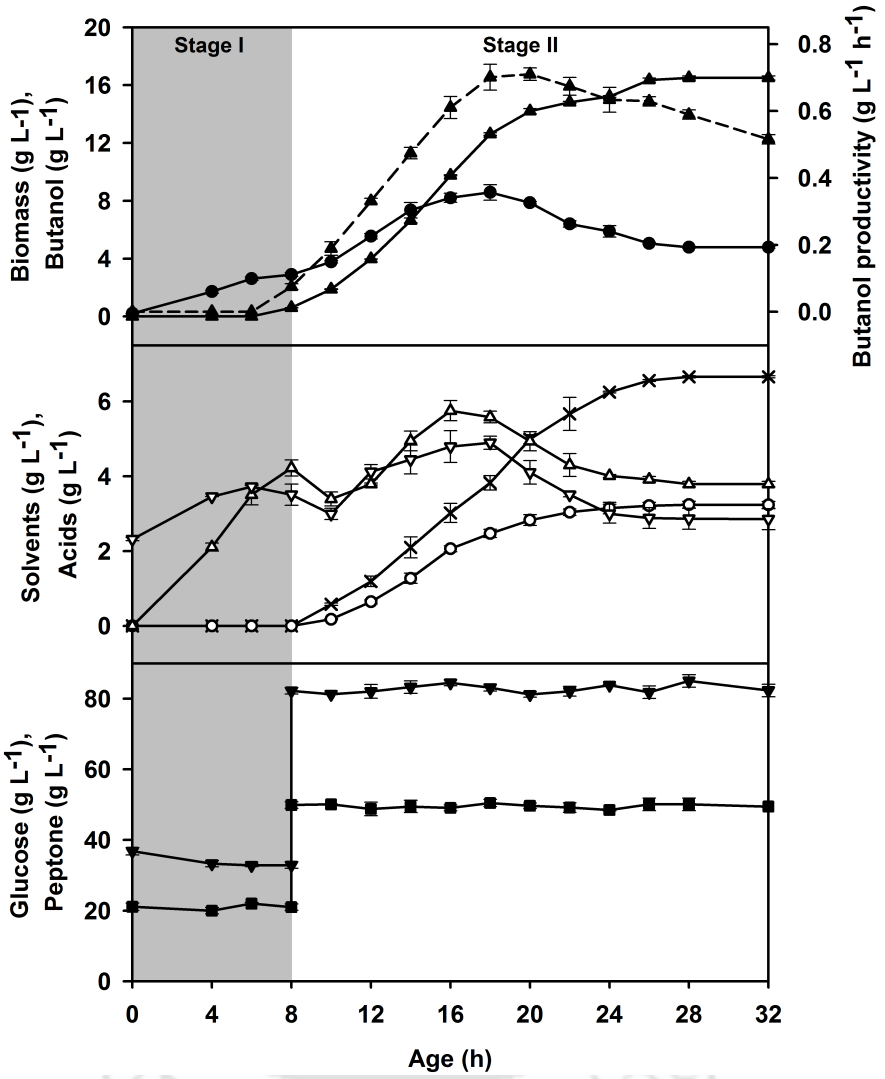



**Fig. 5.4.** Dynamic profile for growth (●), butanol (▲), butanol productivity (▲, - -), acetone (×), ethanol (○), butyric acid (△), acetic acid (▽), glucose (▼) and peptone (■) when *C. acetobutylicum* MTCC 11274 was grown in two stage fed-batch mode with combinatorial use of metal ions.

**Table 5.3.** Comparison of butanol titer and productivity by *Clostridium* sp. using calcium carbonate as inducer without product recovery

| Organism | Substrate | $CaCO_3$ (g $L^{-1}$) | Mode | Butanol productivity (g $L^{-1}$ $h^{-1}$) | Butanol titer (g $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| ***C. acetobutylicum* MTCC 11274** | **Glucose** | **5** | **Fed batch-Mg-Ca** | **0.59** | **16.5** | **This study** |
| *C. beijerinckii* TISTR 1461 | Sweet sorghum juice | 7 | Batch | 0.43 | 15.46 | Sirisantimethakom et al., 2016 |
| *C. acetobutylicum* L7 (Adapted ATCC 824) | Glucose | 4 | Batch-(Zn-Ca) | 0.4 | 16.1 | Wu et al., 2016 |
| ***C. acetobutylicum* MTCC 11274** | **Glucose** | **5** | **Batch** | **0.39** | **14.2** | **This study** |
| *C. acetobutylicum* L7 (A) | Glucose | 4 | Batch | 0.36 | 14.4 | Wu et al., 2016 |
| *C. acetobutylicum* ATCC 824 | Glucose | 4 | Batch | 0.31 | 14.78 | Richmond et al., 2011 |
| *C. beijerinckii* P260 | Glucose | 4 | Batch | 0.29 | 13.89 | Richmond et al., 2011 |
| *C. beijerinckii* NCIMB 8052 | Glucose | 4 | Batch | 0.27 | 16.3 | Han et al., 2013 |
| *C. beijerinckii* NCIMB 8052 | Glucose | 4 | Batch | 0.27 | 13 | Zhang and Ezeji, 2014 |
| *Clostridium* sp. BOH3 | Cassava starch | 1 | Batch-(Ca-Tryptophan) | 0.25 | 17.8 | Li et al., 2015 |
| *Clostridium* sp. BOH3 | Cassava starch | 1 | Batch | 0.16 | 11.8 | Li et al., 2015 |
| *C. acetobutylicum* ATCC 824 | Glucose | 5 | Batch | 0.15 | 10.8 | Yang et al., 2013 |
| *C. acetobutylicum* ATCC 824 | Glucose | 5 | Batch | 0.15 | 10.8 | Yang et al., 2013 |
| *Clostridium* sp. strain HN4 | Food waste | 3 | Batch | 0.12 | 17.64 | Qin et al. , 2018 |
| *C. acetobutylicum* CH02 (A) | Glucose | 4 | Batch | 0.076 | 8 | Gottumakkala et al., 2015 |
| *C. sporogenes* BE01 | Rice straw hydrolysate | 10 | Batch | 0.05 | ~5 | Gottumakkala et al., 2013 |
| *C. acetobutylicum* | Glucose-Xylose | 10 | Batch | ND | 11.5 | El Kanouni et al., 1988 |
| *C. acetobutylicum* (M) | XML-ZL2 | 5 | Batch | ND | 13.19 | Li et al., 2013 |

A-Adapted; M-Mutant; ND-Not determined

## 5.4 Conclusion

A novel two stage fed-batch strategy was demonstrated to achieve high butanol productivity. Maximum butanol titer of 13.1 g $L^{-1}$ was obtained with an overall productivity of 0.55 g $L^{-1}$ $h^{-1}$. The process was further improved by combinatorial use of limitation and supplementation of $MgSO_4$ and $CaCO_3$, respectively, resulting in butanol titer of 16.5 g $L^{-1}$ and overall productivity of 0.59 g $L^{-1}$ $h^{-1}$. This is the first report on combinatorial use of $MgSO_4$ limitation and $CaCO_3$ supplementation resulting in one of the highest butanol productivity and titer in comparison to published literature.

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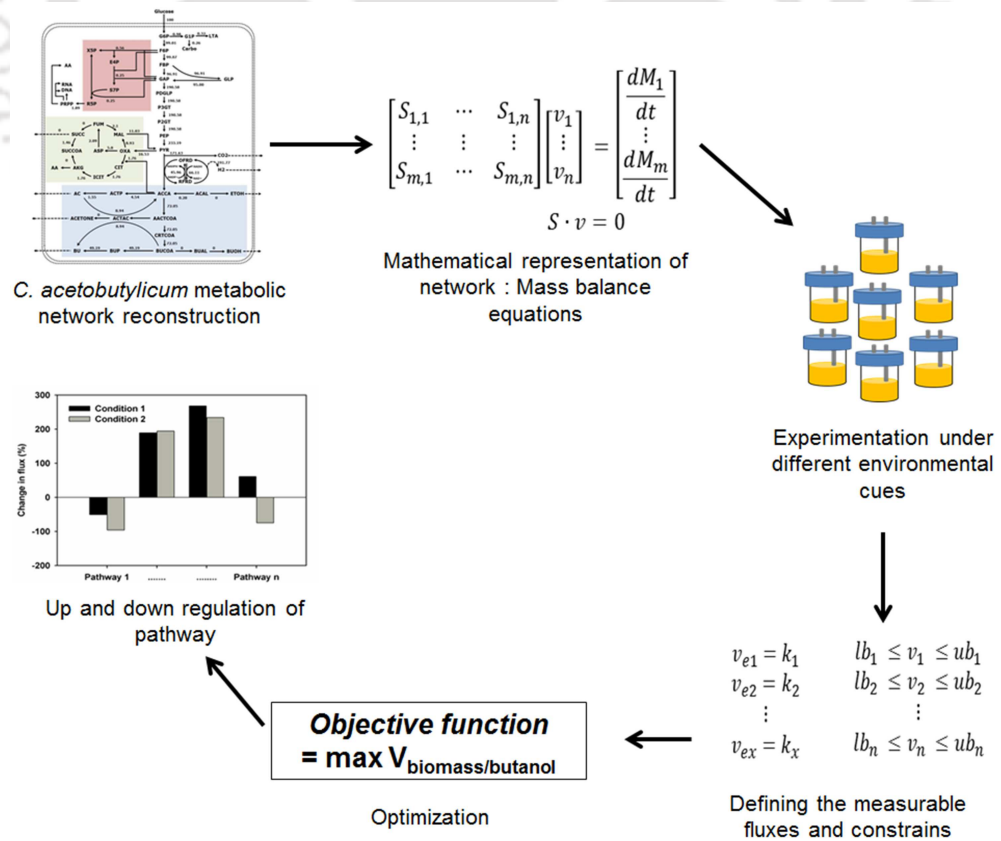
*"Nothing in life is to be feared, it is only to be understood."*

Marie Curie (1867-1934)
Polish physicist and chemist

# 6

# Development of a mathematical model to understand the regulation of butanol synthesis in *Clostridium* sp.




Flux balance analysis to understand the regulations of carbon distribution in butanol production of *C. acetobutylicum* MTCC 11274 under different environmental cues

## 6.1 Background and motivation

Butanol is considered as a promising next generation biofuel due its resemblance to gasoline and superior physiological properties compared to ethanol; however, its production has not reached a commercially viable stage (Zheng et al., 2015). Fermentative production of butanol, typically by *Clostridium* spp., suffers from several limitations such as solvent toxicity, low titer, mixed product profile, and lack of understanding of complex metabolic pathway (Kaushal et al., 2017). To address the above issues, diverse studies have been carried out in terms of mode of cultivation (Xue et al., 2017), strain improvement (Xue et al., 2012), lignocellulosic substrates (Gottumakkala et al., 2013), recovery strategies (Xue et al., 2012), and mathematical modelling (Millat and Winzer, 2017). In addition, nutritional and environmental factors affecting clostridial growth and solventogenesis have been explored to improve growth, induce solventogenesis, modulate pathway towards desired product, increase butanol tolerance, etc. Several studies have been carried out employing environmental cues for example use of different pH strategies (Tashiro et al., 2004), supplementation of inducers (Tashiro et al., 2004; Honicke et al., 2012; Wu et al., 2016), and limitation of trace minerals (Bahl et al., 1986). Nutritional composition of the media and intracellular pool of ATP and NAD(P)H have been reported to affect the temporal behavior and product profile of ABE fermentation (Millat and Winzer, 2017). Analyzing these studies through metabolic modelling will provide insights into the underlying regulatory mechanisms of butanol biosynthesis, which in turn will aid in targeted strain improvement and process development.

One of the widely used mathematical modelling techniques for study of a metabolic pathway is flux balance analysis (FBA) which involves analysis of flow of metabolite across a biochemical network (Orth et al., 2010). These networks, typically in case of genome scale models, incorporate all the biochemical reactions known for a particular organism and the genes encoding the catalyzing enzymes. FBA is then used to understand carbon flux distribution across this network and further describe and predict microbial behavior in terms of specific growth rate or metabolite production rate (Orth et al., 2010). First metabolic model for *Clostridium* sp. (*C. acetobutylicum*) was developed by Papoutsakis (1984) and was further extended by Desai et al. (1999). Genome annotation for *C. acetobutylicum* (Nolling et al., 2001) and *C. beijerinckii* (Wang et al., 2011) paved way for their genome scale modelling in 2008 (Senger and Papoutsakis, 2008a and 2008b; Lee et al., 2008) and 2011 (Milne et al., 2011), respectively. Since then, several modelling endeavors have been undertaken to improve understanding of the complex metabolism of *Clostridium* spp. These include three more genome scale models (McAnulty et al., 2012; Dash et al., 2014; Yoo et al., 2015) which incorporate data from process, flux, and labelling experiments and other regulatory information (Crown et al., 2011; Amador-Noguez

et al., 2011; Wang et al., 2013; Au et al., 2014). All these studies have been carried out under batch and continuous mode of fermentation; however, there are very few reports on flux balance analysis of *C. acetobutylicum* incorporating environmental cues and none on two-stage fed-batch mode of cultivation.

Improvement in titer and solvent tolerance in *C. acetobutylicum* MTCC 11274 via changing media, fermentation mode, and incorporation of metal ions was observed in chapter 5. In the present study, FBA was carried out for the strain to understand the changes in carbon flux distribution towards butanol production when the two-stage fermentation was carried out under different environmental cues. Further, modulation in intracellular carbon flux distribution was predicted when the switch from acidogenesis to solventogenesis occurred. The study involved metabolic network reconstruction followed by linear programming optimization to estimate internal carbon fluxes. Model was validated by comparing model predicted values for objective function (specific growth rate and carbon flux towards butanol production) with the experimental values obtained under different nutritional conditions.

## 6.2 Materials and methods

### 6.2.1 Organism, growth, and maintenance conditions

*C. acetobutylicum* MTCC 11274 was stored as glycerol stocks at -80°C. Revival of glycerol stock and seed preparation was carried out as mentioned in Chapter 3. Anaerobic condition was maintained by intermittent purging of pure nitrogen, while the anaerobicity was confirmed by addition of a redox indicator dye, resazurin at 1 g $L^{-1}$. 10 mL of actively growing culture was used as seed culture. Experiments were carried out in P2 media (Monot et al., 1982) optimized for biomass productivity and butanol productivity. Medium optimized for maximum biomass productivity comprised of (in g $L^{-1}$) glucose: 33.0, peptone: 21.12, $K_2HPO_4$: 0.50, $KH_2PO_4$: 0.50, $MgSO_4.7H_2O$: 0.36, $MnSO_4.H_2O$: 0.018, $FeSO_4.7H_2O$: 0.018, NaCl: 0.018, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001. The medium optimized for maximum butanol productivity comprising (in g $L^{-1}$) glucose: 82.0, peptone: 49.0, $K_2HPO_4$: 0.50, $KH_2PO_4$: 0.50, $MgSO_4.7H_2O$: 0.46, $MnSO_4.H_2O$: 0.023, $FeSO_4.7H_2O$: 0.023, NaCl: 0.023, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001. Batch and two-stage fed-batch fermentation experiments were carried out in 500 mL customized bottles as detailed in Chapter 3 and Chapter 5. All the experiments were conducted in triplicate and the values were measured as mean ± standard error. All the chemicals (AR grade) were purchased from Himedia, India unless otherwise mentioned.

### 6.2.2 Flux balance analysis

Flux balance analysis is a type of constraint based modelling and functions with the underlying assumption that under a particular environmental condition the

microorganism approaches a steady state constrained by stoichiometry of the network and physiochemical and biological properties (Kauffman et al., 2003). It is difficult to gain complete knowledge of all the constraints especially the physiochemical constraints. This results in an underdetermined system which has the possibility of multiple steady state solutions; therefore, in order to obtain an optimal solution, optimization is carried out with respect to a predefined biologically appropriate objective function (Millat and Winzer, 2017). Thus FBA provides flux distribution through the metabolic network, assuming steady state operation, under specified environmental conditions with respect to the imposed constraints. It is important to mention that typically during FBA, the regulation of a reaction or pathway is not considered while predicting flux distribution.

In the first step, metabolic network was reconstructed which was then converted into its mathematical form by representing the stoichiometric coefficients of each metabolite ($m$) involved in every reaction ($n$) in a matrix, designated as stoichiometric matrix ($S$). The flux through each reaction ($n$) was represented as flux vector $v$. Further, application of mass balance and the assumption of pseudo steady state gave rise to the stoichiometric model (Orth et al., 2010): $\boldsymbol{S.v = 0}$. Since size of the stoichiometric matrix $m \times n$ in our study was 152 × 207, the degree of freedom ($f$) was 55 ($n$-$m$), thus making the system under determined. In case of underdetermined model i.e. when the number of unknown variable (fluxes) is more than the equation (metabolites), no unique solution exist. Therefore, linear optimization was used to solve the stoichiometric model with respect to a specified objective function ('*fmincon*'; MATHWORK, Natick, MA) (Kaushal et al., 2018). Choice of objective function was based on the metabolic profile of *Clostridium* sp. which typically exhibits an acidogenic phase and a subsequent solventogenic phase. During acidogenic phase maximal growth and acid formation is achieved, while solventogenic phase is discerned by reduced growth rate, utilization of acids and initiation of solvent production (Jones and Woods, 1986). To obtain a complete picture of clostridial metabolism, FBA was carried out with respect to different objective functions for both the phases, while grown under batch and two-stage fed-batch conditions. For acidogenic phase FBA was carried out at 10 h using the objective function *maximization of biomass* ($M_\mu$); FBA for solventogenic phase was carried out at 20 h using the objective function *maximization of butanol* ($M_B$). Additional constraints were the model inputs in the form of flux values for 15 exchange reactions out of which nine were experimentally determined and for the remaining six upper and lower boundaries were provided. In case of acidogenesis, flux values were experimentally determined for rate of consumption of glucose, acetic acid, and butyric acid; and rate of production of acetic acid, butyric acid, acetone, butanol, and ethanol. In case of solventogenesis, flux values were experimentally determined for specific growth rate and rate of consumption of glucose, acetic acid, and butyric

acid and; and rate of production of acetic acid, butyric acid, acetone, and ethanol.

### 6.2.3 Reconstruction of metabolic network

Reconstruction of metabolic network for *C. acetobutylicum* was carried out using *C. acetobutylicum* ATCC 824 genome annotated in KEGG database as the primary source along with other databases such as MetaCyc (Caspi et al., 2012) and SEED (Overbeek et al., 2005). For the purpose of gap filing, enzymes which were not found in any of the aforementioned databases were added to model based on prior literature. Fig. 6.1 shows the schematic representation of the central metabolic pathways in *C. acetobutylicum*. All the manually added reactions are enlisted in Table 6.1.In the present study, FBA was performed with glucose as the sole carbon source. Glucose metabolism in *C. acetobutylicum* is initiated through the glycolytic pathway where glucose is catabolized to pyruvate and further into acetylCoA and $CO_2$. Conversion of pyruvate to acetyl-CoA is catalyzed by the enzyme pyruvate:

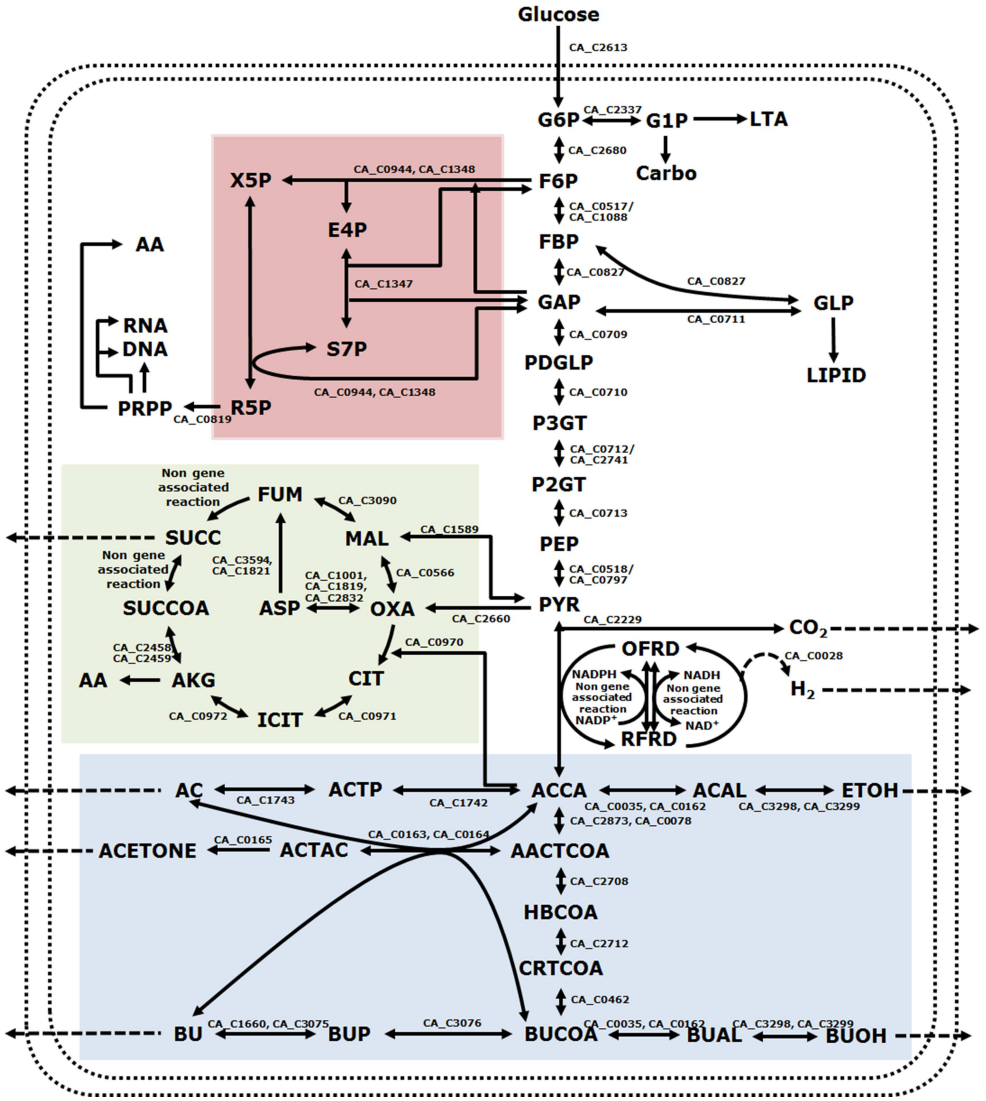



**Fig. 6.1.** Major metabolic pathways in *C. acetobutylicum*. Genes whose products were identified are labeled with the corresponding gene locus while genes with no locus are the ones whose products were not identified.

ferredoxin oxidoreductase (Jones and Woods, 1986) and is also linked with hydrogen production.

Production of hydrogen plays an important role in maintaining redox balance in clostridial spp. This involves the oxidation of ferredoxin coupled with reduction of $NAD(P)^+$ and hydrogen production which are catalyzed by ferredoxin: NAD(P)H oxidoreductase and hydrogenase enzyme, respectively (Jones and Woods, 1986). Till date, ferredoxin: NADH oxidoreductase is not found in any of the clostridial genomes, still it was added to the model on the basis of literature where it was proposed to play a key role as part of the redox balancing pathway (Yoo et al., 2015). This reaction was considered reversible (Fig. 6.1), as evinced from experiments carried out by Gheshlaghi et al. (2009). Significant enzymatic activity of both NADH: ferredoxin oxidoreductase and ferredoxin: NAD reductase was observed in *C. acetobutylicum*. Former enzyme performs the function of ferredoxin reduction by NADH, while the latter catalyzes reduction of NAD by ferredoxin. However, in case of ferredoxin: NADPH oxidoreductase, specific activity of NADPH: ferredoxin oxidoreductase enzyme was found to be low (Gheshlaghi et al., 2009). Hence, the enzyme was considered irreversible with the main role being assumed as the production of NADPH (Jungermann et al., 1973).

Solventogenic phase in *C. acetobutylicum* involves acetone, butanol, and ethanol production along with acid reutilization (Jones and Woods, 1986). The enzyme acetoacetyl-CoA: acetate/butyrate: CoA transferase couples acetic acid and butyric acid reutilization with acetone formation (Jones and Woods, 1986). However, acetone decoupled butyric acid reutilization has been reported by Desai et al. (1999) and hence based on the model developed by Shinto et al. (2007), directionality of the enzymes phosphotransacetylase-acetate kinase (PTA-AK) and phosphotransbutyrylase-butyrate kinase (PTB-BK) was considered reversible for alternate acid reutilization (Fig. 6.1). The end reactions of butanol and ethanol synthesis pathway are catalyzed by alcohol dehydrogenase, an enzyme known to have NADH and/or NADPH cofactor dependence (Gheshlaghi et al., 2009). Since the substrate requirement for generation of both the cofactors is same, NADH was considered as the common redox representative for the purpose of simplification (Villadsen et al., 2011).

Clostridial spp. generate essential precursors for nucleotide and amino acid biosynthesis via pentose phosphate pathway (PPP). These precursors can be produced via two routes, either oxidative route from glucose-6-phosphate (G6P) or non-oxidative route from fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (GAP). Oxidative route of PPP was found to be absent according to $^{13}C$ based studies carried out in *C. acetobutylicum* (Crown et al., 2011) and hence the oxidative reactions were excluded from the metabolic network.

**Table 6.1.** Enzymes manually added in the different biosynthesis pathways

| 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 | 6.2.1.5 | Succinyl-coenzyme A synthetase | 'SCOA + ADP + Pi → Suc + CoA + ATP' | TCA | Dash et al., 2014 |
| 3 | 2.7.4.6 | Nucleoside-diphosphate kinase | ATP + UDP ⟺ ADP + UTP'<br>CDP + ATP ⟺ CTP + ADP' | Nucleic acid biosynthesis | Dash et al., 2014 |
| 4 | 2.3.1.38 | Acetyl coenzyme A-acyl-carrier-protein transacylase | Acetyl-CoA + Acyl-carrier protein ⟺ CoA + Acetyl-[acyl-carrier protein] | Fatty acid biosynthesis | Senger and Papoutsakis, 2008a |
| 5 | 3.1.3.27 | phosphatidylglycerophos-phatase | Phosphatidylglycerophosphate + $H_2O$ ⟺ Phosphatidylglycerol + Orthophosphate | Fatty acid biosynthesis | Senger and Papoutsakis, 2008a |
| 6 | 1.18.1.3 | $NAD^+$-ferredoxin reductase | 2 Reduced ferredoxin + $NAD^+$ + $H^+$ ⟺ 2 Oxidized ferredoxin + NADH | Hydrogen production | Yoo et al., 2015 |
| 7 | 1.18.1.2 | ferredoxin-$NADP^+$ reductase | Reduced ferredoxin + $NADP^+$ ⟺ Oxidized ferredoxin + NADPH + $H^+$ | Ferredoxin reductase | Senger and Papoutsakis, 2008a |
| 8 | 2.7.7.39 | glycerol-3-phosphate cytidylyltransferase | CTP + sn-Glycerol 3-phosphate ⟺ Diphosphate + CDP-glycerol | Glycerophospho-lipid metabolism | Yoo et al., 2015 |
| 9 | 4.2.1.91 | arogenate dehydratase | L-Arogenate ⟺ L-Phenylalanine + $H_2O$ + $CO_2$ | Amino acid biosynthesis | Senger and Papoutsakis, 2008a |

Catalysis of reactions involved in non-oxidative route is carried out by transketolase and transaldolase, therefore these reactions were incorporated in the model. Lack of Rnf complex renders *C. acetobutylicum* unable to derive energy during electron transfer from reduced ferredoxin to NAD and undertake substrate level phosphorylation for energy generation (Millat and Winzer, 2017). In the absence of oxidative phosphorylation, the key role of tricarboxylic acid (TCA) cycle is assumed to be the generation of essential precursor for amino acid synthesis. $^{13}C$ experiments conducted for *C. acetobutylicum* have shown that the TCA cycle is bifurcated from oxaloacetate towards oxidative and reductive route culminating in secretion of succinate (Amador-Noguez et al., 2010; Crown et al., 2011). With the exception of succinyl-CoA synthetase and fumarate reductase, enzymes catalyzing all the other reactions of TCA cycle are annotated in the genome (Fig. 6.1). These two genes are yet to be identified in any of the clostridial spp. (Au et al., 2014). However, the reactions describing conversion of fumarate to succinate and succinyl-CoA to succinate were manually added in the metabolic network (Fig. 6.1) and the directionality was decided on the basis of published literature (Crown et al. 2011; Senger and Papoutsakis, 2008a; Au et al., 2014). Certain other reactions apart from the TCA cycle, for which the genes were not found, were added manually to the network are listed in Table 6.1. Detailed list of all the reactions is provided in the appendix.

### 6.2.4 Biomass composition

Optimized biomass equation developed by Senger and Papoutsakis, (2008a) for *C. acetobutylicum* was used to determine the stoichiometric coefficients of various biomass constituents such as DNA, RNA, protein, carbohydrate, lipid, peptidoglycan, and lipoteichoic acid. The equations for protein, DNA, and RNA composition were taken from Lee et al., (2008); cell wall from McAnulty et al. (2012); and lipoteichoic acid formation from Senger and Papoutsakis (2008a), respectively. The study by Dash et al. (2014) was used to determine the lipid and peptidoglycan compositions, while hexadeconic acid was taken as the fatty acid representative based on work by Senger and Papoutsakis, (2008a). Additional information on biomass composition used for *C. acetobutylicum* is detailed in the appendix.

### 6.2.5 Measureable external fluxes

A model is considered to be as good as the constraints imposed on it, hence additional constraints such as maximum substrate consumption rate and biomass and product formation rate were included in the model. Concentration of biomass i.e. the dry cell weight (DCW) was determined according to the calibration equation 1 OD=0.328 g $L^{-1}$ DCW where OD was measured at 600 nm using UV-visible spectrophotometer as described in chapter 3 (section 3.2.7). The concentrations of glucose, organic acids and solvents were measured in high performance liquid

chromatograph as detailed in Chapter 3 (section 3.2.7). Two forms of maintenance energy were considered: Growth associated (GA) and non-growth associated (NGA). GA maintenance energy was the ATP requirement for biomass synthesis and was kept constant as 40 mmol ATP $g^{-1}$ DCW, which was adapted from Senger and Papoutsakis, (2008a). Model predicted fluxes were fitted to experimental data for determination of NGA maintenance energy.

## 6.3 Results and discussion

### 6.3.1 Nutrient dependent modulation of biomass and product formation in *C. acetobutylicum* MTCC 11274

In Chapter 3 and Chapter 5, nutrient dependent modulations of biomass and solvent synthesis was observed for the strain *C. acetobutylicum* MTCC 11274 when exposed to different media compositions. To gain further insight into the metabolic flexibility and versatility of the organism, metabolic network experiments were designed where the organism was exposed to more diverse fermentation medium or strategies. Understanding the internal flux distribution becomes even more important since the critical balance of $NADH/NAD^+$, $NADPH/NADP^+$, and $Fd_{red}/Fd_{ox}$ ratios and their distribution across various pathways is of direct consequence to the final product outcome. For instance, when grown on biomass supporting media, production of butanol (6.1 g $L^{-1}$) was dominant, followed by biomass (2.9 g $L^{-1}$) and acetone (1.45 g $L^{-1}$), while no ethanol was observed. In contrast, growth on butanol supporting media was characterized by formation of butanol (12.44 g $L^{-1}$) followed by acetone (5.9 g $L^{-1}$), biomass (4.5 g $L^{-1}$), and ethanol (2.3 g $L^{-1}$). Two-stage fed-batch fermentation combined the characteristics of both the media resulting in butanol (13.1 g $L^{-1}$) followed by biomass (6.8 g $L^{-1}$), acetone (6.3 g $L^{-1}$), and ethanol (2.84 g $L^{-1}$). Further nutrient modulations in two-stage fed-batch fermentation by $MgSO_4$ limitation and $CaCO_3$ supplementation resulted in improvement in biomass (8.79 g $L^{-1}$) and butanol (14.56 g $L^{-1}$) titer, respectively. Combinatorial use of $MgSO_4$ limitation and $CaCO_3$ supplementation resulted in butanol (16.35 g $L^{-1}$) followed by biomass (8.82 g $L^{-1}$), acetone (6.45 g $L^{-1}$), and ethanol (3.15 g $L^{-1}$). Hence, flux balance analysis was carried out to unravel the regulation behind the potential metabolic modulation demonstrated by the organism in response to nutrient modulation under batch and two-stage fed-batch fermentation.

### 6.3.2 Flux distribution of C. *acetobutylicum* MTCC 11274 when grown on maximum biomass productivity and maximum butanol productivity media under batch mode of fermentation

FBA was done to ascertain intracellular carbon flux distribution at 10 h and 20 h of batch fermentation on maximum biomass productivity and maximum butanol productivity media. Comparison of the model predicted and experimentally obtained

specific growth rates showed similarity of 96.6% and 96.0% for biomass productivity and maximum butanol productivity media, respectively at 10 h. While in case of butanol flux at 20 h, biomass productivity and maximum butanol productivity media showed 92.86% and 95.31% similarity between experimental and predicted values, respectively (Table 6.2). During batch fermentation using biomass productivity

**Table 6.2.** Comparison of model predicted and experimentally determined specific growth rates ($h^{-1}$) and butanol flux (mmol $g^{-1}$ $h^{-1}$) for different batch and two-stage fed-batch fermentation

| Experiments | Specific growth rate ($h^{-1}$)[a] | | Butanol flux (mmol $g^{-1}$ $h^{-1}$)[b] | |
|---|---|---|---|---|
| | Experimental | Predicted | Experimental | Predicted |
| Biomass batch | 0.348 | 0.36 | 2.693 | 2.9 |
| Butanol batch | 0.096 | 0.1 | 3.971 | 4.166 |
| Two-stage fed-batch (TSFB) | 0.363 | 0.394 | 5.365 | 5.65 |
| $MgSO_4$ Limited TSFB | 0.261 | 0.289 | 4.773 | 5.2 |
| $CaCO_3$ Supplemented TSFB | 0.363 | 0.392 | 5.92 | 6.244 |
| $MgSO_4$ (L)-$CaCO_3$ (S)-TSFB | 0.261 | 0.289 | 5.712 | 6.04 |

[a]Objective function *maximization of biomass* was used for the flux analysis at 10 h of growth
[b]Objective function *maximization of butanol* was used for the flux analysis at 20 h of growth

medium, glucose uptake was the first step in carbon flux partitioning (Fig. 6.2 A and B). Majority of the flux flowed via glycolytic pathway towards pyruvate (182.51%) with intermittent branching towards carbohydrates and lipoteichoic acid (G6P: 1.31%), pentose phosphate pathway (F6P and GAP: 1.83%), and lipid (FBP: 2.54%) (Fig. 6.2A). PPP chiefly provides precursors for nucleic acid and amino acid synthesis, while lipoteichoic acid (LTA) is a cell wall constituent. Incoming flux at pyruvate node was bifurcated into TCA cycle (10.20%) and acetyl CoA node (ACCA: 162.32%). Flux from the ACCA node diverted towards butyric (BU: 47.72%) and acetic acid (AC: 39.16%) production reactions, whereas TCA flux predominately contributes towards synthesis of important biosynthetic precursors like -ketogluterate. Being a typical example of ABE fermentation, 10 h of cultivation exhibited no flux diversion through acetone, butanol, and ethanol formation reactions (Fig. 6.2A, Table 6.3). In *C. acetobutylicum* cultivation, metabolome remodeling and flux diversion from biomass related pathways to solvent related pathways was reported to occur during metabolic switch from acidogenesis to solventogenesis (Amador-Noguez et al., 2011). Similar shift in carbon flux distribution was observed at 20 h of cultivation (Fig. 6.2B). All the flux from carbon uptake went through glycolytic pathway towards pyruvate (200%).

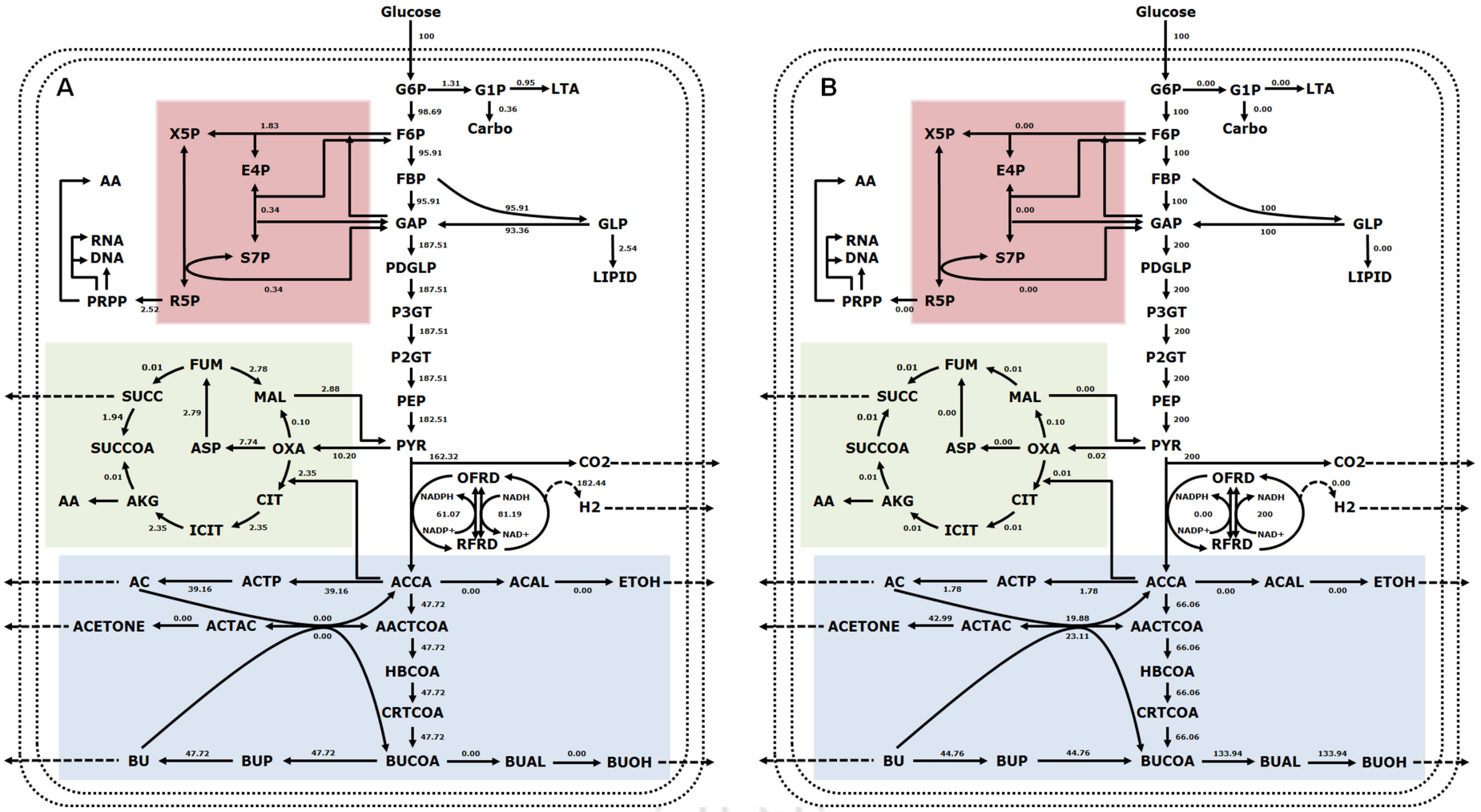



**Fig. 6.2.** Distribution of carbon fluxes under batch fermentation using biomass productivity medium (A) at 10 h with maximization of biomass as objective function and (B) at 20 h with maximization of butanol as objective function. All the flux values were normalized to 100 mmol glucose assimilated and were measured in mmol $g^{-1}$ DCW $h^{-1}$.

**Table 6.3.** ATP balance for batch fermentation using biomass productivity medium and batch fermentation using biomass productivity medium

| Pathways | Biomass batch[a] | | Butanol batch[a] | |
|---|---|---|---|---|
| | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] |
| **ATP balance** | | | | |
| Glycolysis | 174.320 | 189.005 | 175.144 | 185.242 |
| Pyruvate metabolism | -0.098 | -9.520 | 0.000 | -0.436 |
| Acidogenesis | 86.880 | -42.990 | 71.657 | -31.898 |
| TCA Cycle | -12.135 | -5.430 | -11.426 | -7.390 |
| Pentose phospate pathway | -2.516 | 0.000 | -2.418 | -1.446 |
| Nucleic acid metabolism | -18.950 | -13.300 | -18.018 | -12.288 |
| Amino acid metabolism | -35.820 | -24.370 | -33.903 | -20.942 |
| Aminosugar metabolism | -0.893 | 0.000 | -0.864 | -0.513 |
| Carbohydrate metabolism | -0.357 | 0.000 | -0.335 | -0.205 |
| Fattyacid biosynthesis | -18.930 | -0.001 | -19.157 | -10.879 |
| Lipid biosynthesis | -0.047 | -0.020 | 0.000 | 0.000 |
| Maintenance Energy | -0.100 | -13.690 | 0.000 | -0.437 |
| DNA biosynthesis | -0.003 | 0.000 | -0.003 | -0.002 |
| RNA biosynthesis | -0.480 | 0.000 | -0.455 | -0.279 |
| Protein biosynthesis | -53.940 | -0.001 | -50.574 | -30.992 |
| Peptidoglycan biosynthesis | -0.890 | 0.000 | -0.858 | -0.513 |
| Biomass | -116.020 | -0.001 | -108.789 | -66.667 |

[a]Cofactor balance obtained for energy yielding and consuming pathways for batch fermentation using biomass productivity medium and batch fermentation using biomass productivity medium. The flux (mmol $g^{-1}$ $h^{-1}$ ) values are expressed per 100 mmol glucose.
[b]Objective function *maximization of biomass* was used for the flux analysis at 10 h of growth ($M_\mu$ 10 h)
[c]Objective function *maximization of butanol* was used for the flux analysis at 20 h of growth ($M_B$ 20 h)

The solventogenic phase coincided with the stationary phase of growth and no flux was diverted at the crucial nodes of biomass production i.e. G6P (carbohydrate and LTA), F6P and GAP (PPP), and FBP (lipid). Incoming flux at pyruvate node completely diverted to ACCA node from where it bifurcated towards acetoacetyl CoA node (AACTCOA: 66.06%) and a comparatively miniscule amount of 1.78% flowed towards AC (Fig. 6.2B, Table 6.3 and 6.4). From AACTCOA node, flux was partitioned between acetone formation (42.99%) and butyryl CoA node (BUCOA: 66.06%). Total influx at BUCOA node comprised of flux coming from AACTCOA node and reutilization of BU, which further culminated towards butanol formation (BUOH: 133.94%).

Fig. 6.3 shows comparison of percentage contribution of normalized flux values at 10 h and 20 h to total flux (10 h and 20 h cumulative) through different

**Table 6.4.** Balance for the cofactors NADPH and NADH for batch fermentation using biomass productivity medium and batch fermentation using biomass productivity medium

| Pathways | Biomass batch[a] | | Butanol batch[a] | |
|---|---|---|---|---|
| | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] |
| **NADPH balance** | | | | |
| Aminoacid metabolism | -37.950 | -0.001 | -35.786 | -21.806 |
| Aminosugar metabolism | -0.298 | 0.000 | -0.291 | -0.171 |
| Fattyacid biosynthesis | -18.933 | -0.001 | -18.158 | -10.879 |
| Lipid biosynthesis | -2.545 | 0.000 | -2.542 | -1.462 |
| Ferredoxin-$NADP^+$ reductase | 58.092 | 0.002 | 56.194 | 33.380 |
| One carbon pool by folate | 1.633 | 0.000 | 1.581 | 0.939 |
| **NADH balance** | | | | |
| Glycolysis | 187.506 | 199.999 | 187.857 | 192.821 |
| Pyruvate metabolism | 2.881 | 5.430 | 2.663 | 2.071 |
| Acidogenesis | -95.433 | -132.124 | -128.218 | -112.173 |
| Solventogenesis | 0.386 | -267.870 | 0.360 | -211.475 |
| TCA Cycle | 2.250 | -5.429 | 2.537 | 0.875 |
| Nucleic acid metabolism | 1.137 | 0.000 | 1.099 | 0.653 |
| Amino acid metabolism | 0.770 | 0.000 | 0.709 | 0.442 |
| Fattyacid biosynthesis | -18.933 | -0.001 | -19.158 | -10.879 |
| Ferredoxin-$NAD^+$ reductase | -81.187 | 199.995 | -48.434 | 137.307 |

[a]Cofactor balance obtained for energy yielding and consuming pathways for batch fermentation using biomass productivity medium and batch fermentation using biomass productivity medium. The flux (mmol $g^{-1}$ $h^{-1}$ ) values are expressed per 100 mmol glucose.
[b]Objective function *maximization of biomass* was used for the flux analysis at 10 h of growth ($M_\mu$ 10 h)
[c]Objective function *maximization of butanol* was used for the flux analysis at 20 h of growth ($M_B$ 20 h)

metabolic pathways and reactions. Biomass related pathways along with acid production, hydrogen synthesis, and NADP reduction were found to be highly active during 10 h of cultivation (Fig. 6.3, Table 6.4). Flux through the NADP reduction reaction yields NADPH and was linked to the high demand of NADPH at 10 h since most of the biosynthetic reactions (lipids, amino acids, fatty acids, etc.) are catalyzed by NADPH-dependent enzymes (Jones and Woods, 1986). Conversely, at 20 h, cessation of growth impelled the downregulation of anabolic reactions. This in turn resulted in reduced demand for NADPH and hence absence of flux through NADP reduction reaction (Fig. 6.3, Table 6.4).

Acetyl CoA and carbon dioxide release reaction was active at both the time points, though the flux contribution during 20 h was more in comparison to 10 h. This

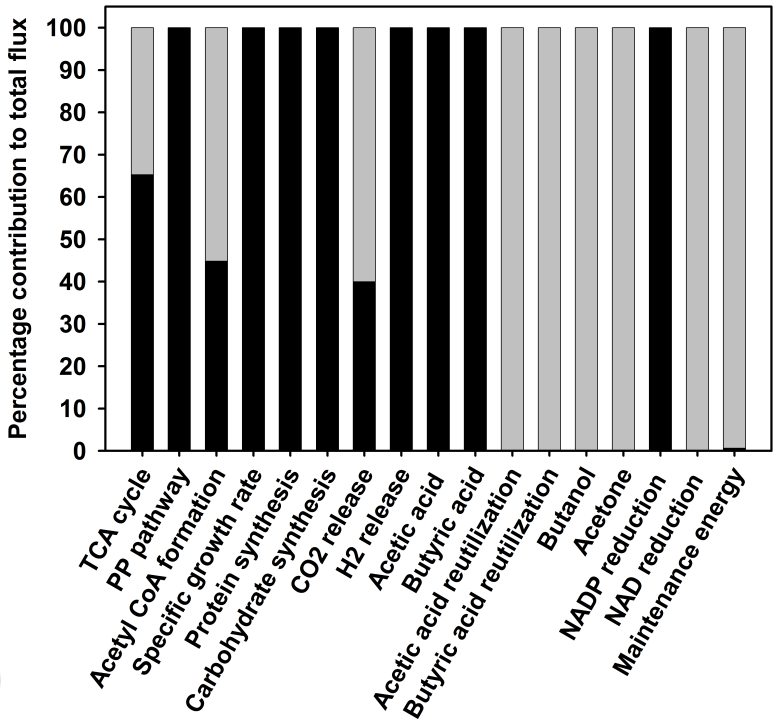



**Fig. 6.3.** Percentage contribution of normalized flux values at 10 and 20 h to total flux (10 h and 20 h cumulative) for batch fermentation using biomass productivity medium. Black and gray bars indicate flux estimates at 10 h with the objective function maximization of biomass and 20 h with maximization of butanol, respectively.

being true since acetyl CoA is a common node between acid and alcohol production pathways and absence of flux in biomass related pathways diverted most of the flux towards product formation at 20 h. Reactions exclusively active at 20 h pertained to acid reutilization, solvent production, NAD reduction, and NGA maintenance energy (Fig. 6.4). While flux through NAD reduction was primarily to fulfill the demand for NADH; NGA maintenance energy flux was required to feed the energy expenditure in metabolite transport, chemotaxis, repair, macromolecule turnover, and stress adaptation which are considered to be of paramount importance for the organisms survival specially at alcohol production stage (Wu et al., 2015).

Like biomass productivity medium, the sole carbon source in the butanol productivity medium was glucose. Hence, the basic carbon distribution pattern was similar in both the batch experiments; however, variation arose in differential activation of pathways due to different initial concentrations of carbon source. At 10 h of cultivation, batch fermentation using biomass productivity media resulted in higher growth as compared to butanol productivity media (Fig. 6.4).

The associated pathways for carbon uptake, TCA, PPP, protein, nucleic acid and carbohydrate synthesis were downregulated in the butanol productivity media (Fig. 6.4, Table 6.3 and 6.4). Being the acidogenic phase, flux towards solvent synthesis reactions was absent in both the experiments. During transition from

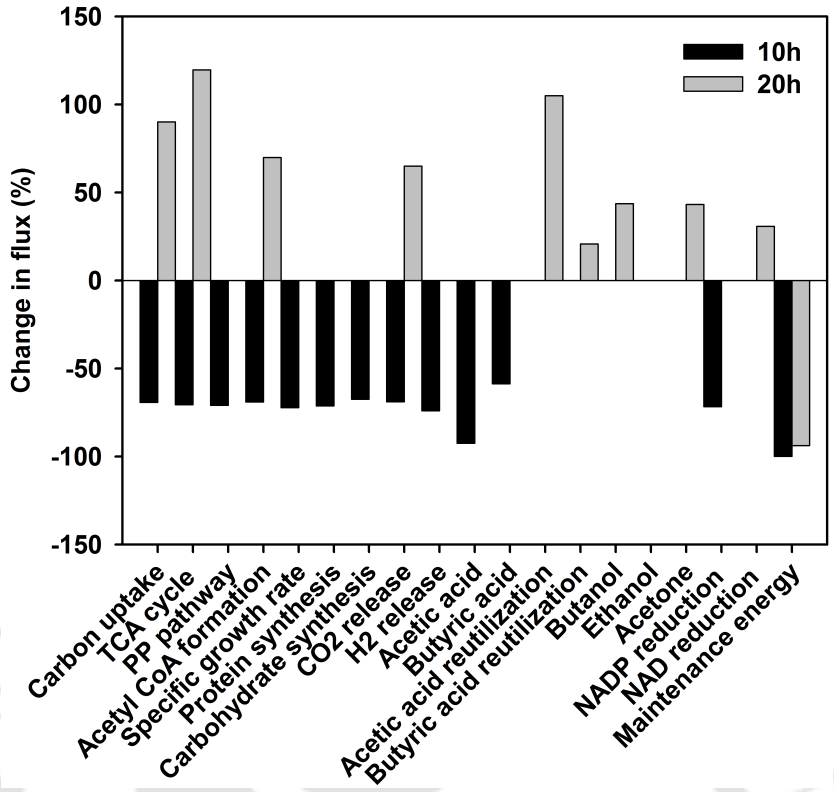



**Fig. 6.4.** Percentage change in absolute flux values under batch fermentation using butanol productivity medium with respect to batch fermentation using biomass productivity medium at 10 h and 20 h of growth. Black and gray bars indicate flux estimates at 10 h with the objective function maximization of biomass and 20 h with the objective function maximization of butanol, respectively.

acid phase to solvent phase, the flux diverted from biomass synthesis pathway to solventogenic reactions. In case of biomass productivity media, previously active pathways of TCA, PPP, protein, nucleic acid, and carbohydrate synthesis carried negligible flux at 20 h (Table 6.3). Conversely, in case of butanol productivity media, flux was observed in both biomass and solvent synthesis pathways but major flux was diverted towards the latter.

### 6.3.3 Flux distribution of *C. acetobutylicum* MTCC 11274 when grown under different two-stage fed-batch fermentations

FBA was carried out at 10 h and 20 h for different two-stage fed-batch fermentations to understand the carbon flux distribution during the individual stages of cultivation. Since the two-stage strategy was developed exploiting the different optimized media, flux distribution at 10 h and 20 h of cultivation under two-stage fed-batch fermentation was compared with 10 h of biomass productivity media and 20 h of butanol productivity media, respectively (Fig. 6.5). It was revealed that the two-stage fed-batch had higher flux in carbon uptake, biomass, and solvent synthesis pathways in comparison to the two batch fermentation experiments. The NGA maintenance energy was lower at both the time points showing the culture

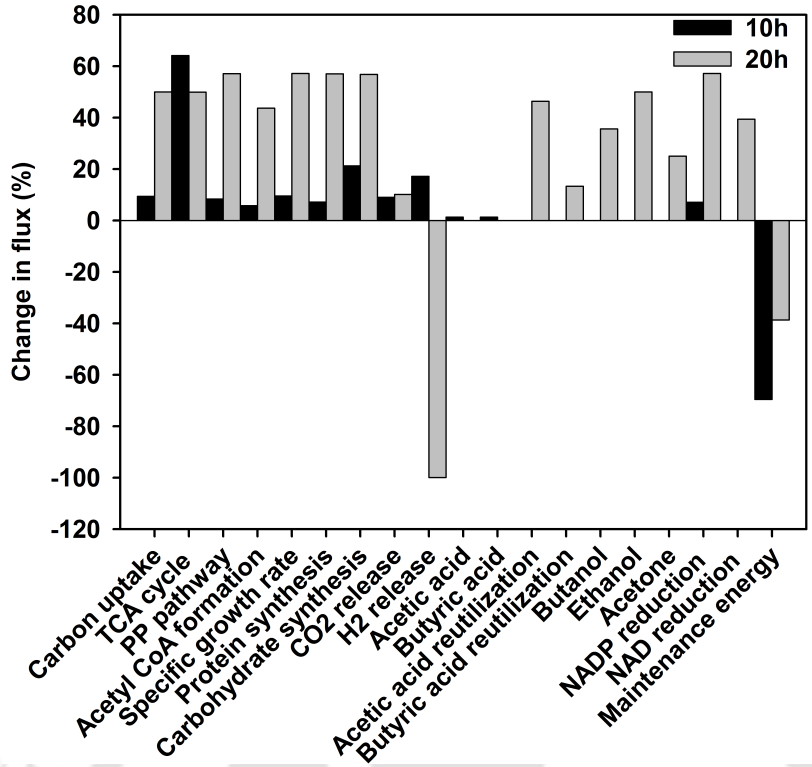



**Fig. 6.5.** Percentage change in absolute flux values under two-stage fed-batch fermentation with respect to batch fermentation using biomass productivity medium at 10 h and two-stage fed-batch fermentation with respect to batch fermentation using butanol productivity medium at 20 h of growth. Black and gray bars indicate flux estimates at 10 h with the objective function maximization of biomass and 20 h with the objective function maximization of butanol, respectively.

was performing better in terms of both growth and production as the nutrients were replenished intermittently (Table 6.3 and 6.5).

Fig. 6.6 A and B illustrate percentage change in absolute flux values under two-stage fed-batch fermentation with respect to magnesium-limited two-stage fed-batch fermentation at 10 h and 20 h of cultivation, respectively. At 10 h, down regulated flux in acetone, butanol, and ethanol pathway depicts the absence of solventogenesis during two-stage fed-batch fermentation. During metabolic switch from acidogenesis to solventogenesis, microbial cells undergo a remodeling of the metabolome and diversion of flux from biomass related pathways to solvent related pathways (Amador-Noguez et al., 2011). As the 10 h time point marks the initiation of solventogenesis for magnesium-limited experiment, a higher flux was observed in TCA, PPP, nucleic acid, protein, and carbohydrate synthesis during magnesium sufficient two-stage fed batch fermentation which was still in acidogenic phase (Fig. 6.6A). Low flux in maintenance energy pathway of magnesium sufficient two-stage fed-batch fermentation can be attributed to the comparatively lower stress experienced by magnesium sufficient cells at early 10 h of growth (Fig. 6.6A, Table 6.5 and 6.6).

**Table 6.5.** ATP balance for two-stage fed-batch fermentation, $MgSO_4$ limited two-stage fed-batch fermentation, $CaCO_3$ supplemented two-stage fed-batch fermentation, and $MgSO_4$ limited-$CaCO_3$ supplemented two-stage fed-batch fermentation

| Pathways | TSFB[a] | | $MgSO_4$ Limited TSFB[a] | | $CaCO_3$ Supplemented TSFB[a] | | $MgSO_4$ (L)-$CaCO_3$ (S)-TSFB[a] | |
|---|---|---|---|---|---|---|---|---|
| | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] |
| **ATP balance** | | | | | | | | |
| Glycolysis | 174.868 | 184.541 | 176.523 | 167.180 | 191.253 | 184.744 | 176.523 | 184.781 |
| Pyruvate metabolism | -0.028 | -0.187 | -0.146 | -11.659 | -0.031 | -0.085 | -0.146 | -0.273 |
| Acidogenesis | 80.255 | -26.342 | 62.589 | -23.331 | 87.775 | -29.408 | 62.589 | -28.274 |
| TCA Cycle | -11.804 | -7.439 | -11.148 | -6.091 | -12.910 | -7.234 | -11.148 | -7.418 |
| Pentose phospate pathway | -2.462 | -1.515 | -2.300 | -0.510 | -2.693 | -1.495 | -2.300 | -1.491 |
| Nucleic acid metabolism | -18.358 | -11.808 | -17.460 | -51.123 | -20.078 | -11.326 | -17.460 | -11.957 |
| Amino acid metabolism | -35.006 | -21.670 | -32.780 | -14.722 | -38.286 | -21.297 | -32.780 | -21.417 |
| Aminosugar metabolism | -0.874 | -0.537 | -0.816 | -0.181 | -0.956 | -0.530 | -0.816 | -0.529 |
| Carbohydrate metabolism | -0.349 | -0.215 | -0.326 | -0.072 | -0.382 | -0.212 | -0.326 | -0.211 |
| Fattyacid biosynthesis | -18.528 | -11.397 | -17.306 | -3.840 | -20.264 | -11.247 | -17.306 | -11.219 |
| Lipid biosynthesis | -0.010 | -0.009 | -0.065 | -14.101 | -0.011 | -0.040 | -0.065 | -0.189 |
| Maintenance Energy | -0.028 | -0.179 | -0.150 | -6.802 | -0.031 | -0.085 | -0.150 | -0.274 |
| DNA biosynthesis | -0.003 | -0.002 | -0.003 | -0.001 | -0.003 | -0.002 | -0.003 | -0.002 |
| RNA biosynthesis | -0.474 | -0.292 | -0.443 | -0.098 | -0.519 | -0.288 | -0.443 | -0.287 |
| Protein biosynthesis | -52.783 | -32.468 | -49.302 | -10.938 | -57.729 | -32.041 | -49.302 | -31.961 |
| Peptidoglycan biosynthesis | -0.874 | -0.537 | -0.816 | -0.181 | -0.956 | -0.530 | -0.816 | -0.529 |
| Biomass | -113.541 | -69.841 | -106.052 | -23.529 | -124.180 | -68.923 | -106.052 | -68.750 |

[a]Cofactor balance obtained for energy yielding and consuming pathways for two-stage fed-batch fermentation, magnesium-limited two-stage fed-batch, calcium-supplemented two-stage fed-batch and magnesium-limited calcium-supplemented two-stage fed-batch. The flux (mmol $g^{-1}$ $h^{-1}$) values are expressed per 100 mmol glucose.

[b]Objective function *maximization of biomass* was used for the flux analysis at 10 h of growth ($M_\mu$ 10 h)

[c]Objective function *maximization of butanol* was used for the flux analysis at 20 h of growth ($M_B$ 20 h)

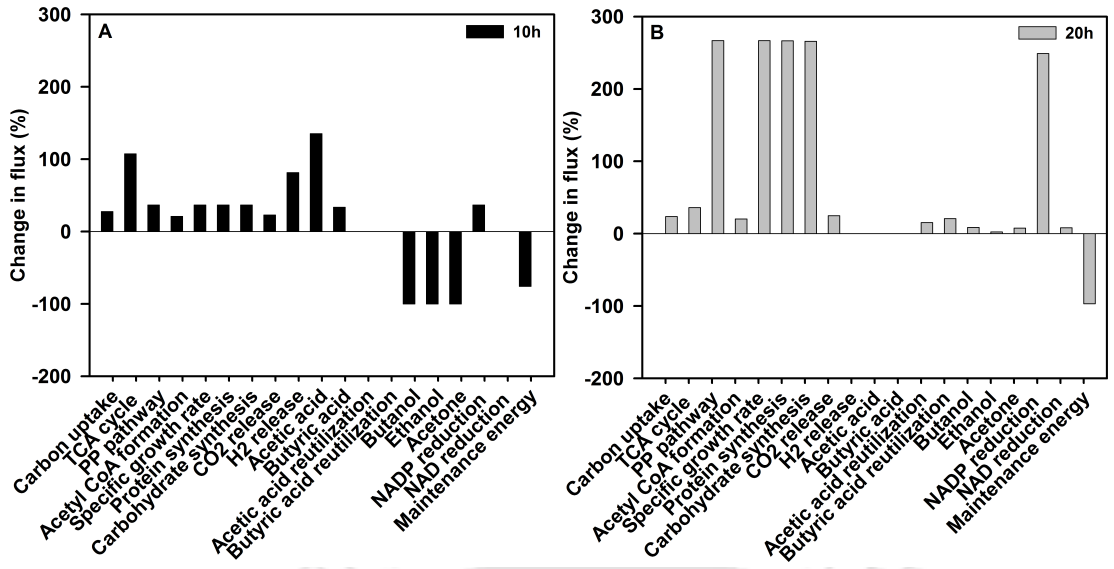



**Fig. 6.6.** Percentage change in absolute flux values under two-stage fed-batch fermentation with respect to $MgSO_4$ limited two-stage fed-batch fermentation at 10 h and 20 h of growth. Black and gray bars indicate flux estimates at 10 h with the objective function maximization of biomass and 20 h with the objective function maximization of butanol, respectively.

Due to early induction of solventogenesis, the magnesium-limited experiment was in the later stage of cultivation and experienced higher butanol stress in comparison to magnesium sufficient experiment. Hence at 20 h of cultivation, upregulated flux was observed in carbon uptake, growth associated, and solventogenic pathways along with down regulated maintenance energy flux during two-stage fed-batch fermentation in comparison to magnesium-limited experiment (Fig. 6.6B, Table 6.5 and 6.6).

Studies on supplementation of metal ions during second stage of two-stage fed-batch fermentation revealed maximum butanol production upon supplementation with 5 g $L^{-1}$ of calcium carbonate. Under calcium supplementation experiment, 10 h of cultivation was same as two-stage fed-batch fermentation because the inducer addition was done upon induction of solventogenesis only. Therefore, comparison between the two experiments was carried out at 20 h of cultivation. Fig. 6.7 shows percentage change in absolute flux values under calcium-supplemented two-stage fed-batch fermentation with respect to two-stage fed-batch fermentation at 20 h of growth. A higher carbon uptake along with higher flux in biomass and solvent synthesis pathways was observed in calcium-supplemented experiment as compared to un-supplemented two-stage fed-batch fermentation. Interestingly, a lower NGA maintenance energy flux was observed which showed that the cells experienced lower stress in contrast to un-supplemented experiment (Table 6.5). This can be attributed to calcium mediated upregulation of heat shock proteins, DNA synthesis, transcription, repair, carbohydrate catabolic enzymes, and stabilizing membrane proteins.

**Table 6.6.** Balance for the cofactors NADPH and NADH for two-stage fed-batch fermentation, $MgSO_4$ limited two-stage fed-batch fermentation, $CaCO_3$ supplemented two-stage fed-batch fermentation, and $MgSO_4$ limited-$CaCO_3$ supplemented two-stage fed-batch fermentation

| Pathways | TSFB[a] | | $MgSO_4$ Limited TSFB[a] | | $CaCO_3$ Supplemented TSFB[a] | | $MgSO_4$ (L)-$CaCO_3$ (S)-TSFB[a] | |
|---|---|---|---|---|---|---|---|---|
| | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] | $M_\mu$ 10 h[b] | $M_B$ 20 h[c] |
| **NADPH balance** | | | | | | | | |
| Aminoacid metabolism | -37.138 | -22.845 | -34.689 | -7.696 | -40.618 | -22.544 | -34.689 | -22.488 |
| Aminosugar metabolism | -0.291 | -0.179 | -0.272 | -0.060 | -0.319 | -0.177 | -0.272 | -0.176 |
| Fattyacid biosynthesis | -18.528 | -11.397 | -17.306 | -3.840 | -20.264 | -11.247 | -17.306 | -11.219 |
| Lipid biosynthesis | -2.490 | -1.532 | -2.326 | -0.516 | -2.724 | -1.512 | -2.326 | -1.508 |
| Ferredoxin-$NADP^+$ reductase | 56.850 | 34.969 | 53.100 | 11.781 | 62.176 | 34.510 | 53.100 | 34.423 |
| One carbon pool by folate | 1.598 | 0.983 | 1.493 | 0.331 | 1.748 | 0.970 | 1.493 | 0.968 |
| **NADH balance** | | | | | | | | |
| Glycolysis | 187.773 | 192.479 | 188.579 | 197.466 | 205.367 | 192.578 | 188.579 | 192.596 |
| Pyruvate metabolism | 1.884 | -4.629 | 2.690 | -12.841 | 2.060 | 1.742 | 2.690 | 1.938 |
| Acidogenesis | -88.409 | -114.286 | -117.431 | -120.660 | -96.693 | -121.341 | -117.431 | -123.274 |
| Solventogenesis | 0.378 | -192.467 | -33.629 | -203.327 | 0.413 | -206.034 | -33.629 | -202.420 |
| TCA Cycle | 7.607 | 1.217 | 1.996 | -3.174 | 8.320 | 1.458 | 1.996 | 1.104 |
| Nucleic acid metabolism | 1.112 | 0.684 | 1.039 | 0.230 | 1.217 | 0.675 | 1.039 | 0.674 |
| Amino acid metabolism | 0.754 | 0.464 | 0.704 | 0.156 | 0.824 | 0.457 | 0.704 | 0.456 |
| Fattyacid biosynthesis | -18.528 | -11.397 | -17.306 | -3.840 | -20.264 | -11.247 | -17.306 | -11.219 |
| Ferredoxin-$NAD^+$ reductase | -93.181 | 127.559 | -27.213 | 145.863 | -101.912 | 141.341 | -27.213 | 139.776 |

[a]Cofactor balance obtained for energy yielding and consuming pathways for two-stage fed-batch fermentation, magnesium-limited two-stage fed-batch, calcium-supplemented two-stage fed-batch and magnesium-limited calcium-supplemented two-stage fed-batch. The flux (mmol $g^{-1}$ $h^{-1}$) values are expressed per 100 mmol glucose.

[b]Objective function *maximization of biomass* was used for the flux analysis at 10 h of growth ($M_\mu$ 10 h)

[c]Objective function *maximization of butanol* was used for the flux analysis at 20 h of growth ($M_B$ 20 h)

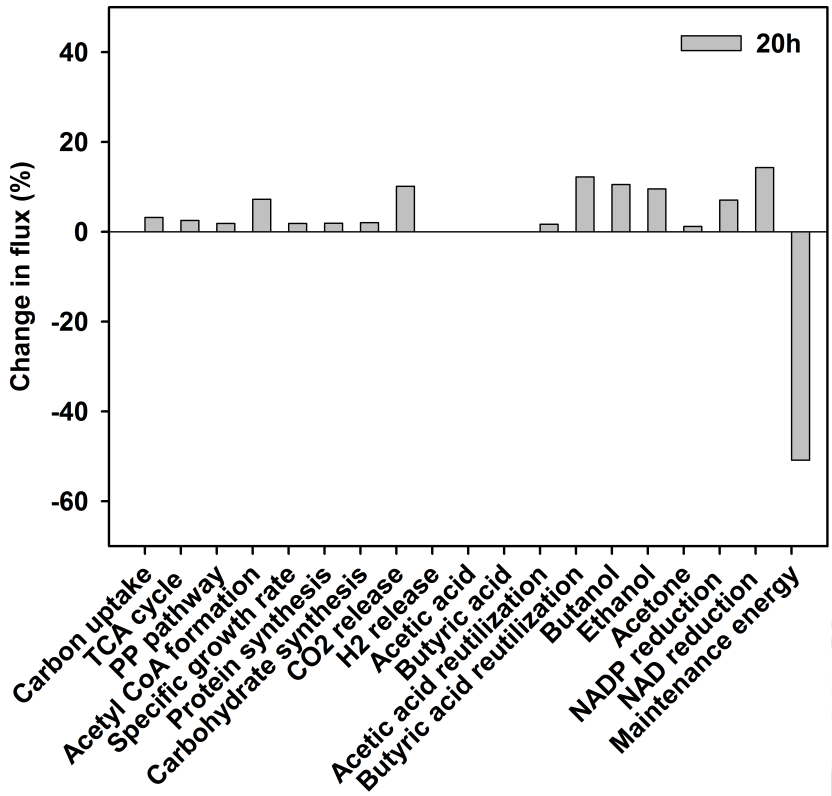



**Fig. 6.7.** Percentage change in absolute flux values under $CaCO_3$ supplemented two-stage fed-batch fermentation with respect to two-stage fed-batch fermentation at 20 h of growth.

Therefore, calcium supplementation aided in higher carbon uptake and better fermentation even in the presence of high butanol concentration which is normally detrimental to the cells.

Two-stage fed-batch fermentation was carried out combining magnesium limitation at stage one with calcium supplementation at the stage two. Similar to previous experiments, FBA was conducted at 10 h and 20 h of cultivation to obtain carbon flux distribution at individual stages (Fig. 6.8). Flux distribution at 10 h of cultivation resembled magnesium-limited experiments due to the presence of flux in solvent production pathways, while at 20 h, presence of calcium retained flux in carbohydrate synthesis reaction, PPP, and TCA (Fig. 6.8). Key highlight of this FBA was that pathways related to biomass and butanol synthesis remained concomitantly active during acidogenesis and solventogenesis under combinatorial usage of magnesium limitation-calcium supplementation under two-stage fed-batch fermentation (Fig. 6.8, Table 6.5 and 6.6). This suggests that butanol synthesis by the use of metal ions in combination under two-stage fed-batch fermentation will be more energy efficient than other mode of operation.

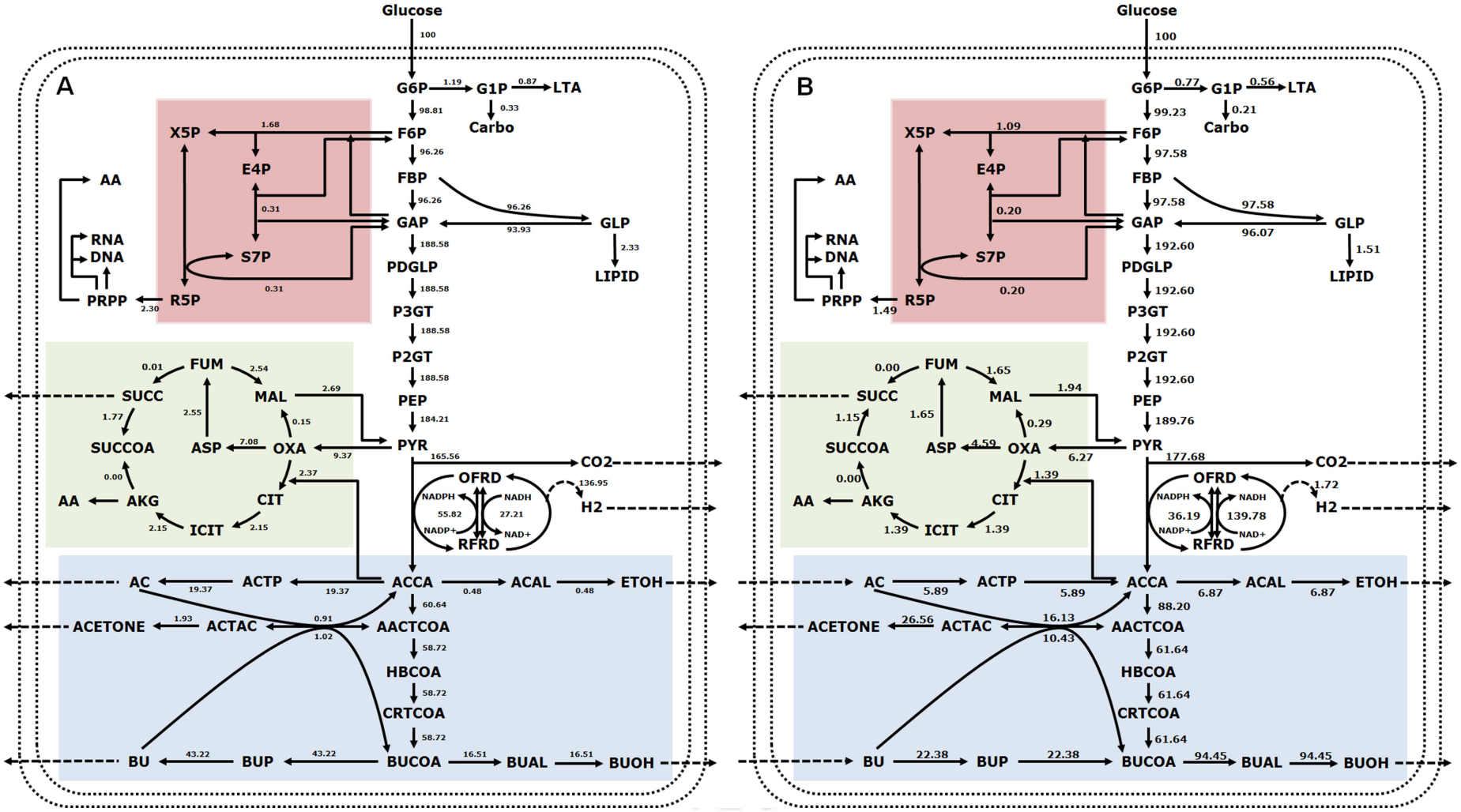



**Fig. 6.8.** Distribution of carbon fluxes under $MgSO_4$ limited and $CaCO_3$ supplemented two-stage fed-batch fermentation (A) at 10 h with maximization of biomass as objective function and (B) at 20 h with maximization of butanol as objective function. All the flux values were normalized to 100 mmol glucose assimilated and were measured in mmol $g^{-1}$ DCW $h^{-1}$.

## 6.4 Conclusion

Flux balance analysis was performed in order to understand the mechanism governing nutritional modulation based metabolic flexibility under two-stage fed-batch fermentation. Upregulated carbon flux toward acid pathways in order to satisfy increased ATP demand required for improved growth in magnesium-limited stage one of two-stage fed-batch fermentation was in turn found to aid early induction of solventogenesis. Calcium mediated upregulation of heat shock proteins, DNA synthesis, repair, carbohydrate catabolic enzymes, and stabilizing membrane proteins maybe responsible for continued higher carbon flux towards growth and butanol pathways, and lowering NGA maintenance energy flux during solventogenesis.

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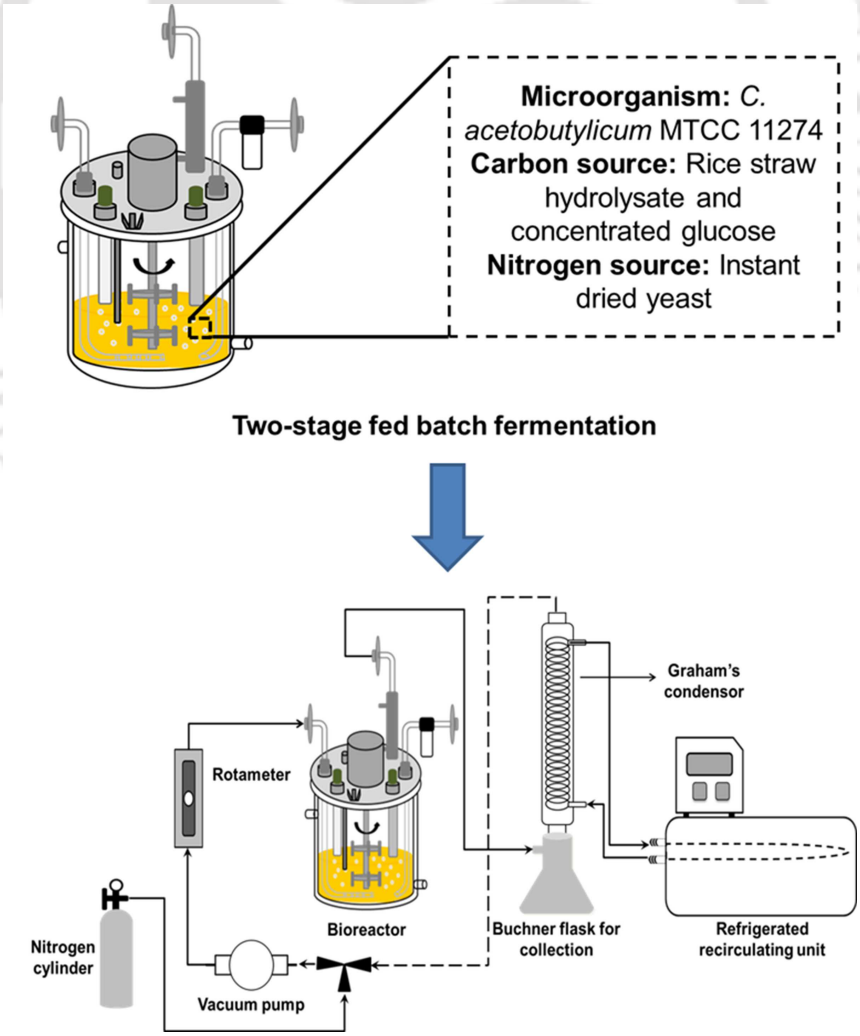
*"In the fields of observation chance favors only the prepared mind."*

Louis Pasteur (1822-1895)
French chemist and microbiologist

# 7

# Development of a bioprocess coupled with *in situ* product recovery




Schematic diagram for two-stage fed-batch fermentation coupled with *in situ* gas stripping

## 7.1 Background and motivation

Butanol production is receiving increasing interest as a response to the demand of green fuels, which are sustainable, renewable, and environment friendly (Xin et al., 2018). Butanol synthesis through ABE (acetone-butanol-ethanol) fermentation was first reported by Pasteur in 1862, gained momentum during World War I, and the first industrial process was demonstrated in 1916 (Sauer, 2016). Despite more than 100 year old process, commercialization of butanol from *Clostridium* spp. remains unachieved due to several bottlenecks like butanol toxicity resulting in low titer and productivity, low yield due to mixed product profile, and high substrate cost (Xin et al., 2018).

Butanol when present in higher concentration increases membrane fluidity which makes it unstable and alters the structure of cell membrane. It also inhibits glucose uptake, thus inhibiting energy generation and increases the membrane permeability which results in release of essential molecules such as ATP, phospholipids, RNA, and proteins from the cell (Dunlop, 2011). Butanol toxicity restricts the titer produced by the organism, which also symbolizes a dilute product in the fermentation broth thus resulting in high downstream processing cost (Jones and Woods, 1986). In addition, sluggish fermentation due to increasing butanol concentration lowers the productivity of the process, in turn adding to the operational cost (Lee, 2016). Development of butanol tolerant strains has not been significantly successful in addressing these bottlenecks (Lee et al., 2016).

Another approach of improving the performance of fermentation process is coupling it with *in situ* product recovery system (Lee et al., 2016). This serves the dual purpose of alleviation of butanol toxicity resulting in continued fermentation and recovery of concentration product. Gas stripping is among the most suitable candidates for *in situ* product recovery technique (Jimenez-Bonilla and Wang, 2018). It provides several advantages in terms of energy efficiency, simplicity, inertness towards cells, selectivity towards desired product, and ease of integration to different modes of operation and other recovery techniques (Jimenez-Bonilla and Wang, 2018; Lee et al., 2016). Xue et al. (2012) demonstrated that integration of fed-batch fermentation with intermittent gas stripping increased the fermentation time from 78 h (batch) to 326 h resulting in improved butanol titer of 113.2 g $L^{-1}$ (approx. 19.1 g $L^{-1}$ in batch fermentation) during cultivation of *C. acetobutylicum* strain JB200 in fibrous bed bioreactor. Further, fed-batch fermentation, using the same culture, integrated with two-stage gas stripping resulted in 48.5 g $L^{-1}$ butanol titer during 201 h cultivation with highly concentrated condensate (Xue et al. 2014). When batch fermentation of *Clostridium beijerinckii* BA101 were coupled with intermittent gas stripping, butanol titer increased from 11.9 g $L^{-1}$ to 16.4 - 46.4 g $L^{-1}$ (Ezeji et al., 2003). Another important factor for sustainable butanol production is the raw

material cost which accounts for approximately 60% of the total capital requirement (Jones and Woods, 1986). A potential alternate for reducting butanol production cost is the use of cheaper substrates such as lignocellulosic biomass, agricultural, and industrial waste (Jones and Woods, 1986). *Clostridium* spp. have been reported to utilize a variety of low cost substrates namely sugarcane bagasse, rice straw, cassava, Jerusalem artichocke, crude glycerol etc. (Kaushal et al., 2017; Moon et al., 2016).

To that end, in the present study, gas stripping was used as an *in situ* product recovery process and was integrated with the two-stage fed-batch strategy developed in section 3.3.1. Further, in order to minimize residual nutrients, modulation of feeding duration was done. *In situ* product recovery and modulated feeding were also carried out for two-stage fed-batch with combinatorial use of metal ion limitation and supplementation. Finally, the carbon and nitrogen sources were replaced with rice straw hydrolyzate and instant dry yeast, respectively to reduce the substrate cost; thus, exhibits the potential of the strategy in developing a suitable bioprocess for butanol production.

## 7.2 Materials and methods

### 7.2.1 Organism, growth, and maintenance conditions

Lyophilized culture *C. acetobutylicum* MTCC 11274 was procured from Institute of Microbial Technology (IMTECH), India, revived and stored as glycerol stocks -80°C. Culture maintenance, revival, and inoculum preparation were carried out as mentioned in section 3.2.1. Two-stage fed batch experiments i.e. with and without combinatorial use of metal ion limitation were carried out as detailed in section 5.2.2 and 5.2.5, respectively. Stage one of two-stage fed batch fermentation was carried out in optimized medium for biomass productivity comprising (in g $L^{-1}$) glucose: 33.0, peptone: 21.12, $K_2HPO_4$: 0.50, $KH_2PO_4$: 0.50, $MgSO_4.7H_2O$: 0.36, $MnSO_4.H_2O$: 0.018, $FeSO_4.7H_2O$: 0.018, NaCl: 0.018, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001. Stage two of two-stage fed batch fermentation was carried out in optimized medium for butanol productivity comprising (in g $L^{-1}$) glucose: 82.0, peptone: 49.0, $K_2HPO_4$: 0.50, $KH_2PO_4$: 0.50, $MgSO_4.7H_2O$: 0.46, $MnSO_4.H_2O$: 0.023, $FeSO_4.7H_2O$: 0.023, NaCl: 0.023, $CH_3COONH_4$: 2.2, para-amino-benzoic acid: 0.01, and biotin: 0.001. The combinatorial strategy was carried out in a similar media with exception of using 0.18 g $L^{-1}$ $MgSO_4.7H_2O$ in stage one and 5 g $L^{-1}$ $CaCO_3$ in stage two. All the chemicals (AR grade) were purchased from Himedia, India unless otherwise mentioned.

### 7.2.2 Intermittent gas stripping coupled two-stage fed-batch process: without and with modulation of feeding duration

*Experiment I:* The two-stage fed-batch process strategy, developed in chapter 5 (section 5.2.2; section 5.3.1), resulted in improved productivity and reduced

fermentation time. However, the titer was limited due to butanol toxicity; to that end intermittent gas stripping was integrated with the two-stage fed-batch fermentation process. Fig. 7.1 describes the *in situ* gas stripping set up for butanol recovery.

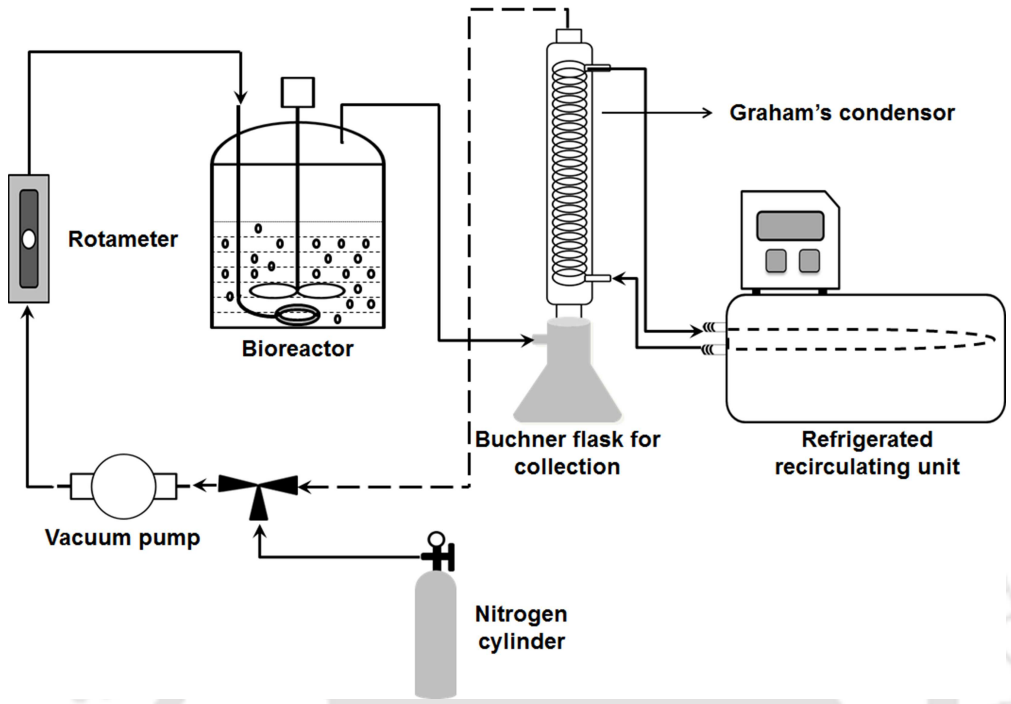



**Fig. 7.1.** Schematic flowchart of the gas stripping set up integrated with fermentation.

In the set-up, the exhaust of the condenser of the bioreactor was connected to the inlet of Graham's condenser (Borosil, India), which was further connected to the inlet of the sparger via a vacuum pump (Tarsons, India). Pure nitrogen (99.99%, Assam Air products, Guwahati, India) was purged at the rate of 2.5 vvm into the fermenter via sparger located at the bottom of the bioreactor vessel, and a continuous mixing was provided for efficient gas mixing and transfer. The exhaust of the fermenter was connected to a condenser (Graham coiled distillate type, internal diameter 62 mm and jacket length 500 mm, Borosil, India) for condensation of the solvents in the gaseous phase. The condenser was continuously supplied with 100% methanol (99.8%, AR grade), using a refrigerated / heating circulator (WCL-P8, Daihan, Korea), maintained at -5°C. Exhaust gas vapors were recirculated through a vacuum pump (Rocker 300, Tarsons, India) into the fermenter sparger. The condensate was collected in a 500 mL Buchner flask attached to the bottom of the condenser and used for quantitative estimation of the solvent extracts. The flask was kept immersed in ice in order to prevent evaporation of the volatile solvents. Solvent laden stripping gas was taken up into the condenser from where the solvents condensed into the collection flask and free gas was again recirculated through the broth (Ezeji et al., 2004).

As and when the concentration of butanol in the broth reached 10 g $L^{-1}$, the

intermittent gas stripping was initiated to recover butanol. The duration of each cycle of gas stripping was 6 h which was optimized based on efficiency of butanol recovery in a different study carried out by a colleague from the same laboratory (data not shown). Experiment was carried out in a 3.0 L automated bioreactor (Bio Console ADI 1025, Applikon Biotechnology, Holland) with 1.0 L optimized medium. The reactor was operated at 37°C and agitator speed of 300 rpm. Prior to inoculation, $N_2$ was flushed through the medium to ensure anaerobic condition. Sampling was carried out at regular time intervals to obtain the dynamic profile of growth, butanol production, and substrate utilization.

*Experiment II:* Intermittent feeding of the key nutrients throughout the entire duration of the fermentation in the Experiment I resulted in significantly high amount of unutilized nutrients (82.9 g glucose and 49 g peptone) at the end of cultivation. This might have resulted in suboptimal economic efficiency of the process. Therefore, with the aim of minimizing the wastage of critical nutrients and in turn improving the cost-effectiveness of the process, experiment II was designed with manipulation of the nutrient feeding duration. Unlike nutrient feeding throughout the entire fermentation period of experiment I, in this experiment the substrate feeding was stopped at 28 h of cultivation. The net amount of glucose which remained unutilized at the end of experiment I was 82.9 g. Therefore, this 28 h time point to stop nutrient feeding was selected based on the calculation of time required to utilize 82.9 g of glucose from the end point of fermentation in experiment I. Hence, the nutrient feeding (both glucose and peptone) was carried out only up to 28 h after which the concentrations of glucose and peptone were not maintained. The cultivation conditions and sampling intervals were kept same as experiment I.

### 7.2.3 Combinatorial use of limitation and supplementation of metal ions in two-stage process engineering strategy with intermittent gas stripping

*Experiment III:* For the purpose of mitigating butanol toxicity mediated cessation of fermentation, the strategy developed in section 5.2.5 was coupled with intermittent product recovery. Intermittent gas stripping was started whenever the butanol concentration in the fermentation broth reached 10 g $L^{-1}$ and carried out for a period of 6 h. The cultivation conditions were kept same as detailed in section 5.2.5. The gas stripping procedure was followed similarly as described in section 7.2.2.

*Experiment IV:* Continual maintenance of nutrients in experiment I resulted in residual glucose and peptone concentration of approximately 80.7 g $L^{-1}$ and 48 g $L^{-1}$, respectively. Therefore, to reduce nutrient wastage at the end of fermentation and to improve process economics, the feeding was stopped at 54 h. The time point to stop feeding (54 h) was decided based on the time required to utilize approximately 80 g glucose from the end point of fermentation in experiment III. Rest of the experimental

and sampling conditions were kept same as experiment III.

### 7.2.4 Two-stage fed-batch fermentation with gas stripping using low cost substrates

With the aim of decreasing the raw material cost in experiment IV, the two-stage fed-batch fermentation was carried out using low cost substrates. Glucose and peptone were replaced with cheaper carbon and nitrogen sources i.e. rice straw hydrolyzate and instant dry yeast (IDY), respectively. Rice straw was procured from local farm in Amingaon district, Guwahati, Assam, India. Rice straw was cut, thoroughly washed, dried overnight, grinded to powdered form, and sieved to obtain uniform particle size (Kaushal et al., 2019). The powdered biomass was mixed with 100 mL 2% (w/v) NaOH solution at 5% solid-to-liquid loading and autoclaved at 121°C for 20 min. Alkaline pretreated biomass was allowed to cool down to room temperature and filtered through muslin cloth. Biomass residue after several cycles of thorough washing was dried overnight, grinded, and sieved (Kaushal et al., 2019). The powdered alkaline pretreated biomass was then mixed with 100 mL 1M $H_2SO_4$ solution (5% solid-to-liquid loading) and autoclaved at 121°C for 20 min. Further, filtration, washing, grinding, and sieving steps were employed similar to alkaline pretreatment. The sequential alkaline-acid pretreated biomass powder was hydrolyzed using a cocktail of two different cellulase enzymes C1 and C2 obtained from Aum enzymes, Gujarat, India and Sigma-Aldrich, USA, respectively (Kaushal et al., 2019). Enzyme hydrolysis using C1 (67.4 U per g biomass) and C2 (94.9 U per g biomass) cocktail was performed with 7.5% solid-to-liquid loading. Hydrolysis was carried out for 96 h at 50°C in a shaking incubator with 120 rpm (Kaushal et al., 2019). It is important to mention that the corresponding optimization and analysis experiments were undertaken by laboratory colleague and published in Kaushal et al. 2019 and do not form the part of this thesis. The optimized conditions were used to generate rice straw hydrolyzate which was then used to perform two-stage fed-batch fermentation experiment described further. The concentrated rice straw hydrolyzate contained 87 g $L^{-1}$ glucose. The two-stage fed batch fermentation, as detailed in Experiment IV, was carried out by replacing the carbon and nitrogen source with rice straw hydrolyzate and IDY, respectively, while maintaining the optimum equimolar carbon and nitrogen concentrations. Stage one was carried out using rice straw hydrolyzate, whereas during transfer to second stage, feed containing concentrated glucose solution along with rice straw hydrolyzate was used in order to prevent dilution of fermentation broth.

### 7.2.5 Analytical methods

Ten mL of fermentation broth was collected and centrifuged at 10,000×*g* for 10 min at 4°C. The pellet and supernatant subsequently obtained were used for estimation of concentration of biomass, substrates, and products as detailed in

section 5.2.6. All the experiments were conducted in triplicates and the values have been represented as mean ± standard error.

## 7.3 Results and discussion

### 7.3.1 Intermittent gas stripping coupled two-stage fed-batch process: without and with modulation of feeding duration

*Experiment I*: While the two-stage fed-batch process engineering strategy resulted in substantial improvement in butanol productivity when compared to batch fermentation, only 13.1 g $L^{-1}$ butanol titer was achieved which was a meager increase with respect to the batch process. This compromised butanol titer maybe attributed to solvent toxicity which resulted in cessation of metabolic activity of the cell. In order to mitigate solvent toxicity and to allow continued butanol biosynthesis, *in situ* product recovery strategy was coupled with two-stage fed-batch process to maintain the butanol concentration below critical level (Abdehagh et al., 2014; Ezeji et al., 2007). Gas stripping is a simple product recovery technique which serves the dual purpose of concentrating the product from fermentation broth and the continuous removal of product which in turn alleviates butanol toxicity. In the present study, coupling of two-stage fed-batch fermentation strategy with *in situ* gas stripping resulted in increased butanol production duration from 24 h to 98 h yielding a maximum cumulative butanol titer of 40.03 g $L^{-1}$ (Fig. 7.2A). This titer was three fold higher when compared to the two-stage fed-batch fermentation without gas stripping (13.1 g $L^{-1}$).

The current strategy also resulted in an improved biomass concentration of 8.7 g $L^{-1}$ ascertaining the fact that mitigation of butanol toxicity leads to prolonged growth along with continuous butanol biosynthesis (Fig. 7.2A). The total fermentation period involved seven gas stripping cycles with an individual duration of 6 h. However, after the third cycle onwards, the rate of butanol production decreased with every subsequent cycle resulting in reduction of overall butanol productivity from 0.55 g $L^{-1}$ $h^{-1}$ (without gas stripping) to 0.41 g $L^{-1}$ $h^{-1}$. It is important to note that cell growth along with the extent of butanol production reduced gradually after approx. 50 h of fermentation (3 cycles of gas stripping) followed by no significant change in butanol titer by the end of approx. 100 h of cultivation period in spite of sufficient amount of glucose in the broth (Fig. 7.2A). This may be attributed to several plausible reasons such as presence of non-productive dormant cells, accumulation of inhibitory compounds (Qureshi et al., 2008) or strain degeneration whereby upon prolonged cultivation microbial strains lose their ability to produce butanol (Jones and Woods, 1986). The key drawback of this strategy was that high amount of residual glucose and peptone remained unutilized at the end of the fermentation which might pose negative impact on economic viability of the process.

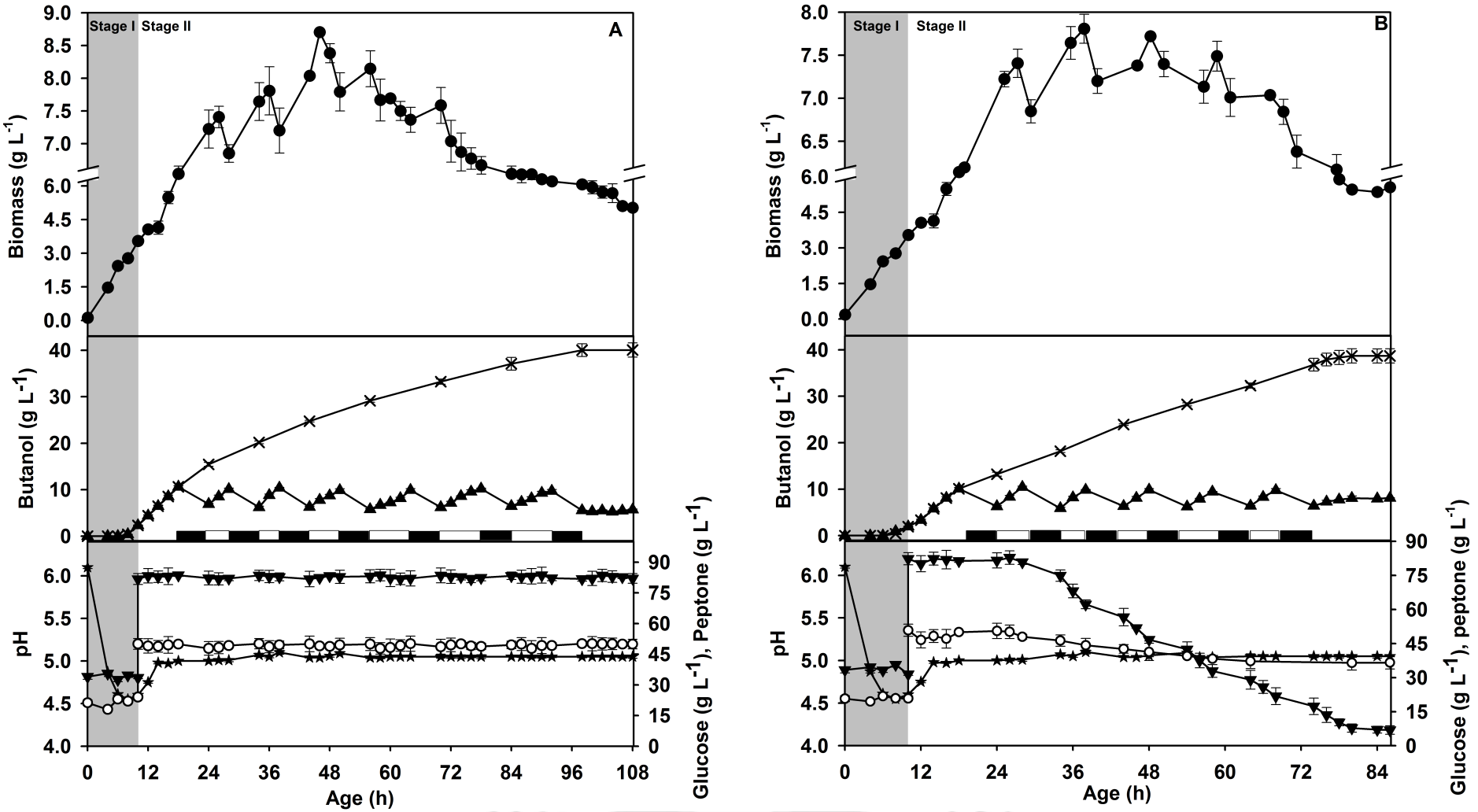



**Fig. 7.2.** Dynamic profile for biomass (•), butanol (▲), cumulative butanol (×), pH (★), glucose (▼) and peptone (○) when *C. acetobutylicum* MTCC 11274 was grown in two stage fed-batch fermentation integrated with intermittent gas stripping with (a) continued feeding (experiment I) and (b) modulated feeding (experiment I). The black bar on x axis represents gas stripping cycles with duration of 6 h each.

*Experiment II*: To minimize residual nutrient concentration at the end of experiment I, experiment II was performed where nutrient feeding was stopped at 28 h (Fig. 7.2B). Interestingly, only 7.76 g of residual glucose remained unutilized at the end of this experiment as opposed to 82.9 g of glucose in case of experiment I (Fig. 7.2B). Therefore, modulation of feeding duration in experiment II resulted in a significant reduction in wastage of residual glucose. Similarly, a reduced amount of 36 g of peptone remained unutilized at the end of the fermentation which was 26.5% lower than that obtained in experiment I (Fig. 7.2B). The cumulative butanol titer and productivity in experiment II remained comparable to experiment I. Hence, maintaining the prolonged solventogenic phase via integration of *in situ* product recovery and modulation of feeding duration offers economic improvement with minimization of unutilized nutrients.

### 7.3.2 Combinatorial use of limitation and supplementation of metal ions in two-stage process engineering strategy with intermittent gas stripping

*Experiment III*: Two-stage fed-batch strategy developed in experiment I resulted in 26% improved butanol titer compared to control fermentation (13.1 g $L^{-1}$) due to improved butanol tolerance. However, the titer could not be increased beyond 16.5 g $L^{-1}$ as the butanol tolerance limit extended up to 1.75% butanol (Fig. 7.3). Therefore, the process was coupled with intermittent gas stripping to mitigate the toxicity effects and accumulate higher butanol (Abdehagh et al., 2014 and Ezeji et al., 2007). Intermittent gas stripping cycle of 6 h duration was initiated whenever the butanol titer approached 10 g $L^{-1}$. Integration of the process strategy with gas stripping resulted in improved butanol titer of 52.6 g $L^{-1}$ which was 218.7% higher than the process without product recovery. The fermentation comprised of eight gas stripping cycles and the duration increased from 26 h to 110 h. Due to the presence of $CaCO_3$, more butanol was produced during the gas stripping cycle and thus resulted in highly concentrated condensate of approximately 200 g $L^{-1}$ with 560 g $L^{-1}$ butanol in the organic layer. Even in the presence of sufficient nutrients, $CaCO_3$ supplementation, and integration of gas stripping, the fermentation efficiency of the culture gradually declined. Decrease in biomass, butanol production, and glucose uptake was observed after 64 h which ultimately resulted in complete cessation at 110 h (Fig. 7.3A). This resulted in reduced overall butanol productivity of 0.48 g $L^{-1}$ $h^{-1}$ at the end of gas striping coupled fed-batch as compared to the process without gas stripping (Fig. 7.3A). This may be ascribed to low cell viability due to continuous exposure to fluctuating butanol concentration from 6-10 g $L^{-1}$ which is known to affect cell membrane stability and induce sporulation (Jones and Woods, 1986).

*Experiment IV*: To minimize residual nutrient concentration at the end of experiment III, experiment IV was performed where nutrient feeding was stopped at 54 h (Fig. 7.3B). At the end of the fermentation, 11.4 g of residual glucose remained

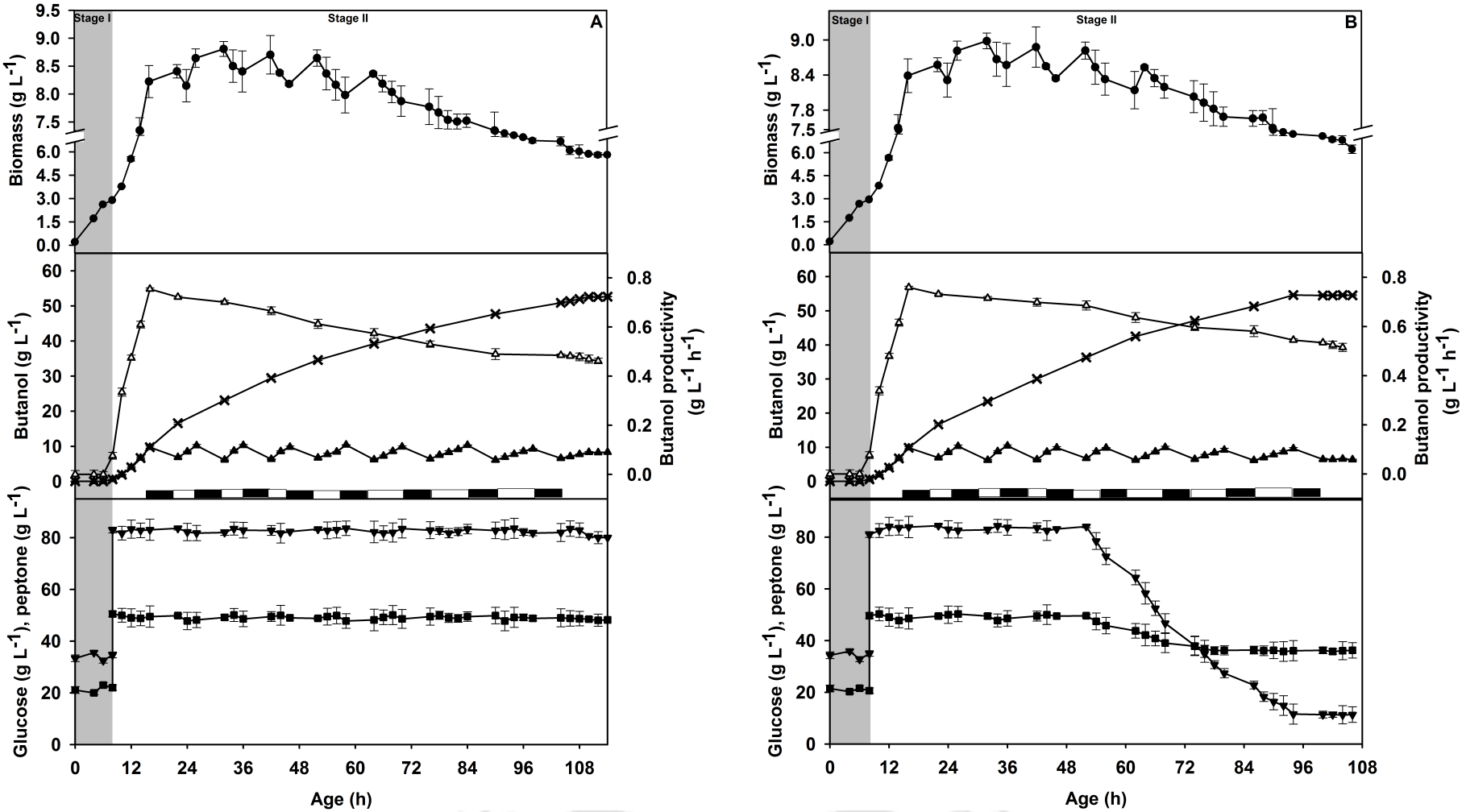



**Fig. 7.3.** Dynamic profile for biomass (•), butanol (▲), cumulative butanol (×), butanol productivity (△), glucose (▼) and peptone (■) when *C. acetobutylicum* MTCC 11274 was grown in two stage fed-batch mode of fermentation with combinatorial use of metal ions coupled to intermittent gas stripping with (a) continued feeding (experiment III) and (b) modulated feeding (experiment IV). The black bar on x axis represents gas stripping cycles with duration of 6 h each.

**Table 7.1.** Comparison of butanol production by different *Clostridium* sp. under fed-batch conditions with *in situ* gas stripping.

| Organism | Type[a] | Substrate | Mode of operation[b] | Recovery method[c] | Productivity ($g\ L^{-1}\ h^{-1}$) | Butanol Yield ($g\ g^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| *C. beijerinckii* BA101 | M | Glucose | Fed-batch | GS | 0.75 | 0.3 | Ezeji et al., 2004 |
| **_C. acetobutylicum_ MTCC 11274** | **WT** | **Glucose** | **Two-stage fed -batch[+]** | **Intermittent GS** | **0.58** | **0.25** | **This study** |
| *C. acetobutylicum* PJC4BK | E | Glucose | Fed-batch | GS | 0.55 | 0.19 | Lee et al., 2012 |
| **_C. acetobutylicum_ MTCC 11274** | **WT** | **Glucose** | **Two-stage fed-batch** | **Intermittent GS** | **0.48** | **0.25** | **This study** |
| *C. acetobutylicum* ATCC 824 | WT | Glucose | Fed-batch | *in situ* extraction-GS-FC | 0.38 | 0.27 | Lu et al., 2016 |
| *C. acetobutylicum* ABE1401 | M | Glucose | Fed-batch | Continuous GS | 0.4 | 0.23 | Cai et al., 2016a |
| *C. acetobutylicum* B3 | M | Glucose | Fed-batch | Permeating-heating-GS | 0.38 | 0.23 | Chen et al., 2014 |
| *C. acetobutylicum* JB200 | M | Glucose | Fed-batch (FBB) | Continuous GS | 0.24 | 0.27 | Xue et al., 2014 |

[a]WT-Wild Type; M-Mutant; E-Engineered; [b]FBB-Fibrous bed bioreactor; [c]GS-Gas stripping, FC-fractional condensation; [+]Two-stage fed batch with feeding duration modulation

unutilized as compared to 80.7 g of glucose in case of experiment III (Fig. 7.3A and B). Therefore, modulation of feeding duration in experiment III resulted in a significant reduction in wastage of residual glucose. Similarly, a reduced amount of 36.2 g of peptone remained unutilized at the end of the fermentation which was 24.8% lower than that obtained in experiment III (Fig. 7.3 A and B). The cumulative butanol titer (54.2 g $L^{-1}$) and productivity (0.58 g $L^{-1}$ $h^{-1}$) in experiment IV improved in comparison to experiment III. With the few exceptions, the overall butanol productivity was comparable to other studies as shown in Table 7.1.

### 7.3.3 Two-stage fed-batch fermentation with gas stripping using low cost substrates

Further, with the aim of decreasing the raw material cost, the two-stage fed-batch fermentation was carried out using low cost substrates. Glucose and peptone were replaced with cheaper carbon and nitrogen sources i.e. rice straw hydrolyzate

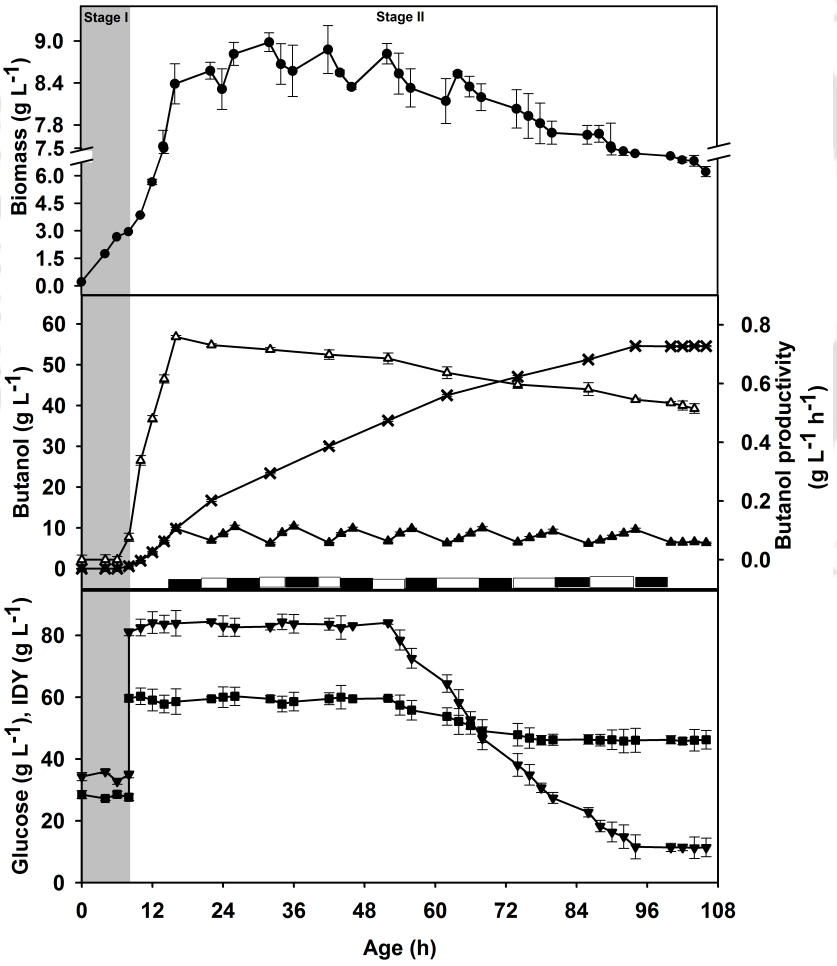



**Fig. 7.4.** Dynamic profile for biomass (●), butanol (▲), cumulative butanol (×), butanol productivity (△), glucose (▼) and IDY (■) when *C. acetobutylicum* MTCC 11274 was grown in two stage fed-batch mode of fermentation with combinatorial use of metal ions coupled to intermittent gas stripping with modulated feeding using low cost substrates. The black bar on x axis represents gas stripping cycles with duration of 6 h each.

**Table 7.2.** 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 | Type[a] | Feedstock | Butanol productivity ($g\ L^{-1}\ h^{-1}$) | Butanol titer ($g\ L^{-1}$) | Butanol Yield ($g\ g^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| *C. acetobutylicum* ABE 1401 | M | Sweet sorghum bagasse | 0.63 | 101.3 | 0.23 | Cai et al., 2015 |
| **_C. acetobutylicum_ MTCC 11274** | **WT** | **Rice straw + Glucose** | **0.58** | **54.2** | **0.25** | **This study** |
| *C. beijerinckii* BA101 | M | Saccharified liquefied cornstarch (SLCS) | 0.41 | 56.2 | 0.25 | Ezeji et al., 2007 |
| *C. acetobutylicum* ABE 1201 | M | Sweet sorghum juice | 0.36 | 112.9 | 0.28 | Cai et al., 2016b |
| *C. acetobutylicum* JB200 | A | Cassava bagasse | 0.35 | 59.81 | 0.25 | Lu et al., 2012 |
| *C. acetobutylicum* strain JB200 | A | Cassava bagasse | 0.29 | 76.44 | 0.23 | Lu et al., 2012 |
| *C. acetobutylicum* ABE 1401 | M | Sweet sorghum bagasse | 0.23 | 84.94 | 0.2 | Cai et al., 2015 |
| *C. acetobutylicum* DSM 792 | WT | Sugarcane-sweet sorghum juices | 0.13 | 18.6 | 0.16 | Rochón et al., 2017 |
| *C. acetobutylicum* ABE-P 1201 | M | Corn stover | 0.09 | 18.6 | 0.19 | Cai et al., 2017 |

[a]WT-Wild Type; M-Mutant; A-Adapted;

and instant dry yeast (IDY), respectively. However, due to low glucose concentration in rice straw hydrolyzate (87 g $L^{-1}$), the adjustment of medium components according to butanol productivity medium would have resulted in approximately 50% dilution of the fermentation broth and loss of active cells. Therefore, glucose was partially replaced with rice straw hydrolyzate so as to maintain highly concentrated feed. A cumulative butanol titer of 54.2 g $L^{-1}$ and overall butanol productivity of 0.58 g $L^{-1}$ $h^{-1}$ were obtained (Fig. 7.4). The butanol titer, yield, and productivity were found to be comparable with other studies when fed batch with gas stripping was performed on low cost substrates (Table 7.2). The experiment resulted in 40% and 100% replacement of carbon and nitrogen source, respectively, with cheaper alternatives. It can be safely put forth that using a highly concentrated sugar hydrolyzate may achieve complete replacement of glucose while maintaining the kinetics of the process. This exhibits the potential of the strategy in developing a suitable bioprocess for butanol production. Fig. 7.5 shows a comparison of butanol titer and productivity data from various two-stage fed-batch fermentations performed depicting stepwise improvement in the process kinetics after employing different nutritional and operational strategy. Hence, maintaining prolonged solventogenic phase via integration of *in situ* product recovery and modulation of feeding duration offers economic improvement with minimization of unutilized nutrients. This exemplifies the potential of the presented strategy in developing an economically feasible process for butanol production.

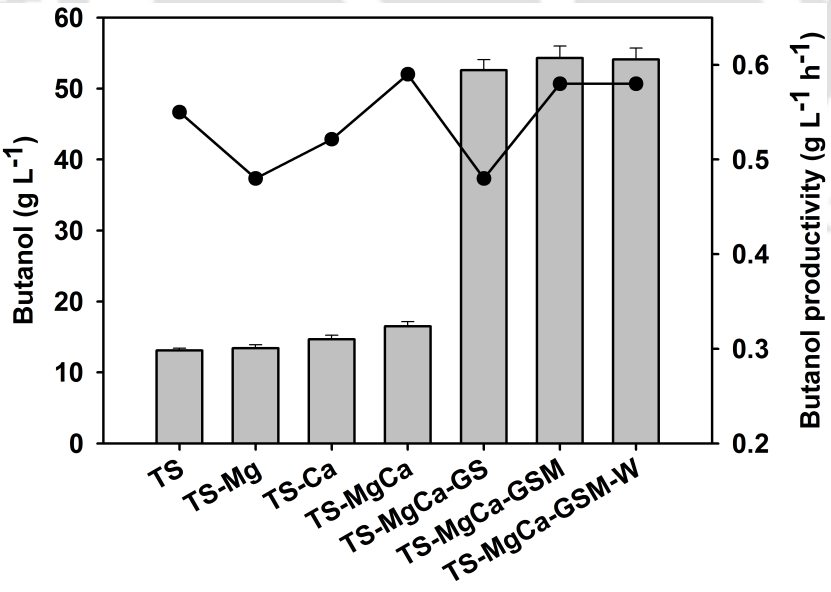



**Fig. 7.5.** Comparison of butanol titer (grey bar) and productivity (black circle, •) in different two-stage fed-batch fermentations. Means that share the same letter do not differ significantly at the 95 confidence level based on the Tukey mean comparison method. (TS-Two-stage fed-batch fermentation; Mg-Magnesium limitation; Ca-$CaCO_3$ supplementation; MgCa-combinatorial $MgSO_4$ limitation and $CaCO_3$ supplementation; GS-Gas stripping; GSM-Gas stripping with modulated feeding duration; W-low cost substrates)

## 7.4 Conclusion

Integration of two-stage fed-batch process with gas stripping, alleviated butanol toxicity and resulted in prolonged fermentation duration with a cumulative butanol titer of 40.3 g $L^{-1}$ and productivity of 0.41 g $L^{-1}$ $h^{-1}$. In order to improve process economics, the feeding duration was modulated resulting in minimal residual nutrients with uncompromised butanol titer (38.68 g $L^{-1}$) and productivity (0.48 g $L^{-1}$ $h^{-1}$). Subsequent coupling of the strategy using combinatorial metal ion limitation and supplementation, with intermittent gas stripping and modulation of feeding duration resulted in maximum cumulative butanol titer of 54.3 g $L^{-1}$, productivity of 0.58 g $L^{-1}$ $h^{-1}$, and minimal residual nutrients. Finally, in order to render enhanced economic feasibility, the process engineering strategy was demonstrated on cheaper substrate combination of rice straw hydrolyzate and instant dry yeast which resulted in a comparable butanol titer and productivity. It can be safely put forth that the strategy is universal in approach and could result in higher productivities when used with a hyper-producer or butanol tolerant strains.

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*"Two roads diverged in a wood, and I – I took the one less traveled by, and that has made all the difference."*

Robert Frost (1874-1963)
American poet

# 8

# Conclusions

Biological production of butanol is faced with bottlenecks such as low butanol titer, productivity, solvent toxicity, lack of understanding of clostridial metabolism and process control, which have hindered its sustainability. In order to address these bottlenecks, biochemical engineering and metabolic modeling approaches were employed to characterize *C. acetobutylicum* MTCC 11274 in detail and to achieve high butanol productivity which is a desired characteristic while designing a large scale production process. Six *C. acetobutylicum* strains were procured from different culture collection centers and screened for butanol production. *C. acetobutylicum* MTCC 11274 showed highest butanol titer of 11.5 g $L^{-1}$ and was selected for further experiments. Screening of suitable carbon and nitrogen sources resulted in the identification of glucose and peptone, respectively, as the most appropriate growth promoting and butanol producing sources. Marginal improvement in butanol titer after carbon and nitrogen screening actuated the need to study the butanol tolerance limit of the strain. The final butanol titer was limited by butanol toxicity; therefore, statistical media optimization was carried out with the objective functions: (i) maximization of biomass productivity and (ii) maximization of butanol productivity. Maximum biomass productivity and butanol productivity obtained after optimization were 103.5% and 63.6% higher than achieved with unoptimized media.

Serial enrichment of butanol tolerance was carried out along with chemical mutagenesis to improve the tolerance limit of the strain. Phase one of serial adaptation was started with exposure of the cells to 0.25% (w/v) butanol and incubating at 37°C for 24 h. This constituted one adaptation cycle and was carried out in the similar way till the stressed cells showed growth similar to control (unstressed cells). Adaptation to 1.0% (w/v) butanol was carried out for 18 cycles; however, no further improvement was achieved in terms of specific growth rate. UV, EMS

and NTG were chosen as mutagens for random mutagenesis experiments for strain improvement. UV mutagenesis was unable to generate any probable solvent tolerant strains. Chemical mutagenesis yielded a number of tentative solvent tolerant strains which were screened and characterized to obtain high butanol producer mutant strain. The eight strains (E1-E8) selected after mutagenesis using EMS on the basis of improved butanol tolerance and the strain (M1) selected after mutagenesis using NTG were characterized in batch fermentation to compare their growth, butanol titer, and butanol productivity in comparison to the wild type (WT) strain MTCC 11274. Maximum butanol titer of 12.85 g $L^{-1}$ was obtained by M1 (NTG mutant) which was similar to WT (12.3 g $L^{-1}$). EMS and NTG treatments were repeated to get improved strains; however, repeated trials did not yield any desirable strains. Therefore, further experiments were carried out using the wild type strain MTCC 11274.

Based on the results obtained from the batch fermentation experiments, a two stage fed-batch process engineering strategy was developed with the aim of improving the volumetric rate of butanol production. The process resulted in an overall productivity of 0.55 g $L^{-1}$ $h^{-1}$ that is highest for fed-batch fermentation without recovery. Two stage fed batch strategy with half concentration of $MgSO_4$ as compared to original resulted in improved titer of 13.41 g $L^{-1}$. Supplementation of $CaCO_3$ in two stage fed batch strategy resulted in butanol titer of 14.66 g $L^{-1}$. Butanol challenge experiments revealed that improved titer was attributed to increased butanol tolerance of the strain in presence of $CaCO_3$. The process was further improved by combinatorial use of limitation and supplementation of $MgSO_4$ and $CaCO_3$, respectively, resulting in butanol titer of 16.5 g $L^{-1}$ and overall productivity of 0.59 g $L^{-1}$ $h^{-1}$. Subsequent coupling of the strategy with intermittent gas stripping and modulation of feeding duration resulted in maximum cumulative butanol titer of 54.3 g $L^{-1}$, productivity of 0.58 g $L^{-1}$ $h^{-1}$, and minimal residual nutrients. Finally, 40% glucose and 100% peptone were replaced with rice straw hydrolysate and instant dry yeast, respectively, to reduce the substrate cost.

Characterization of the strain under different operational conditions and nutritional modulations revealed metabolic versatility during acidogenic and solventogenic phase. Flux balance analysis was performed in order to understand the mechanism governing this metabolic flexibility. Elevated ATP demand due to improved growth was satisfied by upregulated carbon flux towards butyric acid synthesis which in turn aided early induction of solventogenesis during magnesium limited two-stage fed batch fermentation. Continued higher carbon flux towards growth concomitant to butanol pathways and lower NGA maintenance energy may be attributed to upregulation of heat shock proteins, DNA synthesis, transcription, repair, carbohydrate catabolic enzymes, and stabilizing membrane proteins during

calcium supplemented two-stage fed-batch fermentation. Pathways related to biomass and butanol synthesis remained concomitantly active during acidogenesis and solventogenesis under combinatorial usage of magnesium limitation-calcium supplementation in two-stage fed-batch fermentation.

# Engineering Significance

Butanol production from fermentative route remains unachievable in terms of sustainability and economic feasibility. The present study aimed to address the key bottlenecks by employing following engineering interventions resulting in significantly improved butanol titer and productivity.

- Detailed characterization of *C. acetobutylicum* MTCC 11274 resulted in optimization of two different concentrations of media components for achieving maximum biomass productivity and butanol productivity. Taking cue from this, a two-stage fed-batch process was designed with sequential utilization of optimized biomass productivity medium followed by optimized butanol productivity medium.
- Combinatorial use of magnesium sulphate limitation and calcium carbonate supplementation was incorporated into the strategy which further improved the performance of *C. acetobutylicum* MTCC 11274 in the two-stage fed batch process.
- Flux balance analysis aided in understanding the effect of nutrient modulation on the metabolism of the organism during growth and butanol biosynthesis.
- Integration of the two-stage fed batch strategy with *in situ* product recovery process aided in mitigation of solvent toxicity, thereby resulting in high cumulative butanol titer. Modulation of feeding duration resulted in minimizing the amount of residual nutrients at the end of fermentation. Finally, replacement of carbon and nitrogen sources with cheaper alternatives substantiated the potential of the strategy for large scale production.

# Future Prospects

- Assessment of wild type strain *Clostridium acetobutylicum* MTCC 11274 performance at pilot scale and for biobutanol production in continuous fermentation.
- Application of metabolic engineering, such as overexpression of major stress protein genes including *groES*, *dnaKJ*, *hsp18*, and *hsp90*, for development of butanol tolerant strain.
- Screening experiments to evaluate tolerance of *Clostridium acetobutylicum* MTCC 11274 towards toxicity arising from chemicals produced during pretreatment.
- Production of biobutanol from the concentrated hydrolyzate obtained from low cost renewable substrates.
- Genome scale modeling and dynamic flux balance analysis of clostridial metabolism.
- Life cycle analysis of the developed process to assess environmental effects from cradle to grave.

# Appendix

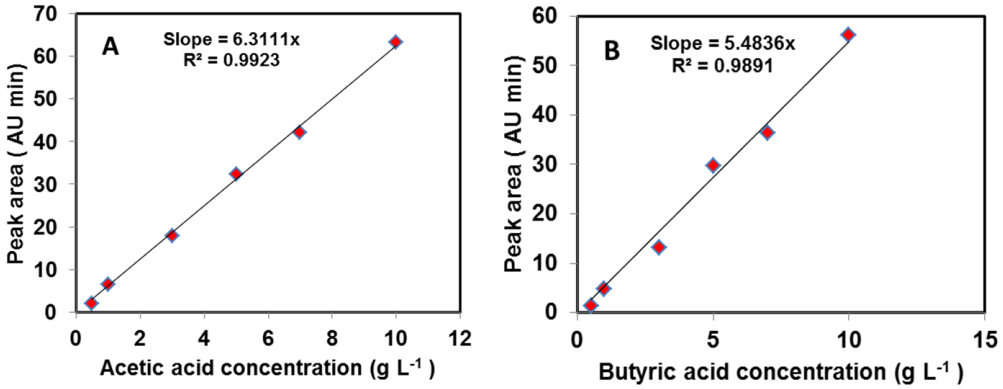


**Fig. A1** Graphical representation of correlation between concentration of acids and their respective absorbance in HPLC

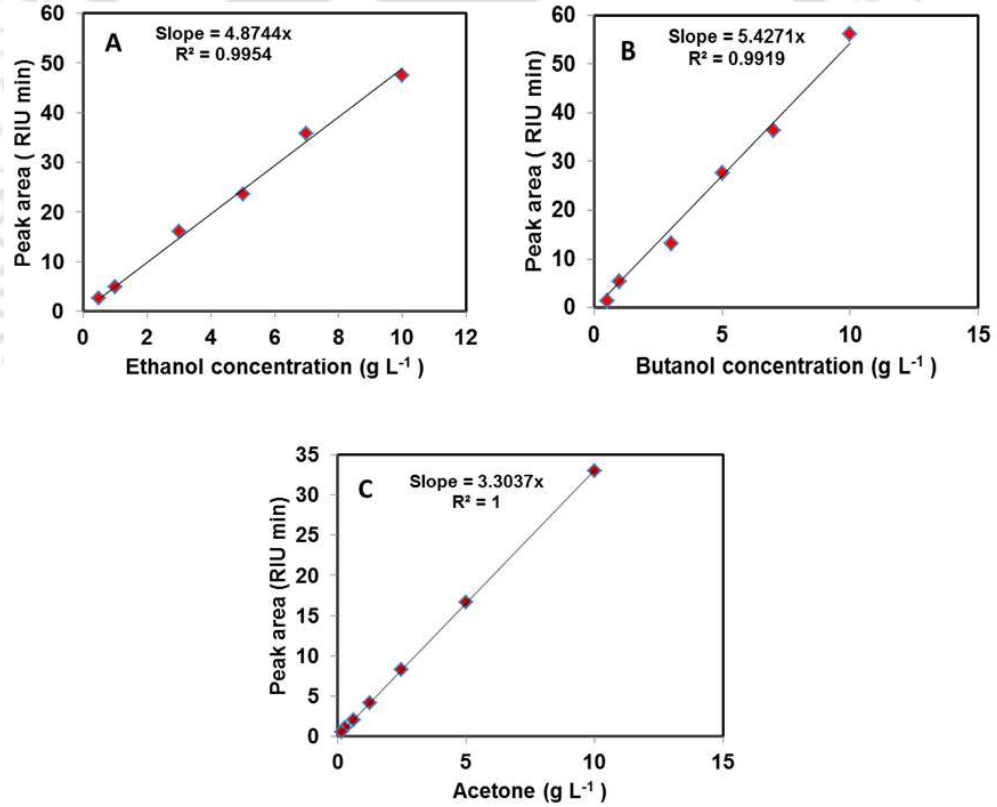


**Fig. A2** Graphical representation of correlation between concentration of solvents and their respective absorbance in HPLC

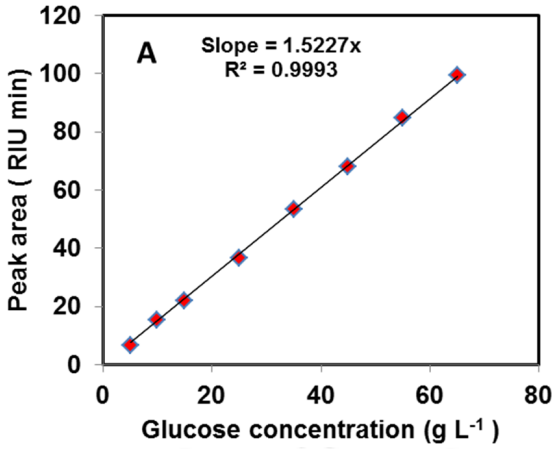


**Fig. A3** Graphical representation of correlation between concentration of glucose and its respective absorbance in HPLC

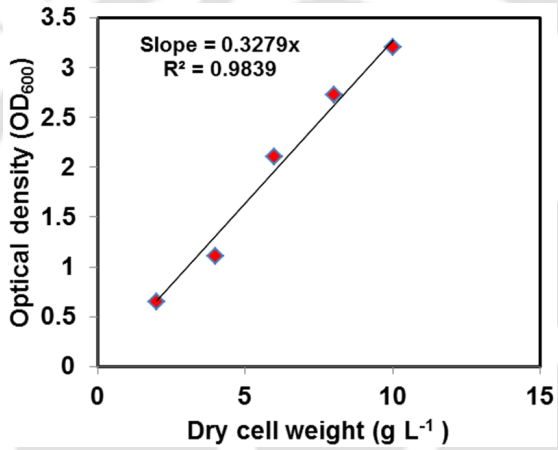


**Fig. A4** Graphical representation of correlation between the dry cell weight and absorbance measured at 600 nm in a spectrophotometer

**Table A1** Macromolecular composition of biomass

| Macromolecular composition | |
|---|---|
| **Component** | **Proportion (% g $g^{-1}$)** |
| Protein | 50 |
| Carbohydrate | 2 |
| RNA | 7 |
| DNA | 1 |
| **Cell wall component** | |
| Peptidoglycan | 21 |
| Lipids | 12 |
| Lipoteichoic Acid | 6 |

| Protein composition | | | |
|---|---|---|---|
| **Amino acids** | **Mole fraction** | **Mol. weight (g $mol^{-1}$)** | **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 $mol^{-1}$)** | **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 $mol^{-1}$)** | **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 $mol^{-1}$)** | **Avg. weight (g)** |
| 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. acetobutylicum* MTCC 11274: List of reactions involved in the central metabolism.

| *S. No.* | *EC Number* | *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 | 3.1.3.11 | fructose-bisphosphatase | | 'FBP --> GLP + GAP' |
| 6 | 4.1.2.13 | fructose-bisphosphate aldolase | | 'GLP + GAP --> FBP' |
| 7 | 4.1.2.13 | fructose-bisphosphate aldolase | | '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 | 2.7.9.2 | phosphoenolpyruvate synthase | **Pyruvate metabolism** | 'Pyr + ATP --> PEP + AMP + Pi' |
| 20 | 1.1.1.38 | malate dehydrogenase | | 'Pyr + CO2 + NADH --> Mal + NAD' |
| 21 | 1.1.1.38 | malate dehydrogenase | | 'Mal + NAD --> Pyr + CO2 + NADH' |
| 22 | 1.2.7.1 | pyruvate:ferredoxin oxidoreductase | | 'Pyr + CoA + OFRD --> ACCA + CO2 + RFRD' |
| 23 | 2.3.1.9 | acetyl-CoA C-acetyltransferase | **Acidogenesis** | '2 ACCA --> CoA + AACTLCoA' |

| | | | | |
|---|---|---|---|---|
| 24 | 2.3.1.9 | acetyl-CoA C-acetyltransferase | | 'CoA + AACTLCoA --> 2 ACCA' |
| 25 | 1.1.1.157 | 3-hydroxyacyl-CoA dehydrogenase | | 'AACTLCoA + NADH --> HBCoA + NAD' |
| 26 | 4.2.1.17 | enoyl-CoA hydratase | | 'HBCoA --> CRTCoA' |
| 27 | 1.3.8.1 | butyryl-CoA dehydrogenase | | 'CRTCoA + NADH --> BUCoA + NAD' |
| 28 | 2.3.1.19 | butyryl transferase | | 'BUCoA + Pi --> BUP + CoA' |
| 29 | 2.3.1.19 | butyryl transferase | | 'BUP + CoA--> Pi + BUCoA' |
| 30 | 2.7.2.7 | butyrate kinase | | 'BUP + ADP --> BU + ATP' |
| 31 | 2.7.2.7 | butyrate kinase | | 'BU + ATP --> BUP + ADP' |
| 32 | 2.3.1.8 | phosphate acetyltransferase | | 'ACCA + Pi --> ACTP + CoA' |
| 33 | 2.3.1.8 | phosphate acetyltransferase | | 'ACTP + CoA --> ACCA + Pi' |
| 34 | 2.7.2.1 | acetate kinase | | 'ACTP + ADP --> AC + ATP' |
| 35 | 2.7.2.1 | acetate kinase | | 'ATP + AC --> ACTP + ADP' |
| 36 | 1.2.1.10 | aldehyde dehydrogenase | **Solventogenesis** | 'BUCoA + NADH --> NAD + BUAL + CoA' |
| 37 | 2.8.3.9 | butyrate-acetoacetate CoA transferase | | BU + AACTLCoA --> ACTAC + BUCoA' |
| 38 | 1.1.1.1 | alcohol dehydrogenase | | 'BUAL + NADH --> BUOL + NAD' |
| 39 | 1.1.1.1 | alcohol dehydrogenase | | 'BUOL + NAD --> BUAL + NADH' |
| 40 | 2.8.3.8 | CoA-transferases | | 'AC + AACTLCoA --> ACTAC + ACCA' |
| 41 | 4.1.1.4 | acetoacetate decarboxylase | | 'ACTAC --> ACETONE + CO2' |
| 42 | 1.2.1.10 | aldehyde dehydrogenase | | 'ACCA + NADH --> ACAL + CoA + NAD' |
| 43 | 1.2.1.10 | aldehyde dehydrogenase | | 'ACAL + CoA + NAD --> ACCA + NADH' |
| 44 | 1.1.1.1 | alcohol dehydrogenase | | 'ACAL + NADH --> ETOH + NAD' |
| 45 | 1.1.1.1 | alcohol dehydrogenase | | 'ETOH + NAD --> ACAL + NADH' |
| 46 | 6.4.1.1 | pyruvate carboxylase | **TCA cycle** | 'Pyr + ATP + HCO3- --> ADP + Pi + OXA' |
| 47 | 1.1.1.37 | (S)-malate:NAD+ oxidoreductase | | 'OXA + NADH --> Mal + NAD' |
| 48 | 4.2.1.2 | fumarate hydratase | | 'Mal --> Fum' |
| 49 | 4.2.1.2 | fumarate hydratase | | 'Fum --> Mal' |

| | | | | |
|---|---|---|---|---|
| 50 | 1.3.5.4 | fumarate reductase | | 'Fum + RFRD --> OFRD + Suc' |
| 51 | 2.3.3.3 | citric-(Re)- synthase | | 'OXA + ACCA --> Cit + CoA' |
| 52 | 4.2.1.3 | aconitate hydratase | | 'Cit --> iCit' |
| 53 | 4.2.1.3 | aconitate hydratase | | 'iCit --> Cit' |
| 54 | 1.1.1.41 | isocitrate dehydrogenase | | 'iCit + NAD --> AKG + NADH + CO2' |
| 55 | 1.2.7.3 | 2-oxoacid oxidoreductase (ferredoxin) | | 'AKG + CoA + OFRD --> RFRD + SCOA + CO2' |
| 56 | 6.2.1.5 | succinyl-coenzyme A synthetase | | 'SCOA + ADP + Pi --> Suc + CoA + ATP' |
| 57 | 6.2.1.5 | succinyl-coenzyme A synthetase | | 'Suc + CoA + ATP --> SCOA + ADP + Pi' |
| 58 | 5.1.3.1 | Ribulose phosphate 3- epimerase | **Pentose phospate pathway** | 'RL5P --> X5P' |
| 59 | 5.1.3.1 | Ribulose phosphate 3- epimerase | | 'X5P --> RL5P' |
| 60 | 5.3.1.6 | Ribose 5 phosphote isomerase | | 'R5P --> RL5P' |
| 61 | 5.3.1.6 | Ribose 5 phosphote isomerase | | 'RL5P --> R5P' |
| 62 | 2.2.1.1 | Transketolase | | 'R5P + X5P --> S7P + GAP' |
| 63 | 2.2.1.1 | Transketolase | | 'S7P + GAP --> R5P + X5P' |
| 64 | 2.2.1.2 | Transaldolase | | 'GAP + S7P --> F6P + E4P' |
| 65 | 2.2.1.2 | Transaldolase | | 'F6P + E4P --> GAP + S7P' |
| 66 | 2.2.1.1 | Transketolase | | 'F6P + GAP --> E4P + X5P' |
| 67 | 2.2.1.1 | Transketolase | | 'X5P + E4P --> F6P + GAP' |
| 68 | 2.7.6.1 | ribose-phosphate diphosphokinase | | 'ATP + R5P --> AMP + PRPP' |
| 69 | 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, 4.3.2.2 | Lumped reaction | **Nucleicacid biosynthesis** | '2 Gln + PRPP + 4 ATP + Gly + fTHF + HCO3- + Asp --> AICAR + 2 Glu + 6 Pi + 4 ADP + THF + Fum' |
| 70 | 2.1.2.3 | AICAR transformylase | | 'AICAR + fTHF --> FPRICA + THF' |
| 71 | 3.5.4.10 | IMP cyclohydrolase | | 'FPRICA --> IMP' |
| 72 | 3.5.4.10 | IMP cyclohydrolase | | 'IMP --> FPRICA' |
| 73 | 1.1.1.205 | IMP dehydrogenase | | 'IMP + NAD --> XMP + NADH' |

| | | | | |
|---|---|---|---|---|
| 74 | 6.3.5.2 | GMP synthase | | 'ATP + XMP + Gln --> AMP + 2 Pi + GMP + Glu' |
| 75 | 2.7.4.8 | guanylate kinase | | 'ATP + GMP --> ADP + GDP' |
| 76 | 2.7.4.8 | guanylate kinase | | 'ADP + GDP --> ATP + GMP' |
| 77 | 2.7.1.40 | pyruvate kinase | | 'GDP + PEP --> GTP + Pyr' |
| 78 | 2.7.1.40 | pyruvate kinase | | 'GTP + Pyr --> GDP + PEP' |
| 79 | 1.17.4.2 | ribonucleoside-triphosphate reductase | | 'GTP + RTRD --> dGTP + OTRD' |
| 80 | 6.3.4.4 | adenylosuccinate synthase | | 'IMP + GTP + Asp --> GDP + Pi + Fum + AMP' |
| 81 | 2.7.4.3 | adenylate kinase | | 'ATP + AMP --> 2 ADP' |
| 82 | 2.7.4.3 | adenylate kinase | | '2 ADP --> ATP + AMP' |
| 83 | 1.17.4.2 | ribonucleoside-triphosphate reductase | | 'ATP + RTRD --> dATP + OTRD' |
| 84 | 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' |
| 85 | 2.7.4.22 | UMP kinase | | 'ATP + UMP --> ADP + UDP' |
| 86 | 2.7.4.22 | UMP kinase | | 'ADP + UDP --> ATP + UMP' |
| 87 | 2.7.4.6 | nucleoside-diphosphate kinase | | 'ATP + UDP --> ADP + UTP' |
| 88 | 2.7.4.6 | nucleoside-diphosphate kinase | | 'ADP + UTP --> ATP + UDP' |
| 89 | 6.3.4.2 | CTP synthase | | 'ATP + UTP + Gln --> ADP + Pi + CTP + Glu' |
| 90 | 3.5.4.13 | CTP deaminase | | 'CTP --> UTP + NH3' |
| 91 | 2.7.4.14 | CMP kinase | | 'ATP + CMP --> ADP + CDP' |
| 92 | 2.7.4.14 | CMP kinase | | 'ADP + CDP --> ATP + CMP' |
| 93 | 2.7.4.6 | nucleoside-diphosphate kinase | | 'CDP + ATP --> CTP + ADP' |
| 94 | 2.7.4.6 | nucleoside-diphosphate kinase | | 'CTP + ADP --> CDP + ATP' |
| 95 | 3.5.4.13 | dCTP deaminase | | 'dCTP --> dUTP + NH3' |
| 96 | 3.6.1.23 | dUTP diphosphatase | | 'dUTP --> dUMP + 2 Pi' |
| 97 | 2.1.1.45 | thymidylate synthase | | 'MLTHF + dUMP --> DHF + dTMP' |
| 98 | 2.7.4.9 | dTMP kinase | | 'ATP + dTMP --> ADP + dTDP' |
| 99 | 2.7.4.6 | nucleoside-diphosphate kinase | | 'dTDP + ATP --> dTTP + ADP' |

| | | | | |
|---|---|---|---|---|
| 100 | 1.17.4.2 | ribonucleoside-triphosphate reductase | | 'CTP + RTRD --> dCTP + OTRD' |
| 101 | 6.3.1.2 | glutamine synthetase | **Aminoacid biosynthesis** | 'Glu + NH3 + ATP --> Gln + ADP + Pi' |
| 102 | 1.4.1.13 | glutamate synthase | | 'Gln + AKG + NADPH --> 2 Glu + NADP' |
| 103 | 2.6.1.1 | aspartate transaminase | | 'OXA + Glu --> Asp + AKG' |
| 104 | 2.6.1.1 | aspartate transaminase | | 'AKG + Asp --> OXA + Glu' |
| 105 | 6.3.5.5 | carbamoyl-phosphate synthase | | 'Gln + HCO3- + 2 ATP --> Glu + CAP + 2 ADP + Pi' |
| 106 | 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' |
| 107 | 4.3.1.17 | L-serine ammonia-lyase | | 'Pyr + NH3 --> Ser' |
| 108 | 4.3.1.17 | L-serine ammonia-lyase | | 'Ser --> Pyr + NH3' |
| 109 | 2.1.2.1 | glycine hydroxymethyltransferase | | 'Ser + THF --> Gly + MLTHF' |
| 110 | 2.1.2.1 | glycine hydroxymethyltransferase | | 'Gly + MLTHF --> Ser + THF' |
| 111 | 2.3.1.30 | serine O-acetyltransferase | | 'ACCA + Ser --> CoA + OAS' |
| 112 | 2.5.1.47 | cysteine synthase | | 'SO4 + ATP + RTRD --> SO3 + AMP + 2 Pi + OTRD' |
| 113 | 2.7.7.4,1.8.99.2 | Lumped reaction | | 'SO3 + 3 NADPH --> H2S + 3 NADP' |
| 114 | 1.8.1.2 | hydrogen-sulfide:NADP+ oxidoreductase | | 'OAS + H2S --> Cys + AC' |
| 115 | 2.6.1.2 | alanine transaminase | | 'Pyr + Glu --> Ala + AKG' |
| 116 | 6.3.5.4 | asparagine synthase | | 'Gln + Asp + ATP --> Asn + AMP + 2 Pi + Glu' |
| 117 | 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' |
| 118 | 2.7.2.4, 1.2.1.11, 1.1.1.3 | Lumped reaction | | 'Asp + ATP + 2 NADPH --> HSer + ADP + 2 NADP + Pi' |
| 119 | 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' |
| 120 | 2.7.1.39, 4.2.3.1 | Homoserine kinase, threonine synthase | | 'HSer + ATP --> Thr + ADP + Pi' |
| 121 | 4.1.2.48 | low-specificity L-threonine aldolase | | 'Thr --> Gly + ACAL' |
| 122 | 4.1.2.48 | low-specificity L-threonine aldolase | | 'Gly + ACAL --> Thr' |

| | | | | |
|---|---|---|---|---|
| 123 | 2.7.2.11,1.2.1.41,1.5.1.2 | Lumped reaction | | 'Glu + ATP + 2 NADPH --> Pro + ADP + 2 NADP + Pi' |
| 124 | 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' |
| 125 | 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' |
| 126 | 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' |
| 127 | 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' |
| 128 | 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' |
| 129 | 5.4.99.5, 1.3.1.12 , 2.6.1.1 | Lumped reaction | | 'Chr + NAD + Glu --> Tyr + AKG + NADH + CO2' |
| 130 | 5.4.99.5, 4.2.1.91,2.6.1.1 | Lumped reaction | | 'Chr + Glu --> Phe + AKG + CO2' |
| 131 | 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' |
| 132 | 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' |
| 133 | 2.6.1.16 | glucosamine 6-phosphate synthase | **Aminosugar biosynthesis** | 'Gln + F6P --> Glu + GS6P' |
| 134 | 5.4.2.10 | phosphoglucosamine mutase | | 'GS1P --> GS6P' |
| 135 | 5.4.2.10 | phosphoglucosamine mutase | | 'GS6P --> GS1P' |
| 136 | 2.3.1.157 | glucosamine-1-phosphate N-acetyltransferase | | 'ACCA + GS1P --> CoA + NAG1P' |
| 137 | 2.7.7.23 | UDP-N-acetylglucosamine diphosphorylase | | 'UTP + NAG1P --> UDPNAG + 2 Pi' |
| 138 | 2.5.1.7 | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | | 'PEP + UDPNAG --> UDPNACG + Pi' |
| 139 | 1.3.1.98 | UDP-N-acetylmuramate dehydrogenase | | 'UDPNACG + NADPH --> UDPNAM + NADP' |
| 140 | 6.3.2.8 | UDP-N-acetylmuramate-L-alanine ligase | | 'ATP + UDPNAM + Ala --> ADP + Pi + UDPNAMA' |
| 141 | 6.3.2.9 | D-glutamate ligase | | 'UDPNAMA + Glu + ATP --> ADP + Pi + UDPNAMAG' |

| | | | | |
|---|---|---|---|---|
| 142 | 6.3.2.4 | D-alanine-D- alanine Ligase | | 'ATP + 2 Ala --> ADP + Pi + DALDAL' |
| 143 | 5.4.2.2 | Phosphoglucomutase | **Carbohydrate biosynthesis** | 'G6P --> G1P' |
| 144 | 5.4.2.2 | Phosphoglucomutase | | 'G1P --> G6P' |
| 145 | 2.7.7.27, 2.4.1.21, 2.4.1.18 | Lumped reaction | | 'G1P + ATP --> Carbo + ADP + 2 Pi' |
| 146 | 6.4.1.2 | acetyl-CoA carboxylase | **Fattyacid biosynthesis** | 'ATP + HCO3- + ACCA --> ADP + Pi + MLCA' |
| 147 | 2.3.1.38 | [ACP]acetyltransferase | | 'ACCA + ACP --> CoA + AACP' |
| 148 | 2.3.1.39 | malonyl transacylase | | 'MLCA + ACP --> CoA + MLACP' |
| 149 | N/A | N/A | | 'AACP + 7 MLACP + 7 NADPH + 7 NADH --> HACP + 7 ACP + 7 NAD + 7 NADP + 7 CO2' |
| 150 | 2.3.1.85 / 3.1.2.14 | fatty-acid synthase | | 'HACP --> ACP + HDA' |
| 151 | N/A | N/A | | 'HDA + CoA --> ACLCA' |
| 152 | 1.1.1.94 | glycerol-3-phosphate dehydrogenase | **Glycero-phospolipid biosynthesis** | 'GLP + NADPH --> G3P + NADP' |
| 153 | 1.1.1.94 | glycerol-3-phosphate dehydrogenase | | 'G3P + NADP --> GLP + NADPH' |
| 154 | 2.3.1.15,2.3.1.51 | Lumped reaction | | '2 ACLCA + G3P --> PA + 2 CoA' |
| 155 | 2.7.1.107 | diacylglycerol kinase | | 'ATP + DAG --> PA + ADP' |
| 156 | 2.7.7.41 | phosphatidate cytidylyltransferase | | 'PG --> DAG + G3P' |
| 157 | 2.7.8.5,3.1.3.27 | Lumped reaction | | 'PG + Lys --> POG' |
| 158 | 3.1.4.3 | phospholipase C | | 'CTP + PA --> CDPDAGLYC + 2 Pi' |
| 159 | N/A | N/A | | 'CDPDAGLYC + G3P --> CMP + PG + Pi' |
| 160 | 2.7.8.- | transferase for other substituted phosphate groups | | 'CDPDAGLYC + PG --> CDL + CMP' |
| 161 | 2.7.7.9 | UDPG phosphorylase | **Lipoteichoic acid Biosynthesis** | 'G1P + UTP --> UDPGlu + 2 Pi' |
| 162 | 2.7.7.9 | UDPG phosphorylase | | 'UDPGlu + 2 Pi --> G1P + UTP' |
| 163 | 2.4.1.157 | 1,2-diacylglycerol 3-glucosyltransferase | | 'UDPGlu + DAG --> UDP + 3D12DAG' |
| 164 | 2.4.1.- | hexosyltransferase | | '3D12DAG + UDPGlu --> DGDG + UDP' |
| 165 | 2.7.8.20 | phosphatidylglycerol-membrane-oligosaccharide | | 'PG + DGDG --> DAG + GPGL' |
| 166 | N/A | N/A | | 'PG + GPGL + Ala --> LTA' |

| | | | | |
|---|---|---|---|---|
| 167 | 2.7.7.39 | glycerol-3-phosphate cytidylyltransferase | **Wall Teichoic acid Biosynthesis** | 'CTP + G3P --> CDPGLY + 2 Pi' |
| 168 | 2.7.7.39 | glycerol-3-phosphate cytidylyltransferase | | 'CDPGLY + 2 Pi --> G3P + CTP' |
| 169 | N/A | N/A | | 'UDPNAG + CDPGLY --> WTA' |
| 170 | 1.18.1.3 | NAD+-ferredoxin oxidoreductase | **ferredoxin-NADP+ reductase** | 'RFRD + NAD --> OFRD + NADH' |
| 171 | 1.18.1.3 | NADH-ferredoxin oxidoreductase | | 'OFRD + NADH --> RFRD + NAD' |
| 172 | 1.18.1.2 | NADP+-ferredoxin oxidoreductase | | 'RFRD + NADP --> OFRD + NADPH' |
| 173 | 1.8.1.9 | thioredoxine reductase | | 'OTRD + NADPH --> RTRD + NADP' |
| 174 | 1.12.7.2 | hydrogenase | | 'RFRD --> OFRD + H2' |
| 175 | 1.5.1.5 | methylenetetrahydrofolate dehydrogenase (NADP) | **One carbon pool by folate** | 'MLTHF + NADP --> METHF + NADPH' |
| 176 | 1.5.1.5 | methylenetetrahydrofolate dehydrogenase (NADP) | | 'METHF + NADPH --> MLTHF + NADP' |
| 177 | 1.5.1.20 | 5,10-methylenetetrahydrofolate reductase (NADPH) | | 'MLTHF + NADPH --> MTHF + NADP' |
| 178 | 3.5.4.9 | cyclohydrolase | | 'METHF --> fTHF' |
| 179 | 3.5.4.9 | cyclohydrolase | | 'fTHF --> METHF' |
| 180 | 1.5.1.3 | Dihydrofolate reductase | | 'DHF + NADP --> FOL + NADPH' |
| 181 | 1.5.1.3 | Dihydrofolate reductase | | 'FOL + NADPH --> DHF + NADP' |
| 182 | 1.5.1.3 | Dihydrofolate reductase | | 'THF + NADP --> DHF + NADPH' |
| 183 | 1.5.1.3 | Dihydrofolate reductase | | 'DHF + NADPH --> THF + NADP' |
| 184 | 3.6.1.15 | nucleoside-triphosphatase | **Maintenance Energy** | 'ATP --> ADP + Pi' |
| 185 | 4.2.1.1 | carbonic anhydrase | **$HCO_3$** | 'CO2 --> HCO3-' |
| 186 | **BIOMASS Precursors** | | **Cell wall biosynthesis** | '0.95 Peptido + 0.05 WTA --> Cellwall' |
| 187 | | | **Lipid Biosynthesis** | '0.497 PG + 0.0622 CDL + 0.0623 POG + 0.0747 DGDG + 0.0125 3D12DAG + 0.207 DAG --> LIPID' |
| 188 | | | **DNA biosynthesis** | '0.345 dATP + 0.115 dGTP + 0.115 dCTP + 0.345 dTTP + 0.0324 ATP --> DNA + 0.0324 ADP + 0.0324 Pi' |

| | | | |
|---|---|---|---|
| 189 | | **RNA biosynthesis** | '0.77374 ATP + 0.29 GTP + 0.214 CTP + 0.225 UTP -> RNA + 0.50274 ADP + 0.50274 Pi' |
| 190 | | **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' |
| 191 | | **Peptidoglycan biosynthesis** | 'UDPNAMAG + 3 ATP + NH3 + Lys + 5 Gly + DALDAL + UDPNAG --> Peptido' |
| 192 | | **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' |
| 193 | | **Exchange reactions** | '--> Glc' |
| 194 | | | '--> NH3' |
| 195 | | | '--> SO4' |
| 196 | | | '--> Pi' |
| 197 | | | 'Biomass -->' |
| 198 | | | 'BUOL -->' |
| 199 | | | 'ETOH -->' |
| 200 | | | 'ACETONE-->' |
| 201 | | | 'BU -->' |
| 202 | | | 'AC -->' |
| 203 | | | 'H2 -->' |
| 204 | | | 'CO2 -->' |
| 205 | | | 'Suc -->' |
| 206 | | | '--> AC' |
| 207 | | | '--> 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 | $\alpha$ 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 |

| Abbreviations and meaning | |
|---|---|
| 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 |
| Glc | Glucose |
| GLP | Glycerone phosphate/Dihydroxy acetone phosphate |
| Gln | Glutamine |
| Glu | Glutamate |
| Gly | Glycine |
| GMP | Guanosine 5’ monophosphate |
| GPGL | Glycerophophoglycoglycerolipid |
| GS1P | Glucosamine 1-phosphate |
| GS6P | Glucosamine 6-phosphate |
| GTP | Guanosine triphosphate |

| Abbreviations and meaning | |
|---|---|
| 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 |

| Abbreviations and meaning | |
|---|---|
| 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. acetobutylicum* MTCC 11274 for different batch and fed-batch fermentations when maximization of biomass (10 h) was used as the objective function

| Reaction No. | Biomass batch | Butanol batch | Two-stage fed-batch | Two-stage fed-batch-Mg-Limited | Two-stage fed-batch-$CaCO_3$ Supplemented | Combinatorial Two-stage fed-batch |
|---|---|---|---|---|---|---|
| 1 | 12.7 | 3.899954 | 13.89 | 10.9 | 13.79 | 10.9 |
| 2 | 5662.23 | 70.10774 | 20.02409 | 162.7621 | 19.97723 | 162.7621 |
| 3 | 5649.696 | 66.25784 | 6.311715 | 151.9923 | 6.363818 | 151.9923 |
| 4 | 12.19326 | 3.745447 | 13.33774 | 10.50851 | 13.24098 | 10.50851 |
| 5 | 5904.117 | 69.54823 | 19.96648 | 161.2988 | 19.93291 | 161.2988 |
| 6 | 5891.937 | 65.8028 | 6.632723 | 150.8065 | 6.695887 | 150.8065 |
| 7 | 0.012957 | 1.14E-06 | 0.003986 | 1.62E-02 | 0.003955 | 1.62E-02 |
| 8 | 6034.011 | 69.05045 | 19.94617 | 159.8231 | 19.93282 | 159.8231 |
| 9 | 6022.153 | 65.40415 | 6.958304 | 149.5843 | 7.039661 | 149.5843 |
| 10 | 6136.88 | 109.6819 | 40.48181 | 349.4579 | 40.27688 | 349.4579 |
| 11 | 6113.067 | 102.3556 | 14.40014 | 328.9027 | 14.38524 | 328.9027 |
| 12 | 6136.881 | 109.6818 | 40.48182 | 349.4579 | 40.27689 | 349.4579 |
| 13 | 6113.068 | 102.3555 | 14.40015 | 328.9028 | 14.38526 | 328.9028 |
| 14 | 6136.882 | 109.6818 | 40.48184 | 349.458 | 40.27691 | 349.458 |
| 15 | 6113.069 | 102.3555 | 14.40017 | 328.9028 | 14.38527 | 328.9028 |
| 16 | 6136.882 | 109.6817 | 40.48185 | 349.458 | 40.27692 | 349.458 |
| 17 | 6113.069 | 102.3554 | 14.40018 | 328.9029 | 14.38528 | 328.9029 |
| 18 | 23.21801 | 7.149617 | 25.43523 | 20.09439 | 25.24893 | 20.09439 |
| 19 | 0.012421 | 1.14E-06 | 0.003948 | 1.59E-02 | 0.003864 | 1.59E-02 |
| 20 | 1566.164 | 59.81191 | 7.159538 | 84.73337 | 7.11567 | 84.73337 |
| 21 | 1566.53 | 59.91577 | 7.421165 | 85.02661 | 7.471733 | 85.02661 |
| 22 | 20.6146 | 6.385391 | 21.79726 | 18.04637 | 21.72533 | 18.04637 |
| 23 | 2235.15 | 64.34675 | 11.09748 | 115.6094 | 11.08986 | 115.6094 |
| 24 | 2229.09 | 61.84643 | 4.957478 | 108.9994 | 4.969856 | 108.9994 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 25 | 6.06 | 2.500229 | 6.14 | 6.4 | 6.12 | 6.4 |
| 26 | 6.06 | 2.500229 | 6.14 | 6.4 | 6.12 | 6.4 |
| 27 | 6.06 | 2.500229 | 6.14 | 6.4 | 6.12 | 6.4 |
| 28 | 2240.487 | 64.36733 | 11.10172 | 100.0432 | 11.0948 | 100.0432 |
| 29 | 2234.427 | 61.86716 | 4.961722 | 95.33228 | 4.974805 | 95.33228 |
| 30 | 2240.487 | 64.3673 | 11.10172 | 100.0432 | 11.0948 | 100.0432 |
| 31 | 2234.427 | 61.86716 | 4.961722 | 95.33228 | 4.974805 | 95.33228 |
| 32 | 1971.335 | 60.03022 | 10.24781 | 88.21599 | 10.25104 | 88.21599 |
| 33 | 1966.362 | 59.73575 | 5.240416 | 86.10472 | 5.251345 | 86.10472 |
| 34 | 1971.335 | 60.03021 | 10.24781 | 88.21599 | 10.25104 | 88.21599 |
| 35 | 1966.362 | 59.73576 | 5.240416 | 86.10472 | 5.251345 | 86.10472 |
| 36 | 5.84E-08 | 9.42E-05 | 9.56E-09 | 1.80E+00 | 5.91E-09 | 1.80E+00 |
| 37 | 5.20E-08 | 5.00E-05 | 8.10E-09 | 1.11E-01 | 5.01E-09 | 1.11E-01 |
| 38 | 1564.881 | 59.84503 | 7.197583 | 87.24697 | 7.207008 | 87.24697 |
| 39 | 1564.881 | 59.84498 | 7.197583 | 85.44697 | 7.207008 | 85.44697 |
| 40 | 5.20E-08 | 4.97E-05 | 8.10E-09 | 9.90E-02 | 5.01E-09 | 9.90E-02 |
| 41 | 7.10E-08 | 6.16E-05 | 1.24E-08 | 2.10E-01 | 7.69E-09 | 2.10E-01 |
| 42 | 1564.889 | 59.838 | 7.171366 | 84.58132 | 7.180945 | 84.58132 |
| 43 | 1564.938 | 59.85222 | 7.22383 | 84.56778 | 7.2331 | 84.56778 |
| 44 | 1564.881 | 59.84502 | 7.197583 | 84.60177 | 7.207008 | 84.60177 |
| 45 | 1564.881 | 59.84499 | 7.197583 | 84.54977 | 7.207008 | 84.54977 |
| 46 | 1.295153 | 0.386048 | 2.117124 | 1.021686 | 2.107705 | 1.021686 |
| 47 | 0.013083 | 1.14E-06 | 0.004071 | 1.64E-02 | 0.00482 | 1.64E-02 |
| 48 | 1.57E+03 | 5.98E+01 | 7.84384 | 8.58E+01 | 7.779983 | 8.58E+01 |
| 49 | 1567.442 | 59.90433 | 8.101395 | 86.07264 | 8.131225 | 86.07264 |
| 50 | 0.001356 | 2.47E-07 | 0.121465 | 9.35E-04 | 0.025555 | 9.35E-04 |
| 51 | 0.298844 | 0.098997 | 1.060688 | 0.233967 | 1.056695 | 0.233967 |
| 52 | 1566.953 | 59.90135 | 7.738401 | 84.8015 | 7.745578 | 84.8015 |
| 53 | 1566.654 | 59.80238 | 6.677712 | 84.56753 | 6.688883 | 84.56753 |
| 54 | 0.298844 | 0.098941 | 1.060688 | 0.233967 | 1.056695 | 0.233967 |
| 55 | 0.000644 | 0.012054 | 0.74152 | 2.41E-05 | 0.7394 | 2.41E-05 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 56 | 1566.421 | 59.81679 | 7.446149 | 84.51982 | 7.455095 | 84.51982 |
| 57 | 1566.667 | 59.87636 | 6.968599 | 84.71328 | 6.978117 | 84.71328 |
| 58 | 1565.772 | 59.80775 | 7.050261 | 84.48458 | 7.060549 | 84.48458 |
| 59 | 1566.048 | 59.88887 | 7.345843 | 84.70124 | 7.354397 | 84.70124 |
| 60 | 1565.772 | 59.80776 | 7.050261 | 84.48458 | 7.060549 | 84.48458 |
| 61 | 1566.048 | 59.88887 | 7.345843 | 84.70124 | 7.354397 | 84.70124 |
| 62 | 1564.885 | 59.83848 | 7.174388 | 84.55776 | 7.183949 | 84.55776 |
| 63 | 1564.928 | 59.8517 | 7.220802 | 84.59178 | 7.23009 | 84.59178 |
| 64 | 1564.885 | 59.83847 | 7.174388 | 84.55776 | 7.183949 | 84.55776 |
| 65 | 1564.928 | 59.85171 | 7.220802 | 84.59178 | 7.23009 | 84.59178 |
| 66 | 1565.729 | 59.88131 | 7.3225 | 84.67884 | 7.331191 | 84.67884 |
| 67 | 1565.496 | 59.81338 | 7.073332 | 84.49621 | 7.083486 | 84.49621 |
| 68 | 0.319527 | 0.094307 | 0.341996 | 0.250676 | 0.339989 | 0.250676 |
| 69 | 0.163443 | 0.048079 | 0.174936 | 0.128224 | 0.17391 | 0.128224 |
| 70 | 0.190997 | 0.056049 | 0.204428 | 0.149841 | 0.203228 | 0.149841 |
| 71 | 1565.469 | 59.8746 | 7.300021 | 84.65813 | 7.308844 | 84.65813 |
| 72 | 1565.278 | 59.81857 | 7.095594 | 84.50829 | 7.105616 | 84.50829 |
| 73 | 0.02443 | 0.007119 | 0.026148 | 0.019166 | 0.025995 | 0.019166 |
| 74 | 0.02443 | 0.007091 | 0.026148 | 0.019166 | 0.025995 | 0.019166 |
| 75 | 1564.901 | 59.84857 | 7.210661 | 84.58404 | 7.220009 | 84.58404 |
| 76 | 1564.877 | 59.84149 | 7.184513 | 84.56487 | 7.194014 | 84.56487 |
| 77 | 1565.469 | 59.87455 | 7.300021 | 84.65813 | 7.308844 | 84.65813 |
| 78 | 1565.278 | 59.81862 | 7.095594 | 84.50829 | 7.105616 | 84.50829 |
| 79 | 0.001356 | 0.000402 | 0.001451 | 0.001063 | 0.001442 | 0.001063 |
| 80 | 0.166567 | 0.048874 | 0.17828 | 0.130675 | 0.177233 | 0.130675 |
| 81 | 1579.134 | 60.02181 | 8.021476 | 86.38853 | 8.036749 | 86.38853 |
| 82 | 1578.191 | 59.75056 | 7.021361 | 85.64241 | 7.042563 | 85.64241 |
| 83 | 0.004067 | 0.001186 | 0.004353 | 0.00319 | 0.004327 | 0.00319 |
| 84 | 0.11992 | 0.035738 | 0.128352 | 0.094079 | 0.127599 | 0.094079 |
| 85 | 1565.135 | 59.8635 | 7.261848 | 84.62486 | 7.270895 | 84.62486 |
| 86 | 1565.015 | 59.82777 | 7.133495 | 84.53078 | 7.143296 | 84.53078 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 87 | 1565.782 | 59.88386 | 7.326681 | 84.68279 | 7.335348 | 84.68279 |
| 88 | 1565.542 | 59.81122 | 7.069196 | 84.49406 | 7.079374 | 84.49406 |
| 89 | 0.03268 | 0.007347 | 0.028303 | 0.029745 | 0.028134 | 0.029745 |
| 90 | 0.008241 | 5.71E-07 | 0.002146 | 1.06E-02 | 0.00213 | 1.06E-02 |
| 91 | 1565.412 | 59.87219 | 7.371161 | 84.96843 | 7.379155 | 84.96843 |
| 92 | 1565.235 | 59.81936 | 7.185907 | 84.82661 | 7.194991 | 84.82661 |
| 93 | 1565.412 | 59.87219 | 7.371161 | 84.96843 | 7.379155 | 84.96843 |
| 94 | 1565.235 | 59.81936 | 7.185907 | 84.82661 | 7.194991 | 84.82661 |
| 95 | 0.004067 | 0.001228 | 0.004353 | 0.00319 | 0.004327 | 0.00319 |
| 96 | 0.004067 | 0.001233 | 0.004353 | 0.00319 | 0.004327 | 0.00319 |
| 97 | 0.004067 | 0.001237 | 0.004353 | 0.00319 | 0.004327 | 0.00319 |
| 98 | 0.004067 | 0.001213 | 0.004353 | 0.00319 | 0.004327 | 0.00319 |
| 99 | 0.004067 | 0.001192 | 0.004353 | 0.00319 | 0.004327 | 0.00319 |
| 100 | 0.005422 | 0.001615 | 0.005804 | 0.004254 | 0.00577 | 0.004254 |
| 101 | 2.860758 | 0.832525 | 3.055246 | 2.248429 | 3.037315 | 2.248429 |
| 102 | 2.165604 | 0.630128 | 2.317885 | 1.69896 | 2.304284 | 1.69896 |
| 103 | 1578.492 | 60.03035 | 7.729717 | 85.19637 | 7.736009 | 85.19637 |
| 104 | 1577.508 | 59.74332 | 6.677353 | 84.42501 | 6.689819 | 84.42501 |
| 105 | 0.144029 | 0.042664 | 0.154157 | 0.112994 | 0.153253 | 0.112994 |
| 106 | 0.02411 | 0.006936 | 0.025805 | 0.018915 | 0.025654 | 0.018915 |
| 107 | 1577.714 | 60.02289 | 7.713924 | 85.17134 | 7.720309 | 85.17134 |
| 108 | 1576.759 | 59.74577 | 6.692456 | 84.42262 | 6.704834 | 84.42262 |
| 109 | 1568.528 | 59.92918 | 7.46748 | 84.83236 | 7.475319 | 84.83236 |
| 110 | 1568.026 | 59.78259 | 6.930777 | 84.43897 | 6.941765 | 84.43897 |
| 111 | 0.366811 | 0.105678 | 0.392604 | 0.287771 | 0.390301 | 0.287771 |
| 112 | 0.366811 | 0.105634 | 0.392604 | 0.287771 | 0.390301 | 0.287771 |
| 113 | 0.366811 | 0.105641 | 0.392604 | 0.287771 | 0.390301 | 0.287771 |
| 114 | 0.366811 | 0.105643 | 0.392604 | 0.287771 | 0.390301 | 0.287771 |
| 115 | 0.277993 | 0.081527 | 0.297541 | 0.218091 | 0.295795 | 0.218091 |
| 116 | 0.029276 | 0.008429 | 0.031335 | 0.022968 | 0.031151 | 0.022968 |
| 117 | 0.103692 | 0.030438 | 0.110984 | 0.081349 | 0.110332 | 0.081349 |

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|---|---|---|---|---|---|---|
| 118 | 0.346943 | 0.100103 | 0.371339 | 0.272184 | 0.36916 | 0.272184 |
| 119 | 0.142936 | 0.041175 | 0.152987 | 0.112136 | 0.152089 | 0.112136 |
| 120 | 0.204007 | 0.058919 | 0.218352 | 0.160048 | 0.217071 | 0.160048 |
| 121 | 1564.938 | 59.85225 | 7.22383 | 84.59412 | 7.2331 | 84.59412 |
| 122 | 1564.889 | 59.83797 | 7.171366 | 84.55567 | 7.180945 | 84.55567 |
| 123 | 0.084384 | 0.024319 | 0.090317 | 0.066201 | 0.089787 | 0.066201 |
| 124 | 0.079217 | 0.022824 | 0.084788 | 0.062148 | 0.08429 | 0.062148 |
| 125 | 0.215265 | 0.062012 | 0.230402 | 0.16888 | 0.22905 | 0.16888 |
| 126 | 0.079217 | 0.022854 | 0.084788 | 0.062148 | 0.08429 | 0.062148 |
| 127 | 0.189433 | 0.054673 | 0.202753 | 0.148614 | 0.201564 | 0.148614 |
| 128 | 0.008611 | 0.002511 | 0.009216 | 0.006755 | 0.009162 | 0.006755 |
| 129 | 0.14638 | 0.04218 | 0.156673 | 0.114838 | 0.155754 | 0.114838 |
| 130 | 0.034442 | 0.009948 | 0.036864 | 0.027021 | 0.036648 | 0.027021 |
| 131 | 0.027554 | 0.007969 | 0.029491 | 0.021617 | 0.029318 | 0.021617 |
| 132 | 0.027554 | 0.00795 | 0.029491 | 0.021617 | 0.029318 | 0.021617 |
| 133 | 0.077578 | 0.023264 | 0.083033 | 0.060862 | 0.082546 | 0.060862 |
| 134 | 1564.924 | 59.83364 | 7.156104 | 84.54535 | 7.165772 | 84.54535 |
| 135 | 1565.001 | 59.85689 | 7.239137 | 84.60621 | 7.248318 | 84.60621 |
| 136 | 0.077578 | 0.023229 | 0.083033 | 0.060862 | 0.082546 | 0.060862 |
| 137 | 0.077578 | 0.023196 | 0.083033 | 0.060862 | 0.082546 | 0.060862 |
| 138 | 0.037794 | 0.011385 | 0.040452 | 0.029651 | 0.040215 | 0.029651 |
| 139 | 0.037794 | 0.011338 | 0.040452 | 0.029651 | 0.040215 | 0.029651 |
| 140 | 0.037794 | 0.01129 | 0.040452 | 0.029651 | 0.040215 | 0.029651 |
| 141 | 0.037794 | 0.011233 | 0.040452 | 0.029651 | 0.040215 | 0.029651 |
| 142 | 0.037794 | 0.011176 | 0.040452 | 0.029651 | 0.040215 | 0.029651 |
| 143 | 1565.336 | 59.87123 | 7.286566 | 84.64613 | 7.295469 | 84.64613 |
| 144 | 1565.17 | 59.8212 | 7.108939 | 84.51593 | 7.118883 | 84.51593 |
| 145 | 0.045309 | 0.013064 | 0.048495 | 0.035546 | 0.048211 | 0.035546 |
| 146 | 2.404482 | 0.747099 | 2.57356 | 1.886365 | 2.558459 | 1.886365 |
| 147 | 0.343497 | 0.106717 | 0.367651 | 0.269481 | 0.365494 | 0.269481 |
| 148 | 2.404482 | 0.747119 | 2.57356 | 1.886365 | 2.558459 | 1.886365 |

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|---|---|---|---|---|---|---|
| 149 | 0.343497 | 0.106734 | 0.367651 | 0.269481 | 0.365494 | 0.269481 |
| 150 | 0.343497 | 0.106665 | 0.367651 | 0.269481 | 0.365494 | 0.269481 |
| 151 | 0.343497 | 0.106564 | 0.367651 | 0.269481 | 0.365494 | 0.269481 |
| 152 | 1566.452 | 59.89917 | 7.371171 | 84.72655 | 7.379576 | 84.72655 |
| 153 | 1566.129 | 59.80005 | 7.025279 | 84.47302 | 7.035713 | 84.47302 |
| 154 | 0.171749 | 0.053247 | 0.183826 | 0.13474 | 0.182747 | 0.13474 |
| 155 | 0.006043 | 3.79E-07 | 0.001428 | 7.09E-03 | 0.001417 | 7.09E-03 |
| 156 | 0.024466 | 0.006035 | 0.021147 | 0.021541 | 0.021021 | 0.021541 |
| 157 | 0.003901 | 0.001392 | 0.004176 | 0.003061 | 0.004151 | 0.003061 |
| 158 | 0.177792 | 0.053037 | 0.185254 | 0.141828 | 0.184164 | 0.141828 |
| 159 | 0.173896 | 0.051278 | 0.181085 | 0.138772 | 0.18002 | 0.138772 |
| 160 | 0.003895 | 0.001553 | 0.004169 | 0.003056 | 0.004145 | 0.003056 |
| 161 | 1565.138 | 59.86412 | 7.262239 | 84.62519 | 7.271284 | 84.62519 |
| 162 | 1565.017 | 59.82717 | 7.133106 | 84.53053 | 7.142909 | 84.53053 |
| 163 | 0.060716 | 0.018686 | 0.064985 | 0.047633 | 0.064604 | 0.047633 |
| 164 | 0.059933 | 0.018236 | 0.064147 | 0.047019 | 0.063771 | 0.047019 |
| 165 | 0.055255 | 0.016633 | 0.059141 | 0.043349 | 0.058794 | 0.043349 |
| 166 | 0.055255 | 0.016385 | 0.059141 | 0.043349 | 0.058794 | 0.043349 |
| 167 | 1564.882 | 59.84532 | 7.198648 | 84.57509 | 7.208066 | 84.57509 |
| 168 | 1564.88 | 59.84469 | 7.196519 | 84.57353 | 7.20595 | 84.57353 |
| 169 | 0.001989 | 0.000619 | 0.002129 | 0.001561 | 0.002117 | 0.001561 |
| 170 | 7487.028 | 60.83902 | 3.032955 | 88.38251 | 3.025776 | 88.38251 |
| 171 | 7497.339 | 62.72793 | 15.97573 | 91.34872 | 15.92099 | 91.34872 |
| 172 | 7.755286 | 2.300394 | 8.300622 | 6.084179 | 8.251916 | 6.084179 |
| 173 | 0.377656 | 0.108837 | 0.404212 | 0.296279 | 0.40184 | 0.296279 |
| 174 | 23.16936 | 5.985957 | 27.05947 | 14.92749 | 27.08247 | 14.92749 |
| 175 | 1566.754 | 59.90253 | 7.388037 | 84.74398 | 7.396343 | 84.74398 |
| 176 | 1566.399 | 59.79837 | 7.008673 | 84.46592 | 7.019205 | 84.46592 |
| 177 | 0.142936 | 0.041191 | 0.152987 | 0.112136 | 0.152089 | 0.112136 |
| 178 | 1566.754 | 59.90252 | 7.388037 | 84.74398 | 7.396343 | 84.74398 |
| 179 | 1566.399 | 59.79838 | 7.008673 | 84.46592 | 7.019205 | 84.46592 |

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|---|---|---|---|---|---|---|
| 180 | 1564.881 | 59.845 | 7.197583 | 84.57431 | 7.207008 | 84.57431 |
| 181 | 1564.881 | 59.84501 | 7.197583 | 84.57431 | 7.207008 | 84.57431 |
| 182 | 1564.879 | 59.84437 | 7.195407 | 84.57272 | 7.204845 | 84.57272 |
| 183 | 1564.883 | 59.84564 | 7.19976 | 84.57591 | 7.209172 | 84.57591 |
| 184 | 0.013111 | 1.14E-06 | 0.003957 | 1.63E-02 | 0.003964 | 1.63E-02 |
| 185 | 4.007107 | 1.223891 | 5.019777 | 3.149269 | 4.993327 | 3.149269 |
| 186 | 0.039784 | 0.011593 | 0.042581 | 0.031211 | 0.042331 | 0.031211 |
| 187 | 0.062622 | 0.018243 | 0.067026 | 0.049129 | 0.066633 | 0.049129 |
| 188 | 0.011788 | 0.003405 | 0.012617 | 0.009248 | 0.012543 | 0.009248 |
| 189 | 0.079567 | 0.022915 | 0.085162 | 0.062422 | 0.084663 | 0.062422 |
| 190 | 1.722117 | 0.495872 | 1.843213 | 1.351036 | 1.832398 | 1.351036 |
| 191 | 0.037794 | 0.011155 | 0.040452 | 0.029651 | 0.040215 | 0.029651 |
| 192 | 0.368367 | 0.106068 | 0.39427 | 0.288992 | 0.391957 | 0.288992 |
| 193 | 12.7 | 3.9 | 13.89 | 10.9 | 13.79 | 10.9 |
| 194 | 3.618451 | 1.055562 | 3.872893 | 2.838748 | 3.850168 | 2.838748 |
| 195 | 0.366811 | 0.105634 | 0.392604 | 0.287771 | 0.390301 | 0.287771 |
| 196 | 0.923846 | 0.274478 | 0.98881 | 0.724776 | 0.983007 | 0.724776 |
| 197 | 0.368367 | 0.106068 | 0.39427 | 0.288992 | 0.391957 | 0.288992 |
| 198 | 0 | 0 | 0 | 1.8 | 0 | 1.8 |
| 199 | 0 | 0 | 0 | 0.052 | 0 | 0.052 |
| 200 | 0 | 0 | 0 | 0.21 | 0 | 0.21 |
| 201 | 6.06 | 2.5 | 6.14 | 4.6 | 6.12 | 4.6 |
| 202 | 5.34 | 0.4 | 5.4 | 2.3 | 5.39 | 2.3 |
| 203 | 23.16936 | 5.985957 | 27.05947 | 14.92749 | 27.08247 | 14.92749 |
| 204 | 20.54327 | 6.374914 | 22.34173 | 18.20006 | 22.36404 | 18.20006 |
| 205 | 0.002 | 0.012 | 0.862985 | 0.000959 | 0.764955 | 0.000959 |
| 206 | 0 | 0 | 0 | 0 | 0 | 0 |
| 207 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A5** Flux distribution (mmole $gDCW^{-1}$ $h^{-1}$) in metabolic network of *C. acetobutylicum* MTCC 11274 for different batch and fed-batch fermentations when maximization of butanol (20 h) was used as the objective function

| Reaction No. | Biomass batch | Butanol batch | Two-stage fed-batch | Two-stage fed-batch-Mg-Limited | Two-stage fed-batch-$CaCO_3$ Supplemented | Combinatorial Two-stage fed-batch |
|---|---|---|---|---|---|---|
| 1 | 2.21 | 4.2 | 6.3 | 5.1 | 6.5 | 6.4 |
| 2 | 6.631436 | 8.473578232 | 2592.857 | 3778.227 | 10.80935 | 10.72885 |
| 3 | 4.421441 | 4.305114839 | 2586.607 | 3773.14 | 4.359809 | 4.378412 |
| 4 | 2.559059 | 4.119658468 | 6.156615 | 7.060523 | 6.347516 | 6.262375 |
| 5 | 6.631485 | 8.432262228 | 2564.247 | 3768.055 | 10.71484 | 10.63667 |
| 6 | 4.421514 | 4.331019324 | 2558.102 | 3762.997 | 4.372848 | 4.391857 |
| 7 | 0.349088 | 1.84E-02 | 0.011805 | 2.002846 | 0.005527 | 0.017565 |
| 8 | 6.6315 | 8.394690848 | 2538.957 | 3758.852 | 10.62998 | 10.5539 |
| 9 | 4.421541 | 4.354858792 | 2532.909 | 3753.821 | 4.386252 | 4.405596 |
| 10 | 7.857731 | 11.55791066 | 7117.051 | 6564.516 | 20.50859 | 20.35889 |
| 11 | 3.43781 | 3.459437454 | 7104.925 | 6554.446 | 7.991033 | 8.032717 |
| 12 | 7.857731 | 11.55791068 | 7117.052 | 6564.516 | 20.50859 | 20.35889 |
| 13 | 3.437811 | 3.459437468 | 7104.926 | 6554.446 | 7.991033 | 8.032718 |
| 14 | 7.857731 | 11.55791068 | 7117.053 | 6564.516 | 20.50859 | 20.35889 |
| 15 | 3.437811 | 3.459437475 | 7104.927 | 6554.446 | 7.991033 | 8.032718 |
| 16 | 7.857732 | 11.55791069 | 7117.053 | 6564.516 | 20.50859 | 20.35889 |
| 17 | 3.437811 | 3.459437482 | 7104.927 | 6554.445 | 7.991033 | 8.032718 |
| 18 | 4.832138 | 8.00133285 | 11.9565 | 10.61592 | 12.33831 | 12.1622 |
| 19 | 0.412238 | 1.83E-02 | 0.01178 | 0.594629 | 0.005512 | 0.017486 |
| 20 | 0.000121 | 6.137538887 | 1979.158 | 3070.4 | 6.697289 | 6.688226 |
| 21 | 0.0001 | 6.224507227 | 1978.866 | 3069.745 | 6.810517 | 6.812286 |
| 22 | 4.419317 | 7.490658485 | 10.76187 | 8.940521 | 11.53583 | 11.37142 |
| 23 | 6.710599 | 8.10218818 | 2185.901 | 3496.534 | 9.708729 | 9.701622 |
| 24 | 4.301138 | 4.386554648 | 2180.601 | 3491.877 | 4.045158 | 4.056848 |

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|---|---|---|---|---|---|---|
| 25 | 1.459461 | 2.355633533 | 3.600021 | 3.076832 | 3.943571 | 3.944774 |
| 26 | 1.459461 | 2.355633534 | 3.600021 | 3.076832 | 3.943571 | 3.944774 |
| 27 | 1.459461 | 2.355633534 | 3.600021 | 3.076832 | 3.943571 | 3.944774 |
| 28 | 4.982964 | 5.504948791 | 1930.878 | 3040.19 | 5.874782 | 6.00672 |
| 29 | 6.001175 | 6.878187611 | 1932.074 | 3040.805 | 7.587752 | 7.438991 |
| 30 | 4.982964 | 5.504948791 | 1930.878 | 3040.19 | 5.874782 | 6.00672 |
| 31 | 6.001175 | 6.878187611 | 1932.074 | 3040.805 | 7.587752 | 7.438991 |
| 32 | 5.520224 | 6.198003288 | 1912.752 | 3023.958 | 6.611065 | 6.52019 |
| 33 | 5.452017 | 6.16446872 | 1913.216 | 3024.534 | 6.809622 | 6.897454 |
| 34 | 5.520224 | 6.198003288 | 1912.752 | 3023.958 | 6.611065 | 6.52019 |
| 35 | 5.452017 | 6.16446872 | 1913.216 | 3024.534 | 6.809622 | 6.897454 |
| 36 | 2.959461 | 4.165633534 | 5.650021 | 4.776832 | 6.243571 | 6.044774 |
| 37 | 0.48179 | 0.436761181 | 0.853765 | 1.085654 | 0.58703 | 0.667729 |
| 38 | 7.003476 | 8.354777057 | 2237.458 | 3528.311 | 10.07196 | 9.950959 |
| 39 | 4.044015 | 4.189143522 | 2231.808 | 3523.534 | 3.828388 | 3.906185 |
| 40 | 0.46821 | 0.923238818 | 0.846235 | 0.494346 | 1.13297 | 1.032271 |
| 41 | 0.95 | 1.359999999 | 1.7 | 1.58 | 1.72 | 1.7 |
| 42 | 5.48606 | 6.316290692 | 1912.509 | 3026.016 | 6.930529 | 6.917921 |
| 43 | 5.486061 | 6.045605172 | 1912.103 | 3025.61 | 6.485432 | 6.492558 |
| 44 | 5.48606 | 6.321001215 | 1912.681 | 3026.096 | 6.938179 | 6.925426 |
| 45 | 5.48606 | 6.041001215 | 1912.261 | 3025.686 | 6.478179 | 6.485426 |
| 46 | 0.000524 | 0.263545673 | 0.395038 | 0.290544 | 0.404974 | 0.401157 |
| 47 | 0.000207 | 2.00E-02 | 0.012374 | 1.86E-01 | 0.005619 | 0.018455 |
| 48 | 5.486197 | 6.150618845 | 1991.271 | 3079.842 | 6.718217 | 6.717208 |
| 49 | 5.485969 | 6.217600028 | 1990.967 | 3079.001 | 6.825826 | 6.822815 |
| 50 | 2.49E-04 | 3.11E-04 | 0.409721 | 8.70E-01 | 5.88E-05 | 1.39E-04 |
| 51 | 0.000275 | 0.056718368 | 0.089058 | 0.024286 | 1.00E-01 | 8.91E-02 |
| 52 | 5.492886 | 6.220879007 | 1913.395 | 3022.458 | 6.788675 | 6.778982 |
| 53 | 5.492611 | 6.164160638 | 1913.306 | 3022.434 | 6.688264 | 6.689885 |
| 54 | 0.000275 | 0.056718369 | 0.089058 | 0.024286 | 1.00E-01 | 8.91E-02 |
| 55 | 0.000266 | 5.23E-05 | 1.14E-05 | 7.18E-07 | 9.75E-03 | 5.00E-05 |

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|---|---|---|---|---|---|---|
| 56 | 5.49289 | 6.16688925 | 1911.788 | 3022.121 | 6.698817 | 6.690925 |
| 57 | 5.49263 | 6.213703096 | 1911.862 | 3022.142 | 6.764057 | 6.764521 |
| 58 | 5.486052 | 6.153976391 | 1910.111 | 3021.827 | 6.663195 | 6.661448 |
| 59 | 5.486068 | 6.206454894 | 1910.193 | 3021.85 | 6.747161 | 6.743915 |
| 60 | 5.486052 | 6.153976391 | 1910.111 | 3021.827 | 6.663195 | 6.661448 |
| 61 | 5.486068 | 6.206454894 | 1910.193 | 3021.85 | 6.747161 | 6.743915 |
| 62 | 5.486057 | 6.176067523 | 1910.055 | 3021.825 | 6.698485 | 6.696109 |
| 63 | 5.486064 | 6.184308025 | 1910.068 | 3021.828 | 6.711669 | 6.709059 |
| 64 | 5.486057 | 6.176067523 | 1910.055 | 3021.825 | 6.698485 | 6.696109 |
| 65 | 5.486064 | 6.184308025 | 1910.068 | 3021.828 | 6.711669 | 6.709059 |
| 66 | 5.486065 | 6.20233 | 1910.16 | 3021.845 | 6.740538 | 6.737411 |
| 67 | 5.486055 | 6.158088375 | 1910.091 | 3021.826 | 6.669757 | 6.667894 |
| 68 | 2.36E-05 | 0.060719006 | 0.095416 | 0.026021 | 9.72E-02 | 9.54E-02 |
| 69 | 1.08E-05 | 0.031058694 | 0.048807 | 0.01331 | 4.97E-02 | 4.88E-02 |
| 70 | 1.16E-05 | 0.036294693 | 0.057035 | 0.015554 | 5.81E-02 | 5.70E-02 |
| 71 | 5.486066 | 6.198348083 | 1910.132 | 3021.84 | 6.73416 | 6.731147 |
| 72 | 5.486054 | 6.16205339 | 1910.075 | 3021.824 | 6.676088 | 6.674112 |
| 73 | 1.70E-06 | 0.004642396 | 0.007295 | 0.001989 | 7.43E-03 | 7.30E-03 |
| 74 | 1.64E-06 | 0.004642397 | 0.007295 | 0.001989 | 7.43E-03 | 7.30E-03 |
| 75 | 5.486061 | 6.182508492 | 1910.064 | 3021.828 | 6.708789 | 6.70623 |
| 76 | 5.48606 | 6.177866095 | 1910.056 | 3021.826 | 6.701361 | 6.698935 |
| 77 | 5.486066 | 6.198348085 | 1910.132 | 3021.84 | 6.73416 | 6.731147 |
| 78 | 5.486055 | 6.162053388 | 1910.075 | 3021.824 | 6.676088 | 6.674112 |
| 79 | 7.02E-07 | 0.000257599 | 0.000405 | 0.00011 | 4.12E-04 | 4.05E-04 |
| 80 | 1.00E-05 | 0.031652299 | 0.049739 | 0.013565 | 5.06E-02 | 4.97E-02 |
| 81 | 5.694344 | 6.277361796 | 1912.024 | 3036.09 | 6.85 | 6.850779 |
| 82 | 5.282066 | 6.082167766 | 1911.735 | 3035.419 | 6.561507 | 6.555365 |
| 83 | 6.44E-07 | 0.000772799 | 0.001214 | 0.000331 | 1.24E-03 | 1.21E-03 |
| 84 | 1.16E-05 | 0.022788063 | 0.03581 | 0.009765 | 3.65E-02 | 3.58E-02 |
| 85 | 5.486066 | 6.191586488 | 1910.095 | 3021.834 | 6.723324 | 6.720505 |
| 86 | 5.486054 | 6.168798425 | 1910.059 | 3021.824 | 6.686864 | 6.684696 |

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|---|---|---|---|---|---|---|
| 87 | 5.486068 | 6.203066086 | 1910.166 | 3021.845 | 6.741725 | 6.738576 |
| 88 | 5.486052 | 6.157351416 | 1910.094 | 3021.826 | 6.668581 | 6.666739 |
| 89 | 0.476345 | 0.021312988 | 0.016547 | 0.383024 | 0.011119 | 0.022032 |
| 90 | 0.476342 | 1.67E-02 | 0.009249 | 0.381034 | 0.003689 | 0.014735 |
| 91 | 5.714547 | 6.203992345 | 1910.036 | 3035.529 | 6.73243 | 6.734107 |
| 92 | 5.260035 | 6.156377386 | 1909.978 | 3034.796 | 6.677607 | 6.670747 |
| 93 | 5.714547 | 6.203992345 | 1910.036 | 3035.529 | 6.73243 | 6.734107 |
| 94 | 5.260035 | 6.156377386 | 1909.978 | 3034.796 | 6.677607 | 6.670747 |
| 95 | 2.37E-06 | 0.0007728 | 0.001214 | 0.000331 | 1.24E-03 | 1.21E-03 |
| 96 | 2.10E-06 | 0.0007728 | 0.001214 | 0.000331 | 1.24E-03 | 1.21E-03 |
| 97 | 1.76E-06 | 0.0007728 | 0.001214 | 0.000331 | 1.24E-03 | 1.21E-03 |
| 98 | 1.48E-06 | 0.0007728 | 0.001214 | 0.000331 | 1.24E-03 | 1.21E-03 |
| 99 | 1.00E-06 | 0.0007728 | 0.001214 | 0.000331 | 1.24E-03 | 1.21E-03 |
| 100 | 2.66E-06 | 0.0010304 | 0.001619 | 0.000441 | 1.65E-03 | 1.62E-03 |
| 101 | 0.47646 | 0.558725921 | 0.861053 | 0.613339 | 0.87098 | 0.866539 |
| 102 | 6.95E-05 | 0.411524603 | 0.646682 | 0.176366 | 6.58E-01 | 6.47E-01 |
| 103 | 5.486081 | 6.273969441 | 1911.384 | 3022.021 | 6.855856 | 6.85065 |
| 104 | 5.486038 | 6.087129293 | 1911.09 | 3021.941 | 6.556912 | 6.557044 |
| 105 | 1.21E-05 | 0.027369563 | 0.043009 | 0.011729 | 4.38E-02 | 4.30E-02 |
| 106 | 4.57E-07 | 0.004581499 | 0.007199 | 0.001963 | 7.33E-03 | 7.20E-03 |
| 107 | 5.486075 | 6.271205775 | 1911.311 | 3022.011 | 6.851392 | 6.846267 |
| 108 | 5.486045 | 6.08985109 | 1911.026 | 3021.934 | 6.561225 | 6.561281 |
| 109 | 5.486073 | 6.22792525 | 1910.44 | 3021.887 | 6.781645 | 6.77778 |
| 110 | 5.486047 | 6.132637314 | 1910.291 | 3021.846 | 6.629185 | 6.628041 |
| 111 | 3.79E-06 | 0.069704249 | 0.109535 | 0.029873 | 1.12E-01 | 1.10E-01 |
| 112 | 4.05E-06 | 0.06970425 | 0.109535 | 0.029873 | 1.12E-01 | 1.10E-01 |
| 113 | 3.94E-06 | 0.06970425 | 0.109535 | 0.029873 | 1.12E-01 | 1.10E-01 |
| 114 | 3.75E-06 | 0.06970425 | 0.109535 | 0.029873 | 1.12E-01 | 1.10E-01 |
| 115 | 1.14E-05 | 0.052826356 | 0.083013 | 0.02264 | 8.45E-02 | 8.30E-02 |
| 116 | 6.44E-07 | 0.00556325 | 0.008742 | 0.002384 | 8.90E-03 | 8.74E-03 |
| 117 | 3.91E-06 | 0.019704362 | 0.030964 | 0.008445 | 3.15E-02 | 3.10E-02 |

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|---|---|---|---|---|---|---|
| 118 | 4.53E-06 | 0.06592873 | 0.103602 | 0.028256 | 1.05E-01 | 1.04E-01 |
| 119 | 1.63E-06 | 0.02716175 | 0.042683 | 0.011641 | 4.35E-02 | 4.27E-02 |
| 120 | 2.86E-06 | 0.03876698 | 0.06092 | 0.016615 | 6.20E-02 | 6.09E-02 |
| 121 | 5.486061 | 6.184845209 | 1910.07 | 3021.829 | 6.712529 | 6.709903 |
| 122 | 5.48606 | 6.17553073 | 1910.055 | 3021.825 | 6.697626 | 6.695266 |
| 123 | 9.69E-07 | 0.016035249 | 0.025198 | 0.006872 | 2.57E-02 | 2.52E-02 |
| 124 | 9.37E-07 | 0.0150535 | 0.023655 | 0.006451 | 2.41E-02 | 2.37E-02 |
| 125 | 1.84E-06 | 0.040906249 | 0.064281 | 0.017531 | 6.54E-02 | 6.43E-02 |
| 126 | 8.10E-07 | 0.015053499 | 0.023655 | 0.006451 | 2.41E-02 | 2.37E-02 |
| 127 | 2.57E-06 | 0.035997499 | 0.056567 | 0.015427 | 5.76E-02 | 5.66E-02 |
| 128 | 3.65E-07 | 0.00163625 | 0.002571 | 0.000701 | 2.62E-03 | 2.57E-03 |
| 129 | 1.44E-06 | 0.02781625 | 0.043711 | 0.011921 | 4.45E-02 | 4.37E-02 |
| 130 | 7.10E-07 | 0.006545 | 0.010285 | 0.002805 | 1.05E-02 | 1.03E-02 |
| 131 | 8.05E-07 | 0.005235999 | 0.008228 | 0.002244 | 8.38E-03 | 8.23E-03 |
| 132 | 7.08E-07 | 0.005235999 | 0.008228 | 0.002244 | 8.38E-03 | 8.23E-03 |
| 133 | 7.41E-06 | 0.014741985 | 0.023166 | 0.006318 | 2.36E-02 | 2.32E-02 |
| 134 | 5.486057 | 6.172818331 | 1910.055 | 3021.824 | 6.693289 | 6.691007 |
| 135 | 5.486064 | 6.187560317 | 1910.078 | 3021.831 | 6.716876 | 6.714173 |
| 136 | 7.37E-06 | 0.014741985 | 0.023166 | 0.006318 | 2.36E-02 | 2.32E-02 |
| 137 | 7.32E-06 | 0.014741985 | 0.023166 | 0.006318 | 2.36E-02 | 2.32E-02 |
| 138 | 3.88E-06 | 0.007181992 | 0.011286 | 0.003078 | 1.15E-02 | 1.13E-02 |
| 139 | 3.72E-06 | 0.007181992 | 0.011286 | 0.003078 | 1.15E-02 | 1.13E-02 |
| 140 | 3.48E-06 | 0.007181993 | 0.011286 | 0.003078 | 1.15E-02 | 1.13E-02 |
| 141 | 3.19E-06 | 0.007181993 | 0.011286 | 0.003078 | 1.15E-02 | 1.13E-02 |
| 142 | 2.92E-06 | 0.007181994 | 0.011286 | 0.003078 | 1.15E-02 | 1.13E-02 |
| 143 | 5.486063 | 6.195965693 | 1910.118 | 3021.838 | 6.730341 | 6.727396 |
| 144 | 5.486058 | 6.164429086 | 1910.068 | 3021.824 | 6.679883 | 6.677839 |
| 145 | 5.67E-07 | 0.00861 | 0.01353 | 0.00369 | 1.38E-02 | 1.35E-02 |
| 146 | 9.90E-05 | 0.456918043 | 0.718014 | 0.195819 | 7.31E-01 | 7.18E-01 |
| 147 | 1.41E-05 | 0.065274006 | 0.102573 | 0.027974 | 1.04E-01 | 1.03E-01 |
| 148 | 9.90E-05 | 0.456918043 | 0.718014 | 0.195819 | 7.31E-01 | 7.18E-01 |

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|---|---|---|---|---|---|---|
| 149 | 1.42E-05 | 0.065274006 | 0.102573 | 0.027974 | 1.04E-01 | 1.03E-01 |
| 150 | 1.41E-05 | 0.065274006 | 0.102573 | 0.027974 | 1.04E-01 | 1.03E-01 |
| 151 | 1.41E-05 | 0.065274006 | 0.102573 | 0.027974 | 1.04E-01 | 1.03E-01 |
| 152 | 5.486066 | 6.210931625 | 1910.235 | 3021.856 | 6.754345 | 6.75097 |
| 153 | 5.486054 | 6.149520776 | 1910.138 | 3021.83 | 6.656087 | 6.654467 |
| 154 | 6.99E-06 | 0.032637003 | 0.051287 | 0.013987 | 5.22E-02 | 5.13E-02 |
| 155 | 0.454505 | 1.50E-02 | 0.007 | 0.719131 | 0.002604 | 0.012073 |
| 156 | 0.454506 | 0.018478933 | 0.012501 | 0.720631 | 0.008205 | 0.017575 |
| 157 | 5.24E-07 | 0.000741369 | 0.001165 | 0.000318 | 1.19E-03 | 1.17E-03 |
| 158 | 0.454512 | 0.047614958 | 0.058286 | 0.733118 | 0.054823 | 0.06336 |
| 159 | 0.454511 | 0.046874779 | 0.057123 | 0.732801 | 0.053638 | 0.062197 |
| 160 | 7.65E-07 | 0.000740179 | 0.001163 | 0.000317 | 1.18E-03 | 1.16E-03 |
| 161 | 5.486062 | 6.191655827 | 1910.095 | 3021.834 | 6.723436 | 6.720614 |
| 162 | 5.486058 | 6.16872922 | 1910.059 | 3021.824 | 6.686753 | 6.684587 |
| 163 | 2.27E-06 | 0.011537678 | 0.018131 | 0.004945 | 1.85E-02 | 1.81E-02 |
| 164 | 2.00E-06 | 0.011388929 | 0.017897 | 0.004881 | 1.82E-02 | 1.79E-02 |
| 165 | 1.61E-06 | 0.010499999 | 0.0165 | 0.0045 | 1.68E-02 | 1.65E-02 |
| 166 | 1.19E-06 | 0.010499999 | 0.0165 | 0.0045 | 1.68E-02 | 1.65E-02 |
| 167 | 5.486061 | 6.180376071 | 1910.06 | 3021.826 | 6.705377 | 6.702879 |
| 168 | 5.48606 | 6.179998072 | 1910.059 | 3021.826 | 6.704772 | 6.702285 |
| 169 | 4.88E-07 | 0.000377999 | 0.000594 | 1.62E-04 | 6.05E-04 | 5.94E-04 |
| 170 | 7.75285 | 9.080534735 | 2472.399 | 4173.168 | 11.34155 | 11.23981 |
| 171 | 3.334888 | 3.31363764 | 2464.363 | 4165.729 | 2.154383 | 2.294178 |
| 172 | 2.07E-04 | 1.473719017 | 2.315844 | 0.631589 | 2.36E+00 | 2.32E+00 |
| 173 | 8.07E-06 | 0.071765049 | 0.112774 | 0.030756 | 1.15E-01 | 1.13E-01 |
| 174 | 0.001165 | 2.50E-01 | 7.46E-05 | 4.13E-06 | 4.03E-04 | 1.10E-01 |
| 175 | 5.486071 | 6.213910828 | 1910.265 | 3021.861 | 6.759127 | 6.755667 |
| 176 | 5.486049 | 6.146557441 | 1910.159 | 3021.832 | 6.651362 | 6.649825 |
| 177 | 1.65E-06 | 0.02716175 | 0.042683 | 0.011641 | 4.35E-02 | 4.27E-02 |
| 178 | 5.486071 | 6.213910828 | 1910.265 | 3021.861 | 6.759127 | 6.755667 |
| 179 | 5.486049 | 6.146557441 | 1910.159 | 3021.832 | 6.651362 | 6.649825 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 180 | 5.48606 | 6.18018707 | 1910.059 | 3021.826 | 6.705075 | 6.702582 |
| 181 | 5.48606 | 6.18018707 | 1910.059 | 3021.826 | 6.705075 | 6.702582 |
| 182 | 5.486059 | 6.179800676 | 1910.059 | 3021.826 | 6.704456 | 6.701975 |
| 183 | 5.486061 | 6.180573476 | 1910.06 | 3021.827 | 6.705693 | 6.703189 |
| 184 | 0.391444 | 1.84E-02 | 0.011248 | 0.34692 | 0.005526 | 0.01751 |
| 185 | 0.000646 | 0.778891973 | 1.204868 | 0.511402 | 1.229529 | 1.210987 |
| 186 | 1.68E-06 | 0.007559997 | 0.01188 | 0.00324 | 1.21E-02 | 1.19E-02 |
| 187 | 8.98E-07 | 0.011899999 | 0.0187 | 0.0051 | 1.90E-02 | 1.87E-02 |
| 188 | 7.61E-07 | 0.00224 | 0.00352 | 0.00096 | 3.58E-03 | 3.52E-03 |
| 189 | 9.85E-07 | 0.015119999 | 0.02376 | 0.00648 | 2.42E-02 | 2.38E-02 |
| 190 | 1.40E-05 | 0.327249996 | 0.51425 | 0.14025 | 5.24E-01 | 5.14E-01 |
| 191 | 2.83E-06 | 0.007181994 | 0.011286 | 0.003078 | 1.15E-02 | 1.13E-02 |
| 192 | 2.95E-06 | 0.069999999 | 0.11 | 0.03 | 1.12E-01 | 1.10E-01 |
| 193 | 2.21 | — 4.2 | 6.3 | 5.1 | 6.5 | 6.4 |
| 194 | 1.46E-04 | 0.687605641 | 1.080524 | 0.294682 | 1.10E+00 | 1.08E+00 |
| 195 | 4.09E-06 | 0.06970425 | 0.109535 | 0.029873 | 1.12E-01 | 1.10E-01 |
| 196 | 6.27E-05 | 0.175556321 | 0.275874 | 0.075235 | 2.81E-01 | 2.76E-01 |
| 197 | 0 | 0.07 | 0.11 | 0.03 | 0.112 | 0.11 |
| 198 | 2.959461 | 4.165633535 | 5.650021 | 4.776832 | 6.243571 | 6.044774 |
| 199 | 0 | 0.28 | 0.42 | 0.41 | 4.60E-01 | 4.40E-01 |
| 200 | 0.95 | 1.36 | 1.7 | 1.58 | 1.72 | 1.7 |
| 201 | 0 | 0 | 0 | 0 | 0 | 0 |
| 202 | 0 | 0 | 0 | 0 | 0 | 0 |
| 203 | 0.001165 | 2.50E-01 | 7.46E-05 | 7.25E-06 | 4.03E-04 | 1.10E-01 |
| 204 | 5.369312 | 8.836980204 | 12.03108 | 9.644848 | 13.24405 | 13.05024 |
| 205 | 0.000515 | 0.000363576 | 0.409732 | 0.869924 | 0.009804 | 0.000189 |
| 206 | 0.4 | 0.82 | 1.2 | 1.04 | 1.22 | 1.3 |
| 207 | 1.5 | 1.81 | 2.05 | 1.7 | 2.3 | 2.1 |

# List of Publications

## Published manuscripts

1. **Saumya Ahlawat**, Mehak Kaushal, Basavaraj Palabhanvi, Gargi Goswami, Debasish Das* (2019). Flux balance analysis of nutritional modulation assisted changes in two-stage fed-batch fermentation by *Clostridium acetobutylicum*. Bioresource Technology Reports. https://doi.org/10.1016/j.biteb.2019.100264
2. **Saumya Ahlawat**, Mehak Kaushal, Basavaraj Palabhanvi, Muthusivaramapandian Muthuraj, Gargi Goswami, Debasish Das* (2019). Nutrient modulation based process engineering strategy for improved butanol production from *Clostridium acetobutylicum*. Biotechnology Progress, 35(2):e2771. (*Impact factor: 2.406*)

## Manuscript submitted

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. (Awaiting submission)

## Publications from collaborative work

### Manuscripts

1. Mehak Kaushal, **Saumya Ahlawat**, Bidhu Bhusan Makut, Gargi Goswami, Debasish Das (2019). Dual substrate fermentation strategy utilizing rice straw hydrolysate and crude glycerol for liquid biofuel production by *Clostridium sporogenes* NCIM 2918. Biomass and Bioenergy, 127: 105257. (*Impact factor: 3.537*)
2. 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: 6.669*)
3. 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:*

*6.669*)

4. Basavaraj Palabhanvi, Muthusivaramapandian Muthuraj, Vikram Kumar, Mayurketan Mukherjee, **Saumya Ahlawat**, Debasish Das* (2017). Continuous cultivation of lipid rich microalga *Chlorella* sp. FC2 IITG for improved biodiesel productivity via control variable optimization and substrate driven pH control. Bioresource Technology, 224: 481-489. (*Impact factor: 6.669*)

*+ represents equal authorship*

** represents corresponding author*

**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).

**Patents 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)
2. 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

1. **Saumya Ahlawat**, Mehak Kaushal, Gargi Goswami, Debasish Das (2018). Nutrient modulation based process engineering strategy for improved butanol production. Bioprocessing INDIA 2018, December 16-18, organized by Indian Institute of Technology Delhi, New Delhi, India.
2. **Saumya Ahlawat** (2018). International Conference on Sustainable Biofuels 2018, February 26-27, organized by Department of Biotechnology, Ministry of Science & Technology, Government of India and Mission Innovation India, New Delhi, India.
3. 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, December 9-11, organized by Indian Institute of Technology Guwahati, Guwahati, India.
4. 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, March 2017, Department of Chemical Engineering, Indian Institute of Technology, Guwahati. (Best poster presentation award).
5. 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, March 2017, Indian Institute of Technology Guwahati, Guwahati.
6. **Saumya Ahlawat**, Mehak Kaushal, Mayurketan Mukherjee, Muthusivarama-pandian 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. Indo-US Workshop on Cell Factories, March 18-20, 2016, organized by Indian Institute of Technology Bombay, Mumbai.
7. **Saumya Ahlawat** (2015). Workshop on Frontier energy research with industry academia partnership, March 20-21, 2015 organized by Center for Energy, Indian Institute of Technology Guwahati, Guwahati.
8. **Saumya Ahlawat** (2015). TEQIP Short term course on solid waste management, January 12-14, 2015, organized by Center for Environment, Indian Institute of Technology Guwahati, Guwahati.

9. **Saumya Ahlawat**, Mehak Kaushal, Muthusivaramapandian Muthuraj, Basavaraj Palabhanvi, Vikram Kumar, Debasish Das (2014). Screening and characterization of 12 *Clostridium* strains for butanol production: Effect of glucose and organic nitrogen source. Bioprocessing INDIA 2014, December 17-20, organized by Institute of Chemical Technology, Mumbai and Indian Institute of Technology Bombay, Mumbai.

10. **Saumya Ahlawat** (2014). National conference on Recent advances in cancer biology and therapeutics, December 5, 2014, held at Indian Institute of Technology Guwahati, Guwahati.

11. Bikash C Maharaj, Minakshi Bhattacharjee, **Saumya Ahlawat**, Muthusivarama-pandian Muthuraj, Basavaraj Palabhanvi, Debasish Das (2013). *Streptococcus* sp. W3: A new isolate as a cell factory for hyaluronic acid production. ICABP 2013, February 21-23, Bharathidasan University, Tamil Nadu, India. (Best poster presentation award).

12. **Saumya Ahlawat** (2012). DBT sponsored workshop on Analysis of Biological Networks, November 6-7, 2012, held at Indian Institute of Technology Guwahati.

13. 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, March 2017, Indian Institute of Technology Guwahati, Guwahati.

14. Hemantika Seel, **Saumya Ahlawat**, Gargi Goswami, Debasish Das (2017). Recent advances in Bio-butanol production by *Clostridium* sp. Research Conclave, March 2017, Indian Institute of Technology Guwahati, Guwahati.

15. 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, November 24-27, 2016, Guwahati University, Guwahati, India.

16. 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, November 24-27, 2016, Guwahati University, Guwahati, India.

17. Mehak Kaushal, **Saumya Ahlawat**, Mayurketan Mukherjee, Muthusivarama-pandian Muthuraj, Debasish Das. (2016) Media engineering & process devel-opment for biobutanol production from non-acetone producing *Clostridium*

sp. using alternate carbon substrate and cheaper nitrogen source. Indo-US workshop on Cell factories, March 18-20, 2016, Indian Institute of Technology Bombay, Mumbai, India.

18. Mayurketan Mukherjee, Anwesha Purakayastha, **Saumya Ahlawat**, Mehak Kaushal, Basavaraj Palabhanvi, Debasish Das. (2016) System biology approach to understand the regulation in metabolic shift from acidogenesis to solventogenesis in *Clostridium acetobutylicum* ATCC 824. Indo-US workshop on Cell factories, March 18-20, 2016, Indian Institute of Technology Bombay, Mumbai, India.

19. 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, March 17-21, 2016, Indian Institute of Technology Guwahati, Guwahati, India.

20. 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, December 17-20, organized by Institute of Chemical Technology, Mumbai and Indian Institute of Technology Bombay, Mumbai.

Underline represents presenting author
+ represents equal contribution

# Vitae

*The author was born on January 17th 1987 in Lucknow, Uttar Pradesh, India. She passed the Secondary School Examination conducted by the Central Board of Secondary Education, Delhi, in 2002. She qualified the Higher Secondary School Examination conducted by Central Board of Secondary Education, Delhi, in 2004. She completed B. Tech in Biotechnology from Sardar Vallabh Bhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, in 2008. She did her M. Tech in Chemical Technology with specialization in Biochemical Engineering from Harcourt Butler Technological Institute (HBTI), Kanpur, Uttar Pradesh, in 2010. She gained academic experience working as a Lecturer in Meerut Institute of Engineering and Technology, Meerut, Uttar Pradesh from January 2011 to June 2012.*

*Saumya Ahlawat joined her Ph.D. Programme in July 2012 at Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India. 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 9.0/10 Cumulative Point Index (CPI). She gave the Open (Ph.D. Synopsis) Seminar on November 16th 2018 and presented her thesis work before the Doctoral Committee and her performance was satisfactory. She presented her final Viva-Voce Seminar on September 27th 2019.*