Sustainable production of biobutanol using *Clostridium acetobutylicum* ATCC 824 as cell factory

A Thesis

Submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

MAYURKETAN MUKHERJEE
(146106025)

Under supervision of

Prof. DEBASISH DAS

Department of Biosciences and Bioengineering
IIT Guwahati

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Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Guwahati 781 039, Assam, India

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biosciences and Bioengineering

STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India under the supervision of Prof. Debasish Das.

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

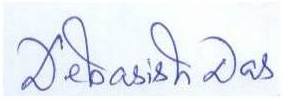
Date: February 2021 **Mayurketan Mukherjee**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biosciences and Bioengineering

CERTIFICATE

It is certified that the work described in this thesis entitled "**Sustainable production of biobutanol using *Clostridium acetobutylicum* ATCC 824 as cell factory**" by Mr. Mayurketan Mukherjee 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 Centre for Energy, Indian Institute of Technology Guwahati, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.



Prof. Debasish Das
Professor
(Thesis Supervisor)
Department of Biosciences & Bioengineering
Indian Institute of Technology Guwahati
Guwahati 781 039, India

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"Feeling gratitude and not expressing it is like wrapping a present and not giving it."

—William Arthur Ward

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Date: 24th February 2021 ***Mayurketan Mukherjee***

Abstract

Continual changes in population patterns associated with increase in global energy demand have led to an adverse effect on environmental health. Currently, the energy supply is met by conventional fossil fuels and coal based electricity. However, the continuous use of these non-replenishing resources has led to a significant reduction in their reserves with many on the brink of exhausting. To that end, tireless efforts have been made towards development of alternate and renewable energy which would aid in the overall environmental health with reduced carbon footprint, decrease in harmful emissions etc. Liquid transportation fuels have been of significant interest lately as majority of the harmful emissions and population is a direct result of enhanced automobile demand. Biobutanol through clostridial fermentation is a potent alternate to the existing petroleum fuels and gasoline owing to its higher energy content, lower volatility, less hygroscopicity and better anti-knocking properties to name a few. However, the commercial implementation of butanol biosynthesis is plagued by severe bottlenecks such as (i) expensive subtrates, (ii) product toxicity and tolerance, (iii) solvent production ratio, (iv) sporulation, (v) low solvent titer and yield and (vi) unknown metabolic regulations.

The present study has been designed and executed with a keen interest on eliminating the major bottlenecks of butanol fermentation, in order to render the developed bioprocess strategies economically feasible and commercially realizable. *Clostridium acetobutylicum* ATCC 824 has been chosen as the platform for butanol biosynthesis. Initially, the strain has been characterized on a wide variety of carbon and nitrogen sources, assessing its maximum butanol potential. Glucose and peptone were screened as suitable carbon and nitrogen source which resulted in a maximum butanol titer of 11.6 g L^{-1} and 11.68 g L^{-1} respectively. After, the initial screening, statistical media optimization

was carried out to optimize the initial concentration of glucose, peptone and trace metal composition with the objective function of maximization of butanol titer. Optimization of media components resulted in a butanol titer of 11.76 g L^{-1}, which was an insignificant improvement of 1.3%. Further, with the objective of enhancing butanol production and elucidating the roles of metal ions, the strain was cultivated under individual starvation of magnesium, manganese, iron and sodium. To that end, magnesium starvation positively influenced butanol production with significant increase in butanol titer (13.72 g L^{-1}) and earlier onset of solvent formation (6 h). Furthermore, zinc supplementation at 10 mg L^{-1} in the optimized media proved beneficial for butanol production. Therefore, a novel medium engineering strategy was developed coupling zinc supplementation in magnesium starved optimized medium which resulted in an enhanced butanol titer of 19.18 g L^{-1} with a maximum productivity of 0.63 g L^{-1} h^{-1}. The novel medium strategy resulted in an improvement of 61.5% and 110% with respect to butanol titer and productivity. With the aim of elucidating the metabolic regulations behind inflected phenotypic response of the organism under the influence of metal ions were captured via obtaining temporal expression profile of the key metabolic enzymes in glycolytic, ethanol, butanol and acetone formation pathways. The elevation in butanol biosynthesis was associated with raised glucose utilization, reduced ethanol production and early induction of solventogenesis. Change in these phenotypic traits of the organism may be attributed to multi-level modulation in central carbon metabolism e.g., upregulation of glycolytic pathway; upregulation in thiolase activity; key intermediate enzyme for biosynthesis of acids and solvent; upregulation in the activity of butyrylaldehyde dehydrogenase & butanol dehydrogenase, the enzymes responsible for butanol biosynthesis and downregulation in alcohol dehydrogenase, redirecting carbon flux from ethanol to butanol.

In order to enhance process feasibility, a fed-batch was demonstrated with intermittent feeding of glucose and zinc which resulted in 24% enhancement in butanol productivity. However, it was inferred that the high butanol titer was detrimental for cellular metabolism and the fermentation terminated at an early stage. Thus, in order to alleviate butanol toxicity the fed-batch was coupled with *in-situ* product recovery through optimized gas stripping parameters which resulted in a cumulative titer of 54.2 g L^{-1} with average productivity of 0.66 g L^{-1} h^{-1}. However, the strategy was demonstrated using expensive laboratory grade peptone and glucose as nitrogen and carbon source respectively.

Hence, present study also demonstrates a novel two-stage sequential bioprocess for production of biobutanol using low cost substrates, corn steep liquor and industrial grade maize starch. Improved butanol titer using low cost substrates was achieved via combinatorial approach of: (i) optimization of initial concentration of corn steep liquor and starch; (ii) hydrolysis of starch into fermentable sugar using industrial grade amylase and (iii) coupling attributes of butanol upregulation via magnesium starvation and zinc supplementation. The strategy resulted in a butanol titer of 16.54 g L^{-1} with a yield of 0.28 g g^{-1}. The produced butanol was further distilled (90% purity) and the qualitative and engine performance was analyzed for its potent application as an alternate transportation fuel. Physicochemical properties of butanol-diesel blends e.g. kinematic viscosity, absolute viscosity, density, flash point, fire point, cloud point and pour point corroborate well with pure diesel. Engine performance analysis revealed enhanced brake thermal efficiency with negligible change in key engine performance parameters under butanol-diesel blends as compared to diesel, depicting suitability of butanol as potent alternate to petroleum fuel. An economic analysis of the developed bioprocess strategies was also performed which revealed that the bioprocess strategy using low cost substrates resulted in

a price of 0.8 USD for every litre of butanol, offering economic feasibility for possible commercial realization which was a significant reduction of 99% with respect to the media using peptone and glucose as nitrogen and carbon source.

Hence, the current study successfully demonstrates suitable and sustainable bioprocess strategies using low cost substrates which aids towards commercial realization of butanol biosynthesis.

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Chapter 1

INTRODUCTION

"Begin at the beginning," the King said gravely, "and go on till you come to the end: then stop."

Lewis Carroll, Alice in Wonderland

CHAPTER 1

Introduction

1.1 Background and motivation

Current energy demands, a function of the demographic transition as well as population explosion, has resulted in numerous problems such as global energy crisis, climate change and human diseases (Li et al. 2019). Fossil oil reserves have permitted the exponential rise of human lifestyle and are consumed at an alarming rate. Thus, the rapid depletion of fuel reserves has triggered a thought process amongst researchers worldwide, to identify and devise a better replacement of the same. This initiative to replace fossil fuels has rightfully diverted the attention towards biomass-based biofuel.

Biofuels produced through biological processes has been achieving increasing attention due to its environment-friendly features (Li et al. 2019). Currently, production of various primary alcohols such as methanol, ethanol and butanol has been explored as potential biofuel molecules for realization at commercial scale. Ethanol has been gaining more attention in comparison to methanol due to its superior fuel properties such as renewability, lesser toxicity and higher energy density (Wyman et al. 2018). Recently, there has been a shift in focus from lower alcohol to higher alcohol (e.g. butanol) in the area of biofuels. Biobutanol, a four carbon straight chain alcohol is considered to be a superior next generation fuel compared to bioethanol as it has higher energy content due to presence of higher carbon atoms, lower volatility and is sparsely hygroscopic (Durre et al. 2007 ; Lee et al. 2008). Higher boiling point of butanol makes it more suitable for motor

engine and it is lesser corrosive in nature, making it suitable for use in the existing fuel pipelines (Ndaba et al. 2015). Biobutanol can also be blended at higher volumetric ratio than bioethanol as it has lower air to fuel ratio and higher octane number ensures its anti-knocking properties (Pfromm et al. 2010). Clostridial strains have been considered as most widely implemented platform for butanol production through Acetone-Butanol-Ethanol (ABE) fermentation route (Moon et al. 2016). However, commercial scale butanol fermentation suffers from several bottlenecks in terms of low butanol titer or productivity, end product toxicity, reduced efficiency in solvent recovery from fermentation broth and high cost of production thus hindering the overall process sustainability (Durre et al. 2007; Moon et al. 2016).

There have been several notable attempts towards increment in butanol titer or productivity from *Clostridium* strains, which includes co-culturing (Bader et al. 2010; Nakayama et al. 2011; Abd-Alla et al. 2012; Luo et al. 2015), cell immobilization (Kong et al. 2015), multistage fermentation (Afschar et al. 1985; Godin and Engasser 1988), cell recycling (Jang et al. 2013a), nutrient limitations (Junelles et al. 1988), process optimization (Liu et al. 2014) and *in situ* product recovery (Qureshi et al. 2005; Staggs et al. 2015). Supplementation or limitation of metal ions from the fermentation medium has been reported to be an effective strategy towards modulation of butanol biosynthesis in *Clostridium* sp. An elevated butanol titer of 20 g L^{-1} has been reported when *C. acetobutylicum* ATCC 10132 was grown in the fermentation medium, supplemented with Na_2CO_3 (Vidhya et al. 2008; U.S. Patent 9,217,163). Supplementation of zinc and calcium was shown to synergistically facilitate carbohydrate uptake and in turn, upregulate butanol titer in *C. acetobutylicum* ATCC 824 (Wu et al. 2016). Zinc associated response of *C. acetobutylicum* was found to be significantly pleotropic in terms of modulation of multiple phenotypic traits such as carbohydrate utilization, glycolysis, acidogenesis and

solventogenesis (Wu et al. 2015). While, different studies in the literatures indicate upregulation of butanol biosynthesis via supplementation or limitation of metal ions, their underlying cellular regulation are not well elucidated.

In spite of development of multiple fermentation strategies and novel hyper producing strains, commercial implementation of biobutanol production remains a distant dream and a missed opportunity owing to its high cost of production due to expensive organic substrates used as carbon and nitrogen source. Substrate selection has been reported to have a strong impact on fermentation performance and overall production cost (Moon et al. 2016). Identifying possible low cost substrates is imperative for designing and demonstrating sustainable bioprocess strategies towards biobutanol production with improved titer. As the clostridial strain is able to utilize a wide range of substrates, identification of locally or logistically available cost-effective carbon sources is important depending upon the country of operation. Wide variety of substrates such as Jerusalem artichoke juice; maltodextrin; sago starch; cassava starch and cassava chips; enzymatically hydrolyzed cassava flour have been employed for biobutanol production (Moon et al. 2016). However, lower biobutanol titer limited below 13 g L^{-1} resulted from these processes using low cost substrates have been found to be the major hurdle toward their commercialization (Luo et al. 2018). Therefore, there is a need to develop sustainable process using low cost substrates without compromising the butanol titer and yield.

1.2 Objectives of the study

The present investigations are carried out in the thesis titled "**Sustainable production of biobutanol using *Clostridium acetobutylicum* ATCC 824 as cell factory**" with the following objectives formulated based upon the current bottlenecks in biobutanol production:

- *Biochemical characterization of Clostridium acetobutylicum ATCC 824 under various environmental cues.*
- *Enzymatic analysis to understand the effects of various cues directed towards butanol upregulation.*
- *Development of a sustainable bioprocess strategy with the proposed regulation.*
- *Performance evaluation of butanol as a potential fuel blend and economic assessment of bioprocess strategy.*

1.3 Approach of thesis

Present study is modelled around addressing key bottlenecks towards commercial realization of butanol namely (i) low butanol titer and productivity, (ii) unknown metabolic regulations due to medium engineering strategies and (iii) high cost of production owing to use of expensive substrates. The bottlenecks along with their possible remedial strategies have been systematically enumerated in the forthcoming thesis chapters.

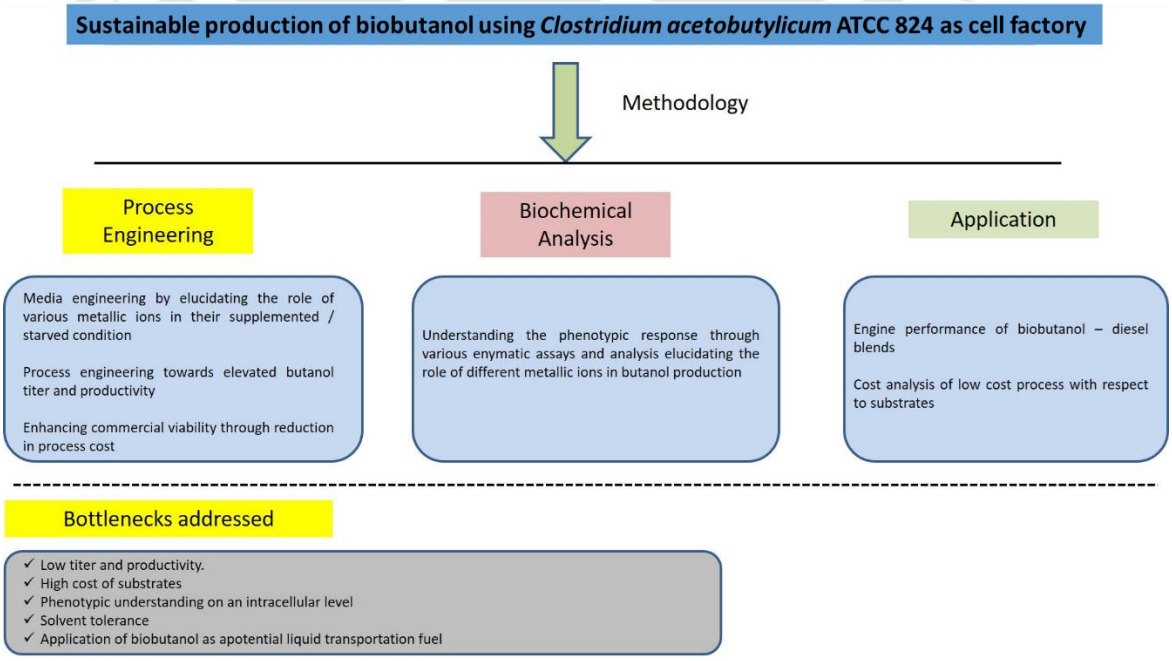

Figure 1.1 Organization of the thesis enumerating the methodology employed for undertaking the proposed strategy in turn addressing the key limitations in butanol biosynthesis

In the first step, the strain was characterized for effects of various carbon and nitrogen sources on growth and butanol titer. These experiments were performed to identify the substrates critical for butanol production. Further, nutritional requirements of the strain were optimized in order to maximize the butanol titer. Secondly, modulation of phenotypic traits of the strain e.g., growth, glucose utilization, butanol production and onset of solventogenesis under the influence of selected metal ions either individually or in combination has been reported. Extensive characterization of the organism was carried out under four different growth conditions: (i) normal medium (control); (ii) medium supplemented with zinc; (iii) medium devoid of magnesium and (iv) medium with zinc supplementation and magnesium starvation. Metabolic regulation behind inflected phenotypic response of the organism under the influence of metal ions was captured via obtaining temporal expression profile of the key metabolic enzymes in glycolytic, ethanol, butanol and acetone formation pathways. A novel process engineering strategy was demonstrated to achieve high butanol titer via culturing the organism in the optimal customized media supplemented with zinc and devoid of magnesium ion. . Furthermore, with the aim of achieving economic feasibility, a low cost process has been demonstrated using corn steep liquor (CSL) as nitrogen source and industrial grade maize starch (starch) as carbon source while maintaining an improved butanol titer. The trade-off between improved titer and utilization of low cost substrate has been achieved via combinatorial approach of (i) optimization of initial concentration of CSL and starch; (ii) hydrolysis of starch into fermentable sugar using industrial grade amylase and (iii) coupling attributes of butanol upregulation via magnesium starvation and zinc supplementation. Biobutanol as a blended fuel (with diesel) and its physicochemical properties has been evaluated and its

engine performance has been assessed with a keen interest on improvement in emission characteristics. Finally, an economic assessment of the proposed bioprocess has been enumerated to highlight the reduction in fermentation cost of butanol with respect to substrates.

1.4 Thesis organization

The thesis consists of **7 chapters** encompassing introduction to conclusions. **Chapter 1** outlines general introduction, objective and scope of the present work along with the approaches required to resolve the major bottlenecks pertaining to butanol biosynthesis. **Chapter 2** is a detailed literature survey on the current depleting fossil reserves resulting in exponential energy demand, and potential of biobutanol as an alternative to current petroleum fuel to combat liquid transportation requirements. The chapter delves in establishing the importance of *Clostridium* sp. as the suitable microbial platform for biobutanol synthesis with in-depth study of its biology, physiology, biochemical and metabolic pathways. Furthermore, the chapter reviews existing medium engineering strategies resulting in various improvements in butanol production. Moreover, it also reports the different strain improvement rationales directed towards maximization of butanol titer. It provides an argument for different process engineering strategies involving modes of cultivation, *in-situ* product recovery and efficient downstream technologies for butanol recovery. The dearth in knowledge in clostridial metabolism with respect to varied phenotypic observations has also been highlighted. **Chapter 3** enumerates the strains potential towards growth and butanol production under different carbon and nitrogen sources. Post screening of carbon and nitrogen sources, the initial concentration of the substrates are statistically optimized with the objective function of maximization of butanol titer. Further, the strains potential of growth and butanol production was estimated under the effect of different metallic ion starvation and

supplementation. **Chapter 4** entails the assessment of butanol improvement as a direct consequence of metallic ion starvation and supplementation. Herein, the difference in the butanol production under zinc supplementation, magnesium starvation or combination of both in the optimized medium is compared and biochemically assessed through various enzymatic assays to correspond the phenotypic variations with their effects on key metabolizing enzymes in butanol metabolism. **Chapter 5** encompasses the various novel medium and process engineering strategies utilized towards improvement in butanol production and sustainability. To that end, the strain is cultivated under different modes of cultivations such as batch, fed-batch, fed-batch with intermittent product removal. Finally, a novel low cost medium engineering strategy has demonstrated towards sustainable butanol production. **Chapter 6** delves mainly towards elucidating the performance of biobutanol as potential fuel blend with diesel and its physiochemical properties have been carried out in a 4- stroke internal combustion engine. Finally, an economic analysis of the novel low cost medium strategy has been performed to highlight the positive implications of using cheap and waste raw materials towards butanol biosynthesis. Finally, **chapter 7** summarizes key research highlights obtained from the present study and elucidates the future prospects stemming out of the key outcomes of the thesis.

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Chapter 2

REVIEW OF LITERATURE

"Research is to see what everybody else has seen and to think what nobody else has thought"

Albert Szent-Gyorgyi, *Nobel laureate in Biochemistry*

CHAPTER 2

Review of Literature

2.1 History of ABE fermentation

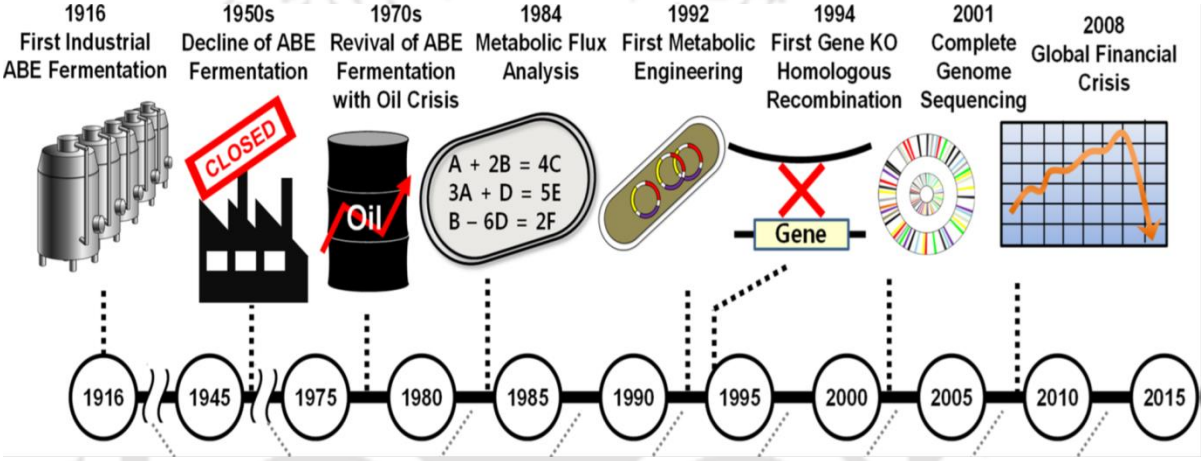

Figure 2.1 Brief history of notable events related to development of ABE fermentation from 1916 to 2015 (adapted from Moon et al., 2016).

ABE fermentation (Acetone – Butanol – Ethanol) was initially developed as a response to alleviate the high acetone demand during World War I (1914 – 1918), which was necessary for the British troops to produce the explosive cordite (Jones and Woods, 1986). Acetone and butanol biosynthesis through microbial fermentation was one of the first large scale bioprocess strategies to be demonstrated industrially, ranking second only to ethanol fermentation in importance (Jones and Woods, 1986). In the initial phases of 1900s, a shortage of natural rubber elevated the academic interest to be streamlined towards development of synthetic rubber. Chaim Weizmann, dedicated most of his research towards development of synthetic rubber, however, in order to develop a technology directed towards synthetic rubber, he discovered a strain to quantifiably

produce butanol and acetone, which later was named as *Clostridium acetobutylicum*. This organism, also known as the Weizmann organism, gradually was designated as the model organism for ABE fermentation and the first taxonomically acceptable description for *C. acetobutylicum* was published in 1926 (McCoy et al., 1926). The process gained impetus during World War I as it effectively hydrolyzed complex sugars to form acetone, which was a major requirement for manufacturing smokeless ammunition *viz.* cordite (Jones and Woods, 1986). Post World War I, the interest gradually declined as the requirement for acetone was diminished, however, butanol served as alternate to lacquers, which was in demand due to the fervent growth in the automobile sector (Jones and Woods, 1986). After, the 2nd World War, there was a steady decline in the ABE fermentation which spiked post the Israel war, 1973, due to the rise in oil prices and hence, researchers have been steadily working towards achieving a suitable platform – substrate combination for successful and robust industrialization (Moon et al., 2016).

2.2 Traditional microbes for ABE production

Clostridium sp. have been the most researched and favored microbial platforms for butanol production along with acetone and ethanol in the ratio of 3:6:1 (A:B:E) (Xin et al., 2018). These are members of the firmicutes genus and are obligate anaerobe, Gram-positive and spore forming organism that is able to ferment a variety of different sugars and convert them to acetic acid, butyric acid and solvents as acetone, butanol and ethanol in the typical ABE-fermentation (Durre et al., 2005). Its genome sequence was recorded and annotated in 2001 (Noelling et al., 2001). It consists of one main chromosome (3.94 Mb) and a mega plasmid pSOL1 (192 kb), which contain 3740 protein-coding open reading frames and 107 RNA genes. The life cycle consists of three distinct phases (Luetke-Eversloh and Bahl, 2011):

• ***Acidogenesis***: In this phase the cells are exponentially growing and the products acetic acid and butyric acid prevail.

• ***Solventogenesis***: In this phase the cells take up the excreted acids and metabolise them to the corresponding alcohols, ethanol and butanol, as well as acetone, in a fixed ratio depending on the substrate. For glucose as substrate the ratio of ABE products is 3:6:1.

• ***Sporulation***: In this phase, productivity ceases and cells transform into a durable state until environmental conditions ameliorate.

A typical feature of the micro-organism is its biphasic nature- characteristically marked by two distinct phases namely; Acidogenic and Solventogenic (Figure 2.2). Acidogenic phase coincides with the exponential phase of growth characterized by increase in biomass and subsequent decrease in pH with acids such as acetic acid, butyric acid and butyrate as the main intermediate metabolites and hydrogen being released in the process (Jones and Woods, 1986; Durre et al., 2007). On reaching a threshold level of acid accumulation, the bacterium undergoes a stress and a shift in the metabolic activity is observed. To avoid the stress, it starts utilizing the acids and readily converts them to solvents namely acetone, butanol and ethanol; thus marking the solventogenic phase. The switch between the two phases can be corresponded with the shift in activity of various enzymes in acid and solvent forming pathways (Lee et al., 2008). Solvent concentration on reaching a toxic level proves to be a hindrance to the growth of the bacterium and stops the growth by sporulation or autolysis of the cells. Sporulation is also hypothesized as a delayed but direct response to the acid stress in the organism but it requires a certain amount of time before it can form spores; thus buying time by solvent formation (Thorn et al., 2013). Solventogenic activity ceases once it sporulates; thus terminating any further growth. Sporulation can also be deciphered as a mechanistic response to solvent toxicity on accumulation of end products.

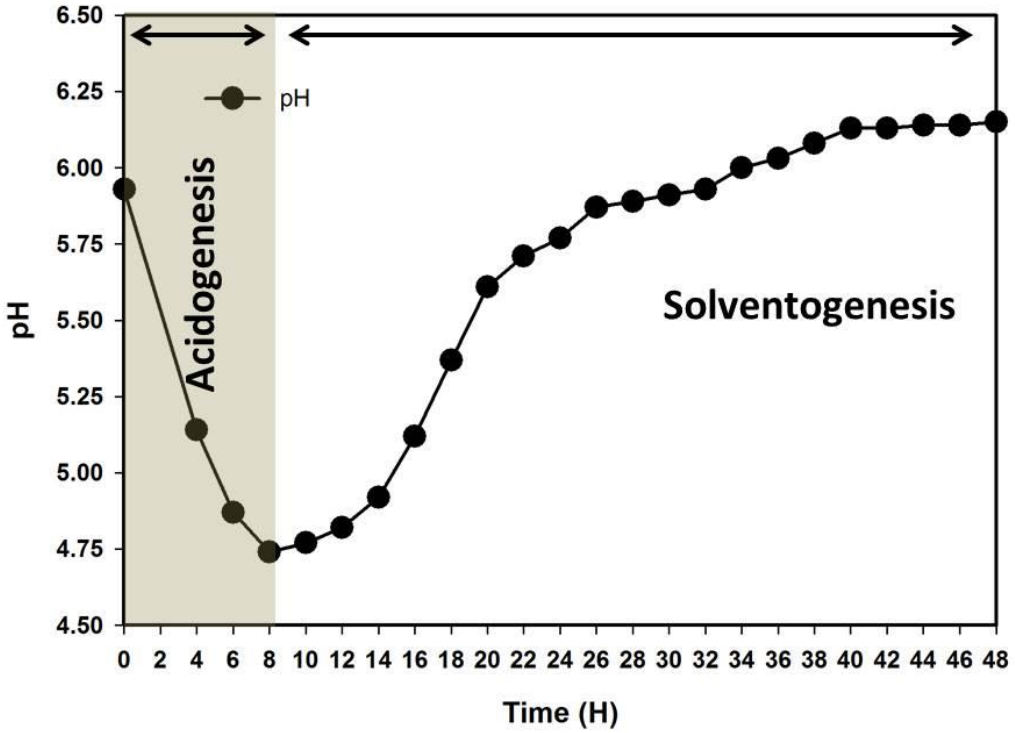

Figure 2.2 Dynamic profile of pH depicting the biphasic nature of *Clostridium* metabolism

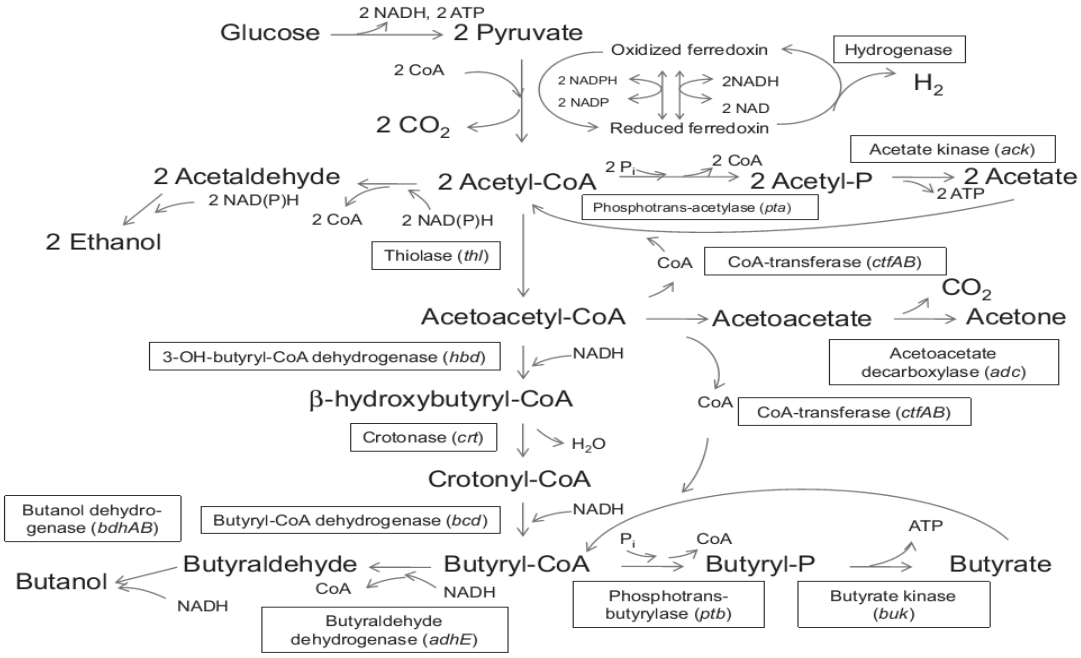

Figure 2.3 Metabolism of *Clostridium* sp. towards utilization of glucose and ABE fermentation (Adapted and modified from Garcia et al., 2011).

Amongst all the butanol producers, *C. acetobutylicum*, particularly its type strain ATCC 824, is the most widely explored in terms of genetic engineering, process development, and mathematical modeling (Xin et al., 2018). It is potent of utilizing a wide gamut of substrates for producing acetone, butanol, and ethanol such as monosaccharides and polysaccharides (glucose, fructose, xylose, starch, pectin, etc.) (Jones and Woods, 1986).

2.3 Regulation of solvent production

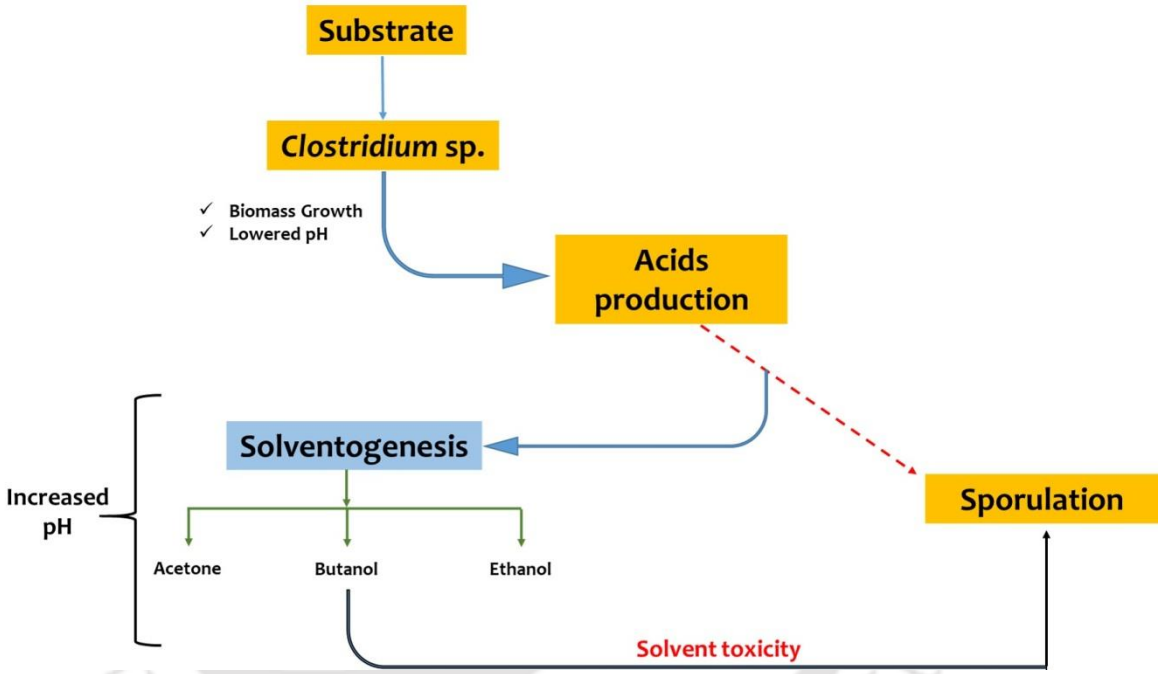

Figure 2.4 Schematic representation of clostridial metabolism and its phases.

Figure 2.4 encapsulates the phenotypic response of the microbe, however, the intrinsic understanding of these key metabolic phases remains a major bottleneck, hindering strain improvement and process design. The genes involved in the formation of solvents are clustered in the 192 kb megaplasmid pSol1 in the form of various operons (Cornillot et al., 1997) with the key genes residing on sol operon. Cytoplasmic operon bdh A/B encodes for butanol dehydrogenase enzyme essential in the butanol formation step. Loss of the megaplasmid results in deficient solvent production, thus, proving its importance to the system. The genes involved in the formation of acetate are formed into

an operon in the order pta-ack. The genes involved in the formation of butyryl-CoA are clustered in the bcs operon in the order *crt-bcd-etfB-etfA-hbd*. *etfA* and *etfB* encode gene products with homology to electron transfer flavoproteins (Bennett and Rudolph, 1995; Boynton et al., 1996). The genes involved in butyrate synthesis, *ptb* and *buk* also form an operon (Cary et al., 1988). Of the genes featuring in sol operon, the adc gene encoding acetoacetate decarboxylase is present in *C.acetobutylicum* as a monocistronic gene (Gerischer and Durre 1990; Petersen et al., 1993).

The sol operon consists of *ctfA* and *ctfB*, (the 2 subunits of butyrate/acetoacetate-CoA transferase) and adhE, one of the 2 acetaldehyde-CoA dehydrogenases found in *C.acetobutylicum*. Also present upstream of *adhE* is orfL which encodes a small peptide of as yet unknown function. The sol operon was found to have 2 promoters, P1 and P2. Reporter studies have shown that P1 is the true promoter and that P2 is an RNA processing site (Thormann et al., 2002). A second *adhE* gene in *C.acetobutylicum* (CAC0035) has been found. As such, this *Clostridium* species has been proven to have found to contain two *adhE* genes (Fontaine et al., 2002). This second gene, *adhE2* was found to be expressed only in "alcohologenic" continuous culture where no acetone is produced as a result of specific substrate combinations of glycerol and glucose (Fontaine et al., 2002). The *adhE2* gene is found on pSOL-1 along with the adc-sol locus. Two other genes encoding butanol dehydrogenases, *bdhA* and *bdhB* are arranged contiguously on the chromosome. The expression profiles of the metabolic genes of *C.acetobutylicum* have been studied by DNA microarray analysis (Alsaker and Papoutsakis, 2005). The expression of the genes involved in acetate and butyrate biosynthesis was found to peak at the transitional stage of growth. Acetate genes had the highest fold up-regulation. The expression of the bcs (butyryl-CoA synthesis) operon was not surprisingly found to increase 1.9 fold in the stationary phase of growth. The *thl* gene (encoding Thiolase)

followed a similar expression pattern. The *bdh* genes were found to be up-regulated at a time point coinciding with the onset of sporulation. There was found to be differential expression between the 2 genes with *bdhA* having much lower levels of expression compared with that of *bdhB*. The genes belonging to the sol operon and *adc*, as expected, were found to be up-regulated in a similar pattern to the *bdh* genes.

Much is known about the genes involved in acidogenesis and solventogenesis in *C.acetobutylicum*. Until recently there has been very little knowledge of the regulatory mechanisms involved in their expression and of the transition between the two stages of growth. Spo0A, the master trannscriptional factor was originally identified in *Bacillus subtilis* and is a key regulator of the sporulation pathway in this organism (Ferrari et al., 1985). *In B.subtilis* Spo0A is phosphorylated by Spo0B which is the last component of a characterised phosphorylation cascade. The activated Spo0A can then bind to a consensus sequence known as a 0A box (Hoch, 1993a; Hoch, 1993b). A recent study identified the most probable consensus sequence as 5′-TTTGTCGAAAA-3′ using two different methods of analysis and found 121 genes directly up-regulated by Spo0A in *B. subtilis*. A homologue of Spo0A has been found in *C. acetobutylicum* and was found to affect the expression of genes at the transitional phase between acidogenic to solventogenic phases of growth. The strain SKO1, which has its spo0A gene disrupted, had lower expression of the sol operon and *adc* (Harris et al., 2002). The phosphorelay system which activates Spo0A in *B.subtilis* is missing in clostridia and there have been various studies into what might activate the *clostridial* Spo0A (Alsaker and Papoutsakis, 2005; Jones et al., 2008). Studies into overexpression of spo0A revealed 123 genes which had their expression altered (Alsaker et al., 2004).

Other transcription factors have been shown to act in concert with Spo0A in *B.subtilis*, including the AbrB and SinR proteins (Grossman, 1995). AbrB acts as an

inhibitor of sporulation in effect acting as an opposite regulator to Spo0A. It modulates catabolism during carbon limitation and acts as a positive and negative regulator of competence during cell growth (Fisher et al., 1994; Hahn et al., 1995). *C.acetobutylicum* carries 3 homologues of Abr (CAC0310, CAC1941 and CAC3641). The use of reporter genes has, however, established that CAC0310 is the true AbrB functional homologue (Scotcher et al., 2005). Expression of CAC0310 is highest during the transitional phase of growth and disruption of CAC0310 delayed solventogenesis and sporulation. There is also a homologue of sinR in *C.acetobutylicum* (Scotcher et al., 2005). This helix-turn-helix DNA binding protein is synthesised during vegetative growth and sporulation in *B.subtilis*. It can form dimers in vivo which directly inhibit sporulation initiators including Spo0A (Cervin et al., 1998; Mandic-Mulec et al., 1995). SinR itself is regulated by SinI which inhibits sinR activity at the onset of sporulation (Bai et al., 1993).

SolR is a possible transcriptional repressor of solventogenesis with a helix-turn-helix DNA binding motif (Nair et al., 1999). Inactivation of solR was found to result in higher levels of butanol and acetone while overexpression of solR resulted in the opposite effect (Nair et al., 1999; Harris et al., 2001). Further studies found that SolR did not bind to any DNA upstream of sol and overexpression of solR in E.coli revealed a different pattern of glycosylated exoproteins to wild type (Thormann and Durre, 2001). This suggests that SolR is not a direct repressor of solventogenesis genes. Furthermore an explanation has been given for the effect of the overexpression of solR on the reduction of solvents produced. It was suggested that the plasmid expressing solR had a Spo0A binding site and so the titration of Spo0A itself could have caused this effect (Thormann and Durre, 2001). The fact that a knockout of solR did increase solvent production does suggest it has some yet to be elucidated role in solvent formation.

2.4 Process engineering strategies towards ABE improvement

In conjunction with unknown metabolic regulations, low butanol titer, yield, and productivity are major challenges towards development of the ABE fermentation process. Both strain and process level improvements have been carried examined to overcome the above challenges. Even after achieving strain level improvement, further improvement at the process level by applying various process strategies becomes imperative to achieve the desired yield.

Substrate selection can have a strong impact on fermentation performance and overall production cost. Identifying cost-effective substrates is vital for the economical ABE fermentation. Initially, low-cost agricultural wastes such as bagasse and rice straw were hydrolyzed by a mixed culture of two cellulolytic fungi to make fermentable sugars (Soni, Das and Ghose 1982). The pretreated bagasse hydrolyzates were used to produce solvent using *C. saccharoperbutylacetonicum*, which produced 16.5 g L^{-1} of butanol; Soni, Das and Ghose 1982; Tashiro et al., 2004). Similarly, various substrates have been employed for butanol production: Jerusalem artichoke juice (Marchal, Blanchet and Vandecasteele 1985); maltodextrin (Formanek, Mackie and Blaschek 1997); Sago starch (Madihah et al., 2001); cassava starch and cassava chips (Thang, Kanda and Kobayashi 2010); enzymatically hydrolyzed cassava flour (Lepiz-Aguilar et al., 2013). The use of multiple carbon sources was also investigated. In a batch fermentation utilizing a mixture of (1:1, w/w) glucose and glycerol, a byproduct of biodiesel production, *C. pasteurianum* produced 21 g L^{-1} of butanol with a yield of 0.23 g g^{-1} (Sabra et al., 2014). A more detailed comparison of fermentation results utilizing various strains and carbon sources can be found in several review papers (Jang et al., 2012c). As the clostridial strain is able to utilize a wide range of substrates, identification of locally or logistically available cost-effective carbon sources is important depending upon the country of operation. Most of cheaper 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 resulting in butanol of 11.7 g L^{-1}. The strain was able to simultaneously utilize glucose and xylose present in the hydrolysate. Most of the wild type strains suffer from carbon catabolite repression due to which they are unable to utilize any 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). Butanol is highly toxic to all microorganisms, including clostridia, and at high concentrations can lead to reduced substrate consumption and decreased overall cellular metabolism. Overcoming butanol toxicity has become a major challenge in the economical production of butanol with fed-batch cultivation demonstrating little to no advantage when compared with batch fermentation for butanol production (Fond et al., 1984). To overcome this problem in terms of bioprocess, various methods of *in situ* recovery processes were extensively studied to further enhance butanol fermentation: gas stripping, liquid-liquid extraction, perstraction, vacuum extraction, pervaporation and adsorption (Abdehagh, Tezel and Thibault 2014; Xue et al., 2014). Gas stripping can be applied to batch, fed-batch (Qureshi, Maddox and Friedl 1992) continuous (Ennis, Qureshi and Maddox, 1987) and immobilized cell fermentations (Ennis, Qureshi and Maddox, 1987). The possibility of employing *in situ* gas stripping for butanol recovery was examined during the batch fermentation of *C.*

saccharobutylicum P262 using sulfate whey permeate as a substrate (Ennis et al., 1986). Compared to batch fermentation, application of *in situ* gas stripping increased the lactose consumption up to 2-fold (58.3 *versus* 29 g L^{-1}), with the final butanol concentration and yield of 11.0 g L^{-1} and 0.19 g g^{-1} lactose, respectively (Ennis et al., 1986). When this approach was applied to a fed-batch fermentation using the *C. beijerinckii* BA101 strain, 151.7 g L^{-1} of butanol could be produced with a yield of 0.30 g g^{-1} glucose and a productivity of 0.75 g L^{-1} h^{-1} (Ezeji, Qureshi and Blaschek 2004). Although the butanol condensate obtained from gas stripping had a greater titer of butanol as compared to the fermentation broth, the condensate still contained a large amount of water, which required additional gas stripping or other separation techniques in order to further concentrate butanol. A two-stage gas stripping fermentation using immobilized cells of *C. acetobutylicum* JB200 made it possible to obtain highly concentrated butanol (420.3 g L^{-1}) from the second stage gas stripping condensate, which was a significant increase over that (175.6 g L^{-1}) obtained from the first stage (Xue et al., 2013). More recently, fed-batch fermentation of *C. acetobutylicum* JB200 coupled with the combination of *in situ* gas stripping and pervaporation using a carbon nanotube composite material resulted in more concentrated butanol of 521.3 g L^{-1} (Xue et al., 2016). Although gas stripping has several advantages as a technique for *in situ* butanol recovery, more studies are needed as its scale up appears still challenging due to increased high gas flow rate and pressure.

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 like 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.

The three major fermentation modes of cultivation 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 microorganism's 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.

2.5 Metabolic Engineering of *Clostridium*: Rationale towards strain improvement for enhanced butanol response.

Another key aspect towards attaining better butanol production is through novel strain development rationales. Extensive research has been deployed in the field of strain engineering to fulfill the demands of end product concentration. Inhibitory limits of butanol can effectively be combated by genetic manipulations and also provides avenue for conducting combinatorial interactions especially for genus like *Clostridium* where much work is still to be done. Three main routes of metabolic engineering are being pursued in order to achieve this goal. The first is to increase solvent production directly by altering metabolic pathways and blocking sporulation, the second is to increase solvent tolerance and the third method is to enable *C. acetobutylicum* to utilise cheaper substrates.

One of the limitations of metabolic engineering with clostridia is the lack of genetic tools available. The presence of the Cac824I restriction system in *C. acetobutylicum* initially prevented the transfer of recombinant vectors prepared in *E. coli*. This was at first overcome by using shuttle vectors such as pFNK-1 which lacks the recognition sites of Cac824I (Mermelstein et al., 1992) and thereafter with the use of an *E.coli* donor carrying the vector pAN1 (Mermelstein and Papoutsakis, 1993), and more recently pAN2 (Heap et al., 2007), which contain the φ3TI methyl transferase gene of the *B.subtilis* phage φ3tI. This protects the plasmid from the restriction by Cac824I. Studies have been conducted using plasmids to overexpress genes involved with solventogenesis. The overexpression of *spo0A* has resulted in increased levels of butanol compared with a plasmid only control strain (Harris et al., 2002). Overexpression of *adc*, *ctfA* and *ctfB* genes has resulted in 90% and 37% higher levels of acetone and butanol, respectively, than the plasmid control strain (Mermelstein et al., 1993).

Progress has also been hampered by the inability to produce knockouts not just in *C. acetobutylicum* but in the whole genus *Clostridium*. Until recently, only a handful of gene knockouts had been made. Four of these were derived through the unstable insertion of a plasmid by a single crossover event and were obtained by labour intensive screening of thousands of transformants (Shimizu et al., 1994; Wilkinson and Young, 1994; Green and Bennett, 1996; Green et al., 1996; Sarker et al., 1999; Liyanage et al., 2001; Harris et al., 2002; Huang et al., 2004; O'Connor et al., 2006; Raju et al., 2006).

Table 2.1 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	ARTP[d] treatment	16.7	12.2	Kong et al., 2016
p(GROE1)	E	*C. acetobutylicum* ATCC 824	groESL overexpression	17.1	13.0	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

2.6 Mathematical approach of understanding *Clostridial* intracellular regulations

Genome Scale Models are designed on the basis of net amount of reactions considered taking into account a certain number of metabolites both final and

intermediate. Senger et al., (2008) designed a network considering 80 transport reactions with 422 intracellular metabolites involving 552 reactions. Development in GSM was done when Lee et al (2008) reconstructed the metabolic network with 502 reactions involving 479 intermediate metabolites. Dash et al (2014) constructed GSM naming it *i*CAC 802 which considered 802 genes, 1137 metabolites spanning 1462 reactions. These metabolic models as designed have opened horizons as they have given an overview of the overall functioning of *Clostridial* system.

Flux balance analysis (FBA) is a mathematical modeling based approach utilized by metabolic engineers to quantitatively simulate microbial metabolism. Modelling of metabolic pathways is useful in analysis and optimization of fermentation process. FBA deals with the study of carbon flux distribution in the metabolic network. First step involves reconstruction of the metabolic network of the organism and simulating it to mimic the natural process. This provides us with the knowledge of predicting and controlling the flux distribution in the metabolic network (Kaufmann et al., 2003). The main focus is on complete understanding of the flux distribution and targeting the nodes to divert the flux towards maximum production of the desired product. Several metabolic flux analysis and flux balance analysis have been carried out in *Clostridium* sp. to provide insight into the solvent production (Desai et al., 1999; Lee et al., 2008; Senger and Papoutsakis, 2008; Milne et al., 2011; McAnulty et al., 2012). However, all studies are directed towards constraint based modelling assuming that the organism achieves steady state at any given environmental condition. The phenomenon this model fails to capture is the dynamics of transition from one steady state to another. A study conducted in microalgae *Chlorella* FC2 IITG captured this transition between light and dark cycles by means of dynamic flux balance analysis (Muthuraj et al., 2013). Such a study has not been

performed in *Clostridium* sp. and can be applied to understand the dynamic change during the transition from acidogenic phase to solventogenic phase.

Kinetic models facilitate the prediction and/or control of microbial process (Kong et al., 2006). Therefore, development and validation of a kinetic model is considered as an important step before a fermentation process is scaled up. Shinto et al (2007) are credited with the development of first kinetic model for *C. saccharoperbutylacetonicum.* However, that model failed to incorporate some metabolites which were proved important in solvent production. This model was then improved upon by Lin et al (2011) who also included enzymatic activities as parameters. Progress has been done in the stoichiometric modelling since the first one was developed by Papoutsakis (1984) which was followed by Desai et al., (1999) describing the role of acid forming pathways in solvent production by the help of metabolic flux analysis. Haus et al., (2011) published a model to capture the pH-induced metabolic shift in *C. acetobutylicum* under phosphate-limited continuous culture considering the changes in gene expression and proteome composition with the changes in external pH. This model was improved upon by Millat et al., (2012) who incorporated pH dependent enzyme kinetics. Despite of progress in the modelling field there are few kinetic models simulating *Clostridium* strain and model describing the complete dynamics of *Clostridium* metabolism is still lacking.

Understanding the potential of *Clostridial* metabolism is a function of its regulatory design. A regulatory model can be designed on the basis of the various omics data- metabolomics, proteomics and transcriptomics data published as of date. Microarray is an important high-throughput technique used to generate omics data. The use of microarray techniques has greatly increased the knowledge of *C.acetobutylicum* gene expression. This powerful technique can be used to look at the regulation of a great number of genes over the normal range of growth or compare the expression of genes over

time under different conditions. Combined with gene overexpression, antisense RNA technology or gene knockout, it can be a useful tool to determine the regulatory effect of specific genes. The majority of this microarray work has been done by the Papoutsakis group currently at the University of Delaware (Alsaker et al., 2004; Alsaker et al., 2005; Borden and Papoutsakis 2007; Jones et al., 2008; Parades et al., 2004; Tomas et al., 2003a; Tomas et al., 2003b; Tummala et al., 2003a; Tummala et al., 2003c) . Some of these studies have already been mentioned previously, with many studies also focusing on the underlying genetic control of sporulation.

The physico-chemical properties of the ABE-products allow an easy detection by gas chromatography (Green et al., 1996). However, since the evaporation of acids may impose some methodological problems, the use of a High-Performance Liquid Chromatography (HPLC) and a refractive index detector is also frequently encountered (Buday et al., 1990). The coupling of a tandem mass spectrometer to the HPLC allows the determination of intracellular metabolites. This procedure requires several preparatory steps, e.g. rapid sampling and rapid quenching (Schaub et al., 2006). Such approaches were used for determining intracellular metabolites of E. coli grown in C^{13}-glucose supplemented medium (Schaub, 2006). For *C. acetobutylicum*, one similar study of a batch culture is published. 121 metabolites were measured using a tandem mass spectrometry after addition of universally labelled C^{13}-glucose. Massive changes in all metabolites during the shift from acidogenesis to solventogenesis were observed. The carbon flux is redirected from biomass growth to solvent production (Amador-Noguez et al., 2011). Online measurements procedures are published for metabolite analysis using a mid-infrared spectroscopy approach (Kansiz et al., 2001) and for redox balance determination using a fluorescent probe (Srivastava and Volesky, 1991).

The analysis of the complete transcriptome of *C. acetobutylicum* allows the temporal resolution nowadays. Numerous such data sets are available: Study of Spo0A overexpression (Alsaker et al., 2004), groESL overexpression (Tomas et al., 2003), ctfAB knockdown (Tummala et al., 2003b) and the transcriptional programme of sporulation (Alsaker and Papoutsakis, 2005, Jones et al., 2008) were performed. Responses to butanol addition (Alsaker et al., 2004) and to several acids (Alsaker et al., 2010) were recorded. Reproduction of array results is usually undertaken by using a real-time PCR approach on few genes (Nolan et al., 2006, Lehmann and Luetke-Eversloh, 2011). The quantities measured by both approaches are in general comparable (Dallas et al., 2005).

Stress response related proteins were detected using pulse-labelled proteins in a batch culture (Terracciano et al., 1988). The proteome study of a phosphate limited chemostat culture analysed 130 proteins and found 52 proteins being up-regulated two-fold during the onset of solventogenesis, and 34 proteins being downregulated by the same factor (Schaffer et al., 2002). A more sensitive proteome protocol was developed and tested in a similar culture, yielding a resolution of over one thousand proteins on a 2D gel (Schwarz et al., 2007a). In a phosphate limited chemostat, 15 proteins could be specifically assigned to acidogenesis and 29 to solventogenesis (Janssen et al., 2010).

2.7 Environmental factors determining butanol production

Understanding of environmental factors responsible for a particular process is of utmost importance before we can divert it in favor of our desired product formation. Several studies have been carried out as an attempt to gain insight into physiological factors controlling the solventogenic phase of *Clostridium* sp. in terms of initiation and duration. Among the key parameters, an important role is played by the operating conditions that promote the switch from acidogenesis phase to the solventogenesis phase. A 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 is associated with the onset of solventogenesis (Millat et al., 2012). A threshold value of the concentration of undissociated acids – acetyl and butyric (Bahl and Gottschalk, 1985) and of the internal pH has been identified as a trigger for the solventogenesis phase (Gottwald and Gottschalk, 1985). Apart from that 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 same (Bahl and Gottschalk, 1985). Changes in incubation temperature and spurts of oxidative stress are also shown to affect butanol production (Jones and Woods, 1986). Another important aspect is related to the maximum butanol concentration that may be reached which is limited by butanol toxicity (Dunlop, 2011). Therefore, factors providing tolerance to the strain can also be targeted for improvement in the production capacity.

2.8 Biobutanol as an alternate transportation fuel

Table 2.2 Comparative qualitative fuel properties

Parameters	Gasoline	Methanol	Ethanol	Butanol
Octane Number	80 – 99	111	108	96
Cetane Number	0 – 10	3	8	25
Evaporation heat (MJ/Kg)	0.36	1.2	0.92	0.43
Combustion energy (MJ/dm^3)	32	16	19.6	29.2
Flammability (%)	0.6 – 0.8	6 – 36.5	4.3 – 19	1.4 - 11

Evident from Table 2.2, butanol is a lucrative alternate to existing non-renewable fuels and is better when compared to ethanol and methanol owing to its qualitative properties. Butanol in comparison to ethanol is more hydrophobic and has a greater energy density. Butanol is less evaporative in nature and thus it is easy to handle and is also less corrosive to engines and pipelines; resulting in its use without any further modifications (Pfromm et al., 2010). Current state of technology focused on improving the yields of butanol in order to make it socio-economically feasible. Butanol is more miscible with gasoline and diesel fuel, has a lower vapor pressure, and is less miscible with water. It is currently used as a feedstock chemical in the plastic industry and as a food grade extractant in the food and flavor industry (Qureshi and Blaschek, 2001). Multiple researchers have highlighted the positive impact on internal combustion engines of butanol when blended with diesel and its suitability in engine performance with improved mechanical and brake thermal efficiency lends credence to its future application as an alternate to petroleum fuel (Rakopoulos et al., 2010). A review of existing literature reveals that vehicle emission performance with n-butanol is inconclusive (Tao et al., 2014); however, existing efforts do signify its potential towards a vehicular fuel candidate. Ethanol has limited solubility in diesel fuel; therefore, phase separation and water tolerance in ethanol–diesel blend fuel are crucial problems. Ethanol fuel has an extremely low cetane number, whereas diesel engines prefer high cetane number fuels, which makes autoignition easy and gives a short ignition delay (Chotwichien et al., 2009). The dynamic viscosity of ethanol is much lower than that of diesel fuel, so that the lubricity is a potential concern of ethanol– diesel blend fuel (Chotwichien et al., 2009). Biodiesel is known to act as an additive or emulsifier due to its potential to improve the solubility of ethanol in diesel fuel over a wide range of temperatures and blend properties. Additionally, the use of a higher alcohol, propanol and butanol, could solve the problem of

fuel instability at low temperature because of more solubility in the diesel. Biodiesel blending with butanol has shown stable engine performance in blends with commercial diesel.

2.9 Bottlenecks

A detailed and in-depth literature survey of the current status of butanol production reveals:

- ABE fermentation is hindered on a commercial platform owing to low titer, productivity and yield.
- Use of expensive and orthodox substrates has been one of the major limitations towards sustainability as it increases the overall production cost, making the return on investment cumbersome.
- A suitable downstream and product recovery needs to be optimized in order to facilitate process economics and separation.
- Dearth of knowledge mapping phenotypic responses to intracellular regulations.
- Application of biobutanol as a platform for vehicular fuels / blends needs to be demonstrated.

2.10 References

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Chapter 3

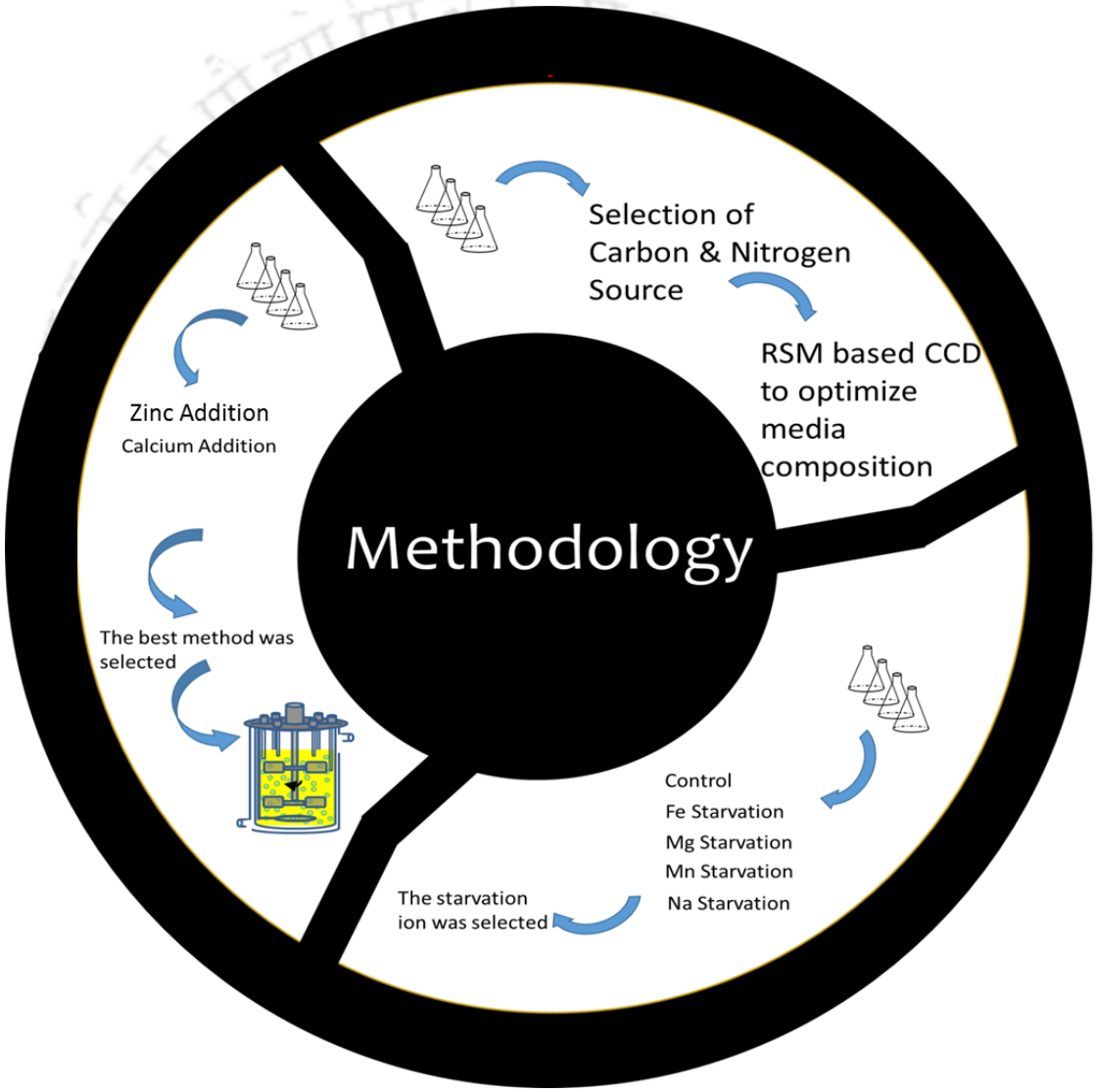
BIOCHEMICAL CHARACTERIZATION OF *CLOSTRIDIUM ACETOBUTYLICUM* ATCC 824 UNDER VARIOUS ENVIRONMENTAL CUES

"What you see is that the most outstanding feature of life's history is a constant domination by bacteria."

Stephen Jay Gould

CHAPTER 3

Biochemical characterization of *Clostridium acetobutylicum* ATCC 824 under various environmental cues



Schematic representation of the methodology obtained for screening of different cues directed towards maximization of butanol production by *Clostridum acetobutylicum* ATCC 824.

3.1 Background and motivation

Continual population growth and simultaneous exponential decline in fossil fuel reserves coupled with adverse climatic changes has kindled research interest towards development of alternate and a more sustainable form of energy (Moon et al. 2016). To that end, research has been continuously pursued, gaining impetus over the last few decades. Studies have been conducted worldwide in order to develop feasible alternate fuel strategies encompassing biomass based biofuels, lignocellulosic and other alternate advanced fuels. Ethanol, amongst the many alternates has been established as a feasible alternate; however ethanol on a commercial aspect falls short due to its high cost of production incurred. Butanol, a primary alcohol, has also been similarly studied since World War II, and it recounts multiple advantages to its credit when compared with the regular gasoline and ethanol. Importance of butanol lies in the fact that it can be a potential replacement of the existing depleting sources of fossil fuels with specific set of advantages when compared to the ethanol blending such as lower air:fuel ratio, lesser tendency to corrode engines and its compatibility to the existing engine infrastructure (Pfromm et al. 2010).

Butanol is inherently produced by *Clostridium acetobutylicum*, a gram-positive, endo-spore forming, and obligate anaerobe under the genus firmicutes (Durre et al. 2007) via utilizing a wide variety of substrates. *Clostridium* displays microbial diversity in terms of habitat, genetic material, metabolism, and nutritional and cultivation requirements (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. *Clostridial* fermentation is typically characterized by two distinct phases, an initial growth associated acid forming phase termed as acidogenesis followed by solventogenesis in which the acids are re-assimilated and are subsequently converted to solvents, namely acetone, butanol and ethanol; hence, the name of the process being abbreviated as ABE fermentation.. However, several limitations in *Clostridium* poses its own set of challenges resulting in lower yield and productivity, which are essential to be annulled, in order to design a sustainable process for commercialization. The challenges include (a) high cost of raw materials, (b) end product toxicity, (c) undesirable ratio of the end products, (d) degeneration of strains, (e) sporulation, and (f) unknown metabolic regulations. To that end, medium engineering is one of the key elements of paramount importance in demonstration of an efficient bioprocess strategy for elevated butanol production.

The present objective was performed to identify the substrates critical for butanol production. Further, nutritional requirements of the strain were optimized in order to maximize the butanol titer. Secondly, modulation of phenotypic traits of the strain e.g., growth, glucose utilization, butanol production and onset of solventogenesis under the influence of selected metal ions either individually or in combination has been reported. Supplementation of zinc and calcium was shown to synergistically facilitate carbohydrate uptake and in turn, upregulate butanol titer in *C. acetobutylicum* ATCC 824 (Wu et al., 2016). On contrary to the supplementation of the metal ions, iron limitation in the medium has been reported to be favorable for butanol production via inhibition of hydrogen formation, which in turn directs carbon flux towards butanol synthesis pathway (Junelles et al., 1988).

3.2 Materials and methods

3.2.1 Microorganism, maintenance and preparation of seed culture

Clostridium acetobutylicum ATCC 824 has been procured from American Type Culture Collection (ATCC), USA and was revived in 10 mL of TYG medium (Annous and Blaschek, 1990) comprising of (g L^{-1}): tryptone 30.0, yeast extract 10.0, glucose 20.0 and cysteine hydrochloride 0.05 in a static incubator (Orbitek, Scigenics Biotech) at 37 °C under strict anaerobic conditions achieved by purging 99.99% pure nitrogen gas. The anaerobicity was confirmed by the addition of resazurin (redox indicator dye) at a concentration of 1 g L^{-1} in the fermentation medium. At an optical density ($O.D_{600}$) of 3.0, 700 µL of the culture was added to 300 µL of 50% (v/v) glycerol in a cryovial (final volume of 1 mL) and stored at -80° C for further studies. The pre-seed culture was prepared at the beginning of any experiment by adding 1 mL of the glycerol stock into 10 mL of TYG medium and was allowed to grow until an $O.D_{600}$ of 3.0 was reached. Subsequently, the seed culture was prepared by transferring 10 mL of the pre-seed culture into 90 mL of TYG medium in a customized air tight bottle. The growth conditions for pre-seed and seed culture preparation were kept similar as mentioned earlier in this section. In all subsequent experiments, 10% (v/v) seed culture was used as inoculum.

3.2.2 Characterization *Clostridium acetobutylicum* ATCC 824 under different carbon and nitrogen sources for its growth and butanol production.

Characterization of *C. acetobutylicum* ATCC 824 was performed with the aim of evaluating the effects of various carbon and nitrogen sources on growth and butanol production. These experiments would in turn facilitate towards designing a production medium for enhanced butanol biosynthesis. The study was conducted in pre-defined P2 media (Monot et al. 1982) which comprises (in g L^{-1}) of glucose 50.0, tryptone 30.0, yeast extract 10.0, K_2HPO_4 0.50, KH_2PO_4 0.50, CH_3COONH_4 3.22, para-amino-benzoic acid

0.01,biotin 0.001 and trace element of 10 mL L^{-1} containing (in g L^{-1}) $MgSO_4.7H_2O$ 0.20, $MnSO_4.H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, NaCl 0.01. The effect of carbon sources was studied by substituting glucose in the P2 media with equimolar carbon concentration of seventeen different carbon sources. These included three pentoses (xylose, ribose and arabinose), five hexoses (dextrose, galactose, fructose, sodium gluconate and mannose), three disaccharides (lactose, sucrose and maltose), three sugar alcohols (sorbitol, mannitol and glycerol) and three polysaccharides (starch, cellulose and dextrin). The carbon source which supported maximum butanol synthesis was selected and used for all subsequent characterization and optimization experiments. The effect of nitrogen sources was further studied by replacing tryptone and yeast extract in the media with ten different organic and inorganic nitrogen sources containing equimolar nitrogen concentration. These included tryptone, yeast extract, peptone, protease peptone, beef extract, urea, glycine, ammonium acetate, ammonium chloride and ammonium sulphate. The best nitrogen source resulting maximum butanol production was selected and used in further experiments. The fermentation batch was carried out in 250 mL customized air tight cultivation flasks with a working volume of 50 mL for 72 h at 37°C in static, anaerobic condition maintained by purging 99.9% pure gaseous nitrogen while the anaerobic condition was confirmed by the addition of resazurin at 1 g L^{-1}. Samples were assessed at regular intervals for determining the maximum biomass growth and butanol production. The experiments were performed in duplicate and the results were expressed as mean ± standard error.

3.2.3 Maximization of butanol biosynthesis through statistical media optimization.

With the objective function of maximization of butanol titer, the initial ranges of carbon source, nitrogen source and trace elements were optimized employing a Central Composite Design (CCD) based Response Surface Methodolgy (RSM). The actual values and coded values of the above selected parameters used in CCD based RSM experimental

design is depicted in Table 3.1. Coded values of +α, +1, 0, and –1,−α correspond to high, medium, and low levels of the variables respectively. A 3^5 quarter factorial CCD was generated using Minitab stat 16.1.1 (Minitab Inc., Pennsylvania, USA) and was employed to optimize the butanol titer. The CCD predicted 18 experiments which included eight factorial points, six axial points and four replicates of center point to search linear, quadratic and interaction effect of parameters on butanol titer. RSM is a mathematical modeling technique which utilizes a polynomial equation to model the interaction among the variables. Under RSM, the linear, quadratic, and interaction effects between the selected medium components and butanol titer were mathematically expressed in the form of a quadratic polynomial Eq. (3.1).

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

Where Y is the butanol titer (g L^{-1}) as model response, X_i is the i^{th} parameter, k is the total number of parameters and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients. All the 18 experiments were performed in duplicate and similar cultivation conditions were followed as mentioned in the previous section.

Table 3.1 Actual values and coded values of the variables employed in CCD-RSM based optimization

Factors		Levels code and corresponding values				
Code	Name	-1.68	-1	0	1	1.68
X_1	Initial glucose concentration (g L^{-1})	30	50	80	110	130
X_2	Initial peptone concentration (g L^{-1})	16.36	30	50	70	83.63
X_3	Initial trace concentration (mL L^{-1})	3.18	10	20	30	36.18

3.2.4 Screening of metal ions based on their supplementation or starvation effect on the organism

Metal ions were screened via elucidating their effect of either starvation or supplementation on growth and butanol production in *C. acetobutylicum* ATCC 824. While, four metal ions magnesium (Mg), manganese (Mn), sodium (Na) and iron (Fe) were chosen for their individual starvation effect, two metal ions zinc (Zn) and calcium (Ca) were chosen for their individual supplementation effect on the organism. In order to capture the individual starvation effect, metallic ions added as a part of the trace elements, were starved from the production media one at a time. Experiment to capture effect of zinc supplementation was carried out via addition of $ZnSO_4.7H_2O$ as the exogenous source of zinc in the production medium at three different concentrations of 0.001 g L^{-1}, 0.01 g L^{-1} and 0.1 g L^{-1}. Effect of calcium ion was evaluated by supplementing $CaCl_2.2H_2O$ at three concentrations of 0.1 g L^{-1}, 0.4 g L^{-1} and 1 g L^{-1} in the production media.

3.2.5 Analytical methods

Samples were collected and centrifuged at 10,000 rpm for 10 min (Multifuge X3R, Thermofisher Scientific, Germany) at regular intervals. Biomass growth was measured by resuspending the pellet in distilled water followed by determining absorbance at 600 nm using UV-Vis spectrophotometer (Cary 100, Varian, Australia). The supernatant was used for estimation of glucose, acids (acetic acid and butyric acid) and solvents (acetone, ethanol and butanol). Glucose, acids and solvents were analyzed in HPLC (Ultimate 3000, Dionex, Thermofisher Scientific, Germany) using Rezex ROA column (300 x 7.8 mm, Phenomenex) and mobile phase of 0.005 N H_2SO_4 with a flowrate of 0.5 mL min^{-1}. While, acids were detected in UV detector at 210 nm, glucose and solvents were detected in Refractive Index detector (RID). The column oven was kept at room temperature and RID at 37°C.

3.3 Results and discussions

3.3.1 Screening and characterization of the strain under different carbon and nitrogen sources for biomass growth and butanol production

Alterations in primary carbon and nitrogen sources have been reported to present significant variations towards clostridial metabolism and solvent formation (Al-Shorgani et al. 2011). The present study details the effect of seventeen different carbon sources on the strains potential of growth and butanol production. *C. acetobutylicum* ATCC 824 was found to produce highest butanol titer of 11.6 g L^{-1} when grown on glucose as the sole carbon source along with mannose (8.5 g L^{-1}) and starch (8.3 g L^{-1}) also showing promising butanol titer (Figure 3.1A). Another key inference evident from carbon screening was that the strain was not potent to grow on pentoses along with lactose, sorbitol, sucrose, CMC, gluconate etc. showing lower butanol production (Figure 3.1A). *C. acetobutylicum* ATCC 824 was cultivated on glucose, xylose, and glucose-xylose blends simulating microalgae based carbohydrates and demonstrated its potential in using the same for butanol production (g L^{-1}) of 13.03, 8.89 and 11.5 respectively (Wang et al. 2014). 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 *C. acetobutylicum* ATCC 824.

It was quite evident from the observation that organic nitrogen sources proved to be a better platform for both biomass growth and butanol production (Figure 3.1B). 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). However, peptone (11.68 g L^{-1}) showed slightly better butanol production as compared to the rest of the platforms. Glycine and urea showed no butanol production, hence rendering them as

improbable as potent nitrogen source for the strain's metabolism. Inorganic nitrogen sources such as ammonium acetate, ammonium sulphate and ammonium chloride demonstrated lesser butanol production as compared to the organic sources. It has been reported that growth and solvent production in *Clostridium acetobutylicum* varies with the carbon to nitrogen (C: N) ratio (Welsh et al., 1987). In the current experiment the C: N ratio remained same as all the experimental media contained equimolar total nitrogen concentration. Therefore it can be concluded that butanol production depends upon the nitrogen content and not on the type of organic nitrogen source.

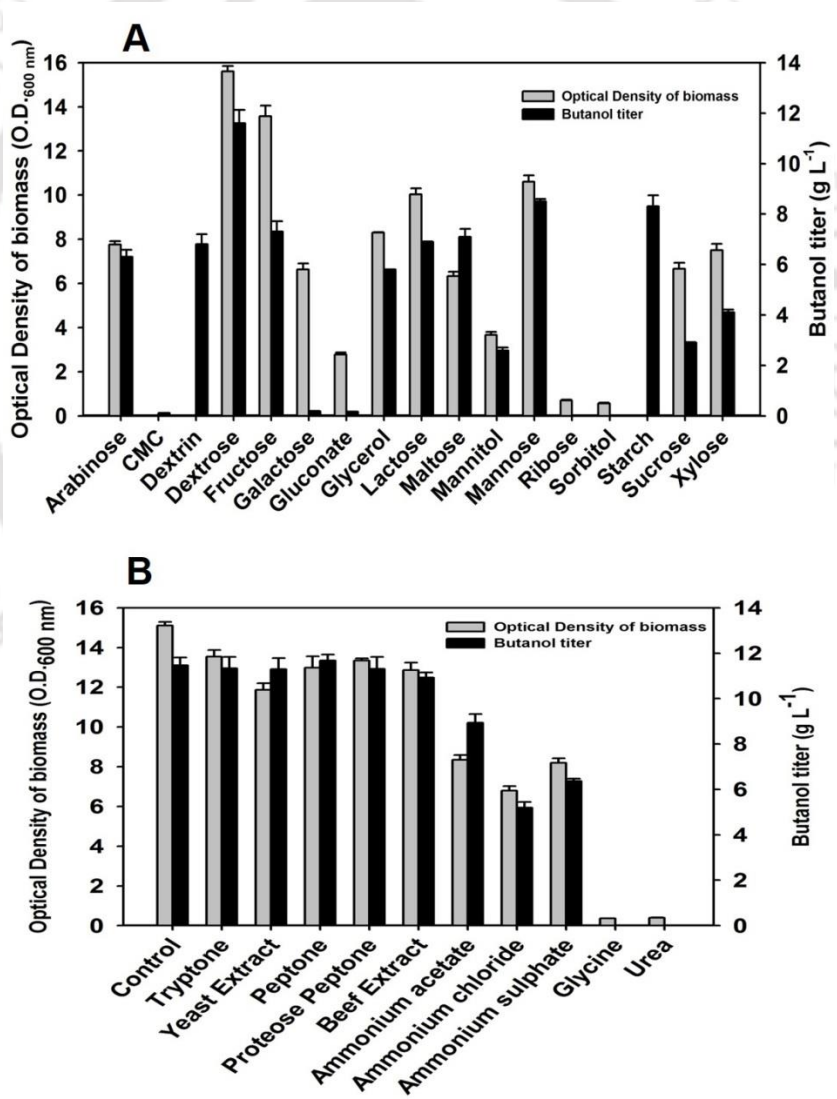


Figure 3.1 Characterization of *C. acetobutylicum* ATCC 824 in terms of biomass (O.D.) and butanol titer (g L^{-1}) when grown under different (A) carbon and (B) nitrogen sources.

Therefore, glucose and peptone were selected as the carbon and nitrogen sources respectively for further medium optimization directed towards maximization of butanol titer.

3.3.2 Maximization of butanol biosynthesis through statistical media optimization.

With the objective function of maximization of butanol titer, a CCD based RSM was designed and demonstrated to optimize the initial concentrations of carbon, nitrogen and trace elements required for the strain to exhibit optimal growth and butanol production (Table 3.2).

Table 3.2 Full factorial central composite design matrix of three variables in coded and natural units along with the observed and predicted response, butanol titer (g L^{-1}). All the experiments were conducted in duplicate and the data were expressed as mean ± standard error.

Standard Order	Glucose (g L^{-1}) X_1	Peptone (g L^{-1}) X_2	Trace (mL L^{-1}) X_3	Butanol (g L^{-1})	
				Observed	Predicted
1	50.00	30.00	10.00	6.97 ± 0.09	6.81
2	110.00	30.00	10.00	9.84 ± 0.066	10.08
3	50.00	70.00	10.00	7.20 ± 0.074	7.44
4	110.00	70.00	10.00	9.20 ± 0.023	9.01
5	50.00	30.00	30.00	4.80 ± 0.015	5.11
6	110.00	30.00	30.00	9.30 ± 0.026	9.18
7	50.00	70.00	30.00	7.90 ± 0.011	7.78
8	110.00	70.00	30.00	9.89 ± 0.008	10.16
9	29.54	50.00	20.00	3.10 ± 0.017	2.98
10	130.45	50.00	20.00	7.80 ± 0.034	7.73
11	80.00	16.36	20.00	8.70 ± 0.062	8.59
12	80.00	83.63	20.00	10.01 ± 0.014	9.94

13	80.00	50.00	3.18	10.80 ± 0.027	10.77
14	80.00	50.00	36.81	10.45 ± 0.033	10.30
15	80.00	50.00	20.00	11.51 ± 0.046	11.51
16	80.00	50.00	20.00	11.50 ± 0.028	11.51
17	80.00	50.00	20.00	11.50 ± 0.032	11.51
18	80.00	50.00	20.00	11.50 ± 0.011	11.51

The performed experiments were retrofitted to RSM to decode and optimize the experimental results and also to study the analysis of variance (ANOVA), in order to elucidate the significance of the effect of the parameters, individually or in their combinatorial form (Table 3.3). At the four central points of the CCD, a maximum butanol titer of 11.5 g L^{-1} was obtained.

Table 3.3 Analysis of variance (ANOVA) for the selected quadratic model for butanol titer

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	9	95.16	95.16	10.57	197.2	0
Linear	3	29.64	29.64	9.88	184.28	0
Glucose (X_1)	1	27.17	27.17	27.17	506.8	0
Peptone (X_2)	1	2.20	2.20	2.20	41.06	0
Trace (X_3)	1	0.26	0.26	0.26	4.97	0.056
Square	3	61.66	61.66	20.55	383.34	0
X_1*X_1	1	53.29	59.76	59.76	1114.53	0
X_2*X_2	1	6.88	7.95	7.95	148.33	0
X_3*X_3	1	1.5	1.5	1.5	27.91	0.001
Interaction	3	3.86	3.86	1.29	23.98	0
X_1*X_2	1	1.43	1.43	1.43	26.63	0.001
X_1*X_3	1	0.32	0.32	0.32	6.12	0.039
X_2*X_3	1	2.10	2.10	2.10	39.19	0
Residual Error	8	0.43	0.43	0.05		
Lack-of-Fit	5	0.43	0.43	0.09	3431.02	0

Pure Error	3	0.96	0.96	0.19	
Total	17	95.6			
R^2	0.995	Adjusted R^2	0.99	Predicted R^2	0.96

The analysis of variance exhibited a regression correlation coefficient (R^2) of 0.995 with respect to the objective function of maximization of butanol titer, indicating high significance for all the CCD experiments to be expressed in the model. The linear and quadratic effects of all the three parameters were found to be significant towards modulation of butanol titer (Figure 3.2).

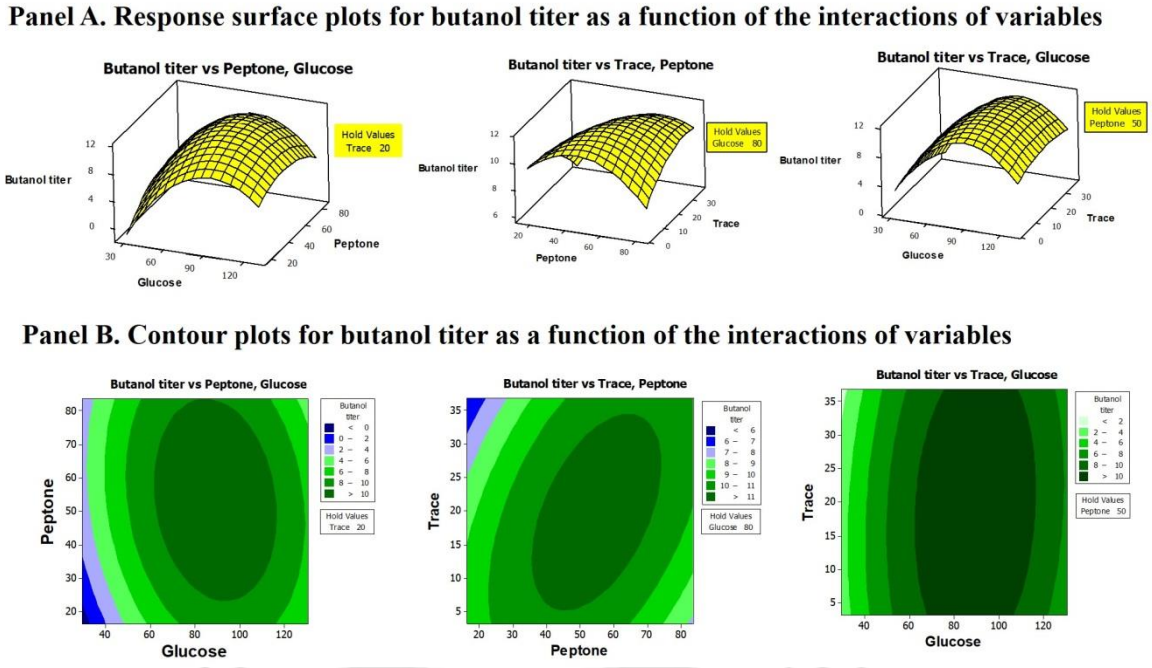


Figure 3.2 Response surface and contour plots depicting the interaction of the variables towards maximization of butanol titer.

On performing the predicted 18 experiments, it was observed that a maximum of 11.76 g L^{-1} of butanol was obtained which was not significant to what has been obtained previously. This result may be attributed to the inherent toxicity of the cells to butanol concentration. The initial concentrations of glucose, peptone and trace elements as predicted via RSM-CCD were used for all subsequent experiments unless mentioned otherwise. The optimized values have been enumerated in Table 3.4.

Table 3.4 Optimized values of media components for maximization of butanol titer

Optimized parameters	Maximization of butanol titer
Initial glucose concentration (g L^{-1})	89.68
Initial peptone concentration (g L^{-1})	53.06
Initial trace concentration (mL L^{-1})	20

With the further aim of understanding the effects of various metallic ions on butanol production, different metal ions were screened. Starvation or supplementation of metallic ions has been reported to regulate butanol biosynthesis in clostridial strains. To that end, the strain was characterized to evaluate its growth and butanol biosynthesis potential under the individual starvation effect of four metal ions e.g., magnesium (Mg), manganese (Mn), sodium (Na) and iron (Fe) and supplementation effect of two metal ions e.g., zinc (Zn) and calcium (Ca).

3.3.3 Screening of metal ions based on their supplementation or starvation effect on growth and butanol titer in *Clostridium acetobutylicum* ATCC 824.

The role of commonly added metallic ions as a part of trace element composition was studied to understand the effect of the ions on butanol titer. The rationale was so developed as to understand any prevailing effects of the metal ions and also to investigate whether their starvation would incur any differential pattern of substrate consumption as well as metabolite formation. Iron starvation has commonly been reported to positively regulate butanol biosynthesis and enhance end product titer. Such a result is observant due to the blocking of *hydrogenase* activity which inhibits the production of hydrogen gas and thus, help in diverting carbon flux towards butanol synthesis pathway (Junelles at al. 1988). Another similar study in *C. beijerenckii* showed enhanced production of hydrogen gas and simultaneous decrease of butanol yield due to modulation of iron concentration in the culture medium (Wu et al. 2016).

Among the four metal ions starved, butanol formation was observed to be upregulated with a highest titer of 13.5 g L^{-1} in case of magnesium followed by 12.01 g L^{-1} in case of sodium (Figure 3.3). Surprisingly, in comparison to the control, growth performance of the organism was found to be better in all four metal ion starved media However, a peripheral decline in butanol formation was recorded for both manganese and iron starved medium in comparison to the control. Another significant finding of magnesium starvation is the reduction in initiation of solventogenesis, thus reducing the lag phase and enhancing butanol productivity along with titer increment (Figure 3.3B). Generally, it has been observed that at around 12-14 hours of fermentation time, butanol production begins. However, it was seen that magnesium starvation significantly reduced butanol induction time to 6 hours.

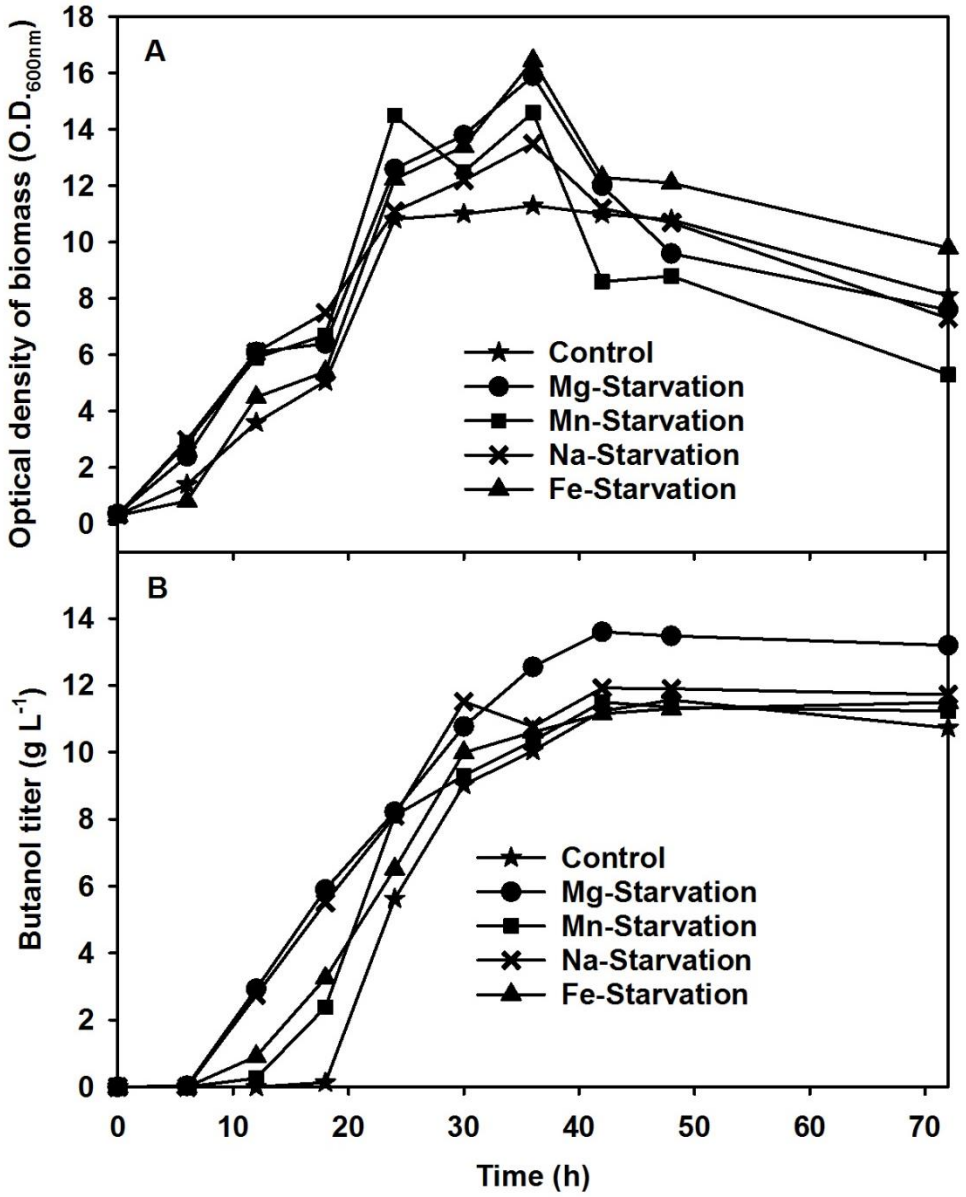


Figure 3.3 Effect of starvation of metal ions on (A) Optical density of biomass and (B) Butanol titer (g L^{-1}).

Response to starvation of key metallic ions such as iron have not been well studied towards improvement of biomass and some literature reports deem the importance of presence of these ions for growth and metabolite production. In an earlier study, *Lactobacillus plantarum* has been shown to grow under iron deficient condition (Archibald, 1983). In contrary to the results obtained in the present study, growth and butanol production from *C. acetobutylicum* ATCC 824 was found to be significantly compromised when $MgSO_4$ or $FeSO_4$ was devoid from the fermentation medium (Monot

et al. 1982). Presence of $MnSO_4$ was found to be neither beneficial nor detrimental for growth and butanol formation if not in excess (Monot et al. 1982). However, in another study, an improved butanol titer was reported for C. acetobutylicum strain ATCC 824 when grown in batch culture under iron limited condition (Junelles et al. 1988).

The strain was further characterized by exogenous supplementation of Zn and Ca under three different concentrations for its potential effect on growth and butanol titer. While calcium supplementation resulted in a reasonable improvement, an analogous growth was observed under zinc addition when compared to the control batch (Figure 3.4). Supplementation of either zinc or calcium in the control medium resulted in an improved butanol titer of 15.4 g L^{-1} and 13 g L^{-1} respectively. However, similar to the present study, supplementation of 4 g L^{-1} $CaCO_3$ or 0.001 g L^{-1} $ZnSO_4$ in the fermentation medium resulted in stimulation of both cell growth and butanol formation in *C. acetobutylicum* L7 (Wu et al., 2013; Wu et al., 2016).

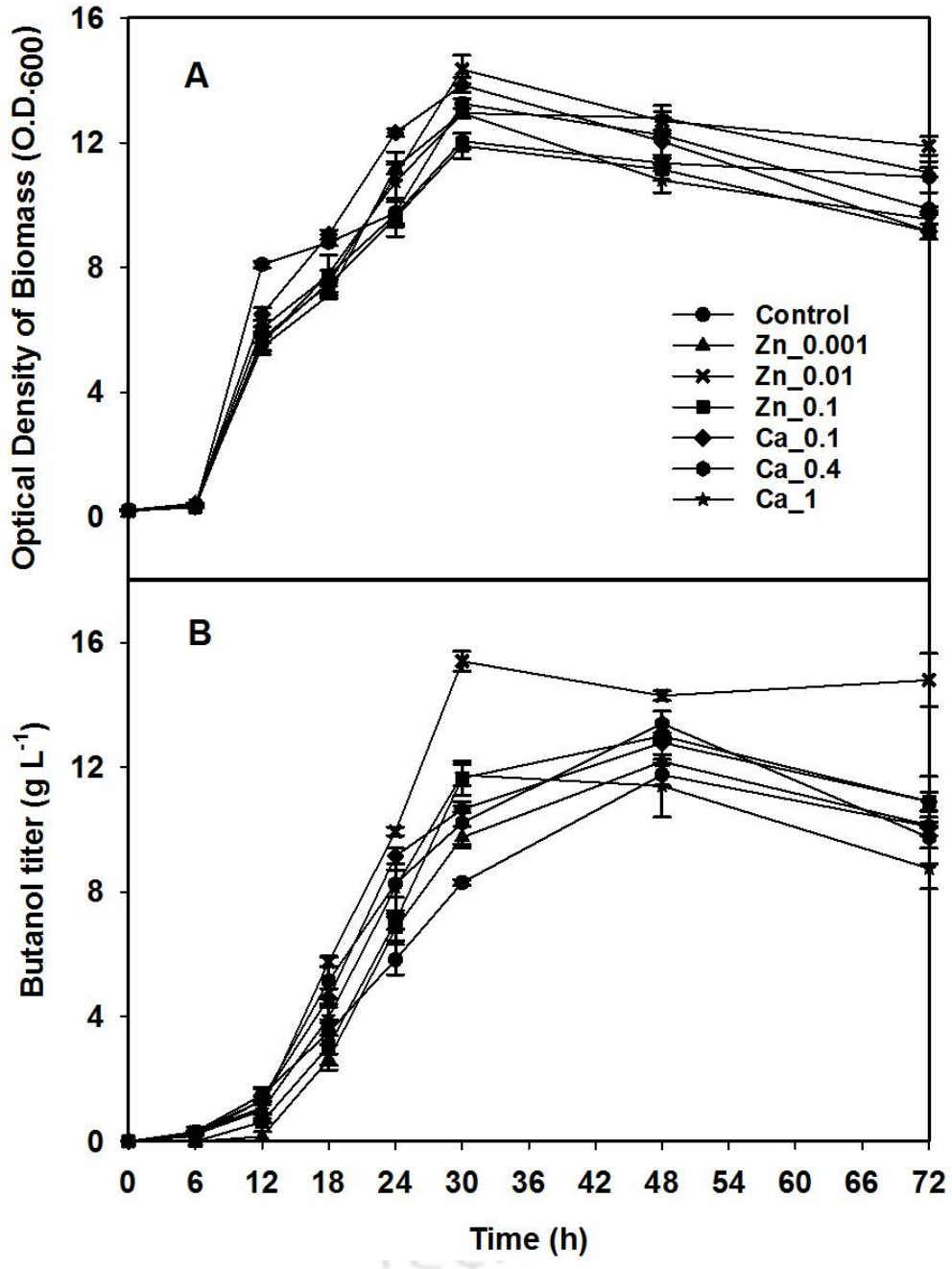


Figure 3.4 Effect of supplementation of metal ions on (A) Optical density of biomass and (B) Butanol titer (g L^{-1}).

Therefore, zinc supplementation at a concentration of 0.01 g L^{-1} resulted in an improvement of 31% in terms of butanol titer as compared to control optimized medium. Mg starvation in the medium has been reported for the first time through this study to positively influence cellular growth and butanol production. Figure 3.5 conclusively

illustrates a comparative growth and butanol profile for all the starvation and supplementation strategies. Zn supplementation and Mg starvation has been selected for further experimentation and characterization to elucidate their positive role towards upregulation in butanol biosynthesis.

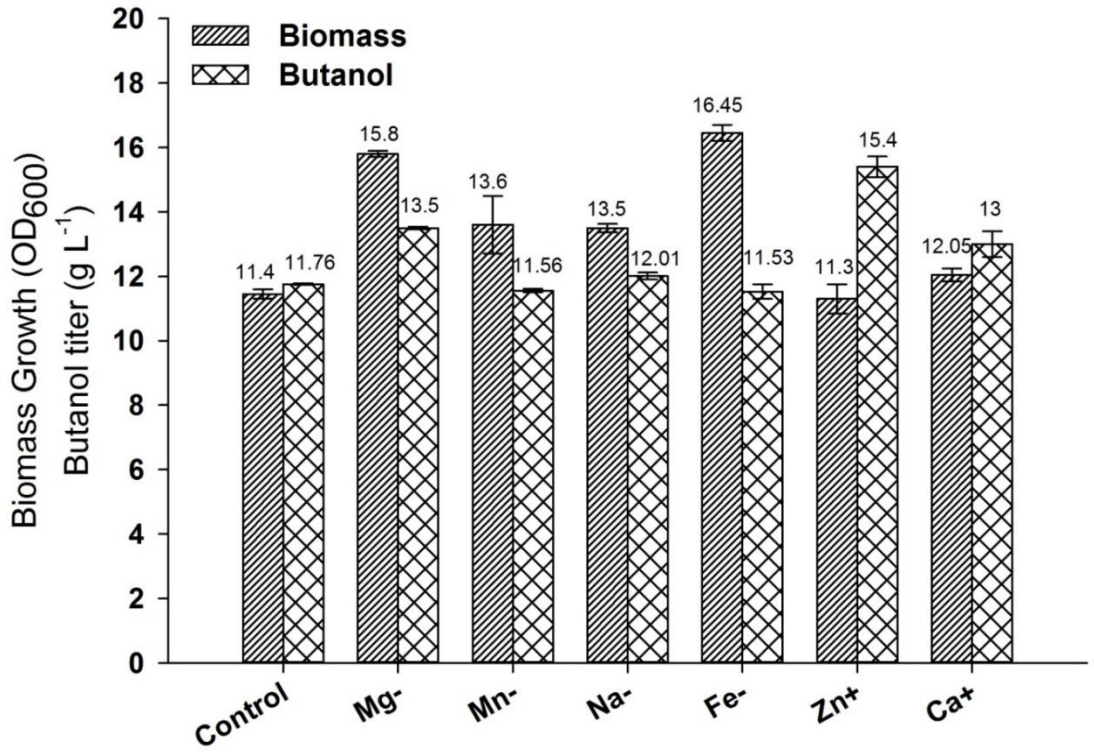


Figure 3.5 Comparison of growth and butanol titer in *C. acetobutylicum* ATCC 824 when grown on control, magnesium starved, manganese starved, sodium starved, iron starved, zinc supplemented (0.01 g L^{-1}) and calcium supplemented (0.4 g L^{-1}) media. Cultivation in production media was considered as control.

3.4 Conclusion

- ✓ Initial screening for carbon and nitrogen sources revealed the strains potential for maximum butanol production in **glucose** and **peptone** respectively.
- ✓ Statistical medium optimization of initial concentration of substrates towards maximization of butanol titer resulted in a **maximum butanol titer of 11.76 g L^{-1}**.
- ✓ Mg starvation and Zn supplementation individually resulted in elevation of **14.7%** and **31%** in butanol titer.
- ✓ Mg starvation also revealed **earlier onset of solvent formation** at 6 h compared to 12 h in control optimized medium.

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Chapter 4

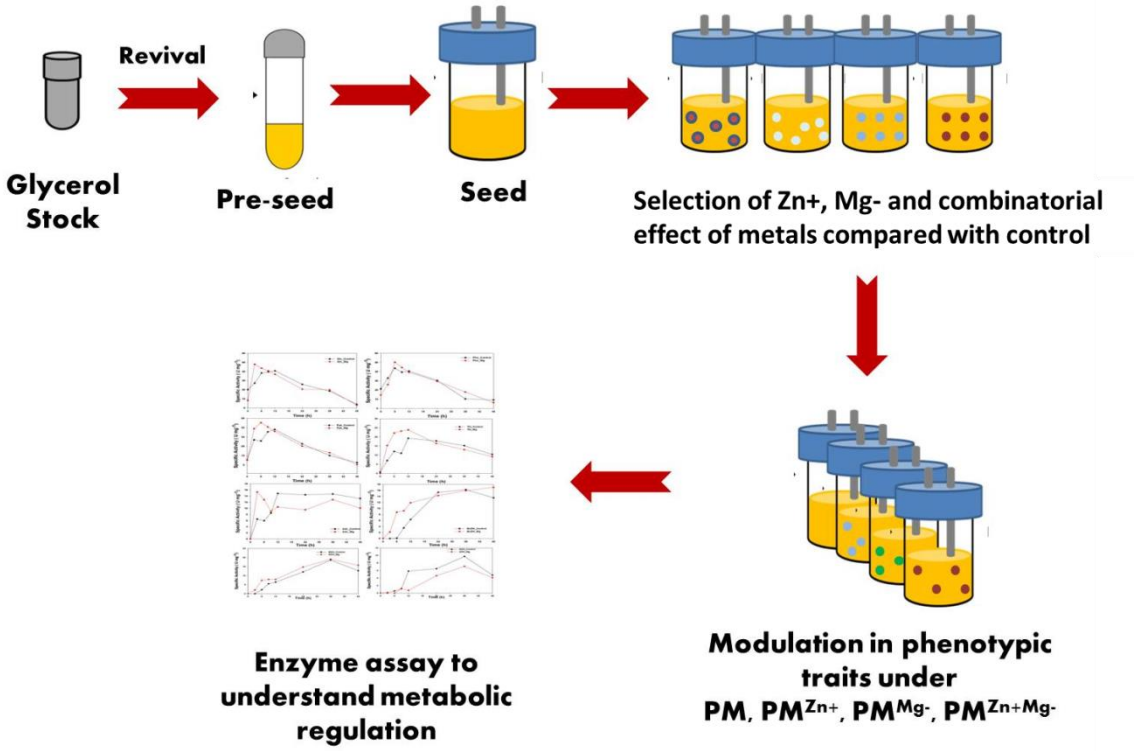
ENZYMATIC ANALYSIS TO UNDERSTAND THE EFFECTS OF VARIOUS CUES DIRECTED TOWARDS BUTANOL UPREGULATION.

"Enzymes are things invented by biologists that explain things which otherwise require harder thinking."

Jerome Lettvin, Scientist

CHAPTER 4

Enzymatic analysis to understand the effects of various cues directed towards butanol upregulation.



Elucidating the role of selected metallic ions towards carbon metabolism by *Clostridium acetobutylicum* ATCC 824 through enzymatic activity analysis.

4.1 Background and motivation

Extensive utilization of fossil fuels with technological development has resulted in numerous problems such as global energy crisis, climate change and human diseases. This clearly depicts the growing concerns to have sustainable technology for alternate and renewable energy. Biofuels produced through biological processes has been achieving increasing attention due to its environment-friendly features (Li et al., 2019). Currently, production of various primary alcohols such as methanol, ethanol and butanol has been explored as potential biofuel molecules for realization at commercial scale. Ethanol has been gaining more attention in comparison to methanol due to its superior fuel properties such as renewability, lesser toxicity and higher energy density (Wyman et al., 2018). Recently, there has been a paradigm shift from lower alcohol to higher alcohol (e.g. butanol) in the area of biofuel research. This can be attributed to various advantages which butanol offers over lower alcohols e.g., can be blended with base fuel without phase separation, can be transported & distributed using existing infrastructure, less corrosive and higher energy content per unit mass (Li et al., 2019). Clostridial strains have been considered as most widely implemented platform for butanol production through Acetone-Butanol-Ethanol (ABE) fermentation route (Moon et al., 2016). However, commercial scale fermentation process suffers from several bottlenecks in terms of low butanol titer or productivity, end product toxicity, reduced efficiency in solvent recovery from fermentation broth and high cost of production thus hindering the overall process sustainability (Durre et al., 2007; Moon et al., 2016).

The supplementation of zinc (Zn) and magnesium (Mg) starvation has been observed to elevate butanol response substantially as identified in the previous chapter (Chapter 3). Zinc associated response of *C. acetobutylicum* has been reported to be significantly pleotropic in terms of modulation of multiple phenotypic traits such as

carbohydrate utilization, glycolysis, acidogenesis and solventogenesis (Wu et al., 2015). Zinc plays significant roles towards stimulating metabolic activities with respects to sugar utilization, cell growth and acids re-assimilation as well as initiation of solventogenesis (Wu et al. 2013), implying it might be functionally synergistic for ABE fermentation. Nevertheless, more information with a desirable trait that derive from considerable impacts of examining conditions or available nutrients on more productive fermentation should be well taken into consideration to maximize the performance of ABE fermentation and thus propose a substantial scope for better understanding of the functional mechanisms of sugar utilization, central carbon metabolism and stress response (Wu et al. 2016).

The present study reports modulation of phenotypic traits of *Clostridium acetobutylicum* ATCC 824 e.g., growth, glucose utilization, butanol production and onset of solventogenesis under the influence of selected metal ions either individually or in combination. Extensive characterization of the organism was carried out under four different growth conditions: (i) normal medium (control); (ii) medium supplemented with zinc; (iii) medium devoid of magnesium and (iv) medium with zinc supplementation and magnesium starvation. Metabolic regulation behind inflected phenotypic response of the organism under the influence of metal ions was captured via obtaining temporal expression profile of the key metabolic enzymes in glycolytic, ethanol, butanol and acetone formation pathways. Eight enzymes have been selected on the basis of their functionality from the various literatures and their activity has been mapped towards unearthing a better understanding and possible hypothesizing the role of selected metal ions in the optimized medium.

4.2 Materials and methods

4.2.1 Microorganism, maintenance and preparation of seed culture

Clostridium acetobutylicum ATCC 824 has been procured from American Type Culture Collection (ATCC), USA and was revived in 10 mL of TYG medium (Annous and Blaschek, 1990) comprising of (g L^{-1}): tryptone 30.0, yeast extract 10.0, glucose 20.0 and cysteine hydrochloride 0.05 in a static incubator (Orbitek, Scigenics Biotech) at 37 °C under strict anaerobic conditions achieved by purging 99.99% pure nitrogen gas. The anaerobicity was confirmed by the addition of resazurin (redox indicator dye) at a concentration of 1 g L^{-1} in the fermentation medium. At an optical density ($O.D_{600}$) of 3.0, 700 µL of the culture was added to 300 µL of 50% (v/v) glycerol in a cryovial (final volume of 1 mL) and stored at -80° C for further studies. The pre-seed culture was prepared at the beginning of any experiment by adding 1 mL of the glycerol stock into 10 mL of TYG medium and was allowed to grow until an $O.D_{600}$ of 3.0 was reached. Subsequently, the seed culture was prepared by transferring 10 mL of the pre-seed culture into 90 mL of TYG medium in a customized air tight bottle. The growth conditions for pre-seed and seed culture preparation were kept similar as mentioned earlier in this section. In all subsequent experiments, 10% (v/v) seed culture was used as inoculum.

4.2.2 Characterization of the strain's metabolism and phenotype under different media composition

In order to capture modulation in phenotypic response of the organism under the influence of zinc supplementation and magnesium starvation (as screened previously in Chapter 3) either individually or in combination, an extensive characterization was carried out under four different growth conditions: (i) production medium (PM) which is considered as control; (ii) production medium supplemented with zinc (PM^{Zn+}); (iii) production medium devoid of magnesium (PM^{Mg-}) and (iv) production medium with zinc

supplementation and magnesium starvation (PM^{Zn+Mg-}). In case of PM^{Zn+} batch, $ZnSO_4.7H_2O$ was exogenously supplemented into the production medium at a concentration of 0.01 g L^{-1} and for PM^{Mg-} batch, magnesium was removed from the trace metal composition of production medium. Production medium is defined as the medium composition optimized preciously in Chapter 3. For detail characterization under zinc supplementation, $ZnSO_4.7H_2O$ concentration of 0.01 g L^{-1} was chosen based on the initial screening experiments where maximum butanol titer was obtained at 0.01 g L^{-1}, out of three concentrations considered as detailed in the previous chapter. Characterization of *C. acetobutylicum* ATCC 824 was carried out in customized 250 mL cultivation flasks for 48 h (till stationary phase) at 37 °C in static and anaerobic condition maintained by purging 99.9% pure gaseous nitrogen while anaerobicity was confirmed by the addition of resazurin at a concentration of 1 g L^{-1} in the fermentation medium. Dynamic profile for growth of the organism, glucose utilization and formation of extracellular metabolites (acids, alcohols and solvents) were obtained by analysing the samples at regular interval. All the experiments were performed in duplicate and the data were expressed as mean ± standard error.

4.2.3 Enzyme assays to understand the modulation of metabolic pathways under individual and combined effect of zinc supplementation or magnesium starvation

With the aim of understanding metabolic regulation resulting in modulated phenotypic response of the organism under the influence of zinc supplementation or magnesium starvation or both, temporal expression profiles of the selected metabolic enzymes were obtained under four different conditions detailed in section 4.2.2. Eight key enzymes intrinsic to the glycolytic pathway (glucokinase, phophofructokinase and pyruvate kinase), intermediate metabolism (thiolase) and solvent formation (acetoacetate decarboxylase, alcohol dehydrogenase, butyrylaldehyde dehydrogenase and butanol

dehydrogenase) were selected for the study. In order to obtain temporal expression profile of these enzymes, samples were collected at regular intervals of 3 h for first 12 h of cultivation and thereafter, samples were withdrawn at an interval of 12 h till the termination of the batch. Cells were collected in 50 mL centrifugation tubes under aseptic conditions and harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. Harvested cells were then washed with lysis buffer composed of 50 mM MOPS at a pH of 7.0 containing 1 mM dithioerythritol (DTT) (Kuit et al. 2012). Further, the cell pellets were resuspended in lysis buffer to achieve an O.D. of 50. Resuspended cells were sonicated (Sonics Vibracell) for 10 cycles with 5 sec pulse and 20 sec cooling period for 10 min. Sonicated cells were centrifuged at 15,000 rpm for 20 min at 4 °C to discard the cell debris. Supernatant was then used for performing enzyme activity assays. Total protein concentration in the crude cell free extract was determined using Bradford reagent with bovine serum albumin (BSA) as standard.

In order to measure the activity of Glucokinase (*Glc*), a reaction mixture containing 60 mM Tris, 20 mM magnesium chloride, 4.0 mM adenosine 5'-triphosphate, 12.0 mM glucose, 0.9 mM ß-nicotinamide adenine dinucleotide phosphate, 10 units glucose 6-phosphate dehydrogenase and crude cell free extract was incubated at 30 °C for 5 min followed by measuring the absorbance at 340 nm (Goward et al. 1986). For Phosphofructokinase (*Pfk*), a reaction mixture containing 50 mM Tris-HCl at pH 8, 5 mM $MgCl_2$, 2 mM ATP, 5 mM fructose 1-phosphate, 5 mM phosphoenol pyruvate, 20 µg pyruvate kinase and cell free extract was used. The reaction mixture was incubated at 30 °C for 10 min and the absorbance was measured at 340 nm (Hengartner and Harris, 1975). Pyruvate kinase (*Pyk*) was estimated by the method given by Zhou et al. (2013) where the reagent mixture containing 50 mM Tris-HCl (pH 7.0), 2 mM PEP, 0.15 mM NADH, 5 mM $MgCl_2$, 4 U/mL lactate dehydrogenase and cell free extract was used. The enzyme

activity was measured in terms of decrease in amount of NADH over time by recording the absorbance at 340 nm. Thiolase (*Thl*) was estimated by adding the crude cell free extract to an assay mixture of 100 mM Tris hydrochloride (pH 7.4), 1.0 mM acetyl-CoA, 0.2 mM NADH, 1 mM dithiothrerythritol and 2 U of 3-hydroxyacyl-CoA dehydrogenase. The assay mixture was incubated at 30 °C for 2 min and the absorbance was measured at 340 nm as reported by Wiesenborn et al. (1988). Acetoacetate decarboxylase (*Adc*) was assayed by adding the crude cell free extract to a reaction mixture comprised of 0.3 M lithium acetoacetate in 0.1 M phosphate buffer at pH 5.9 at 30 °C. The absorbance was measured at 270 nm as a function of decrease in optical density of the reaction mixture (Tagaki et al. 1968). Alcohol dehydrogenase (*Adh*) activity was obtained by measuring the optical density of the reaction mixture at 340 nm after incubation for 3-4 min at 25 °C (Gold et al. 1996). The reaction mixture comprised of cell free extract, 50 mM Tris-HCl, 1 M ethanol and 40 mM NAD^+. Butanol dehydrogenase (*Bdh*) was assayed using a reaction mixture of 11 mM butyrylaldehyde, 0.23 mM NADPH, 77 mM Tris-HCl at pH 7.8. The activity was measured by recording the absorbance at 340 nm after addition of cell free extract to the above reaction mixture (Durre et al. 1987). Butyrylaldehyde dehydrogenase (*Budh*) activity was estimated as per Durre et al. (1987), where the crude cell free extract was added onto a reaction mixture consisting of 0.2 mM butyryl-CoA, 1 mM dithioerythritol, 0.27 mM NADH, 72 mM semicarbazide hydrochloride, 67 mM Tris-HCl at pH 6.0. The activity was measured as the change of absorbance at 340 nm.

4.2.4 Analytical Methods

Samples were collected and centrifuged at 10,000 rpm for 10 min (Multifuge X3R, Thermofisher Scientific, Germany) at regular intervals. Biomass growth was measured by resuspending the pellet in distilled water followed by determining absorbance at 600 nm using UV-Vis spectrophotometer (Cary 100, Varian, Australia). The supernatant was used

for estimation of glucose, acids (acetic acid and butyric acid) and solvents (acetone, ethanol and butanol). Glucose, acids and solvents were analyzed in HPLC (Ultimate 3000, Dionex, Thermofisher Scientific, Germany) using Rezex ROA column (300 x 7.8 mm, Phenomenex) and mobile phase of 0.005 N H_2SO_4 with a flowrate of 0.5 mL min^{-1}. While, acids were detected in UV detector at 210 nm, glucose and solvents were detected in Refractive Index detector (RID). The column oven was kept at room temperature and RID at 37 °C. The absorbance of reaction mixture in all enzyme activity assay was measured using UV-Vis spectrophotometer (Cary 100, Varian, Australia).

4.3 Results and discussions

4.3.1 Modulation in phenotypic traits of the organism under individual and combinatorial effect of zinc supplementation or magnesium starvation

C. acetobutylicum has been used as the potential producer of biobutanol through anaerobic ABE fermentation. Biobutanol is produced from acetyl-coA involving complex branched metabolic pathways. There exists two distinct phases of fermentation in the batch cycle: acidogenic phase in which the bacterium grows rapidly and produces organic acids, followed by the solventogenic phase in which the organic acids get converted into solvents. Therefore, any modulation in butanol biosynthesis under the influence of metal ion supplementation or starvation is expected to be linked with changes in various phenotypic response of the organism e.g., growth, extent of glucose utilization, formation of acids or solvents and onset of solventogenesis. With the aim of capturing the change in these phenotypic traits under the influence of zinc supplementation and magnesium starvation, either individually or in combination, extensive characterization of *C. acetobutylicum* ATCC 824 was performed on four different culturing media e.g., PM, PM^{Zn+}, PM^{Mg-} and PM^{Zn+Mg-}.

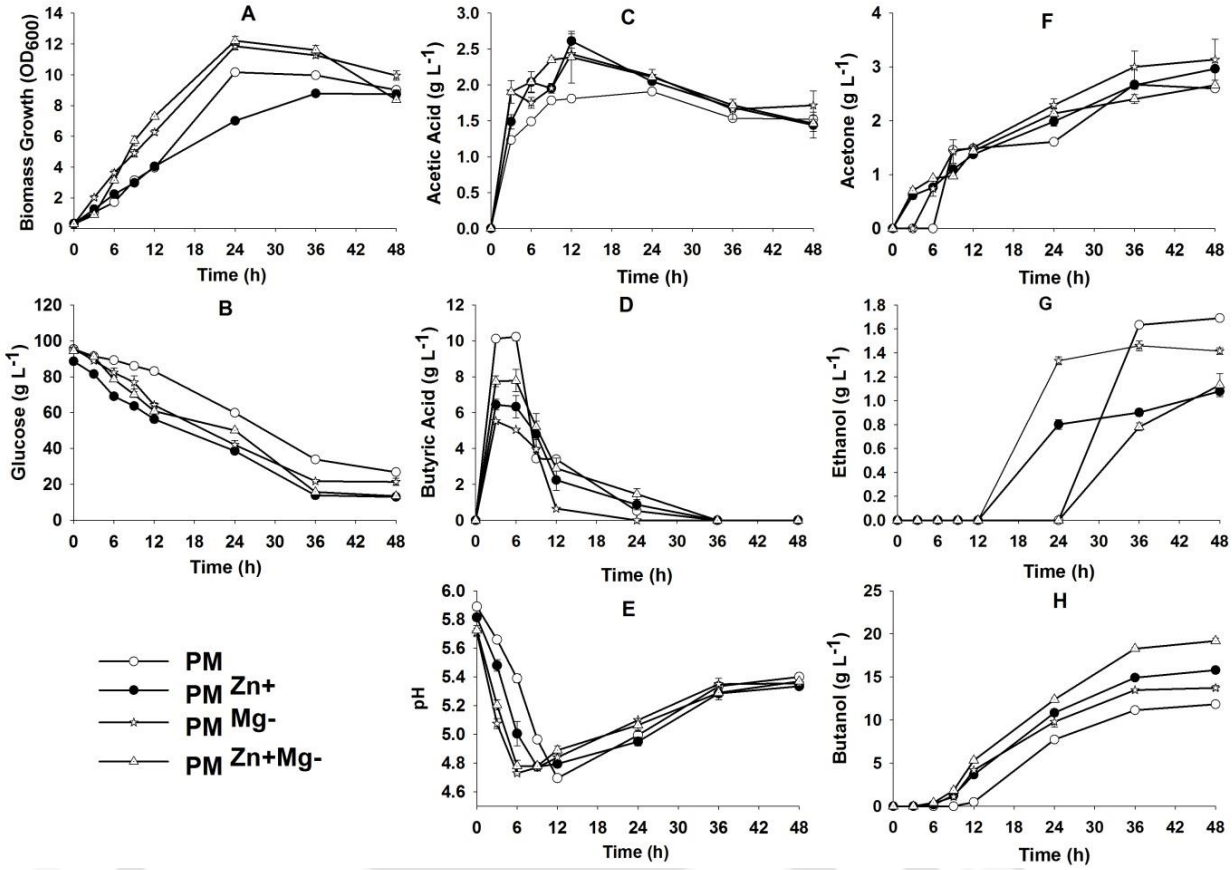


Figure 4.1 Dynamic profiles of (A) growth (OD_{600}), (B) glucose (g L^{-1}), (C) acetic acid (g L^{-1}), (D) butyric acid (g L^{-1}), (E) pH, (F) acetone (g L^{-1}), (G) ethanol (g L^{-1}) and (H) butanol production (g L^{-1}) in *C. acetobutylicum* ATCC 824.

While growth on PM^{Mg-} media was found to be marginally advantageous with highest specific growth rate (μ) of 0.074 h^{-1}, growth on PM^{Zn+} (μ = 0.068 h^{-1}) and PM^{Zn+Mg-} (μ = 0.069 h^{-1}) medium remain uncompromised when compared with the control PM media (μ = 0.07 h^{-1}) (Figure 4.1). Interestingly, rate and extent of glucose utilization was observed to be improved when organism was subjected to either zinc supplementation or magnesium starvation or combination of both (Figure 4.1). For instance, rate of glucose utilization increased from 1.43 g L^{-1} h^{-1} in control media to 1.57 g L^{-1} h^{-1} and 1.55 g L^{-1} h^{-1} for zinc supplemented and magnesium starved media respectively. The improvement in glucose utilization rate was even more prominent in case of PM^{Zn+Mg-} medium with the highest utilization rate of 1.69 g L^{-1} h^{-1}. Further, glucose utilization towards growth and formation of various metabolic products (acids and solvents) significantly elevated by 1.1,

1.08 and 1.18 fold for PM^{Zn+}, PM^{Mg-} and PM^{Zn+Mg-} media respectively with respect to the control media (Figure 4.2).

These upregulations in extent of glucose utilization without perceptible enhancement in the growth clearly point towards stimulation in formation of acids or solvents under individual or combinatorial effect of zinc supplementation and magnesium starvation. Indeed, we observed an elevated and faster accumulation of acetic acid in the culture broth under the condition of PM^{Zn+} or PM^{Mg-} or PM^{Zn+Mg-} in comparison to the control (Figure 4.1). This observation corroborates well with the fall in pH of the fermentation broth in case of metal ion modulated media at much faster rate followed by attainment of critical pH of 4.7 resulting in early induction of solventogenesis (Figure 4.1). For instance, commencement of butanol biosynthesis in case of metal ion modulated media was found to be 6 h earlier than the control media (Figure 4.1). Phenomenon of early solventogenesis in case of metal ion modulated media was also evident from the comparative dynamic profile of acetone and ethanol for all the four batches (Figure 4.1). Unlike acetic acid, formation of butyric acid in the metal ion modulated media was found to be lower in comparison to the control till 6 h of cultivation (Figure 4.1). This observation of lower butyric acid formation in the early phase of fermentation of the metal ion modulated media can be attributed to the early initiation of butanol biosynthesis where a fraction of the butyric acid has been continuously converted into butanol. Formation of acetic acid (Figure 4.2B), acetone (Figure 4.2D) and butanol (Figure 4.2F) was found to be significantly upregulated under the influence of zinc supplementation, magnesium starvation and combined zinc supplemented-magnesium starved batch when compared with the control. However, the effect of metal ion modulation was more prominently reflected in butanol titer with an increment from 11.83 g L^{-1} in case of control media to 15.79 g L^{-1} under zinc supplementation, 13.72 g L^{-1} under magnesium starvation and 19.18

g L^{-1} under the combinatorial effect of zinc supplementation and magnesium starvation. Interestingly, ethanol synthesis was observed to be downregulated in all the three media condition PM^{Zn+} or PM^{Mg-} or PM^{Zn+Mg-} when compared with the control.

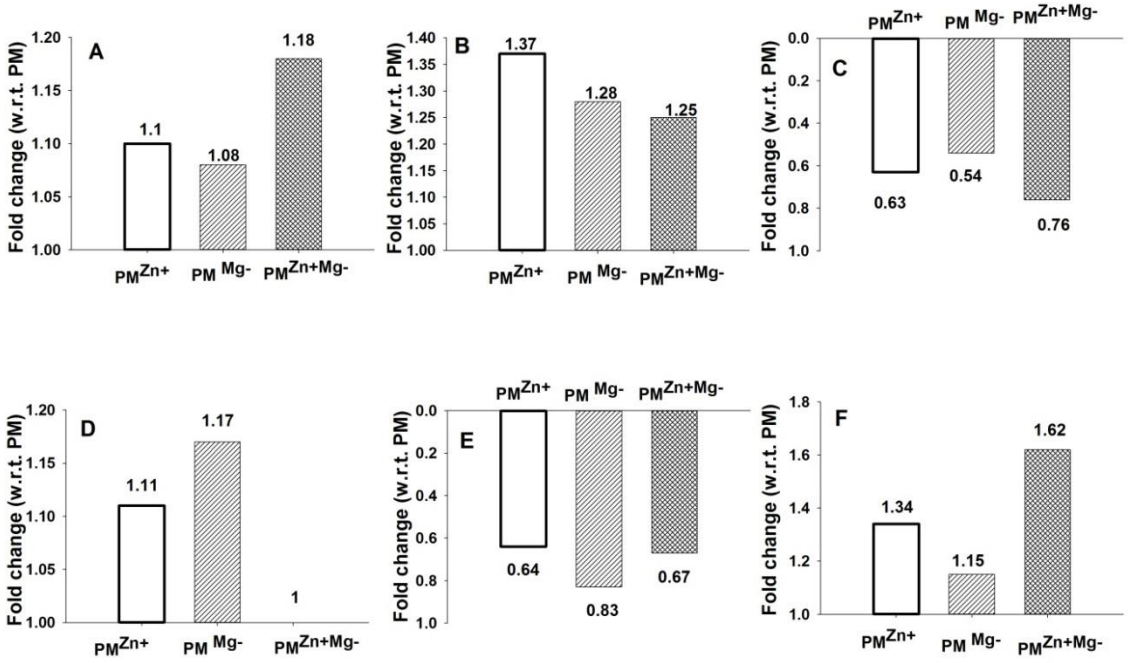


Figure 4.2 Fold change in (A) glucose consumption (g L^{-1}), (B) net acetic acid production (g L^{-1}), (C) net butyric acid production (g L^{-1}), (D) acetone production (g L^{-1}), (E) ethanol production (g L^{-1}) and (F) butanol production (g L^{-1}) for PM $^{Zn+}$, PM $^{Mg-}$ and PM $^{Zn+Mg-}$ batches with respect to control batch (PM).

Elevation in butanol biosynthesis in *C. acetobutylicum* ATCC 824 upon supplementation of $ZnSO_4.7H_2O$ in the fermentation medium may be attributed to various cellular processes where zinc plays an important role. Zinc remains one of the most abundant transition metals in proteins, affecting both of their structure and function (Auld and Bergman, 2008). Therefore, zinc as a cofactor plays crucial roles in regulating gene expressions and in turn, biological functions of many metalloenzymes including alcohol dehydrogenase and other enzymes in glycolytic pathway (Auld and Bergman, 2008; Maret et al. 2011; Maret et al. 2001; Mccall et al. 2000). Further, zinc supplementation has been shown to exhibit elevated ethanol titer from self-flocculating yeast cells SPSC01 through elevated stress tolerance against high temperature and high ethanol concentration (Xue et

al. 2010; Zhao et al. 2009). In a previous study, *in vitro* activity of butanol dehydrogenase, a key enzyme for butanol biosynthesis in clostridial strains has been shown to be positively regulated by the presence of zinc in the buffer solution (Walter et al. 1992). Besides, its alcohol specific functionality, zinc is a key player in regulating intracellular metabolism via gene expression of zinc-dependent DNA binding domain (Zhao et al. 2009). However, no studies on effect of magnesium starvation on ABE fermentation in *Clostridium* strains have been reported till now.

4.3.2 Decoding the modulation in central carbon metabolism under individual and combined effect of zinc supplementation or magnesium starvation through enzymatic activity assays

In order to understand the individual and combinatorial effect of zinc supplementation or magnesium starvation on central carbon metabolism to reprogram metabolic network, temporal expression profiles of key metabolic enzymes were obtained under four different growth conditions: PM, PM^{Zn+}, PM^{Mg-} and PM^{Zn+Mg-}. Specific activity of the key enzymes in the clostridial metabolic pathway responsible towards glucose metabolism, acid reassimilation and solvent formation were selected and studied (Table 4.1; Figure 4.3). The specific enzymes selected for this study have been screened on the basis of their change in activity reported previously (Wu et al. 2015).

Table 4.1 Selected metabolic enzymes for investigation under different medium engineering strategy (Adapted for the study from Tomas et al. 2004; Wu et al. 2015)

Sl.No	Enzyme	Operon	Function	Modulation under zinc supplementation
1	Glucokinase	Glycolytic enzymes	Conversion of glucose to glucose 6 phosphate	Not reported
2	Phosphofructokinase	Glycolytic enzymes	Conversion of fructose 6 phosphate to fructose biphosphate	Negligible
3	Pyruvate kinase	Glycolytic enzymes	Pyruvate and ATP formation from PEP	Elevated expression
4	Thiolase	Intermediate	Acetyl CoA converted to Acetoacetyl CoA (precursor of acetone) and acid reasimmilation	1.75 fold upregulated
5	Acetoacetate decarboxylase	Solvent formation	Acetone formation	Elevated expression
6	Alcohol dehydrogenase	Solvent formation	Ethanol production	Not reported
7	Butyrylaldehyde dehydrogenase	Solvent formation	Butyrlaldehyde formation (Precursor for butanol)	Elevated expression
8	Butanol dehydrogenase	Solvent formation	Butanol formation	Not reported

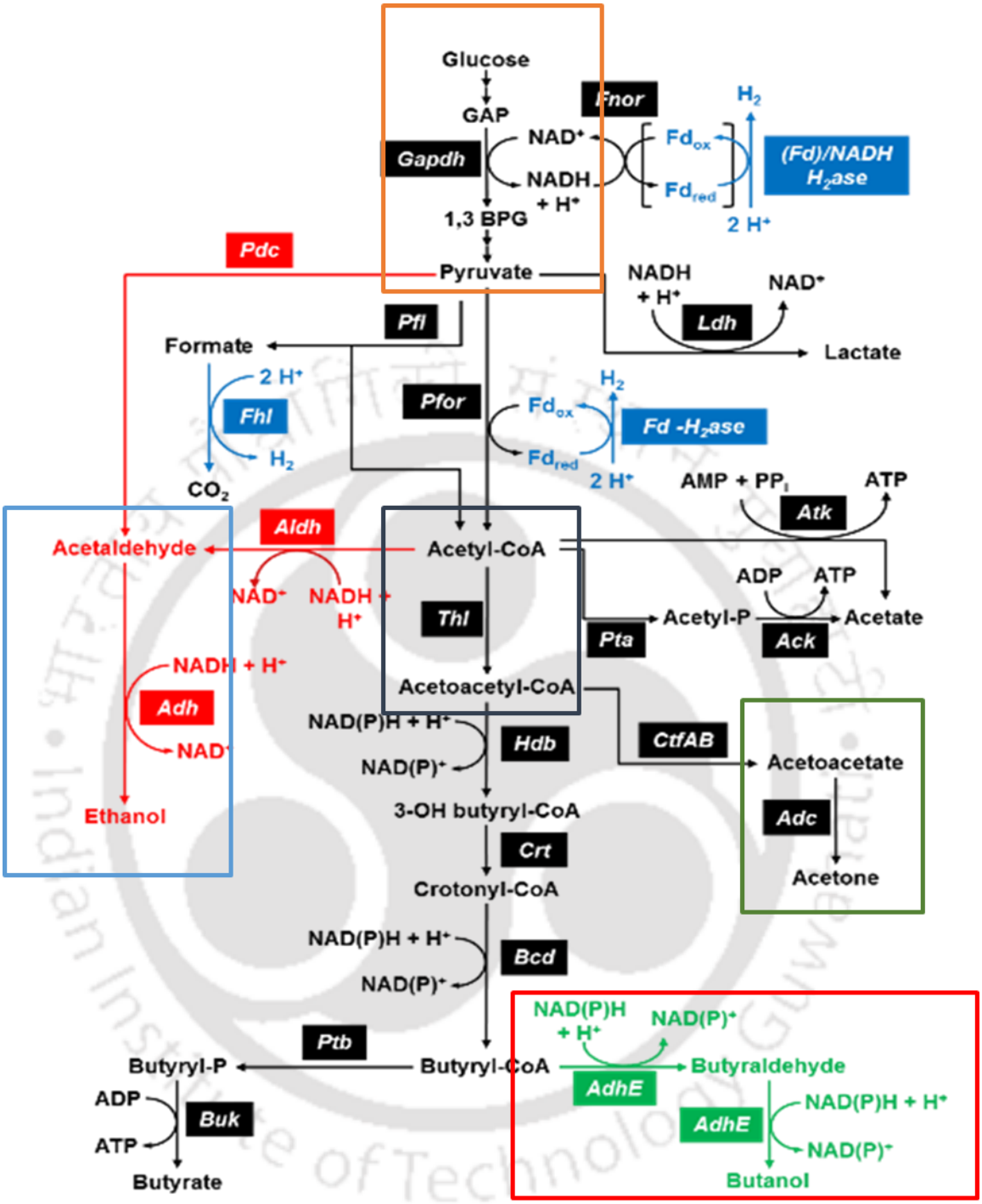


Figure 4.3 Schematic representation of central carbon metabolism of *Clostridium acetobutylicum* ATCC 824 directed towards butanol biosynthesis (Adapted and modified from Mazzoli et al. 2012)

Specific activity of the three key enzymes in the glycolytic pathway responsible towards glucose metabolism e.g., glucokinase, phosphofructokinase and pyruvate kinase was found to be upregulated throughout the entire course of fermentation under the condition of zinc supplementation or magnesium starvation or combination of both when compared with the control (Figure 4.4). This elevated activity of the key glucose metabolic enzymes resulted in improved glucose utilization and in turn, enhanced production of butanol in metal ion modulated batches as compared to the control. Transcriptomic analysis of zinc associated response of *C. acetobutylicum* revealed an enhancement of glucose utilization via upregulation of first glycolytic gene *glcK* encoding a putative glucokinase (Wu et al. 2015). Mutant strain of *C. acetobutylicum* ATCC 824 with simultaneous overexpression of genes *pfkA* and *pykA* encoding the synthesis of 6-phosphofructokinase and pyruvate kinase respectively exhibited an enhancement in butanol production along with an increase in intracellular pool of ATP and NADH (Ventura et al. 2013).

Furthermore, temporal activity profile of an intermediate enzyme thiolase was probed as it plays a key role in biosynthesis of acids and solvent via catalytic conversion of acetyl Co-A into acetoacetyl Co-A. Similar to the glycolytic enzymes, thiolase activity in all three metal ion modulated batch was found to be upregulated during the entire period of cultivation as compared to the control (Figure 4.4). Irrespective of metal ion modulation in the culture media, activity of thiolase increased linearly until 12 h of cultivation followed by a gradual decrease till the end of the batch (Figure 4.4). An upregulation in thiolase encoding gene *thlA* was reported when *C. acetobutylicum* ATCC 824 was cultivated using zinc supplemented medium (Wu et al. 2015). Surprisingly, in the same study, *thlB* encoding acetyl-CoA acetyltransferase was found to be downregulated in presence of zinc in the culture medium (Wu et al. 2015).

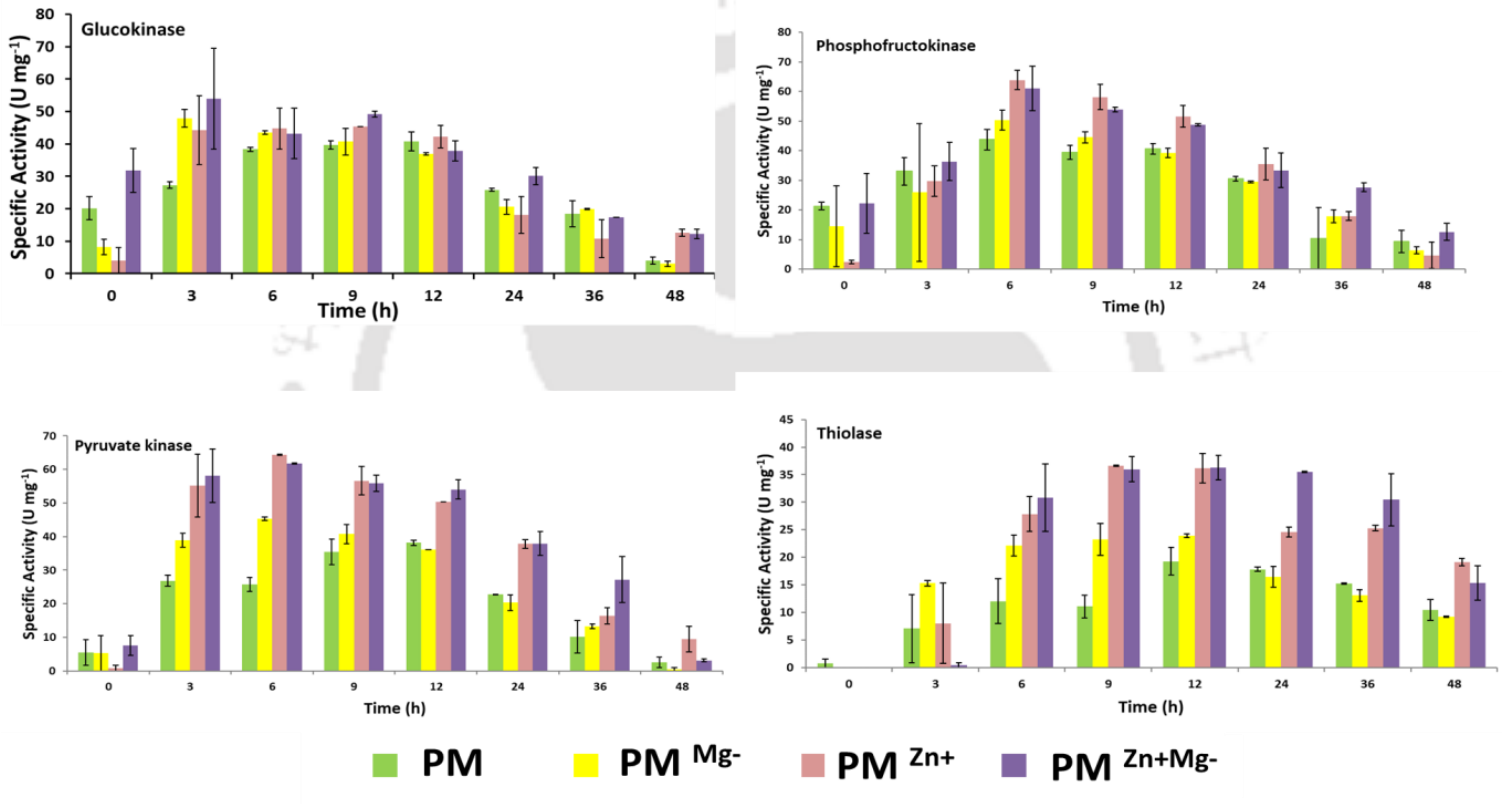


Figure 4.4 Dynamic profiles of specific activities (U mg^{-1}) of (A) glucokinase, (B) phosphofructokinase, (C) pyruvate kinase, (D) thiolase. PM represents growth in production media (control), PM^{Zn+} represents growth in production media supplemented with zinc, PM^{Mg-} represents growth in production media devoid of magnesium and PM^{Zn+Mg-} represents growth in production media supplemented with zinc & devoid of magnesium.

It is important to note that out of two thiolase encoding genes, only *thlA* has been reported to be physiologically relevant for transition from acidogenesis to solventogenesis (Winzer et al. 2000; Grimmler et al. 2011), while very little is known about *thlB* regulation on clostridial metabolism. Similar to the present study, activity of thiolase increased steadily with the progression of cultivation followed by attainment of maximum activity in the early stationary growth phase of *C. acetobutylicum* ATCC 824 (Wiesenborn et al. 1988). Further, an engineered thiolase enzyme has been reported to result in improved butanol production by *C. acetobutylicum* (Mann et al. 2013). Beside, thiolase also plays an indirect role in acid re-assimilation (Wiesenborn et al. 1988). Therefore, an upregulation in the activity of thiolase is expected to increase intracellular carbon flux towards synthesis of C_4 compounds and in turn, a significant upturn in butanol biosynthesis, as observed in the present study.

Further, two enzymes butyrylaldehyde dehydrogenase and butanol dehydrogenase, directly linked to the butanol biosynthesis was upregulated in all three metal ion modulated batches as compared to the control (Figure 4.5). Butanol synthesis was found to be concomitant with linear increase in activity of these two enzymes till the end of the batch. One of the key observations to be highlighted is that the early induction (3 h) of these enzymes as compared to the control (6 h) which support an early solventogenesis in all metal ion modulated batches. Overexpression of *bdhA* and *bdhB* points towards vital role of zinc in upregulation of these genes responsible for encoding butanol dehydrogenase and in turn improved butanol formation in *C. acetobutylicum* ATCC 824 (Walter et al. 1992). Furthermore, activity of acetoacetate decarboxylase responsible for acetone formation was found to be upregulated (Figure 4.5), which was consistent with the rapid and elevated acetone formation in the entire metal ion modulated batches as compared to the control. Similar observation in terms of the upregulated expression of *adc*

gene encoding acetoacetate decarboxylase has been reported when *C. acetobutylicum* ATCC 824 was exposed to zinc supplemented media (Wu et al. 2013). Downregulation of alcohol dehydrogenase, the key enzyme for ethanol biosynthesis under the influence of zinc supplementation or magnesium starvation or both (Figre 4.5), supports reduction in ethanol biosynthesis in all metal ion modulated batches as compared to the control. This result points towards redirection of carbon flux from ethanol biosynthesis pathway to the formation of butanol under the influence of metal ions. Upregulation in the activity of glucokinase, phosphofructokinase, pyruvate kinase, thiolase, acetoacetate decarboxylase, butyrylaldehyde dehydrogenase, butanol dehydrogenase and downregulation in the activity of alcohol dehydrogenase was observed to be most significant when zinc supplementation and magnesium starvation was implemented in combination rather than individually. Hence, an improved butanol titer with the highest value of 19.18 g L^{-1} was achieved in case of PM^{Zn+Mg-} as compared to the individual effect of zinc supplementation or magnesium starvation.

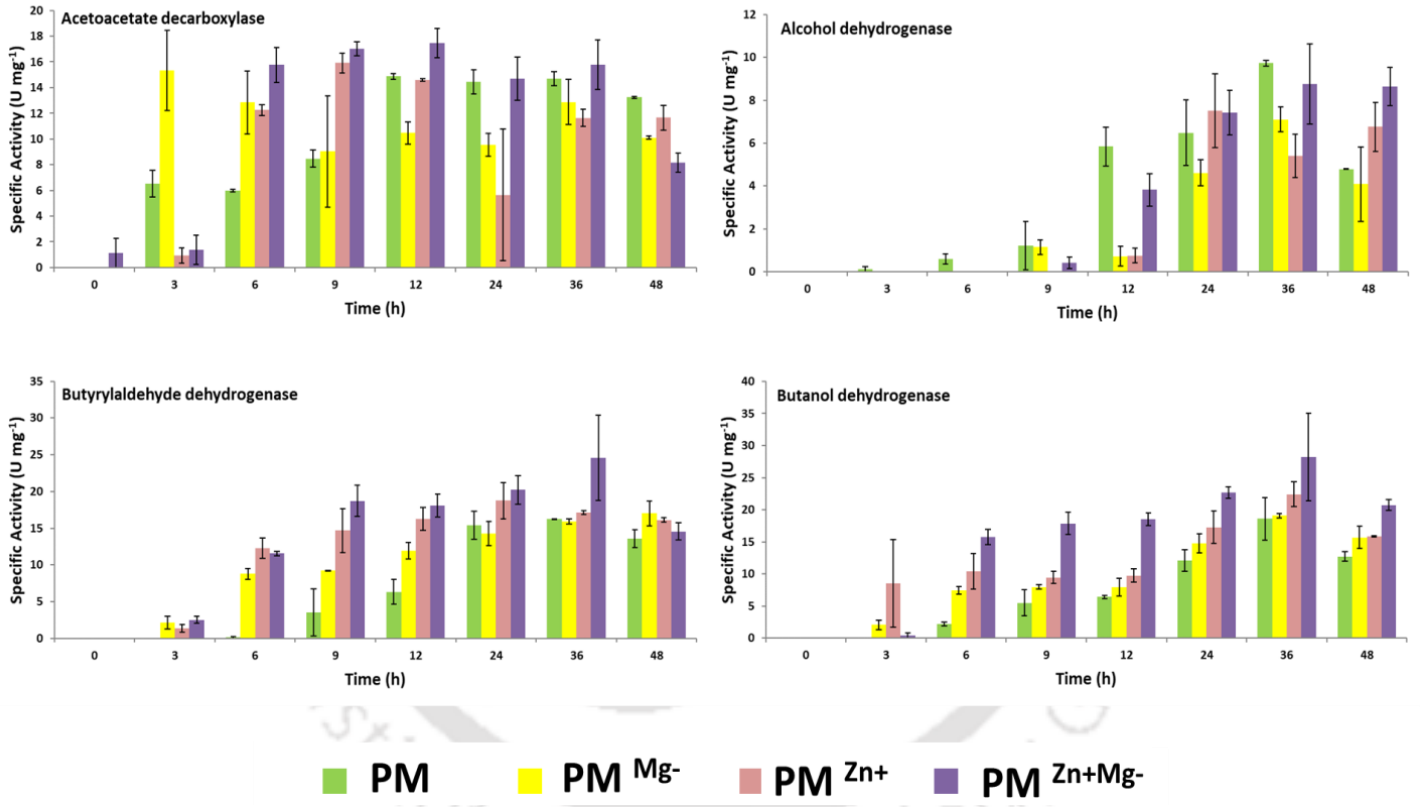


Figure 4.5 Dynamic profiles of specific activities (U mg^{-1}) of (A) acetoacetate decarboxylase, (B) alcohol dehydrogenase, (C) butyrylaldehyde dehydrogenase, (D) butanol dehydrogenase. PM represents growth in production media (control), PM^{Zn+} represents growth in production media supplemented with zinc, PM^{Mg-} represents growth in production media devoid of magnesium and PM^{Zn+Mg-} represents growth in production media supplemented with zinc & devoid of magnesium.

While there have been few reports on zinc associated response of *C. acetobutylicum* in terms of enhanced butanol production or carbohydrate utilization, to date no studies on the effect of magnesium starvation on ABE fermentation and its underlying metabolic regulation has been reported. Based on the results obtained in the present study, elevation in butanol titer in *C. acetobutylicum* ATCC 824 under the influence zinc supplementation or magnesium starvation may be attributed to the multiple level metabolic regulation: enhanced glucose utilization via upregulation of glycolytic pathway; upregulation in the activity of thiolase which play a key role in the biosynthesis of acids and solvents; upregulation in the activity of butyrylaldehyde dehydrogenase & butanol dehydrogenase, the enzymes directly linked to butanol biosynthesis and repression in ethanol biosynthesis pathway enabling redirection of carbon flux from ethanol to butanol.

4.4 Conclusion

- ✓ The study successfully captures modulation in butanol biosynthesis along with other phenotypic traits e.g., glucose utilization, formation of acids or solvents and onset of solventogenesis under the influence of either zinc supplementation or magnesium starvation or combination of both.
- ✓ Maximum butanol titer of 19.18 g L^{-1} was obtained in PM^{Zn+Mg-} which was 61% increase as compared to PM. Phenotypically glucose utilization, net acetic acid, acetone and butanol production was observed to be upregulated under metal ion modulation batches.
- ✓ In order to understand the phenotypic variations, enzyme activity assays of 8 different and metabolically important enzymes was performed. Key enzymes such as glucokinase, phosphofructokinase, pyruvate kinase (glycolysis), thiolase (intermediate), acetoacetate decarboxylase (acetone), butyrylaldehyde dehydrogenase

and butanol dehydrogenase (butanol) were significantly upregulated under metal ion starvation / supplementation or both as compared to PM.

- ✓ Downregulation of alcohol dehydrogenase highlights the lowered ethanol production under metal ion effect.

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Chapter 5

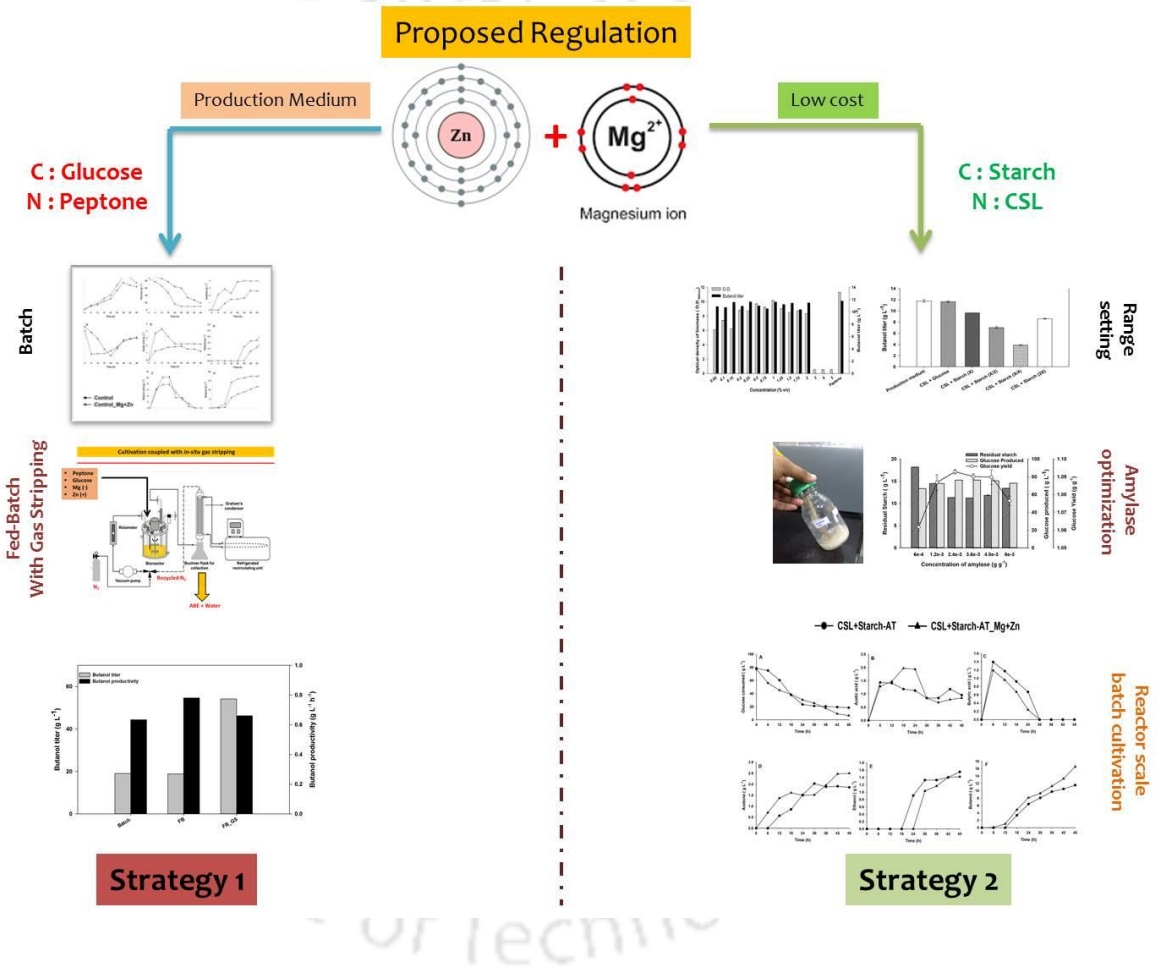
DEVELOPMENT OF A SUSTAINABLE BIOPROCESS STRATEGY WITH THE PROPOSED REGULATION

"The greatest threat to our planet is the belief that someone else will save it."

Robert Swan,

CHAPTER 5

Development of a sustainable bioprocess strategy with the proposed regulation



Development and demonstration of sustainable bioprocess strategy coupled with magnesium starvation and zinc supplementation

5.1 Background and motivation

Concomitant rise in population and exponential decline in fossil fuel reserves coupled with adverse climatic changes has kindled research interest on prospective sustainable alternatives to traditional fuel resources. Recently, researchers have been focusing on developing modified strains and sustainable process strategies towards elevated and economic bio-butanol production as a possible alternative to fossil fuels (Moon et al., 2016; Ndaba et al., 2015). The most widely used platform for ABE fermentation from a wide variety of substrates is *Clostridium* sp. (Moon et al., 2016). It has been continually studied over the past 100 years, through multiple rationales directed towards strain improvement and process enhancement coupled with media engineering, however, of much success has been attained towards its possible industrial implementation. Butanol production is majorly hindered via low solvent titer and productivity, high cost of substrates and difficult downstream technologies (Kaushal et al., 2017). To that end, varied literatures suggest that multiple routes have been carried out in terms of mode of cultivation (Ezeji et al., 2004; 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 different environmental and medium engineering cues affecting *Clostridium* sp. in terms of its central carbon metabolism, differential transition from acidogenesis to solventogenesis, modulating the metabolic pathway towards desired product, imparting solvent tolerance etc. (Wu et al., 2016). 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).

In spite of development of various fermentation strategies and novel hyper producing strains, commercial implementation of biobutanol production remains a distant dream and a missed opportunity owing to its high cost of production (Moon et al., 2016; Sauer et al., 2016). Costly substrates such as analytical grade peptone and glucose have been predominantly used towards biobutanol production. These chemicals increase the production cost and in turn render the whole process economically infeasible. To that end,

substrate selection has been reported to have a significant impact on fermentation performance and overall production cost. Identifying possible low cost substrates is imperative for designing and demonstrating sustainable process strategies towards biobutanol production with improved titer. As clostridial strains are able to utilize a wide range of substrates, identification of locally or logistically available cost-effective carbon sources are important depending upon the country of operation. Wide variety of substrates such as Jerusalem artichoke juice (Marchal et al., 1985) ; maltodextrin (Formanek et al., 1997) ; Sago starch (Madihah et al., 2001) ; cassava starch and cassava chips (Thang et al., 2010) ; enzymatically hydrolyzed cassava flour (Lepiz Aguilar et al., 2013) have been employed for biobutanol production. However, lower biobutanol titer below 13 g L^{-1} resulted from these processes involving low cost substrates have been found to plague the positive efforts (Luo et al., 2018). Therefore, there is a need to develop sustainable process using low cost substrates without compromising the butanol titer and yield.

The present study reports and demonstrates a novel medium engineering strategy wherein high butanol titer of 19 g L^{-1} with maximum productivity of 0.63 g L^{-1} h^{-1} was achieved via zinc supplementation in optimized production medium, completely devoid of any exogenous source of magnesium on a reactor scale. Furthermore, a bioprocess strategy herein is proposed with the intermittent feeding of limiting nutrients coupled with *in-situ* product recovery achieved through optimized gas stripping parameters. However, the strategy, demonstrated using expensive laboratory grade peptone and glucose as nitrogen and carbon source respectively makes it economically cumbersome and challenging. Therefore, with the aim of achieving economic feasibility a low cost process has been demonstrated using corn steep liquor (CSL) as nitrogen source and industrial grade maize starch (starch) as carbon source while maintaining an improved butanol titer. The trade-off between improved titer and utilization of low cost substrate has been achieved via

combinatorial approach of (i) optimization of initial concentration of CSL and starch; (ii) hydrolysis of starch into fermentable sugar using industrial grade amylase and (iii) coupling attributes of butanol upregulation via magnesium starvation and zinc supplementation.

5.2 Materials and methods

5.2.1 Microorganism, maintenance and preparation of seed culture

C. acetobutylicum ATCC 824 had been procured from American Type Culture Collection, USA and the cultivation conditions for initial revival and storage were followed as mentioned earlier in Chapter 4. The lyophilized culture obtained was revived in TYG medium composed of (g L^{-1}): tryptone 30.0, yeast extract 10.0 glucose 20.0, and cysteine hydrochloride 0.05 (Annous and Blaschek, 1990) and stored as glycerol stocks of active cultures at -80° C for further use.

The glycerol stock was revived at the beginning of any experiment by adding 1 ml from the stock into 10 ml of TYG medium and was allowed to grow up to an O.D. (600 nm) of 3.0. Further, the seed culture was prepared by transferring 10 ml of revival culture into 90 ml of TYG medium in a customized air tight bottle and incubating till biomass O.D. (600 nm) of 3.0 was reached. The growth condition for revival and seed preparation was kept similar as mentioned earlier in this section. In all subsequent media optimization and reactor studies, 10 % (v/v) seed culture was used as inoculum, unless mentioned otherwise.

5.2.2 Performance evaluation of *Clostridium acetobutylicum* ATCC 824 when grown in optimized production medium supplemented with zinc and devoid of magnesium in reactor scale.

A process strategy has been demonstrated towards elevated butanol response due to supplementation of zinc and starvation of magnesium in the optimized production

medium on reactor scale (Chapter 3). It has been demonstrated that zinc supplementation (0.01 g L^{-1}) and starvation of magnesium resulted in an improved butanol titer due to the significant upregulation in key metabolic enzymes pertaining to the glucose utilization, acids reassimilation and solvent production (Wu et al, 2015). Therefore, in the current study the optimized medium obtained from the previous chapter was further cultivated in a 7.5 L automated bioreactor (BioFlo 115, New Brunswick, Eppendorf, Germany) with a working volume of 2.5 L to assess biomass growth, glucose utilization, acids and solvent production. Strict anaerobic conditions were maintained by purging pure N_2 gas into the reactor, operated under 200 rpm agitation at a temperature of 37° C. In order to assess the effect of nutritional stress and further elevate butanol productivity for elevating feasibility, the microorganism was grown under fed-batch mode of cultivation where, glucose and zinc were fed intermittently to maintain the initial concentration. However, it was observed that similar titer was observed at the end of fermentation owing to butanol toxicity.

5.2.3 Demonstration of a sustainable bioprocess strategy combining intermittent feeding of limiting nutrients and *in-situ* gas stripping.

It was inferred from the batch cultivation studies in the previous sections and literature reports, that butanol at a higher concentration exerts toxic stress which in turn results in the loss of cell viability, hindering further product formation. In order to, lower the butanol toxicity stress as experienced by the microorganism an effective *in situ* product recovery mechanism has been devised. To that end, gas stripping has been extensively practiced owing to its cost effectiveness and efficient removal of butanol from the fermentation broth. This removal process helps in concentrating the end product as well. With the objective function of maximization of butanol recovery from the broth, key gas stripping parameters such as temperature of the condenser, duration of stripping,

flowrate of stripping gas were optimized employing a Central Composite Design (CCD) based Response Surface Methodolgy (RSM). The actual values and coded values of the above selected parameters used in CCD based RSM experimental design is depicted in Table 5.1. Coded values of +α, +1, 0, and –1,−α correspond to high, medium, and low levels of the variables respectively. A 3^5 quarter factorial CCD was generated using Minitab stat 16.1.1 (Minitab Inc., Pennsylvania, USA) and was employed to optimize the butanol recovery.

The CCD predicted 18 experiments which included eight factorial points, six axial points and four replicates of center point to search linear, quadratic and interaction effect of parameters on butanol titer. RSM is a mathematical modeling technique which utilizes a polynomial equation to model the interaction among the variables. Under RSM, the linear, quadratic, and interaction effects between the selected medium components and butanol titer were mathematically expressed in the form of a quadratic polynomial Eq. (5.1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad (5.1)$$

Where Y is the butanol recovery as model response, X_i is the i^{th} parameter, k is the total number of parameters and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients. All the 18 experiments were performed in 3 L automated bioreactor (Applikon) with a model ABE mixture in 3:6:1 ratio in water (1 L working volume) containing 8 g L^{-1} of butanol. The threshold concentration of butanol was experimentally achieved, defined as the critical concentration of butanol beyond which there was a sharp decline in the specific growth rate of biomass.

Table 5.1 Actual values and coded values of the variables employed in CCD-RSM based optimization for gas stripping.

Factors		Levels code and corresponding values				
Code	Name	-1.68	-1	0	1	1.68
X_1	Temperature of condenser (° C)	-7.04	-5	-2	1	3.04
X_2	Flowrate of stripping gas (vvm)	0.313	1.25	2.625	4	4.94
X_3	Stripping duration (h)	1.95	4	7	10	12.05

Furthermore, in order to maximize butanol titer and productivity by sustaining the cellular viability over a longer period of time, a bioprocess strategy has been demonstrated in a 7.5 L automated bioreactor (BioFlo 115, New Brunswick, Eppendorf, Germany) with a working volume of 2.5 L involving intermittent feeding of glucose and zinc, in zinc supplemented and magnesium starved production medium along with optimized *in-situ* removal of solvents from fermentation broth (Figure 5.1). The feeding strategy developed helps in maintaining the initial concentration (w/v) of glucose and zinc in the fermentation broth. Samples from the reactor was withdrawn at every 4 h interval and the amount of glucose and zinc consumed by the organism, was replenished in the media. The cultivation parameters have been followed as mentioned for batch cultivation.

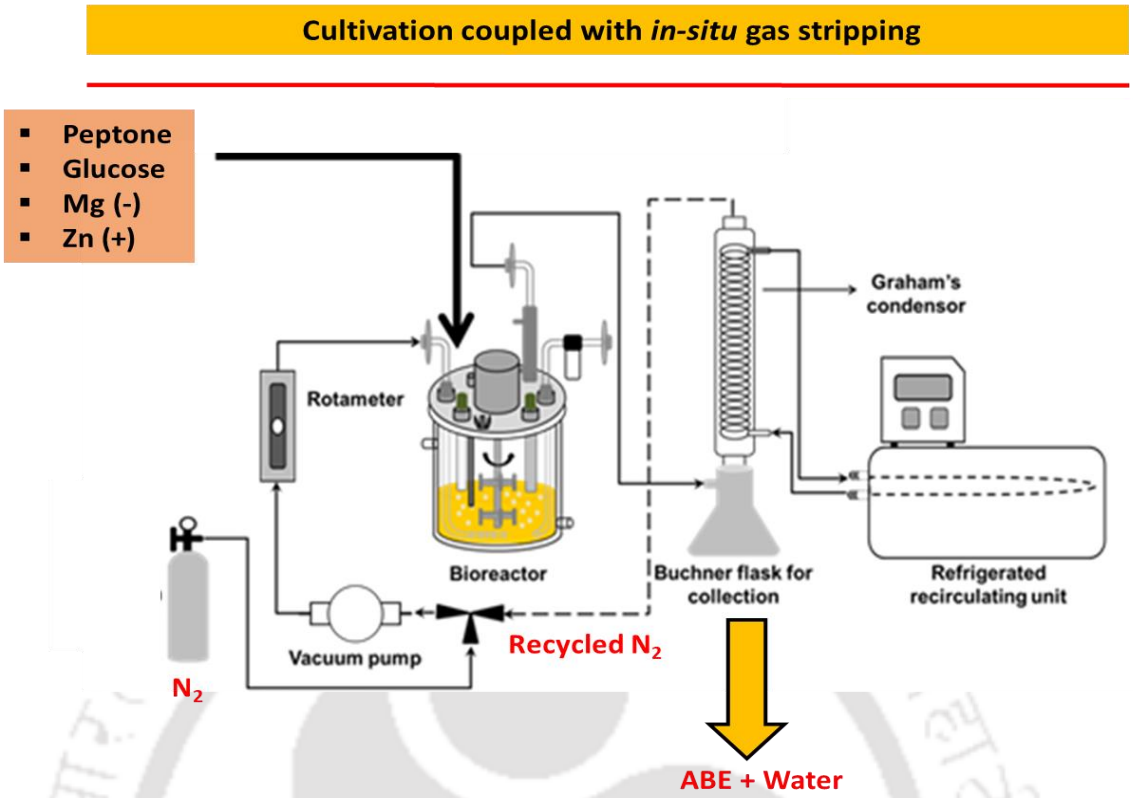


Figure 5.1 Schematic representation of fed-batch strategy coupled with optimized *in-situ* gas stripping.

5.2.4 Performance evaluation of the strain under starch and corn steep liquor as cheaper carbon and nitrogen source respectively.

Substrate cost is one of the major hindrances towards commercial implementation of butanol biosynthesis (Moon et al., 2016). To that end, multiple researches leading to development of sustainable media strategies has been undertaken by substituting either carbon or nitrogen or both towards butanol production (Kaushal et al, 2019). In an earlier study, a novel medium engineering strategy was demonstrated for *C. acetobutylicum* ATCC 824 resulting in a higher butanol titer of 19 g L^{-1} owing to zinc supplementation in magnesium starved optimized medium. While, process engineering strategy resulted in high butanol titer from wild type strain, economic feasibility of the process remains doubtful with respect to high cost of analytical grade carbon and nitrogen source glucose and peptone, respectively. With the aim of achieving economic feasibility, glucose and

peptone present in the optimized production medium was replaced with industrial grade starch (Bharat Traders, Guwahati, India) and CSL (generous gift from Sayaji Group, Gujrat, India), respectively. In the first step, the strain was characterized under fifteen different concentrations of CSL ranging from 0.05% to 5%, (v/v). The concentration of CSL resulting in highest butanol titer was considered for subsequent characterization. In the next step, the strain was characterized under four different concentrations of starch: X, X/4, X/2 and 2X expressed in g L^{-1}. The term 'X' represents 81.1 g L^{-1} of starch containing equimolar carbon as present in optimized glucose concentration of 89.6 g L^{-1} in the production media. All characterization experiments under starch were carried out with optimal concentration of CSL as obtained from the experiments in the first step. The experiments were carried out in 250 mL customized air tight cultivation flasks with a working volume of 50 mL for 72 h at 37°C in static, anaerobic condition maintained by purging 99.9% pure gaseous nitrogen while anaerobic condition was confirmed by the addition of resazurin at 1 g L^{-1}. Samples were assessed at regular intervals for determining the growth of the organism and butanol production. The experiments were performed in duplicate and the results were expressed as mean ± standard error.

5.2.5 Dual optimization of butanol titer and yield towards enhanced sustainability using low cost substrates.

The ranges of initial CSL concentration, initial starch concentration in media were determined using outcomes of preliminary studies (Table 5.2) with the dual objective function of enhanced butanol titer and yield. Post characterization of the strain at different starch concentrations, gelatinization of starch was observed at higher concentrations. Therefore, in order to develop a suitable medium strategy, and demonstrate successfully at reactor scales, lower concentrations of starch were taken as inputs for statistical

optimization. Such an approach would inevitably lower the butanol production, however, would facilitate in smoother reactor operations.

Table 5.2 Parameters, their code and level values used in CCD-RSM based optimization

Factors		Levels code and corresponding values				
Code	Name	-1.68	-1	0	1	1.68
X_1	Initial starch concentration (g L^{-1})	26.9	30	37.5	45	48.1
X_2	Initial CSL concentration (% v/v)	0.034	0.2	0.6	1	1.17

The CCD was formulated using statistical software Minitab stat.16.1.1 (Minitab Inc., Pennsylvania, USA) with three factors and five levels CCD. The CCD required 11 experiments were predicted to search linear, quadratic and interaction effect of parameters on biomass and butanol productivity. Where, Y is the butanol titer (g L^{-1}) and butanol yield as model response, Xi is the i^{th} parameter, k is the total number of parameters and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad (5.2)$$

A batch mode of cultivation in shake flask level (250 ml anaerobic set up bottles with 50 ml working volume) was performed to assess the effects of Mg starvation / Zn supplementation, individually as well as in their combinatorial form to determine whether the metallic ions play an important role in upregulating butanol production and yield in the optimized CSL and starch media. Following the experiments on shake flask level, batch cultivation was performed in 7.5 L automated bioreactor (New Brunswick, Eppendorf) to study the batch kinetics under zinc supplemented and magnesium starved CSL and starch enriched media. The conditions of cultivation were maintained similarly for all the experiments as mentioned earlier.

5.2.6 Hydrolysis of starch using industrial grade amylase.

With the aim of achieving improved butanol titer using cheaper industrial grade carbon source, starch was pretreated with amylase before fermentation. Optimal amylase loading was obtained to achieve maximum glucose yield by subjecting 81.1 g L^{-1} of starch to six different amylase loading ranging from 0.6 to 6 mg g^{-1} (amount of amylase used to hydrolyse 1 g of starch). The optimization of amylase loading was performed in 250 mL conical flasks with a working volume of 100 mL containing 81.1 g L^{-1} of starch, subjected to treatment for 24 h with intermittent sampling, kept at 37 °C under 150 rpm shaking. Hydrolysis was continued till concentration of glucose in three successive sampling points remained constant. Glucose yield (GY, g g^{-1}) was calculated as follows (equation 3).

$$\mathrm{GY}(\mathrm{g\,g^{-1}}) = \frac{\text{Amount of glucose produced}}{\text{Amount of starch consumed}} \qquad (3)$$

The optimal amylase loading resulting in maximum glucose yield was used for the subsequent experiments.

5.2.7 Demonstration of a novel low cost bioprocess strategy for improved butanol production.

A bioprocess was demonstrated for production of butanol in bioreactor using CSL and starch as cheaper nitrogen and carbon source, respectively. The bioprocess consists of two sequential stages of hydrolysis of starch followed by fermentation. In the first stage, hydrolysis of starch was carried out in a 5 L conical flask employing optimal amylase loading and hydrolysis parameters were kept similar as described in the previous section. In the second stage, butanol fermentation was carried out in a 7.5 L bioreactor with a working volume of 2.5 L using optimized production media supplemented with glucose obtained from first stage and optimal concentration of CSL obtained from the section 5.2.4. Finally, a sustainable bioprocess was demonstrated with low cost substrates coupled with zinc supplementation and magnesium starvation. Fermentation was carried

out under strict anaerobic conditions maintained by purging pure N_2 gas into the reactor, operated under 200 rpm agitation at a temperature of 37 ° C. Sampling was done regular time interval for analysis of growth, substrate utilization, formation of acids and solvents.

5.2.8 Analytical Methods

The samples collected at regular time interval were centrifuged at 10,000 rpm for 10 minutes (Multifuge X3R, Thermofisher Scientific, Germany). The growth was quantified by re-dissolving the pellet in distilled water for determination of absorbance at 600 nm using UV-Vis spectrophotometer (Cary 100, Varian, Australia). The supernatant was analysed for glucose, acids and solvents in HPLC equipped with UV-Vis and Refractive Index (RI) detector (Shimadzu) and Rezex ROA column (300 x 7.8 mm, Phenomenex). Acids were detected in UV detector at 210 nm, while glucose and solvents were detected in RI detector. H_2SO_4 (0.005 N) was used as mobile phase with a flowrate of 0.5 mL min^{-1}. The column oven was kept at 28°C and RI detector at 37°C. Starch was quantified spectrophotometrically by assessing the color change due to anthrone reagent at 630 nm (Harris, 1958). Zinc was quantified by atomic absorption spectroscopy (AAS) (Varian, Australia).

5.3 Results and discussions

5.3.1 Performance evaluation of *Clostridium acetobutylicum* ATCC 824 when grown in optimized production medium supplemented with zinc and devoid of magnesium in reactor scale.

The current industrial benchmark for butanol titer is estimated to be 22 – 28 g L^{-1} (Moon et al., 2016), butanol yield at the laboratory scale is reported to be 14 - 18 g L^{-1} in wild type *Clostridium* strains (Moon et al., 2016) and 16 - 21 g L^{-1} (Jang et al., 2012) in recombinant variants of *Clostridium*. There have been continuous efforts to address some of these limitations via process engineering strategies to enhance butanol production (Jang

et al., 2012); downstream processing to reduce solvent toxicity (Ezeji et al., 2003) and metabolic engineering to develop butanol hyper producing strains (Mermelstein & Papoutsakis, 1993). In-spite of numerous attempts to genetically manipulate *Clostridium* sp. for improved butanol titer, limited success has been reported so far. This may be attributed to difficulties in expressing enzymes like *Bacillus subtilis* α-acetolactate synthase, *Escherichia coli* acetohydroxyacid isomeroreductase, *E. coli* dihydroxy acid dehydratase, *L. lactis* ketoacid decarboxylase, and *E. coli* or *L. lactis* alcohol dehydrogenases converting pyruvate to isobutanol due to the lack of an inducible expression system in *C. cellulolyticum*. Moreover, the existence of complex metabolic regulation in *C. acetobutylicum* that renders the metabolic engineering of wild type strains difficult. Hence, there has been ever increasing efforts towards achieving commercially viable butanol titer via process engineering strategies and efficient downstream processing (Moon et al., 2016). For instance, co-culturing of *C. acetobutylicum* with *Saccharomyces cerevisiae* resulted in an improvement of 35% in butanol titer with respect to mono-culture producing 15.7 g L^{-1} butanol with a yield of 0.23 g g^{-1} glucose (Luo et al., 2015). Bankar et al., (2012) reported a high titer of 16.9 g L^{-1} with a productivity of 1.69 g L^{-1} h^{-1} by employing an extraction system of oleyl alcohol and decanol mixture (4:1) integrated with two stage continuous fermentation of *C. acetobutylicum*. Extensive research in the field of process engineering and downstream processing has resulted in improved butanol production. European patent EP 2238257 A2 reported a high yielding strain *C. acetobutylicum* ATCC 10132 wherein the supplementation of 0.5% (w/v) of Na_2CO_3 resulted in an elevated butanol titer of 20 g L^{-1}. Researchers in the past have also tried to elucidate the effects of various metallic ions on elevating butanol titer. The synergistic effects of zinc and calcium supplementation in *C. acetobutylicum* ATCC 824 resulted in an elevated butanol titer of 16.1 g L^{-1} (Wu et al., 2016). Junelles et al., (1988) reported

iron limitation to be favorable for butanol production by inhibiting hydrogen formation and redirecting carbon flux towards butanol synthesis pathway. Butanol titer of 12.6 g L^{-1} was reported under iron limited conditions as compared to 9.4 g L^{-1} achieved under iron sufficient condition (Junelles et al., 1988).

In the present study, demonstration of the novel medium engineering strategy was demonstrated on a reactor scale using peptone as nitrogen source, glucose as carbon source, supplementing of exogenous zinc and completely devoid of any magnesium & the performance of the strain was compared when cultivated in optimized medium (Figure 5.2). A maximum butanol titer of 19.18 g L^{-1} was achieved when the control production medium was supplemented with 10 mg L^{-1} zinc and completely devoid of any exogenous supply of magnesium. This was a drastic improvement of 61% from control medium and is the highest titer to be ever reported for wild type strain ATCC 824. Another key inference from this study was the comparative earlier onset of solvent formation from 6 h as compared to 12 h in control medium. Furthermore, the maximum productivity obtained was 0.63 g L^{-1} h^{-1}, which was an improvement of 110% as compared to control medium.

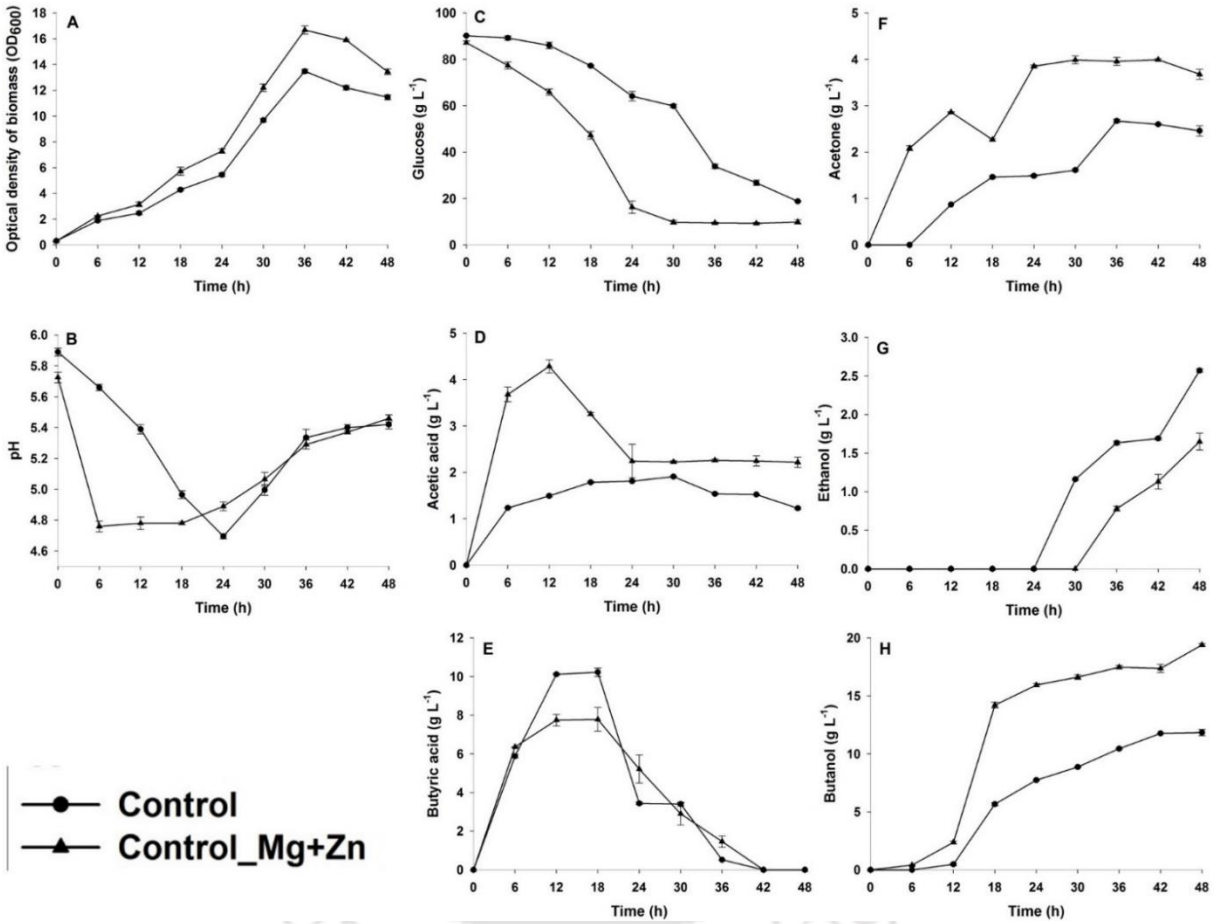


Figure 5.2 Dynamic profiles of (A) growth, (B) pH, (C) glucose utilization, (D) acetic acid, (E) butyric acid, (F) acetone, (G) ethanol and (H) butanol in *C. acetobutylicum* ATCC 824.The organism was grown in optimized media (Control) and optimized media with zinc supplementation and magnesium starvation (Control_Mg+Zn).

A significant upregulation in growth and glucose utilization was observed under metallic ion influence which can be credited to the upregulation in the activity of glycolytic enzymes, leading to rapid glucose consumption. It was also inferred that an elevated and faster accumulation of acetic acid in the culture broth under the metallic ion mediated condition as compared to control. This observation corroborates well with the fall in pH of the fermentation broth in case of metal ion modulated media at much faster rate followed by attainment of critical pH of 4.7 resulting in early induction of solventogenesis.

Elevation in butanol biosynthesis in *C. acetobutylicum* ATCC 824 upon supplementation of $ZnSO_4.7H_2O$ in the fermentation medium may be attributed to various cellular processes where zinc plays an important role (as discussed and experimentally proven in Chapter 4). Zinc as a cofactor plays crucial roles in regulating gene expressions and in turn, biological functions of many metalloenzymes including alcohol dehydrogenase and other enzymes in glycolytic pathway (Auld and Bergman, 2008; Maret et al., 2011; Maret et al., 2001; Mccall et al., 2000). Besides, its alcohol specific functionality, zinc is a key player in regulating intracellular metabolism via gene expression of zinc-dependent DNA binding domain (Zhao et al., 2009). However, no studies on effect of magnesium starvation on ABE fermentation in *Clostridium* strains have been reported till now. The elevated butanol response obtained as a result of the novel media engineering strategy reports one of the highest butanol titers for wild type *C. acetobutylicum* ATCC 824 strain (Table 5.3).

Table 5.3 Comparison of butanol titer for wild type *Clostridium acetobutylicum* strains when cultivated under glucose as sole carbon source.

Strain	Type	Substrate	Mode of cultivation	Butanol titer (g L^{-1})	Reference
C. acetobutylicum **ATCC 824**	**W**	**Glucose**	**Batch**	**19.12**	**This study**
C. acetobutylicum L7	A	Glucose	Batch	16.1	Wu et al., 2016
C. acetobutylicum MTCC 11274	W	Glucose	Batch	14.2	Ahlawat et al., 2019
C. acetobutylicum L7	W	Glucose	Batch	14.4	Wu et al., 2016
C. acetobutylicum ATCC 824	W	Glucose	Batch	14.78	Richmond et al., 2011
C. beijerinckii P260	W	Glucose	Batch	13.89	Richmond et al., 2011
C. beijerinckii NCIMB 8052	W	Glucose	Batch	16.3	Han et al., 2013

5.3.2 Demonstration of a sustainable bioprocess strategy combining intermittent feeding of limiting nutrients and *in-situ* gas stripping directed towards elevated butanol productivity.

In order to further accentuate and improve butanol titer and productivity for process feasibility, a fed-batch strategy was designed with intermittent feeding of glucose and zinc into the production medium to maintain their optimal concentrations. However, it was observed that the microbe ceased metabolic activity after attaining a butanol titer around 18.67 g L^{-1} with a maximum productivity of 0.78 g L^{-1} h^{-1} which was 24% improvement as compared to batch cultivation (Figure 5.3).

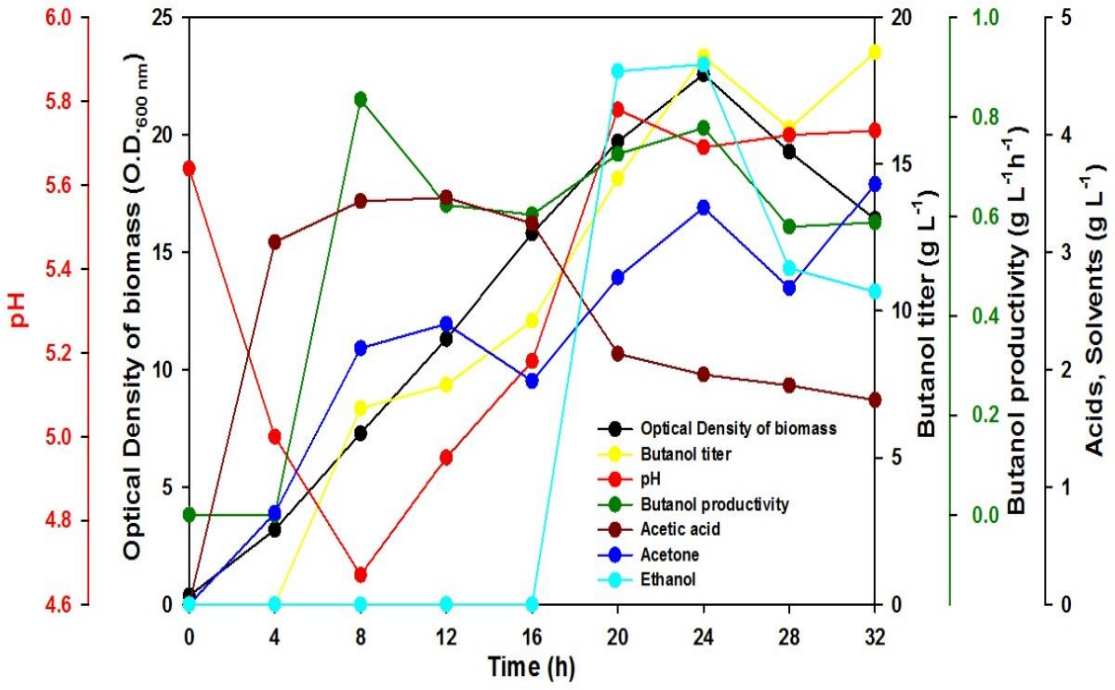


Figure 5.3 Dynamic profiles for pH, growth, acetic acid, butyric acid, acetone, ethanol, and butanol production and butanol productivity using *C. acetobutylicum* ATCC 824 with zinc supplementation in optimized media, devoid of magnesium, under fed-batch mode of cultivation.

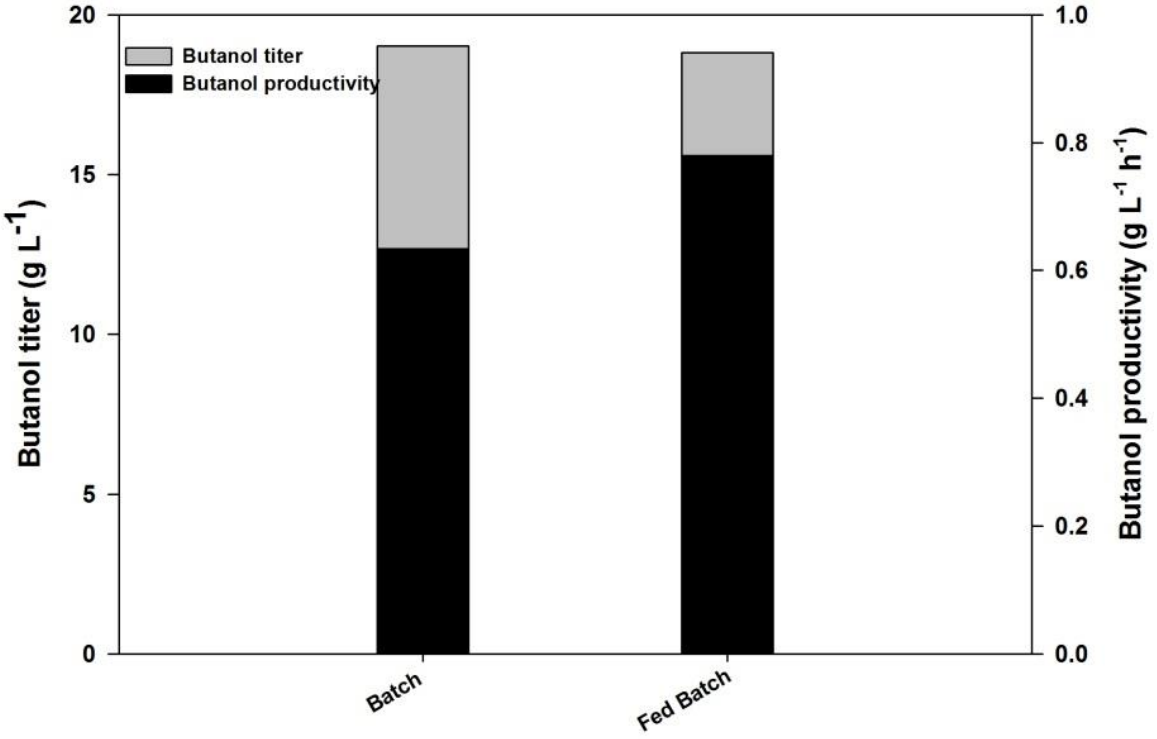


Figure 5.4 Comparison between batch and fed-batch mode of cultivation of *C. acetobutylicum* ATCC 824 with zinc supplementation in optimized media, devoid of magnesium.

However, there was a differential observation as the cellular growth was hampered marking a decrease in the microbial growth beyond 24th hour and butanol production was also stagnated. Such an observation incurred in nutrient sufficient conditions as glucose was maintained throughout at its initial concentration can be attributed to cellular mortality due to butanol toxicity stress. This may be attributed to the dissolution of the megaplasmid pSol1, which hosts the *sol* operon essential for the production of solvents. The megaplasmid is reported to be affected and rendered inactive under high butanol stress. In order to combat such an inference, *in-situ* product recovery method has been proposed. In order to, minimize butanol toxicity; *in-situ* butanol recovery mechanism has been optimized. To that end, before optimization of the gas stripping process another fed-batch under similar growth conditions were operated to determine the threshold butanol

concentration for initializing gas stripping of butanol from fermentation broth. Specific growth rate of the cells was taken as the parameter for determining the starting concentration of butanol. Figure 5.5 depicts that there is a sharp decline in the specific growth rate beyond 8 g L^{-1} of butanol titer. Thus, the starting concentration of butanol for gas stripping was kept at 8 g L^{-1} for optimization as well as further experiments. Beyond 8 g L^{-1} the specific growth rate of the cells reduced by 90% of the initial when butanol formation was initiated in the fed-batch.

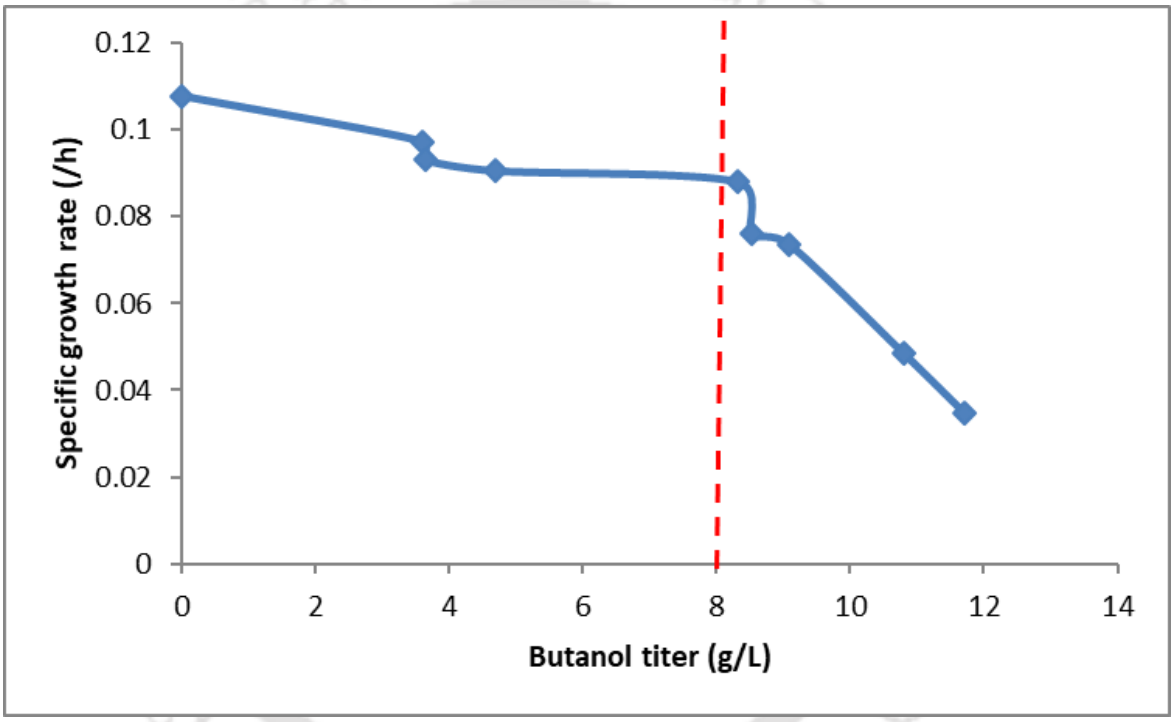


Figure 5.5 Specific growth rates of cells in fed-batch mode of cultivation with respect to butanol titer.

The CCD based RSM was performed with the objective function of maximization of butanol recovery efficiency from the broth. The optimization experiments were performed with an ideal mixture of acetone, butanol and ethanol in a ratio of 3:6:1 mimicking the fermentation broth. The butanol concentration was initially kept at 8 g L^{-1}.

Table 5.4 Tabular representation of CCD design of media parameters containing experimental and predicted values for butanol recovery efficiency (%).

StdOrder	Temperature of the condenser (°C)	Flowrate of stripping gas (vvm)	Stripping duration (h)	Experimental Butanol Recovery Efficiency (%)	Predicted Butanol Recovery Efficiency (%)
1	-5	1.25000	4.0000	53.2500	53.0535
2	1	1.25000	4.0000	20.0000	9.9413
3	-5	4.00000	4.0000	71.2500	57.5574
4	1	4.00000	4.0000	21.8750	12.8202
5	-5	1.25000	10.0000	45.0000	55.2662
6	1	1.25000	10.0000	25.0000	39.9041
7	-5	4.00000	10.0000	51.5000	62.7701
8	1	4.00000	10.0000	44.3750	45.7830
9	-7.04	2.62500	7.0000	60.8750	56.9120
10	3.04	2.62500	7.0000	4.1250	6.3747
11	-2	0.31253	7.0000	51.1250	42.8404
12	-2	4.93747	7.0000	45.0000	51.5713
13	-2	2.62500	1.9546	18.2927	38.5002
14	-2	2.62500	12.0454	90.0000	68.0792
15	-2	2.62500	7.0000	75.0000	75.5220
16	-2	2.62500	7.0000	75.7500	75.5220

Table 5.5 Depicting the optimized values of parameters of gas stripping for maximization of butanol recovery efficiency.

Optimized parameters	Maximization of butanol titer
Temperature of the condenser (°C)	(-)4.2
Stripping duration (h)	8.2
Flowrate of stripping gas (vvm)	3.2

On performing the sixteen predicted experiments, the optimized parameters were obtained (Table 5.5) after analyzing the experimental values in the software. The response optimizer predicted a maximum butanol recovery efficiency of 80.43% with a R^2 of 0.983. The optimized parameters were then validated in duplicate with ideal mixture in the 3 L automated bioreactor with a working volume of 1 L, 78% butanol recovery efficiency was observed. The experimental validation was nearly similar to the predicted model response.

Fed-batch mode of cultivation with intermittent gas stripping of *C. acetobutylicum* ATCC 824 was performed with the objective function of elevating butanol productivity and in turn enhancing the process feasibility. It was inferred that there were 4 phases of gas stripping each beginning when the concentration of butanol was 8 g L^{-1} as previously optimized. The stripping conditions were kept same as per the optimization performed previously. A cumulative titer of 54.16 g L^{-1} was observed after duration of 82 hours with an average productivity of 0.66 g $L^{-1}h^{-1}$ (Figure 5.6). The productivity slightly improved as compared with the batch cultivation with an elevated butanol response (Figure 5.7). Similar results were reported by Ahlawat et al., (2019) towards fed-batch mode of cultivation of *C. acetobutylicum* MTCC 11274. Compared to batch fermentation, application of *in situ* gas stripping increased the lactose consumption up to 2-fold (58.3 versus 29 g L^{-1}), with the final butanol concentration and yield of 11.0 g L^{-1} and 0.19 g g^{-1} lactose, respectively (Ennis et al., 1986). When this approach was applied to a fed-batch fermentation using the *C. beijerinckii* BA101 strain, 151.7 g L^{-1} of butanol could be produced with a yield of 0.30 g g^{-1} glucose and a productivity of 0.75 g L^{-1} h^{-1} (Ezeji, Qureshi and Blaschek, 2004).

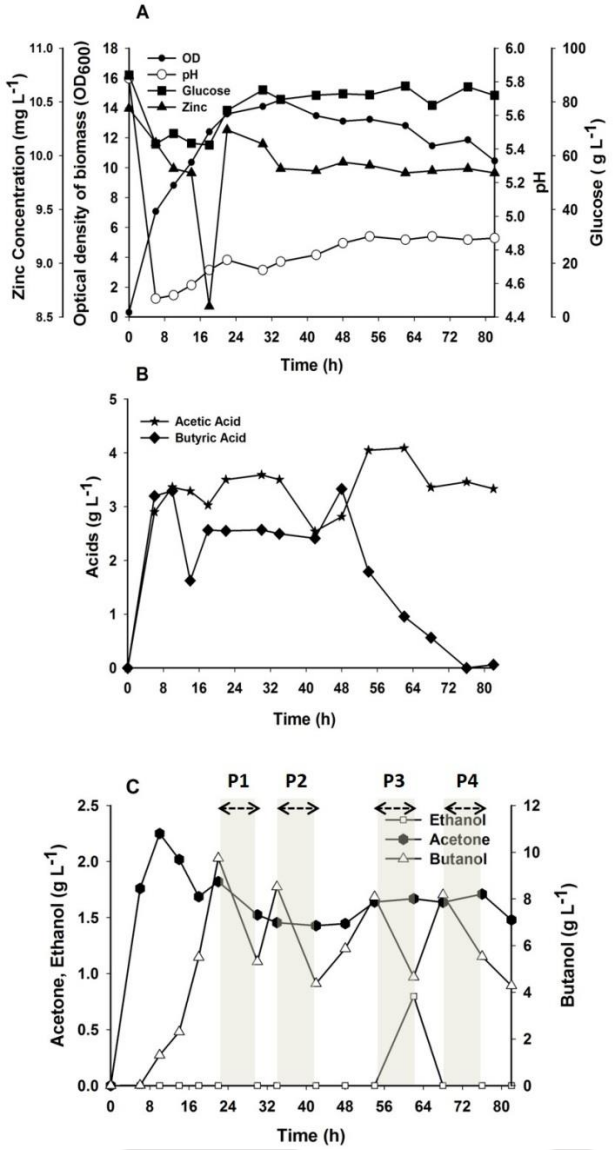


Figure 5.6 Dynamic profiles of (A) growth, pH, glucose & zinc; (B) acetic acid & butyric acid; (C) acetone, ethanol & butanol titer. The organism was grown in their optimized media with zinc supplementation and magnesium starvation. Glucose and zinc were maintained at their initial concentration with intermittent feeding coupled with gas stripping. P1, P2, P3 and P4 represent the various phases of gas stripping cycles

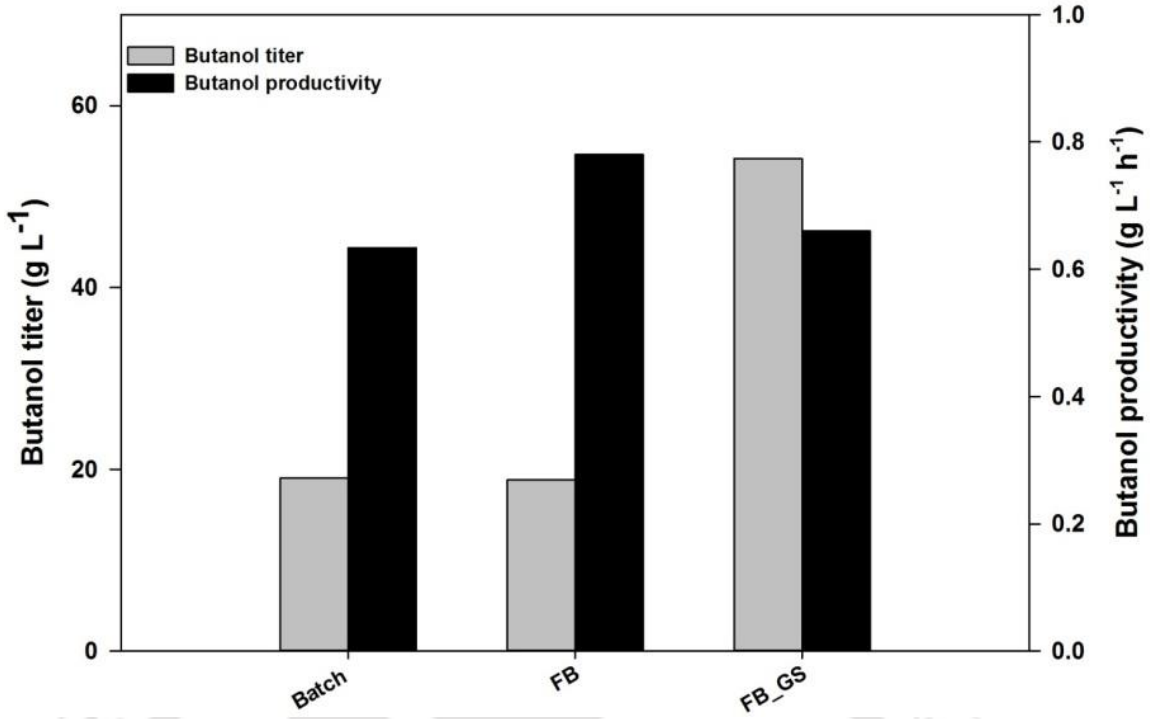


Figure 5.7 Comparison between different modes of cultivation in terms of butanol titer and productivity.

In spite of development of multiple fermentation strategies and novel hyper producing strains, commercial implementation of biobutanol production remains a distant dream and a missed opportunity owing to its high cost of production. Costly substrates such as analytical grade chemicals *viz.* peptone and glucose have been predominantly used towards biobutanol production. These chemicals increase the production cost and in turn render the whole process economically infeasible. To that end, substrate selection has been reported to have a strong impact on fermentation performance and overall production cost (Moon et al., 2016). Identifying possible low cost substrates is imperative for designing and demonstrating sustainable process strategies towards biobutanol production with improved titer. As the clostridial strain is able to utilize a wide range of substrates, identification of locally or logistically available cost-effective carbon sources is important depending upon the country of operation.

5.3.3 Growth and butanol production profile of *C. acetobutylicum* ATCC 824 using CSL and starch.

The present study reports a novel low cost process, demonstrated using corn steep liquor (CSL) as nitrogen source and industrial grade maize starch (starch) as carbon source while maintaining an improved butanol titer. With the change in CSL concentration from 0.05% to 2% (v/v), the butanol titer was observed to vary from 10.88 g L^{-1} to 11.48 g L^{-1} with negligible butanol production at CSL concentrations beyond 3% (v/v) (Figure 5.8A). The highest butanol titer of 11.63 g L^{-1} was obtained at a CSL concentration of 0.25 % (v/v). This butanol titer was comparable with the butanol titer (11.76 g L^{-1}) obtained under previously optimized production medium using analytical grade peptone as nitrogen source. Therefore, the strain exhibited an uncompromised butanol titer when nitrogen source peptone in the optimized production medium was replaced with cheaper substrate CSL at a concentration of 0.25 % (v/v). However, biomass growth observed under CSL supplementation was comparatively lesser than peptone, which can be attributed to possible presence of various inhibitors in the CSL, a waste byproduct of corn milling industry. It has been observed that at a CSL concentration of more than 3% (v/v) cellular growth and metabolism of *C. acetobutylicum* ATCC 824 was significantly hampered with negligible butanol production. Similar findings were corroborated by Parekh et al., (1998) wherein clostridial growth was severely compromised at higher concentrations of CSL due to high amounts of organic acids. Further, the phenomenon of wet milling of corn requires draining of the aqueous phase to produce CSL, which in turn removes certain nutrients and hence, might also affects cellular growth (Campos et al., 2002). In a batch culture containing 60 g L^{-1} glucose and 16 g L^{-1} CSL solids, 16.5 g L^{-1} butanol was produced by *C. beijerenckii* BA101 as compared to 10.7 g L^{-1} butanol using parental strain *C. beijerinckii* 8052 (Parekh et al., 1998).

Starch is a polysaccharide comprising of glucose monomers joined by α-1,4 linkages presents itself as a possible alternate to the expensive laboratory grade carbon sources such as glucose because of its high abundance and low cost (Ndaba et al., 2015). *C. acetobutylicum* ATCC 824 is inherently capable of metabolizing starch through its amylolytic activity, which ensures their growth and butanol fermentation via degrading starch into readily utilizable sugars like glucose (Annous and Blaschek, 1991). Highest butanol titer of 9.63 g L^{-1} was obtained when medium was supplemented with 81.1 g L^{-1} starch along with 0.25 % (v/v) CSL (Figure 5.8B). This result records a downfall in butanol titer by 18.1% when compared with the optimal production medium. Madihah et al., (2001) had presented a similar butanol titer of 8.38 g L^{-1} from gelatinized sago starch for *C. acetobutylicum* P262. Butanol titer was found to be further compromised with any deviation in starch concentration from 81.1 g L^{-1} (Figure 5.8B). The lower butanol titer obtained in the present study may be attributed to supplementation of both the cheaper commercial grade substrates (81.1 g L^{-1} starch and 0.25 %, v/v CSL) together in the fermentation medium in contrast to other literature reports. Another factor which might have negatively influenced the butanol titer is gelatinization of starch when supplemented at higher concentration. In the present study, starch gelatinization was observed at higher starch concentration of 81.1 g L^{-1} and 162.2 g L^{-1}, while it was absent at lower concentration of 40.55 g L^{-1} and 20.27 g L^{-1}. Therefore, results from characterization of *C. acetobutylicum* ATCC 824 on CSL and starch as cheaper substrates points towards scope of developing economically feasible bioprocess, albeit butanol titer needed to be improved beyond 9.63 g L^{-1}. With this as primary objective, process engineering strategy was employed in the next steps.

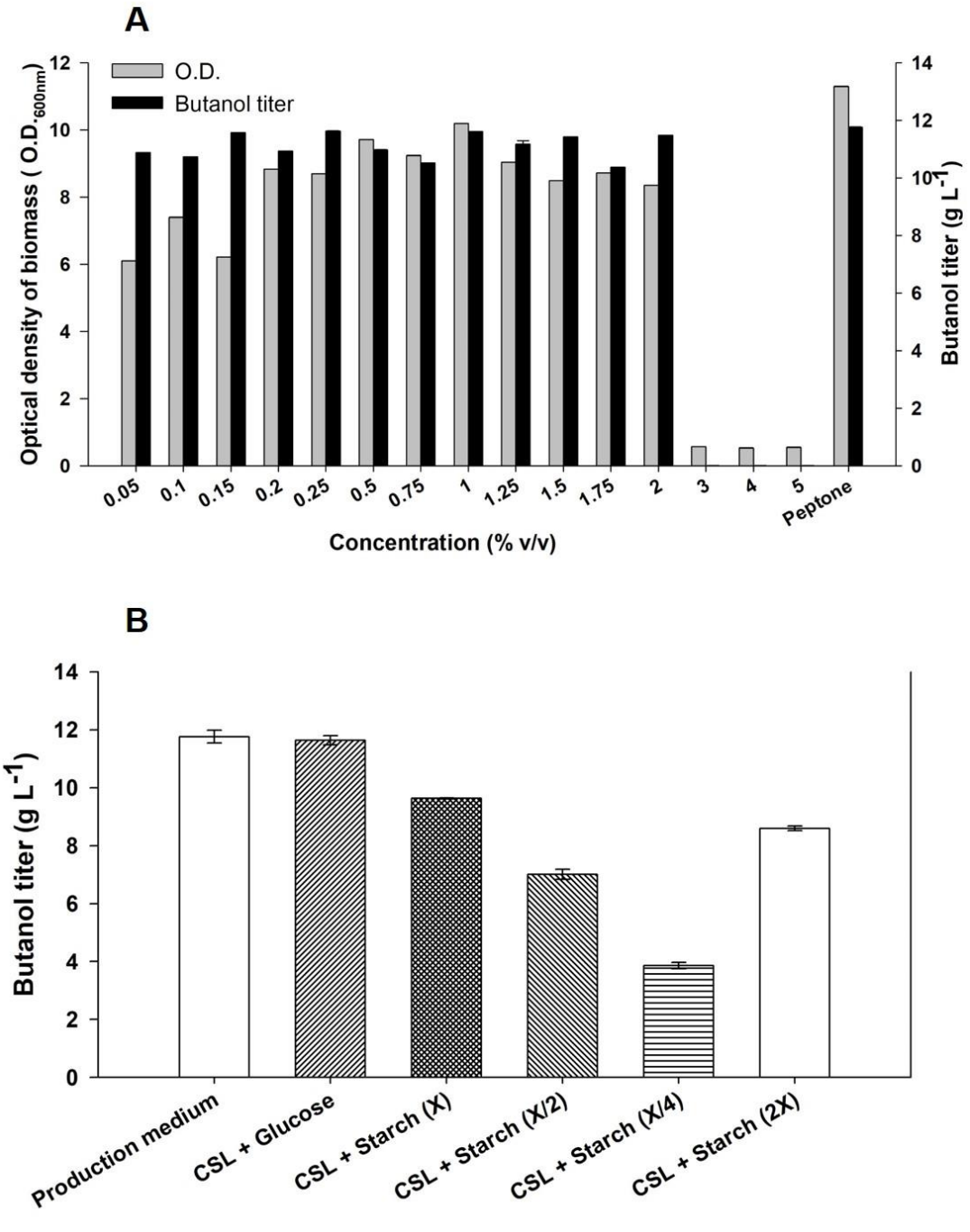


Figure 5.8 Characterization of (A) growth and butanol titer in CSL as the sole nitrogen source and (B) butanol titer under different initial concentrations of starch as carbon source of *C. acetobutylicum* ATCC 824.

5.3.4 Dual optimization of butanol titer and yield towards enhanced sustainability using low cost substrates.

The present investigation relates to the use of CSL and industrial grade maize starch as suitable replacement for costlier nitrogen and carbon sources respectively, commonly used in practice. This is the first report where a media engineering strategy has been developed for low cost butanol production involving CSL and industrial grade starch. Furthermore, statistical media optimization has been performed with multi objective optimization for maximization of butanol titer and yield. Post optimization, process engineering strategy in terms of the individual effect of magnesium starvation or zinc supplementation and their combinatorial effect on butanol titer and yield has been investigated. It was observed that starch when supplemented at a concentration of 81.1 g L^{-1} (X) produced the highest butanol titer of 9.63 g L^{-1}. However, gelatinization was observed at such high concentrations of starch, which would ultimately render the process infeasible and difficult to scale up at reactor level. Therefore, a similar range setting experiment was carried out with the objective of identifying a concentration range of starch, which could produce a comparable butanol titer *sans* gelatinizing. From the range setting experiment, it was observed that concentration of starch below 50 g L^{-1} showed no gelatinization (Figure 5.9). Therefore, the suitable concentration of starch was considered to be in the range of 30–45 g L^{-1} and this was considered for the dual objective optimization of the substrates, CSL and starch towards maximization of butanol titer and yield.

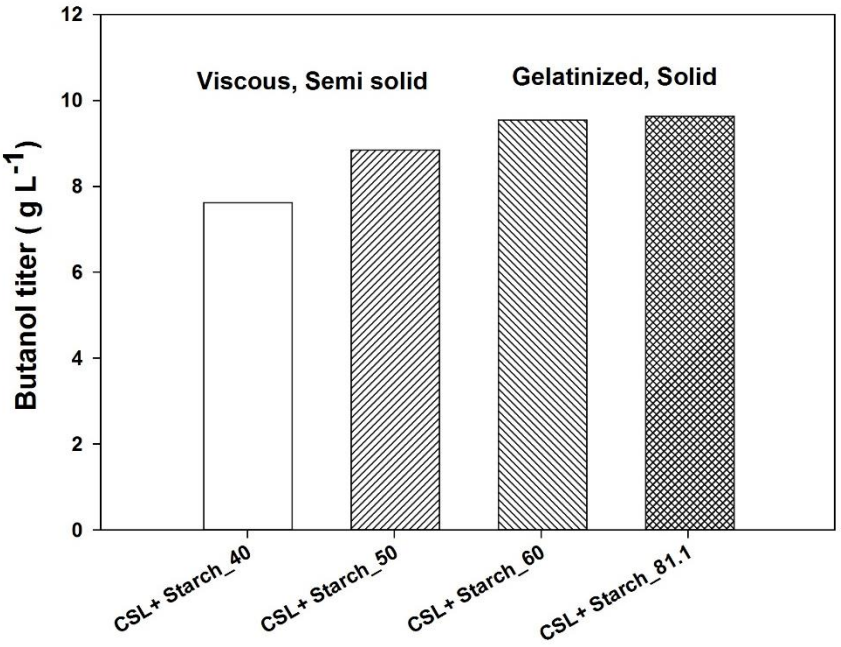


Figure 5.9 Butanol titer of *C. acetobutylicum* ATCC 824 grown under different concentrations of industrial grade maize starch and their subsequent state in the media.

Most of the process engineering strategies developed so far have limited success towards elevating the yield. Therefore, butanol yield was also considered as an important parameter for media optimization, as it aids in the overall sustainability of the process. Following selection of starch and CSL as the carbon and nitrogen source respectively, media optimization was performed with the dual objective function of maximization of butanol titer and yield. A set of 11 experiments was predicted via the Minitab 16.1.1 software and was performed to determine the effects of the different concentration of the substrates on butanol titer and yield respectively (Table 5.6).

Table 5.6 Tabular representation of CCD design of media parameters containing experimental and predicted values for butanol titer and yield.

Sl. No.	CSL (% v/v)	Starch (g L^{-1})	Experimental butanol titer (g L^{-1})	Experimental butanol yield (g g^{-1})	Predicted butanol titer (g L^{-1})	Predicted Butanol yield (g g^{-1})
1	0.20	30.00	4.74	0.25	5.48	0.26
2	1.00	30.00	4.47	0.26	5.46	0.27
3	0.20	45.00	7.74	0.27	7.82	0.27
4	1.00	45.00	7.02	0.25	7.36	0.25
5	0.03	37.50	7.19	0.27	6.84	0.26
6	1.17	37.50	7.22	0.26	6.50	0.25
7	0.60	26.90	5.89	0.28	4.89	0.26
8	0.60	48.10	7.96	0.24	7.89	0.25
9	0.60	37.50	7.79	0.28	7.92	0.28
10	0.60	37.50	7.78	0.28	7.92	0.28
11	0.60	37.50	8.18	0.28	7.92	0.28

The experimental observations were validated with respect to the predicted values and were analyzed using the Minitab 16.1.1 software. The optimal values of starch and CSL as predicted for maximization of butanol titer and yield are as appended in Table 5.9.

Table 5.7 Optimized concentrations of CSL and starch towards maximization of butanol titer and yield.

Optimized parameters	Maximization of butanol titer and yield
Initial CSL concentration (% v/v)	0.53
Initial starch concentration (g L^{-1})	39.5

Figure 5.10 demonstrates the combinatorial effect of CSL and starch at their respective optimal concentrations on butanol titer and yield. The optimized parameters predicted a maximum butanol titer of 8.15 g L^{-1} with a yield of 0.278 g g^{-1} of starch. Validation experiments were carried out and it was observed that a butanol titer of 8.1 g L^{-1} with a yield of 0.272 g g^{-1} of starch was obtained.

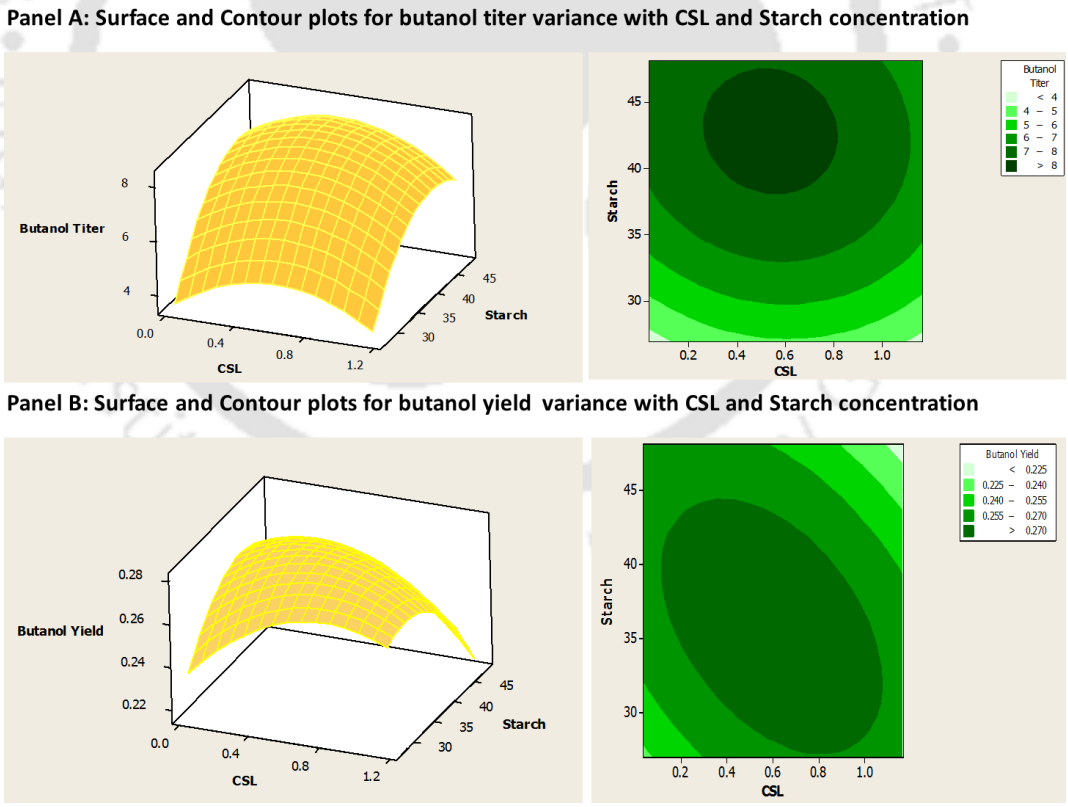


Figure 5.10 Surface and contour plots elucidating the effects of different concentrations of CSL and starch on butanol titer (Panel A) and yield (Panel B).

Metallic ions in their starved or supplemented forms have been reported to positively regulate butanol production. In order to further enhance butanol titer and yield, the effects of starvation and supplementation of metallic ions were investigated in the CSL and starch optimized media (as mentioned earlier). To that end, two separate experiments were performed to investigate the individual effect of magnesium ion starvation and zinc supplementation at a concentration of 0.01 g L^{-1} on butanol biosynthesis. An increment in butanol titer and yield was observed with both magnesium starvation and zinc supplementation (Figure 5.11). Further, an experiment was executed to evaluate the combinatorial effect of zinc supplementation in magnesium starved medium containing optimized concentrations of CSL and starch. A maximum butanol titer of 10.4 g L^{-1} was achieved with an improved yield of 0.31 g g^{-1} of starch. This was a significant improvement in comparison to the media containing optimal concentrations of CSL and starch but devoid of any metallic ion supplementation or starvation (control), which resulted in a butanol titer of 8.1 g L^{-1} with a yield of 0.272 g g^{-1} of starch.

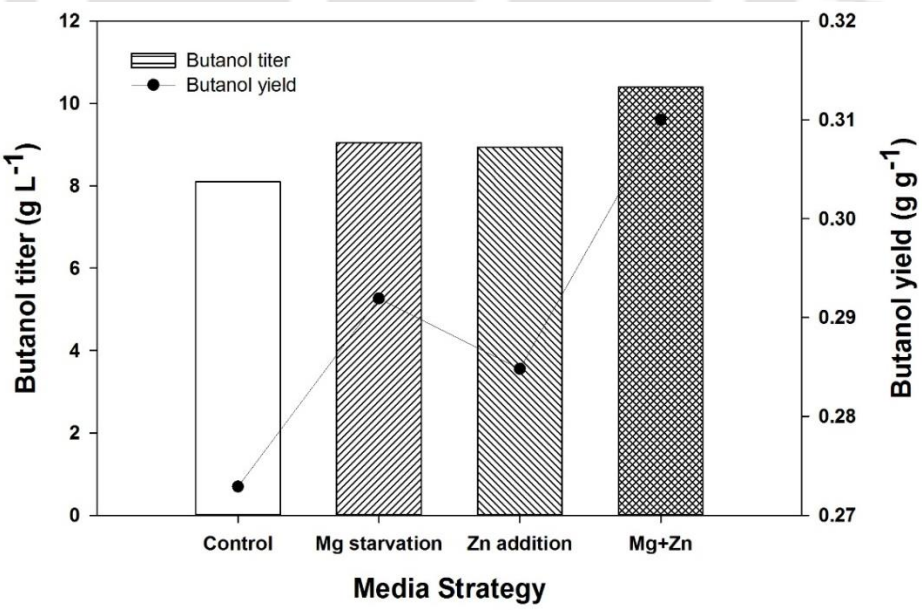


Figure 5.11 Comparative profile of butanol titer and yield for control media, medium with magnesium starvation, medium with zinc supplementation and medium with both magnesium starvation and zinc supplementation.

Finally, a low cost butanol biosynthesis was demonstrated in a 7.5 L automated bioreactor (Biojenik, India) using a customized medium containing CSL as the nitrogen source, industrial grade maize starch as the carbon source supplemented with zinc and devoid of magnesium. The strategy resulted in a butanol titer of 10.4 g L^{-1} with a yield of 0.31 g g^{-1} (Figure 5.12).

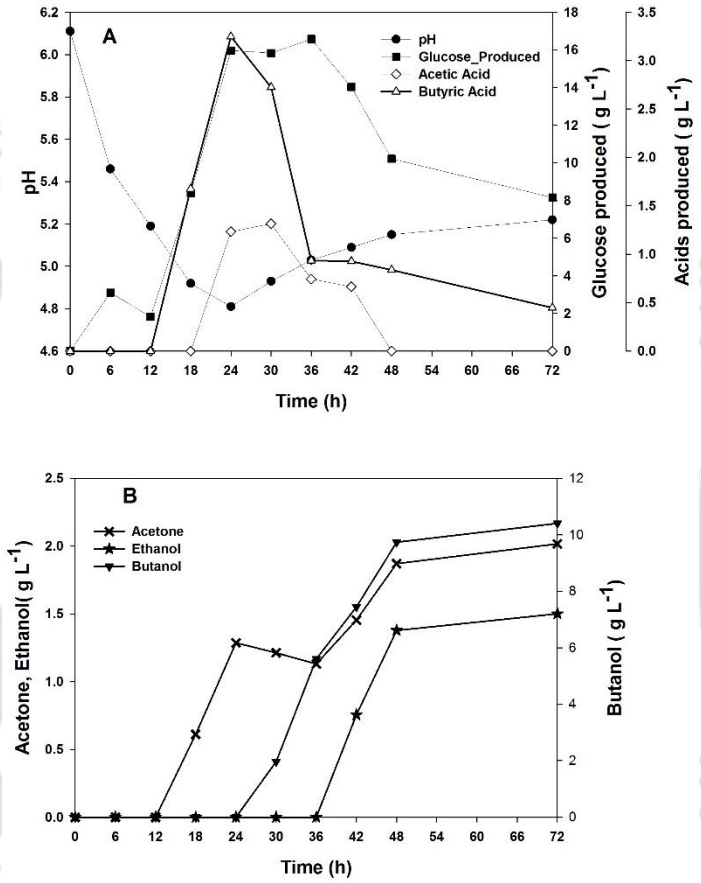


Figure 5.12 Dynamic profile of (A) pH, glucose & acids and (B) acetone, ethanol & butanol production in a low cost customized medium containing CSL as the nitrogen source, industrial grade maize starch as the carbon source supplemented with zinc and devoid of magnesium. The organism was cultivated under batch mode in a 7.5 L automated bioreactor.

The novel media engineering strategy helped in demonstrating a sustainable bioprocess towards improved butanol production by using cheaper raw materials, which effectively reduces the overall process cost. CSL and starch nourished media coupled with zinc supplementation and magnesium starvation is reported for the first time for butanol production from *C. acetobutylicum* ATCC 824 under batch mode of cultivation. However,

the butanol titer obtained was significantly lower. Therefore, industrial grade crude amylase was used for breakdown of starch in order to avoid gelatinization.

5.3.5 Optimization of amylase loading for hydrolysis of starch into readily utilizable sugar

Gelatinization or formation of highly viscous solutions after autoclaving of starch at higher concentration (Ashwar et al., 2015) poses challenge towards possible scale up and further bioreactor operation. While *Clostridium* sp. have been reported to possess high amylolytic activity, hydrolysis of starch into readily utilizable sugar is expected to result in better utilization of carbon source and in turn, improved butanol production. It was observed in our study that starch at concentrations higher than 50 g L^{-1} gelatinizes and becomes viscous which is even more prominent at higher starch concentration of 81.1 g L^{-1}. Different studies demonstrated starch hydrolysis via pre-treatment with concentrated acid, alkali or both in order to release readily utilizable simple sugars such as glucose, maltose etc. (Maiti et al., 2018; Kheyrandish et al., 2015). However, the use of concentrated acids or alkali results in production of furan compounds which are detrimental to cellular growth (Maiti et al., 2018). In view of the low cost and easy availability, the current study examines and optimizes the use of industrial grade amylase for starch degradation into readily utilizable sugars. With the increase in amylase loading from 0.6 mg to 2.4 mg per gram of starch, glucose yield and amount of glucose released increased linearly with concomitant decrease in residual starch concentration (Figure 5.13). Further increase in amylase loading beyond 2.4 mg per gram of starch, amount of glucose released and residual starch concentration was observed to be similar. However, glucose yield was significantly lower at the terminal amylase loading of 0.6 mg per gram of starch (Figure 5.13), depicting presence of excess hydrolytic enzyme. The highest glucose yield of 1.092 g g^{-1} was achieved at amylase loading of 2.4 mg per gram of starch

after 24 h of incubation, which is 99.27% of the theoretical glucose yield (1.1 g g^{-1}) from starch. Crude amylase extracts prepared from *Bacillus subtilis* resulted in 96.25% conversion of starch to glucose in 72 hours (Kunamneni et al., 2005). Madihah et al., (2001) reported a maximum specific activity of amylase at 50 g L^{-1} concentration of sago starch in the production medium required for butanol.

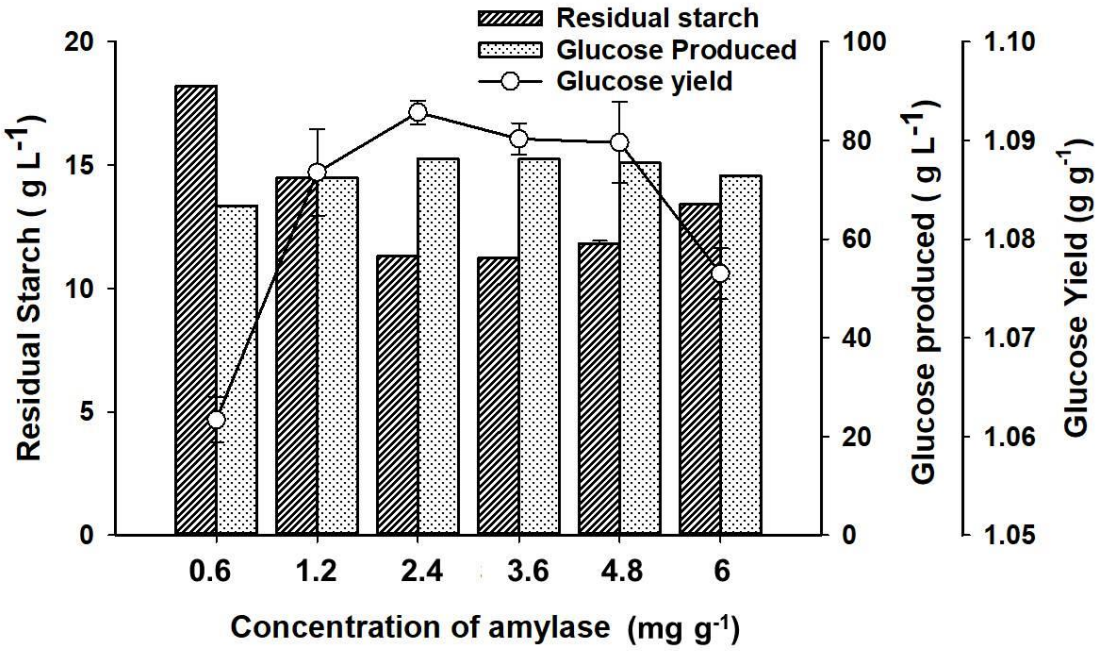


Figure 5.13 Optimization of amylase loading for pretreatment of starch with the objective function of maximization of glucose yield.

5.3.6 Bioprocess strategy for improved butanol production using low cost substrates

With the aim of achieving improved butanol titer using CSL and industrial grade starch, a sequential two stage bioprocess strategy was implemented. In the first stage, hydrolysis of starch was carried out using optimal amylase loading of 2.4 mg per gram of starch. In the second stage, butanol production was carried out in a bioreactor using optimized production media supplemented with glucose obtained from first stage and optimal CSL concentration of 0.25 % (v/v). With the aim of increasing the butanol titer further, attribute of metal ion modulated butanol production in *Clostridium acetobutylicum* ATCC 824 was integrated with sequential two stage bioprocess strategy. In this strategy,

while first stage of starch hydrolysis remained same as explained before, the second stage of butanol production was carried out in a bioreactor using optimized production media supplemented with glucose obtained from first stage, optimal CSL concentration of 0.25 % (v/v), 10 mg L^{-1} of zinc supplementation and magnesium starvation.

Two stage bioprocess strategy (CSL+$Starch_{AT}$) resulted in an improvement in butanol titer from 9.63 g L^{-1} to 11.52 g L^{-1} which, might be attributed to higher availability of ready to utilize mono sugar glucose obtained from starch hydrolysis by industrial grade amylase. Butanol titer was found to be elevated further to 16.54 g L^{-1} in case of low cost medium with zinc supplementation and magnesium starvation (CSL+$Starch_{AT}$+Zn-Mg) (Figure 5.14F). This elevation in butanol titer in case of CSL+$Starch_{AT}$+Zn-Mg batch as compared to CSL+$Starch_{AT}$ batch might be attributed to the combinatorial effect of improvement in glucose utilization and upregulation in enzymes involve in butanol biosynthesis. The consumption of glucose in case of metal ion modulated low cost medium was significantly upregulated by 18% as compared to the batch consisting of only CSL and glucose obtained from amylase pretreated starch (Figure 5.14A). In an earlier study, the authors have demonstrated that improvement in glucose utilization in *C. acetobutylicum* ATCC 824 under the influence of zinc supplementation and magnesium starvation is linked to upregulation in the specific activity of glycolytic enzymes such as glucokinase, phosphofructokinase and pyruvate kinase. Previously Wu et al., (2015) had reported similar upregulation in glucose utilization due to upregulation in *glcK* encoding a putative glucokinase enzyme. The improvement in butanol titer achieved in the present study well corroborates with previous findings where a maximum of 19.18 g L^{-1} butanol was obtained under the combinatorial effect of zinc supplementation and magnesium starvation. This was attributed to the significant upregulation in the activity of key metabolic enzymes in butanol biosynthesis butyrylaldehyde dehydrogenase and butanol

dehydrogenase. Further, formation of acetic acid (Figure 5.14B) and butyric acid (Figure 5.14C) followed by their subsequent reassimilation was significantly upregulated in metal ion modulated batch. These observations are in tandem with previous findings from laboratory grade optimized medium, where the combinatorial approach of zinc supplementation in magnesium starved production medium showed an elevation in acetic acid and butyric acid reassimilation towards improved solvent formation. In the present study, while ethanol synthesis remained unaltered (Figure 5.14E), formation of acetone (Figure 5.14D) was upregulated under the influence of metal ion modulation due to upregulation of acetoacetate decarboxylase enzyme.

C. beijerenckii BA101, hyper butanol producing mutant strain resulted in an improved butanol titer of 25.7 g L^{-1} from cassava flour (Lepiz-Aguilar et al., 2013). However, in case of wild type clostridial strains, butanol titer obtained in the present study is comparable to literature reports (Table 5.10). Wild type strain *C. saccharoperbutylacetonicum* produced butanol in the range of 16.4 to 16.9 g L^{-1} (Table 5.10) when grown in a medium consisting of bagasse, cassava chips or cassava starch, which is similar to 16.54 g L^{-1} of butanol obtained in the present study (Thang et al., 2010). Madihah et al., (2001), previously reported a low cost medium strategy using sago starch as the carbon source resulting in a butanol titer of 8.38 g L^{-1} from batch cultivation of *C. acetobutylicum* P262. Use of inexpensive carbon and nitrogen sources was highlighted by Kaushal et al., (2019), wherein rice straw hydrolysate and crude glycerol & instant dry yeast were used as carbon and nitrogen source resulting in a butanol titer of 11.7 g L^{-1} using *C. sporogenes* NCIM 2918. Therefore, the present process engineering strategy involving low cost substrates resulted in an improved butanol titer using wild type *Clostridium acetobutylicum* strain.

Table 5.8 Comparison of butanol titers produced by *Clostridium* sp. cultivated using low cost substrates

Strain	Type	Substrate	Mode of cultivation	Butanol titer (g L^{-1})	Reference
C. acetobutylicum ATCC 824	**W**	**Corn Steep Liquor + Industrial grade starch**	**Batch**	**16.54**	**This study**
C. acetobutylicum ATCC 824	W	Napier grass	Batch	9.5	He et al., 2017
C. acetobutylicum ATCC 824	W	Bamboo	Batch	10.4	Kolawole et al., 2015
C. acetobutylicum P262	W	Sago starch	Batch	8.38	Madihah et al., 2001
C. sporogenes NCIM 2918	W	Crude glycerol + Rice straw hydrolysate + instant dry yeast	Batch	11.7	Kaushal et al., 2019
C. saccharoperbutylacetonicum ATCC 27022	W	Bagasse	Batch	16.5	Moon et al., 2016
C. saccharoperbutylacetonicum N1-4	W	Cassava chips	Batch	16.4	Thang et al., 2010
C. saccharoperbutylacetonicum N1-4	W	Cassava starch	Batch	16.9	Thang et al., 2010

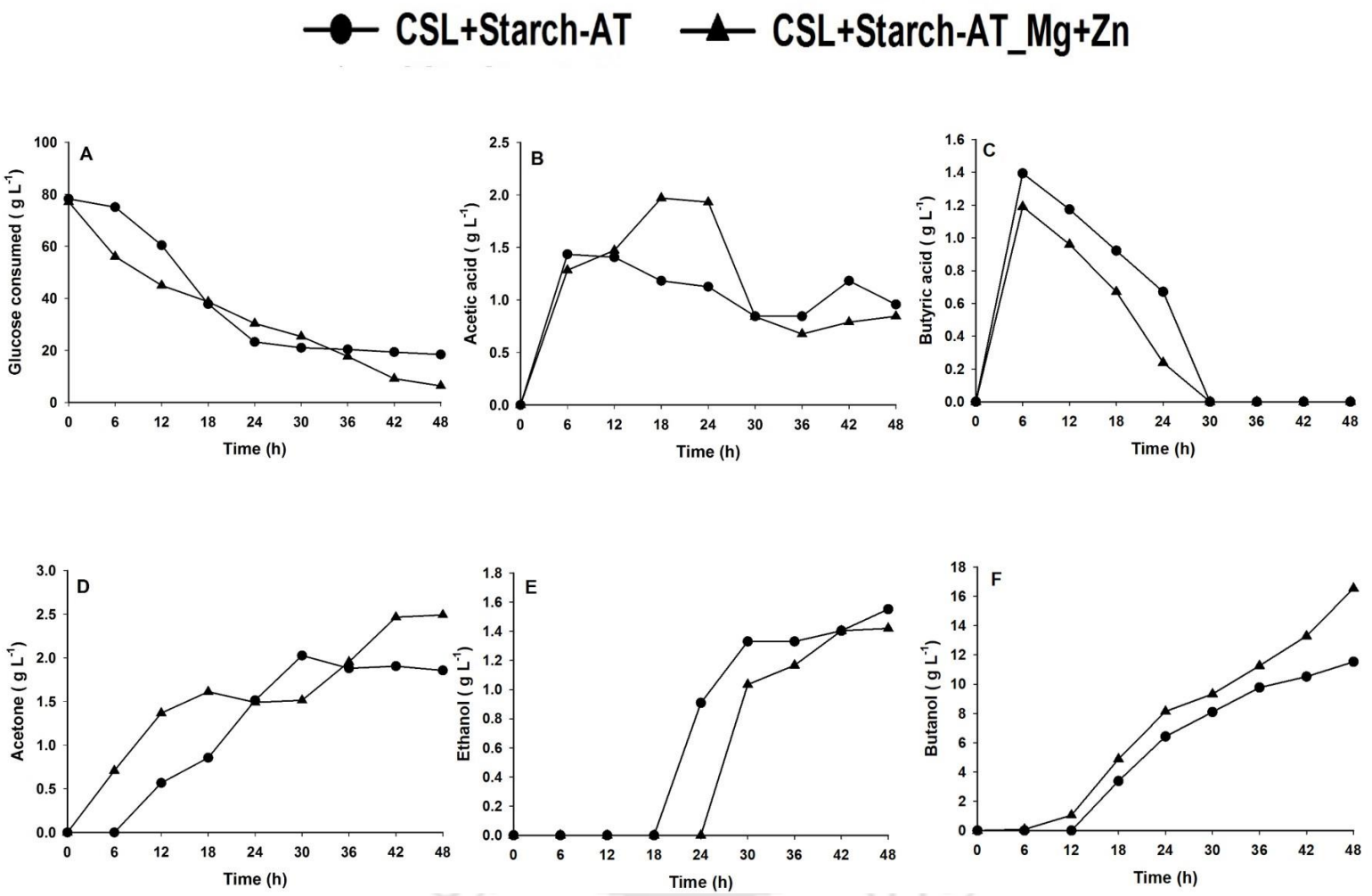


Figure 5.14 Dynamic profiles of (A) glucose, (B) acetic acid, (C) butyric acid, (D) acetone, (E) ethanol and (F) butanol produced under batch mode cultivation. CSL+Starch$_{AT}$ batch refers to medium with CSL and amylase treated starch. CSL+Starch$_{AT}$+Zn-Mg referes to batch with magnesium starvation and zinc supplementation in CSL and amylase treated starch optimized medium.

5.4 Conclusion

- ✓ Fed-batch cultivation resulted in 24% improvement in butanol productivity. However, the fermentation was hindered by solvent toxicity.
- ✓ In order to alleviate solvent toxicity, gas stripping was optimized.
- ✓ It was inferred that 8 g L^{-1} of butanol was detrimental to growth of the strain.
- ✓ Fedbatch coupled with gas stripping resulted in cumulative titer of 54.2 g L^{-1} with an overall productivity of 0.66 g L^{-1} h^{-1}.
- ✓ Low cost medium engineering strategy was developed using CSL and starch as nitrogen and carbon source.
- ✓ The novel low cost medium strategy resulted in a butanol titer of 16.54 g L^{-1}.

Table 5.9 Overview of the butanol production achieved via different process engineering strategies

Strain	Substrates	Mode	Strategy	Titer	Yield	Overall Productivity	Downstream
C. acetobutylicum ATCC 824	Glucose and Peptone	Batch (5 L)	Zinc supplementation with Magnesium starvation	19.18 g/L	0.25 g/g	0.40 g/L/h	-
C. acetobutylicum ATCC 824	Glucose and Peptone	Fed-Batch (5 L)	Zinc supplementation with Magnesium starvation	54.16 g/L	0.26 g/g	0.66 g/L/h	Novel *in-situ* gas stripping
C. acetobutylicum ATCC 824	Starch and CSL	Batch (5 L)	Zinc supplementation with Magnesium starvation	10.4 g/L	0.31 g/g	0.14 g/L/h	-
C. acetobutylicum ATCC 824	Amylase treated Starch and CSL	Batch (5 L)	Zinc supplementation with Magnesium starvation	16.54 g/L	0.28 g/g	0.35 g/L/h	-

5.5 References

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Chapter 6

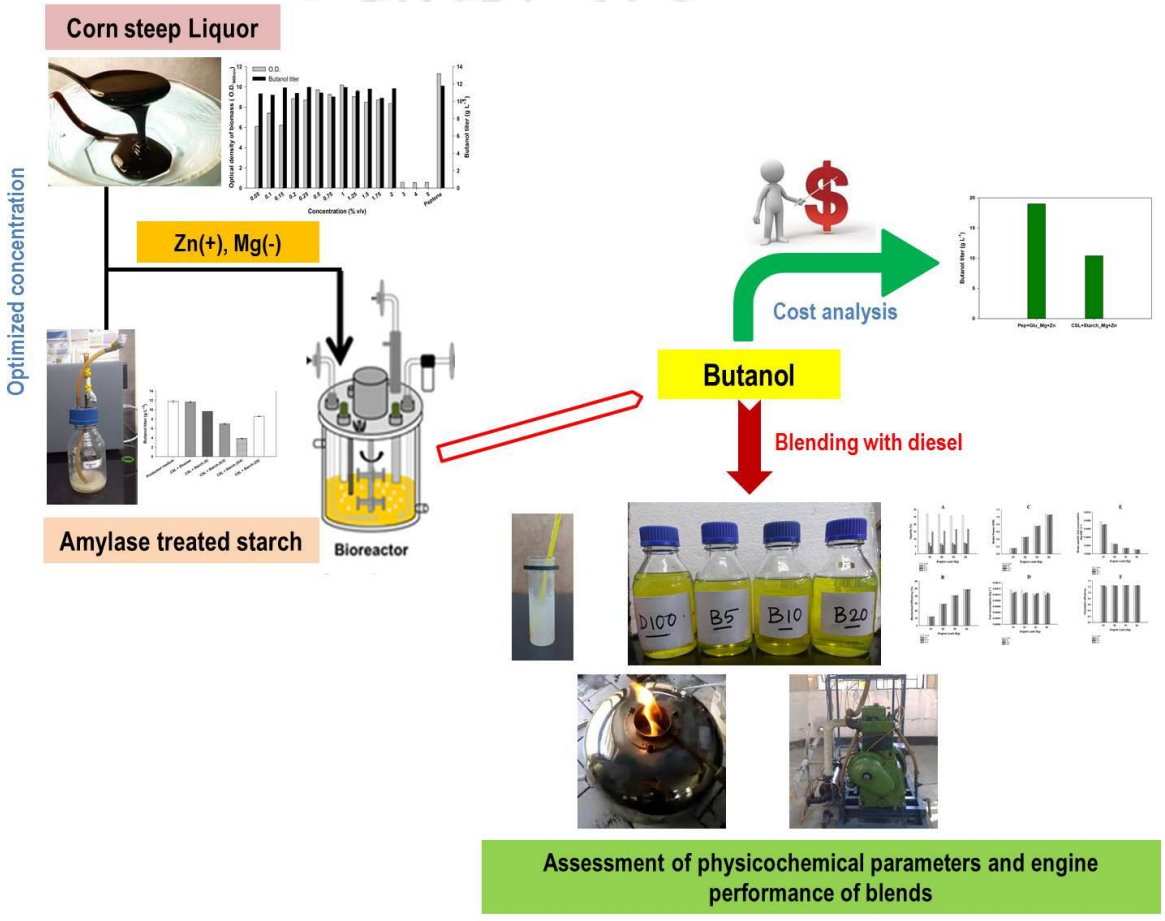
PERFORMANCE EVALUATION OF BUTANOL AS A POTENTIAL FUEL BLEND AND ECONOMIC ASSESSMENT OF BIOPROCESS STRATEGY

"I think the cost of energy will come down when we make this transition to renewable energy."

Al Gore, Ex-VP of USA

CHAPTER 6

Performance evaluation of butanol as a potential fuel blend and economic assessment of bioprocess strategy



Assessment of engine performance and physicochemical properties of butanol – diesel blends and comparative cost analysis of the bioprocess strategy developed

6.1 Background and motivation

The impending global mandate towards development of a suitable alternate transportation fuel has made researchers worldwide strive tirelessly towards development of sustainable technologies for cleaner fuel generation (Sauer et al., 2016). Similarly, upward trending of fuel prices, continual threats towards depletion of conventional energy resources and deteriorating global climate has been the major motivations towards re-commercialization of butanol biosynthesis (Grassi et al., 2018; Patakova et al., 2018). Butanol is considered to be potent next generation biofuel due to its resemblance to gasoline and superior physicochemical properties compared to ethanol and methanol (Zheng et al., 2015). However, the high cost of substrates and costly fermentation processes have proven to be the major hindrances towards commercial realization of butanol biosynthesis (Wu et al., 2016).

It is envisioned that by the year 2050, 25% of the global transportation will be fuelled by biofuels (Pereira et al., 2014). Biofuels provide a suitable platform for reduction of dependency on petroleum fuel, reducing the overall global carbon footprint with minimal changes in the existing vehicular design and logistics. Furthermore, a thriving biofuel sector strengthens the employment scenario via creating newer profiles and portfolios in the urban and rural sectors (Pereira et al., 2014). By the year 2012, 10% of the global energy supply was provided by biofuels with around 3% of it owing to transportation fuel. In 2010, the US Environmental Protection Agency (EPA) published the Renewable Fuel Standard Program (RFS II) Final Rule. The new renewable fuel standards increase the total volume of renewable fuel required to be blended into transportation fuel to 36 billion gallons by 2022 (Tao et al., 2014). Additionally, in tandem to production of biofuels such as ethanol and butanol, a newer biorefinery approach has been targeted towards facilitated commercialization of these processes. As far as,

butanol's viability as a transportation fuel is concerned, it contributes towards cleaner emissions by reducing soot emissions and unburned hydrocarbons in the tail pipe exhaust (Qureshi and Blaschek, 2001). Butanol has research and motor octane numbers of 113 and 94 compared to 111 and 92 for ethanol (Ladisch, 1991). Some of the advantages of butanol as a fuel have been reported in the literature (Ladisch, 1991) including a vapor pressure for pure butanol of 0.63 psi as compared to 2.25 psi for ethanol, and a heat of vaporization of 141.3 kcal kg^{-1} for butanol, compared to 204.1 kcal kg^{-1} for ethanol. Additionally, the high boiling point (118.8 °C) and lower vapor pressure for butanol may affect cold starting. It is more miscible with gasoline and diesel fuel, has a lower vapor pressure, and is less miscible with water. It is currently used as a feedstock chemical in the plastic industry and as a food grade extractant in the food and flavor industry (Qureshi and Blaschek, 2001). Multiple researchers have highlighted the positive impact on internal combustion engines of butanol when blended with diesel and its suitability in engine performance with improved mechanical and brake thermal efficiency lends credence to its future application as an alternate to petroleum fuel (Rakopoulos et al., 2010). A review of existing literature reveals that vehicle emission performance with n-butanol is inconclusive (Tao et al., 2014). Another key aspect of butanol biosynthesis and its subsequent commercialization, is delving deeper into the process economics taking into account the cost of raw materials, capital expenditure, operational overtures and hence, projecting a realistic global estimate for possible marketing butanol as the reality it promises a possibility.

In the present study, a novel media engineering coupled with sustainable bioprocess strategy has been demonstrated towards elevated butanol production using industrial grade starch and waste CSL as substrates (explained in Chapter 5). The present chapter delves mainly towards elucidating the performance of biobutanol as potential fuel

blend with diesel and its physiochemical properties have been carried out in a 4- stroke internal combustion engine. It highlights biobutanol as a key alternative to existing petroleum fuels for its suitability in operation in engines and has better properties as compared to lower alcohols with carbon content such as methanol and ethanol. Furthermore, a cost analysis has been performed with respect to substrates to highlight the impact of using cheaper and industrial grade resources such as CSL and starch.

6.2 Materials and methods

6.2.1 Preparation of diesel – butanol blends

Butanol produced through fermentation using low cost substrate was blended with diesel in three different concentrations namely B5 (Butanol:Diesel = 5:95), B10 (Butanol:Diesel = 10:90) and B20 (Butanol:Diesel = 20:80) and examined for their performance as transportation fuel in single cylinder 4- stroke internal combustion engine (Model: TV1, Kirloskar, India). The physiochemical properties and engine performance of the blended fuels was compared with pure diesel (referred as D100 hereafter). The diesel was purchased from a local petroleum pump at the established market rate.

6.2.2 Evaluation of physicochemical properties of diesel – butanol blends

Physicochemical properties such as kinematic viscosity, cloud point, pour point, flash point and fire point were estimated for D100, B5, B10 and B20. Kinematic viscosity was measured at 40 °C by using an interfacial viscometer (Anton Paar physica MCR 301 rheometer) with sample volume of 0.5 mL. Density of the butanol – diesel blends along with pure diesel oil was measured by density meter (Anton Paar DMA 4500M) with a sample volume of 1 mL. Absolute viscosity of the samples were quantified as per the following equation

$$\text{Absolute Viscosity } (\mu) = \text{Kinematic viscosity } (\nu) * \text{density } (\rho) \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Cloud point and pour point were determined in an apparatus, which consists of a flat bottom tube enclosed in an air jacket. The jacket is placed in a chiller containing methanol and being operated at -30 °C. 50 mL of the sample is placed in a glass tube, which is placed inside the apparatus. A thermometer is placed inside the tube to determine the temperature of the sample. As the samples are gradually allowed to cool inside the chiller, the tube is regularly examined for its appearance to determine the cloud and pour point. The temperature at which the samples present a foggy whitish appearance is denoted as cloud point. The samples are further allowed to cool to a temperature beyond which the sample freezes and does not flow even if kept in horizontal position. The temperature where the sample ceases to flow is denoted as pour point. Both cloud and pour point is expressed in terms of °C.

Flash and fire point was estimated in an apparatus comprising of a cylindrical cup wherein 50 mL of the sample was placed (Koehler, USA). The cup was held in a metallic heater powered by means of an electrical heater. A thermometer was inserted on the top edge of the cup. A rheostat was used to regulate the heating available to the cup. The sample was intermittently stirred for uniform heating. A test flame was introduced over the surface of the cup to determine the temperature at which a distinct flash was observed. Beyond the flash point, the samples were allowed to be heated until the vapors continuously burn, which is noted to be the fire point of the sample. Both flash and fire point is expressed in terms of °C.

6.2.3 Operational specifications of single cylinder 4- stroke diesel internal combustion engine.

Engine (Figure 6.1) testing was performed using a single cylinder 4- stroke internal combustion engine with the complete specifications as mentioned in Table 6.1. The engine was water-cooled and operated at constant speed of 1500 rpm with a maximum capacity of

7 BHP (5.2 KW). The engine was attached to a portable flue gas analyzer (AVL, India) for determining the various exhaust gas composition such as carbon monoxide (CO), carbon dioxide (CO_2), oxygen (O_2), hydrocarbon (HC) content and nitrogen oxides (NO_x). For all diesel – butanol blends and pure diesel, engine was operated under a single engine speed with 5 different operational load (%) conditions to determine the various operational parameters such as brake power, mechanical efficiency, volumetric efficiency, fuel consumption and brake specific fuel consumption and air to fuel ratio.

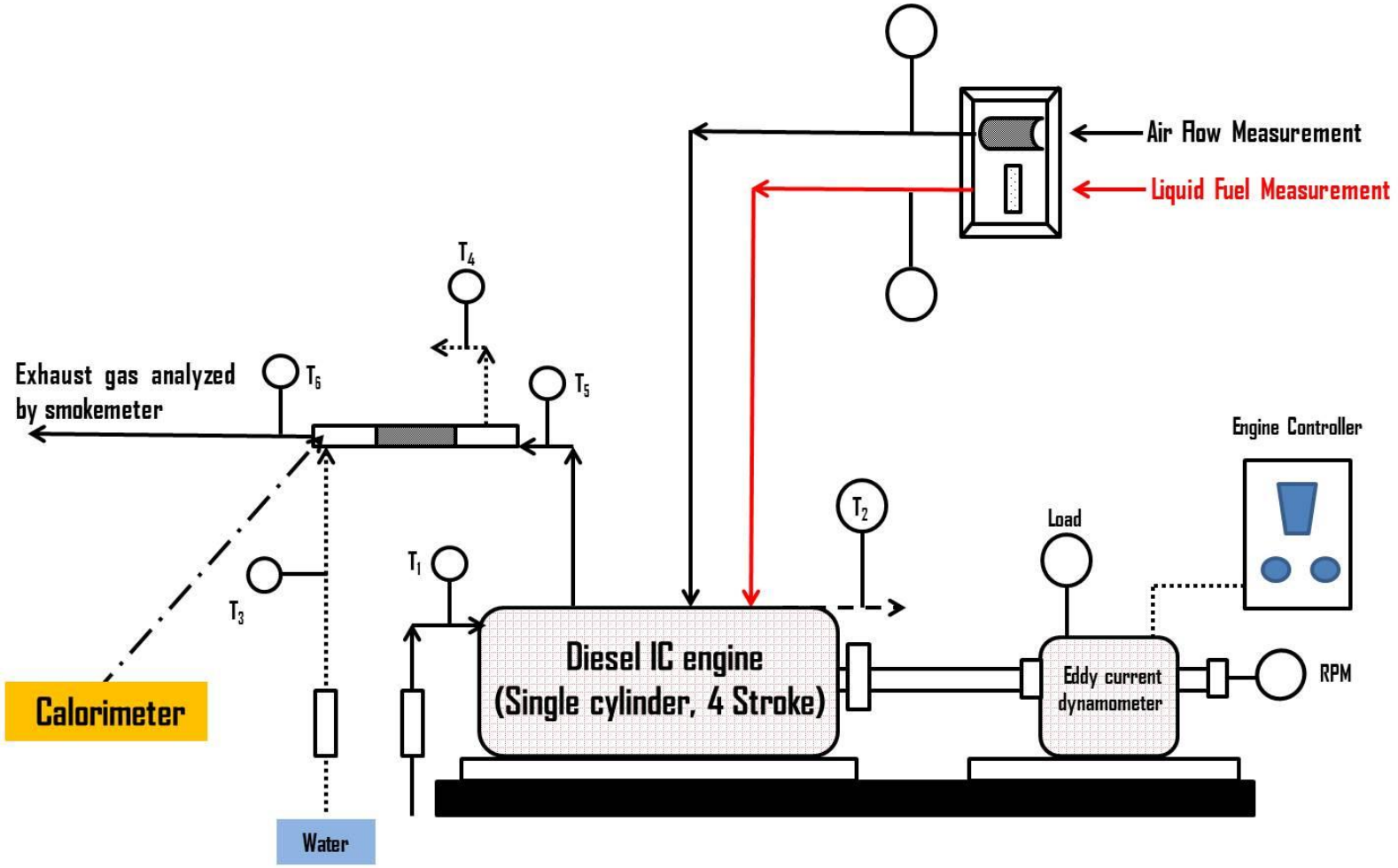


Figure 6.1 Schematic representation of the engine setup for performance analysis of pure diesel and butanol – diesel blend samples.

Table 6.1 Specifications of 4- stroke internal combustion diesel engine.

Make	**Kirloskar, Model – TV1**
Type	Vertical, Single cylinder, 4 –stroke, DI diesel engine
No. of cylinders	01
Rated power	5.2 KW @ 1500 rpm
Coolant	Water
Bore stroke	87.5 * 110 mm
Swept volume	0.661 L
Compression ratio	17.5:1
Speed	1500 rpm, constant
Injection pressure	210 bar
Combustion chamber	Hemi-spherical bowl-in-piston type
Dynamometer	Eddy current (Make: Saj; Model: AG10)
Governor	Mechanical (centrifugal)
Air flow	Orifice meter and manometer
Fuel flow	Fuel measuring unit, range 0 – 450 mL
Speed indicator and sensor	Digital, non-contact type
Load indicator	Model: AX-271, 0 – 100 Kg, 230 V AC
Temperature indicator	Digital, Multipoint
Temperature sensor	K – type thermocouple
Rotameters	Engine cooling, 40 – 400 LPH, calorimeter, 10 – 100 LPH
Software	Engine soft: Engine performance analysis software
Pressure Transducer	
Make and type	PCB make, Piezo electric (15000 psi)
Resolution and response time	0.1 psi, 2 µs
Crank angle sensor	360° degree encoder with a resolution of 2°

6.2.4 Comparative economic analysis of the bioprocess strategies developed for butanol biosynthesis

Cost analysis of butanol fermentation has been performed with 1 L of butanol being the functional unit and accordingly cost of substrates; cost incurred towards fermentation and distillation has been estimated. Water cost has been assumed negligible. The capital expenditure has not been included in the calculations as the existing facility has been used in the entirety of the study. The fermentation cost of producing 1 L of butanol using low cost substrates as demonstrated in the present study was compared with the process using laboratory grade glucose and peptone as carbon and nitrogen source, respectively (Chapter 3, 4 and 5). Table 6.2 formulates the cost of raw materials required for production of 1 L of butanol using both low cost industrial grade substrates and laboratory grade chemicals. All the fermentation experiments were carried out in a 7.5 L automated bioreactor (New Brunswick, Eppendorf, Germany) with a working volume of 2.5 L. Therefore, initially, the cost of raw materials was calculated on the basis of the working volume which has been further scaled up for working volume theoretically required for production of 1 L butanol. The reactor is assumed to be equipped with a Rushton 6 blade impeller for mixing of the nutrients in the fermentation broth (Table 6.3). The power incurred towards mixing due to agitation in the reactor has been calculated as per the following equations:

$$\text{Batch volume (V)} = \pi\,(\text{Teq}^3) / 4 \quad \ldots\ldots (2)$$

Where T_{eq} is the equivalent diameter of the reactor.

The agitator diameter (D) is calculated as per the following:

$$D = 0.4 * \text{Teq} \quad \ldots\ldots (3)$$

The bulk velocity (V_c) is calculated as follows:

$$V_c = \text{Scale of agitation} * 6 \quad \ldots\ldots (4)$$

$$Pumping\ rate\ (Q) = V_c \frac{(\pi \cdot T_{eq}^2)}{4} \quad \ldots\ldots (5)$$

$$Power\ (P) = N_p * \rho * N^3 \cdot D^5 \quad \ldots\ldots (6)$$

Where,

Power Number (N_p) = 5.5

The power factor of the mixer has been considered to be functional at a loading of 80%.

$$Motor\ power = \frac{P}{Power\ factor} \quad \ldots\ldots (7)$$

Table 6.2 Cost of substrates used for production of 1 L of butanol

Low cost media strategy							
Source	**Material**	**Cost (Rs per Kg)**	**Cost (Rs per g)**	**Amount used (g)**	**Amount for 2.5 L broth (g)**	**Cost for 2.5 L (Rs.) ****	**Cost for 50 L (Rs.) ****
Alibaba	CSL	30	0.03	2.5	0.46	0.01	0.28
Alibaba	Starch	7	0.007	81.1	202.75	1.42	28.38
India Mart	Amylase	45	0.04	0.19	0.48	0.02	0.43
	Water	0	0	0		0	0
India Mart	Mn	35	0.03	0.01	0.02	0.000875	0.017
India Mart	Fe	7	0.007	0.01	0.02	0.000175	0.003
India Mart	Na	4.5	0.004	0.01	0.02	0.0001125	0.002
India Mart	Zn	42	0.04	0.01	0.02	0.00105	0.02
India Mart	PABA	140	0.14	0.01	0.02	0.0035	0.07
India Mart	Acetate	20	0.02	3.22	8.05	0.16	3.22
Alibaba	Phosphate	35	0.03	1	2.5	0.08	1.75
					Total	**1.70**	**34.18**

Control production medium with Peptone and Glucose							
Source	**Material**	**Cost (Rs per Kg)**	**Cost (Rs per g)**	**Amount used (g)**	**Amount for 2.5 L broth (g)**	**Cost for 2.5 L broth (Rs.) ****	**Cost for 42.5 L broth (Rs.) ****
Himedia	Peptone	2700	2.7	53.5	133.75	361.12	6139.12
Himedia	Glucose	700	0.7	89.6	224	156.8	2665.6
	Water	0	0	0	0	0	0
Himedia	Mn	1600	1.6	0.01	0.02	0.04	0.68
Himedia	Fe	664	0.66	0.01	0.02	0.016	0.28
Himedia	Na	500	0.5	0.01	0.02	0.012	0.21
Himedia	Zn	1500	1.5	0.01	0.02	0.037	0.64
Himedia	PABA	1006	1.01	0.01	0.02	0.025	0.43
Himedia	Acetate	860	0.86	3.22	8.05	6.92	117.69
Himedia	Phosphate	1500	1.5	1	2.5	3.75	63.75
					Total	**528.72**	**8988.40**

****** *The cost of raw materials was calculated on the basis of the working volume (2.5 L) which has been further scaled up for working volume theoretically required for production of 1 L butanol.*

Table 6.3 Parameters required for calculation of power input incurred towards agitation.

Parameters	Values	Unit
Volume	0.05	m^3
Type	Rushton 6 Blade	-
D/T	0.4	-
Scale for 200 rpm	3	-
Density	1200	kg/m^3
Viscosity	1000	cP

6.3 Results and discussions

6.3.1 Qualitative analysis of diesel - butanol blends

In order to assess suitability of butanol for blending with petroleum as a potent transportation fuel, various physicochemical properties such as kinematic viscosity, absolute viscosity, density, pour point, cloud point, flash point and fire point were experimentally determined for B5, B10, B20 and compared with D100. In comparison to D100 kinematic viscosity, absolute viscosity and density was found to decrease marginally with the increase in butanol percentage in the blend (Table 6.4, Figure 6.2). The relative decrease in kinematic and absolute viscosity for the blended samples as compared to D100 can be attributed to the phenomenon of solvation that the butanol molecules undergo upon blending with higher hydrocarbons (diesel) wherein the molecular interactions weaken due to increased spaces in the hydroxyl groups (Brandao et al., 2018). It has been reported that the lower viscosities of transportation fuels are more favored in automobile engines attributed to their enhancement in formation of sprayed droplets for the injection system, in turn providing higher fuel blend dispersion and efficient penetration capacities inside

the engine ignition chamber (Brandao et al., 2018). Chotwichien et al., (2009) has reported a similar kinematic viscosity of 2.73 cSt at 40°C for B5 butanol-diesel blend.

Cloud and pour point are essential cold flow properties intrinsic to liquid transportation fuels, which assess the feasibility of the fuel for its use in automobile engines at lower temperature. All the blends exhibited similar cloud point (-1 °C to -2 °C) and pour point (-8 °C to -10 °C) when to D100 with a cloud point and pour point of -3 °C and -12 °C, respectively (Table 6.4, Figure 6.2). Butanol blended with diesel at 5, 7.5 and 10 (%, v/v) resulted in a cloud point (°C) in the range of -3.3 to -3.6 whereas the pour point (°C) was in the range of -20 to -21 which corroborates with the present study (Lapuerta et al., 2018).

Table 6.4 Comparative physicochemical properties between diesel and diesel - butanol blends

Parameter	D100	B5	B10	B20
Kinematic Viscosity (cSt at 40°C)	2.86	2.36	2.28	2.08
Absolute Viscosity (cP)	2.42	1.97	1.89	1.72
Density (g cc^{-1})	0.846	0.833	0.828	0.826
Cloud Point (°C)	-3	-1	-2	-1
Pour Point (°C)	-12	-8	-10	-9
Flash Point (°C)	54	38	36	35
Fire Point (°C)	60	40	37	36

Flash point is defined as the temperature needed for the ignition of the fuel whereas fire point is defined as the ambient temperature at which the fuel starts to burn continuously (Rakopolous et al., 2010) In the present study, a significant reduction in both

flash point (35 °C to 38 °C) and fire point (36 °C to 40 °C) was observed for butanol-diesel blends as compared to D100 with a flash and fire point of 54 °C and 60 °C, respectively (Table 6.4, Figure 6.2). Flash point has a significant dependence on the volatility and the vapour pressure of the blend (Kalaghatgi et al., 2015). When the vapour pressure increases, the flash point decreases. It has been reported that when a small amount of alcohol is blended with diesel, the flash point effectively decreases, as the vapour pressure of the blend is lower than that of pure diesel (Ibrahim, 2016). Therefore, the observed reduction in flash and fire point indicating better flammability and volatility is mainly due to the presence of volatile alcohol such as butanol in the blend (Chotwichien et al., 2009). Butanol blending at 10% with diesel and biodiesel resulted in a lowered flash point of 42.5°C, compared to 58°C of pure diesel samples (Tuccar et al., 2014). Therefore, the lowered flash and fire point for the blended fuels are advantageous to the functioning of the engine and are better equipped than pure diesel. It can be positively inferred from the present analysis that butanol blending with diesel is advantageous and a suitable renewable alternate to existing petroleum fuels since its physicochemical properties are similar or better compared to pure diesel.

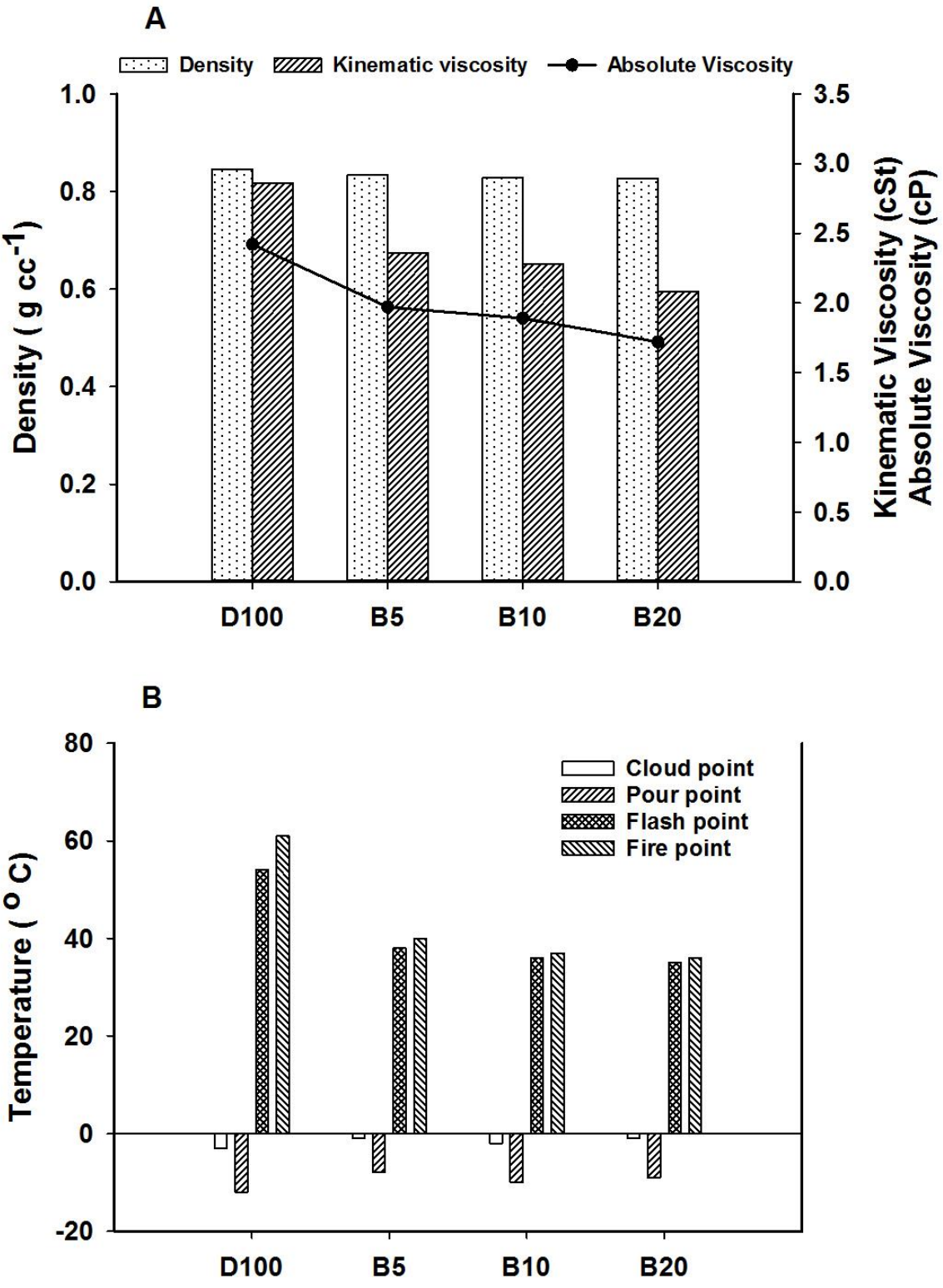


Figure 6.2 Comparison between the physicochemical properties of the butanol diesel blends and pure diesel. (A) Comparison between density (g cc^{-1}), absolute viscosity (cP) and kinematic viscosity (cSt) & (B) cloud point (°C), pour point (°C), flash point (°C) and fire point (°C) of B5, B10, B20 and D100.

6.3.2 Performance evaluation of butanol as potential fuel blend with diesel in a single cylinder 4- stroke diesel internal combustion engine

Butanol as a potential alternate liquid transportation fuel has been assessed by investigating the performance of different butanol – diesel blends in a single cylinder 4-stroke diesel internal combustion engine. Pure diesel (D100) and the three butanol – diesel blends namely B5, B10 and B20 were checked for their engine performance in terms of brake power, fuel consumption, mechanical efficiency, exhaust gas composition, brake thermal efficiency, air to fuel ratio and brake specific fuel consumption.

An improvement in the quality of exhaust gas was marked in terms of reduction of CO, CO_2, hydrocarbon and NOx for all butanol-diesel blends when compared to pure diesel (Figure 6.3). With the increase in engine load (%), CO emission was found to decrease with concomitant increase in CO_2 emission for both blends and pure diesel. CO is produced as a result of incomplete combustion of fuel inside the engine. Increasing engine load upregulates combustion efficiency of the engine resulting in enhanced complete combustion of fuel and its simultaneous conversion into CO_2 (Figure 6.3B). Similar pattern of CO emission was reported by Rakopoulous et al., (2010), where concentration of CO in the emission was lowered for butanol–diesel blend as compared to neat diesel. The exhaust gas emissions cumulatively can be labeled as soot emitted by the engine based on their physicochemical attributes. The CO emissions qualitatively followed a similar pattern to the soot concentration from engine exhaust as reported by Rakopoulous et al., (2010). NO_x emission was found to decrease gradually with the increase in butanol percentage in the blend (Figure 6.3D). The lowered NO_x emissions can be attributed to the operation of the engine under a leaner fuel blend due to the lower calorific value and higher heat of evaporation of butanol when blended with diesel (Rakopoulous et al., 2010). Reduction in hydrocarbon emission in case of butanol – diesel blend corroborates

with the findings of Gu et al., (2012), which elucidated that combustion of gasoline – butanol blends lowered hydrocarbon, NO_x and CO emissions in a SI engine. Pure butanol when blended with diesel has been shown to significantly reduce smoke opacity, soot content, hydrocarbon, CO and NO_x emissions (Dernotte et al, 2010). It was also reported that increasing butanol blending with diesel decreases the emission of greenhouse gases without negatively affecting engine performance (Wang et al., 2016). The lower values of smoke opacity in butanol-blended samples have been attributed to the presence of oxygen in the samples leading to lowered soot generation (He et al., 2017).

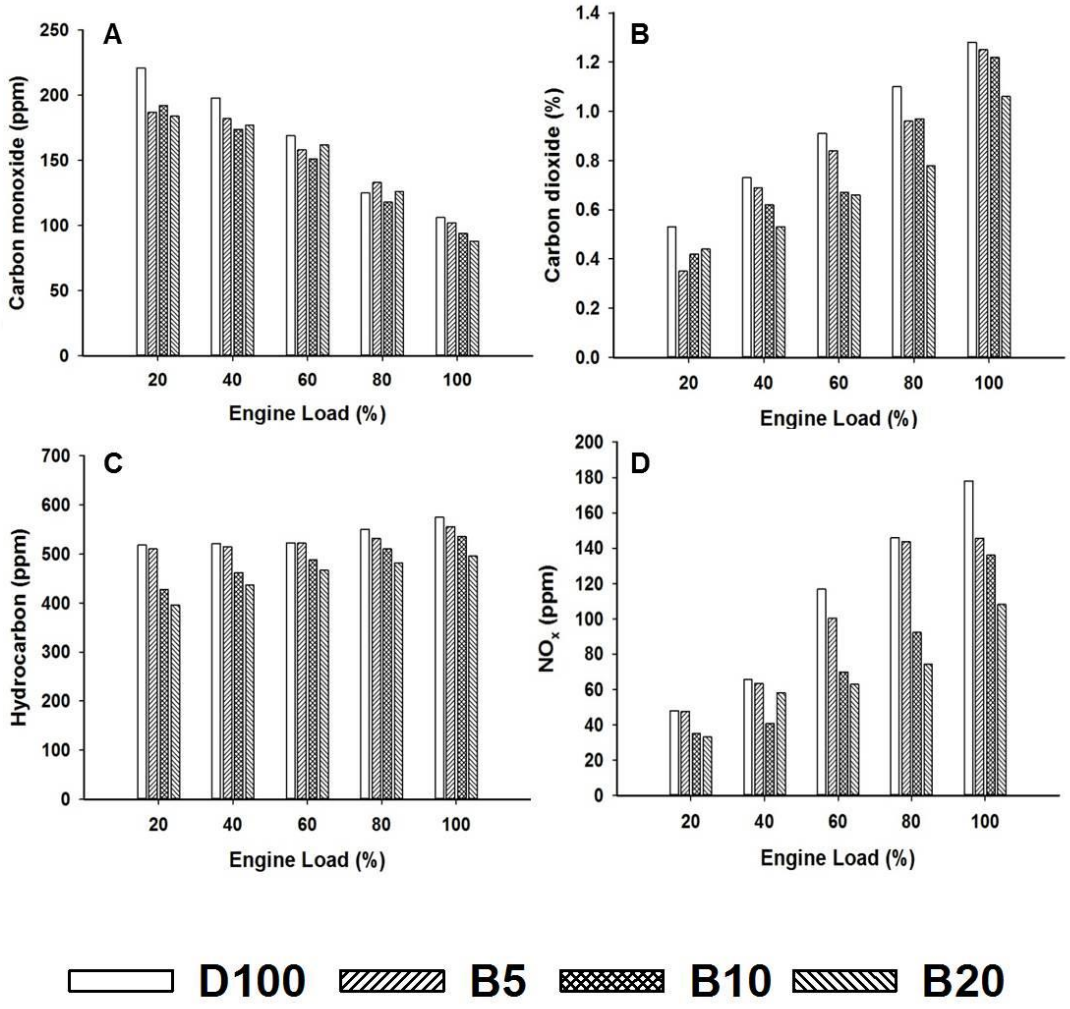


Figure 6.3 Comparison between (A) carbon monoxide (ppm), (B) carbon dioxide (%), (C) hydrocarbon (ppm) and (D) NO_x emissions from the IC engine when operated under five different load (%) conditions with D100, B5, B10 and B20.

Brake power and mechanical efficiency remained unchanged for pure diesel and blended samples (Figure 6.4A and E). This signifies that the engine was able to perform adequately under different butanol blends when compared to D100. A maximum of approximately 30% mechanical efficiency was achieved with all the samples under a 100 % engine load of 16 Kg. 35% (v/v) of butanol blending in diesel was reported to be mechanically efficient to run an IC engine at a maximal load capacity operating at 9000 rpm (Wang et al., 2016). For all the blended samples, fuel consumption was found to be marginally lower at lower engine load (up to 60%) when compared to D100 (Figure 6.4B). However, fuel consumption was similar for all blended fuels and pure diesel at higher engine load (Figure 6.4B). Figure 6.4C demonstrates the comparative brake specific fuel consumption for D100, B5, B10 and B20. Brake specific fuel consumption was observed to be lowered with increase in engine load which is directly proportional to the fuel blend mass flow rate. However, it is imperative to note that the consumption pattern was similar in case of the entire fuel blends compared to pure diesel (D100). It is observed that for all the butanol - diesel fuel blends, the brake thermal efficiency is slightly higher than that for the corresponding neat diesel, with the increase being higher with increasing percentage of butanol in the blend (Figure 6.4D). Increase in brake thermal efficiency of the engine under B5, B10 and B20 as compared to D100 can be testified to the lower cetane number of the blends as compared to pure diesel, which, in turn leads internal combustion of the engine at constant fuel volumes with negligible heat loss due to exhaust gas temperatures (Rakopolous et al., 2010) (Figure 6.4D). Air to fuel ratio was observed to decrease with increment in engine load for all the fuel samples including butanol – diesel blends as well as neat diesel (Figure 6.4F). At lower load capacity, the air to fuel ratio of B5, B10 and B20 were observed to be slightly higher till 40% of engine load capacity. The gradual decrease of air to fuel ratio with increase in engine load capacity is attributed to enhanced

fuel mass flow rates at higher load conditions with the air intake in the system being continually unchanged (Ibrahim Al-Hasan et al., 2008). With increase in butanol concentration in diesel, the air to fuel ratio was also observed to slightly reduce owing to the significant higher heat of vaporization of butanol as compare to diesel (Ibrahim Al-Hasan et al., 2008). Chen et al., (2013) similarly reported gradual decrease in the air to fuel ratio with increasing butanol concentration in diesel owing to the lower energy density of butanol as compared to neat diesel.

Enhanced brake thermal efficiency under butanol – diesel blends ensures suitability of butanol (upto 20% blending with pure diesel) as a promising and potential alternate transportation fuel blend, as it increases the overall engine performance and efficiency with negligible change in key engine performance parameters such as brake power and mechanical efficiency with reduced soot emissions from the engine exhaust.

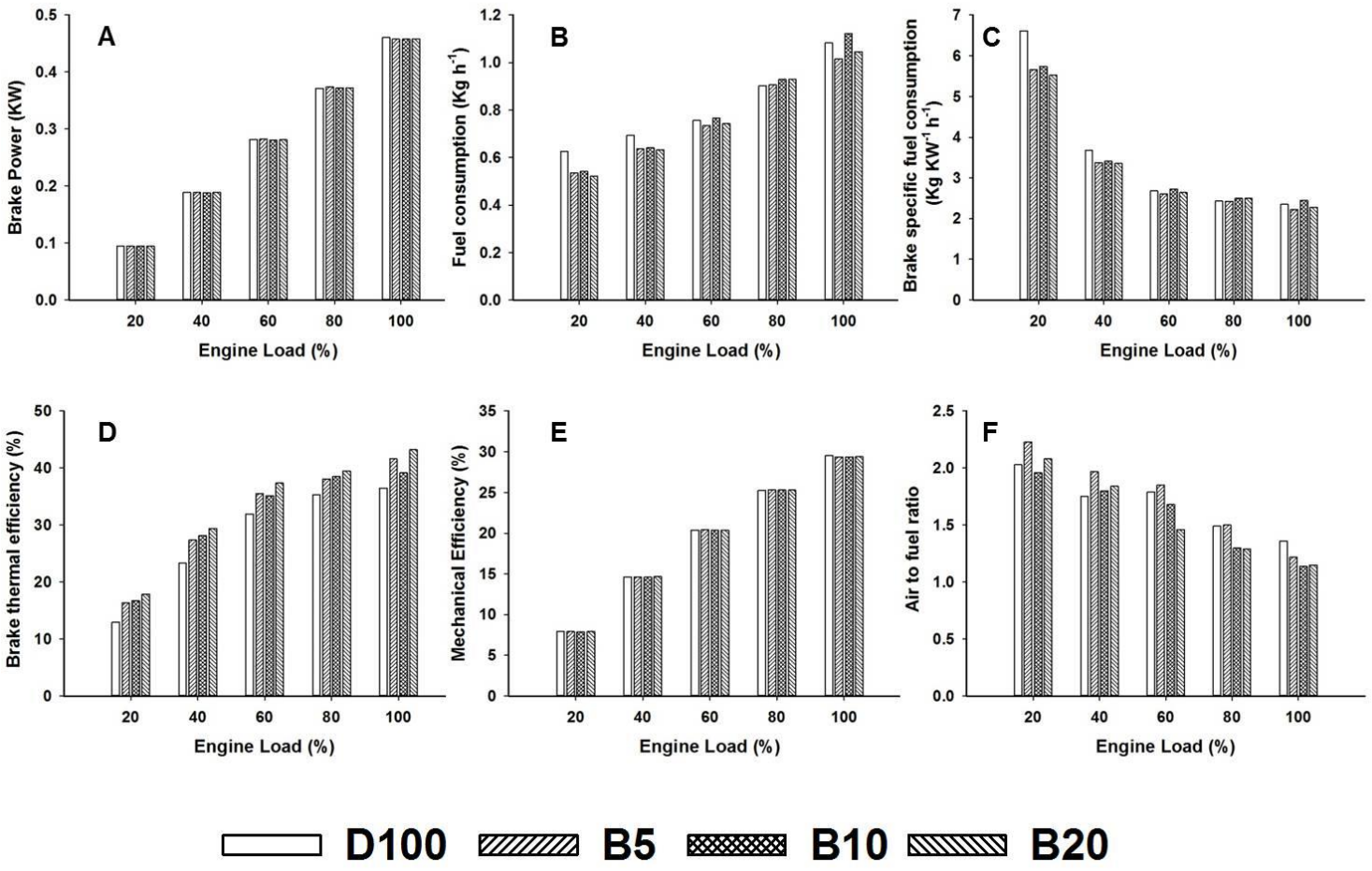


Figure 6.4 Comparison between (A) brake power (KW), (B) fuel consumption (Kg h^{-1}), (C) brake specific fuel consumption (Kg Kw^{-1} s^{-1}), (D) brake thermal efficiency (%), (E) mechanical efficiency (%) and (F) air to fuel ratio of IC engine when operated with D100, B5, B10 and B20 under different sets of engine load (%).

6.3.3 Economic analysis of the bioprocess strategies developed for butanol biosynthesis

In order to evaluate and understand the effect of cheaper substrates on the cost of butanol production, a detailed economic analysis has been carried out. The quantity of substrates required to produce 1 L of butanol has been considered as the major parameter for this estimation. A novel process engineering strategy towards elevated butanol titer successfully demonstrated using laboratory grade chemicals such as peptone and glucose, which resulted in a butanol titer of 19 g L^{-1}. Studies have reported that a titer in excess of 19 g L^{-1} is pre-requisite for industrial scale and commercial realization (Luo et al., 2018). However, it has been enumerated that, 70% of the total butanol production cost is attributed to the substrates. The use of expensive and orthodox carbon sources for the production of biobutanol has proven to be one of the key limiting factors towards its commercial establishment. In this study, use of low cost and commercially available industrial grade substrates resulted in a price of 0.8 USD for every litre of butanol (0.97 USD Kg^{-1} of butanol) (Table 6.5). The obtained cost analysis highlights significant reduction of 99.5 % in butanol cost as achieved by using laboratory grade chemicals, which resulted in a price of 126.16 USD per litre of butanol (152.65 USD Kg^{-1} of butanol) (Table 6.6 and Figure 6.5). Qureshi and Blaschek (2001) had proposed the cost of 1 Kg butanol in a grass rooted plant to be around USD 0.73, which might increase to a higher forecast of USD 1.07 depending upon the price fluctuations of corn. Qureshi et al., (2013) further illustrates a grass-rooted plant for the conversion of wheat to butanol is estimated at USD 1.3 per Kg of butanol. Starch has been reported to be favorable for butanol production, due to the amylolytic activity of *Clostridium* sp. (Jesse et al., 2002; Campos et al., 2002). Clostridial strains are capable of fermenting a wide spectrum of substrates as they are uniquely versatile owing to the multiple enzymes present which can easily break complex carbon molecules into simple

sugars (Qureshi et al., 2012). However, the market demand of feedstock is comparatively high, which make them infeasible for butanol production. Kaushal et al., (2019) reported a sustainable butanol production platform *C. sporogenes* NCIM 2918 utilizing lignocellulosic hydrolysate and crude glycerol for efficient total alcohol production. However, use of lignocellulosic biomass requires cumbersome pre-treatment processes which make the overall bioprocess unrealistic on larger scale.

The current study highlights economic feasibility and the potential realization of the two-stage bioprocess for butanol production as a potential replacement of transportation fuel using low cost substrates CSL and amylase treated starch.

Table 6.5 Cost analysis for 1 L butanol produced using low cost media engineering strategy

Specification		Amount	Unit	Reference
Total Culture Volume		50	L	Experimental Data
Maximum Butanol Titer		16.5	g L^{-1}	Experimental Data
Total Butanol Produced		825	g	
	Or	1.018518519	L	Density of Butanol =810 kg/m^3
	Or	1018.518519	mL	
Batch Time		48	h	Experimental Data
Mixing/Agitation		200	rpm	Experimental Data
Temperature		37	°C	Experimental Data
Cost incurred for fermentation				
Water pumping / Circulation		0.864	KWh or unit	18 w circulation pump used
Aeration		Nil		NA
Agitation		0.05	KW	https://checalc.com/solved/agitator.html
Electrical energy incurred for agitation		2.4	KWh or unit	
Rate of electricity		6.65	INR/unit	https://www.bijlibachao.com/news/domestic-electricity-lt-tariff-slabs-and-rates-for-all-states-in-india-in.html
Cost of mixing		15.96	INR	
Cost of water pumping		5.7456	INR	
Raw Material cost		34.18	INR	Table 6.2

A. Total cost for Fermentation		**55.88**	**INR**	
		Cost incurred for distillation		
Volume of distillation		50	L	
Time required for distillation		30	mins	
	Or	0.5	h	
Electrical energy Incurred for Heating		0.225	KWh or unit	Heater of 0.48 KW used
Cost of Heating		1.49625	INR	
B. Total cost during distillation		**1.50**	**INR**	
Grand Total (A+B)		**57.38**	**INR**	
	Or	0.80	USD	1 INR = 0.014 USD
	Or	0.97	USD Kg^{-1} of butanol	

Table 6.6 Cost analysis for 1 L butanol produced laboratory grade substrates

Specification	Amount	Unit	Reference
Total Culture Volume	42.5	L	Experimental Data
Maximum Butanol Titer	19.12	g L^{-1}	Experimental Data
Total Butanol Produced	812.6	g	
Or	1.003209877	L	Density of Butanol =810 kg/m^3
Or	1003.209877	mL	
Batch Time	48	h	Experimental Data
Mixing/Agitation	200	rpm	Experimental Data
Temperature	37	℃	Experimental Data
Cost incurred for fermentation			
Water pumping / Circulation	0.864	KWh or unit	18 w circulation pump used
Aeration	Nil		NA
Agitation	0.05	KW	https://checalc.com/solved/agitator.html
Electrical energy incurred for agitation	2.4	KWh or unit	
Rate of electricity	6.65	INR/unit	https://www.bijlibachao.com/news/domestic-electricity-lt-tariff-slabs-and-rates-for-all-states-in-india-in.html
Cost of mixing	15.96	INR	
Cost of water pumping	5.7456	INR	

Raw Material cost		8988.40	INR	Table 6.2
A. Total cost during Fermentation		**9010.11**	**INR**	
		Cost incurred for distillation		
Volume of distillation		50	L	
Time required for distillation		30	mins	
	Or	0.5	h	
Electrical energy Incurred for Heating		0.225	KWh or unit	Heater of 0.48 KW used
Cost of Heating		1.49625	INR	
B. Total cost during distillation		**1.50**	**INR**	
Grand Total (A+B)		**9011.6**	**INR**	
	Or	126.16	USD	1 INR = 0.014 USD
	Or	152.65	USD Kg^{-1} of butanol	

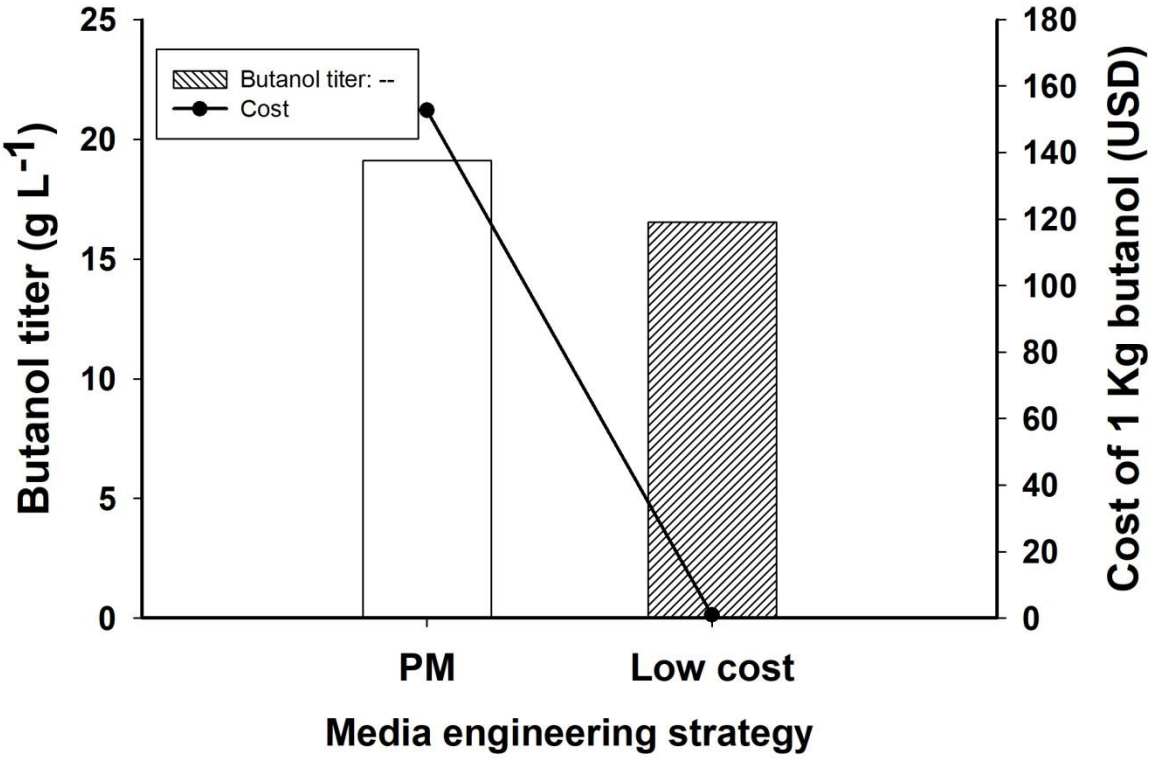


Figure 6.5 Comparison of cost required to produce 1 Kg of butanol (USD). PM stands for medium composed of peptone and glucose whereas low cost signifies medium supplemented with CSL and starch.

6.4 Conclusion

- ✓ Estimation of physicochemical properties revealed reduced kinematic viscosity, absolute viscosity and density in diesel – butanol blends as compared to D100.
- ✓ Flash and fire points reduced in the range of 35 – 40 % in case of blended samples compared to D100, signifying better engine combustibility.
- ✓ Similar pour and cloud points were estimated for blended samples compared to D100.
- ✓ Engine performance was evaluated which showcased significant reduction in opacity signaling lower emissions of harmful gases such as CO, HC and NO_x.
- ✓ B5, B10and B20 had suitable properties towards engine operation for its possible operation as transportation fuel alternate.
- ✓ Low cost medium strategy revealed a reduction of 99% in butanol production cost where 1 Kg of butanol is estimated to cost around USD 0.97 as compared to 152.65 USD Kg^{-1} for production medium with peptone and glucose.

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Chapter 7

CONCLUSIONS

"The future is green energy, sustainability, renewable energy."

Arnold Schwarzenegger, Actor and Politician

CHAPTER 7

Conclusions

Butanol biosynthesis is maligned by several bottlenecks such as low titer and yield, high substrate cost, lack of knowledge about intracellular regulations and cumbersome process technologies have hindered its industrialization. With the overall objective of developing and demonstrating a sustainable bioprocess strategy for butanol production, the present study was executed with *C. acetobutylicum* ATCC 824 as the biological platform. The strain was characterized for its growth and butanol production on a wide variety of carbon and nitrogen sources, wherein, glucose and peptone yielded the highest butanol titer (11.6 g L^{-1} and 11.68 g L^{-1}). Glucose and peptone were found to produce the highest butanol titer as it has been reported widely, since glucose and peptone help in the nourishment and metabolism of the cell. Following, statistical optimization was performed to optimize the initial concentrations of the substrates with the objective function of maximization of butanol titer, which resulted in a minimal improvement in the butanol production. It was hypothesized that the butanol production was hindered due to the solvent toxicity. To that end, the strain was further characterized under different stress conditions of metal ion starvation / supplementation. Zinc supplementation and magnesium starvation resulted in an improvement of 31% and 14.7% in butanol response. Zn supplementation was found to improve butanol performance of the strain through modulating key enzyme activities. The micronutrient Zn plays significant role towards ABE fermentation. Zn has been previously reported as well as inferred in the present study to enhance glucose utilization and earlier onset of solventogenesis. Transcriptional

analysis reveals that under zinc supplemented medium glucose specific PTS and glucose transporting genes were significantly upregulated. Further, multiple genes pertaining to glycolysis, acids reassimilation and butanol production were upregulated, which positively impacted butanol response. Significant higher ATP generation was also inferred due to improved glycolysis and earlier initiation of solventogenesis which results in redirection of carbon flux towards butanol production pathway. Combinatorial effect of zinc supplementation in magnesium starved optimized medium resulted in the highest butanol of 19.12 g L^{-1} which is the highest reported for this wild type strain. The efforts were then concentrated towards elucidating the role of zinc and magnesium towards elevated butanol response and changes in solvent formation pattern. Temporal profiling of important enzyme activities revealed upregulation in glycolytic enzyme activity, thiolase upregulation and butanol metabolism which corroborated well with the phenotypic observations of elevated glucose uptake, rapid acid re-assimilation and improved butanol production in case of metal ion modulated medium.

With the proposed novel media strategy, further bioprocess was demonstrated via coupling intermittent feeding of glucose and zinc and *in-situ product* recovery which resulted in an improved cumulative butanol titer of 54.2 g L^{-1} with average productivity of 0.66 g L^{-1} h^{-1}. In order to eliminate the expensive substrates, low cost and industrial grade chemicals such as corn steep liquor and industrial grade starch were used as nitrogen and carbon source. To that end, a novel low cost medium engineering strategy was developed using CSL and amylase treated starch coupled with zinc supplementation and magnesium starvation resulting in an improved butanol titer of 16.54 g L^{-1}.

Biobutanol as a potential transportation fuel was also investigated through qualitative analysis of its physicochemical properties and engine performance. Lowered kinematic viscosity of butanol and diesel blends point towards better engine function

whereas, enhanced brake thermal efficiency of the blends in comparison to neat diesel strengthens its potent use as an alternate to petroleum. Butanol blending with diesel provides for a leaner and cleaner vehicular fuel option which can be industrialized and marketed as it does not require any modifications in the existing engine configuration. Finally, the bioprocess strategies were economically assessed and cost analysis reveals that there was a significant reduction of 99% in cost of 1 L butanol when grown using low cost substrates as compared to laboratory grade chemicals with minimal alterations in capital expenditure.

The present study highlights and answers key solutions to some of the major bottlenecks via (i) designing novel medium strategies towards improved butanol titer, yield and productivity, (ii) alleviating solvent toxicity through metal ion influence, (iii) predicting experimentally possible intracellular regulations owing to metal ion supplementation / starvation and (iv) developing economically feasible bioprocess through using cheaper substrates for butanol production.

Engineering significance

Butanol biosynthesis, though in practice for over more than 100 years, has not met with significant industrial success and is hindered by several bottlenecks. The current study ensued hopes to be a step in the forward direction with the main objective of overcoming the bottlenecks, rendering the overall strategy industrially relevant and lucrative.

The major technological advances as proposed are:

- Significant improvement in butanol titer and productivity through design of a novel medium engineering rationale.
- Elucidating the possible effects of zinc supplementation / magnesium starvation or the combination of both on improvement in butanol production through biochemical assays. Differential activities of the specific enzymes help in decoding the major regulators responsible for central carbon metabolism and butanol production, in view, addressing the void in unknown intracellular regulations.
- Demonstration of the strain's potential of growth and solvent production on low cost substrates, highlight the significance and industrial relevance of the approach undertaken via mitigating the expensive and conventionally used substrates for butanol fermentation.
- The assessment of the fermented butanol in blends with diesel provide enough traction to the claim that butanol can be a potential alternate to the existing petroleum fuels in the automobile sector. Use of butanol blends would help in reduction of the global carbon footprint and GHG in the atmosphere, thus improving the environmental health and overall living conditions.

Future Scope of the work

- ✓ Scale up of the novel low cost medium engineering strategy to assess commercial scale butanol production with product recovery.
- ✓ Techno-economic feasibility of the butanol biosynthetic process.
- ✓ Demonstration of the medium engineering strategy with novel microbe – substrate platforms for sustainable butanol production
- ✓ Transcriptional analysis to further probe the combinatorial effect of Mg starvation and zinc supplementation in media.
- ✓ Assessment of proposed media regulation on newer and novel substrate – microbe combinations for butanol production.

List of Publications

Patents filed at Indian Patent Office:

1. **Mayurketan Mukherjee**, Saumya Ahlawat, Mehak Kaushal, Gargi Goswami, Debasish Das. Improved culture media for butanol synthesis using *Clostridium acetobutylicum* ATCC 824. (Application No. 201731028507, filed on August 10th 2017; published online).
2. **Mayurketan Mukherjee**, Gargi Goswami, Debasish Das. Media and process for biosynthesis of butanol (Application No. 201931005755, filed on 13th February 2019)

Publications from thesis work:

1. **Mukherjee, M.**, Sarkar, P., Goswami, G., & Das, D. (2019). Regulation of butanol biosynthesis in *Clostridium acetobutylicum* ATCC 824 under the influence of zinc supplementation and magnesium starvation. Enzyme and Microbial Technology, 129, 10352. doi.org/10.1016/j.enzmictec.2019.05.009. (Impact factor: 3.553)
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Publications from collaborative work:

1. Sarkar, P., **Mukherjee, M.**, Goswami, G., & Das, D. (2019). Adaptive Laboratory Evolution induced novel mutations in *Zymomonas mobilis* ATCC ZW658: A potential platform for co-utilization of glucose and xylose. (Accepted for publication in Journal of Industrial Microbiology & Biotechnology) (Equal author contribution)
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List of Conferences / Workshop

List of conferences

1. **Mayurketan Mukherjee**, Payel Sarkar, Gargi Goswami and Debasish Das. Development of a cost effective media engineering strategy for improved butanol production. DBT National Workshop on Bioenergy-2018, 6-7th July 2018, held at Indian Institute of Technology, Roorkee, India
2. **Mayurketan Mukherjee**, Anwesha Purkayastha, Saumya Ahlawat, Mehak Kaushal, Gargi Goswami and Debasish Das**.** Novel medium engineering strategy directed towards enhancing butanol production from *Clostridium acetobutylicum* ATCC 824. Bioprocessing INDIA 2017, 9-11th December, held at Indian Institute of Technology,Guwahati, India. **(Secured Best Poster)**
3. **Mayurketan Mukherjee**, Anwesha Purkayastha, Saumya Ahlawat, Mehak Kaushal and Debasish Das**.** Investigating the effects of organometallic ions on *Clostridium acetobutylicum* ATCC 824 metabolism. Research Conclave 2017, held Indian Institute of Technology, Guwahati.
4. **Mayurketan Mukherjee**, Anwesha Purkayastha, Saumya Ahlawat, Mehak Kaushal and Debasish Das. Effect of metallic ions on butanol production from *Clostridium acetobutylicum* ATCC 824. 57th International Annual Conference of The Association of Microbiologists of India, held at Guwahati University, Guwahati, India.
5. **Mayurketan Mukherjee**, Anwesha Purakayastha, Saumya Ahlawat, Mehak Kaushal, Basavaraj Palabhanvi and Debasish Das. 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', held at Indian Institute Of Technology Bombay, Mumbai.

List of workshop attended

1. Attended the 2nd Instructional School of National Network on Mathematics and Computational Biology held at IISc Bangalore from 22nd May 2016 to 1st June 2016.

Vitae

The author was born in a Bengali household on 11th November, 1989 in Kolkata, West Bengal, India. He successfully completed his school education in the year 2008, appearing the higher secondary examination from M. P. Birla Foundation H. S. School under Council for the Indian School Certificate Examinations (CISCE). Academically, he completed his B. Tech and M. Tech from West Bengal University of Technology in the year 2012 and 2014 respectively.

Mayurketan Mukherjee joined the Ph.D. program in Bioprocess Development Lab under the supervision of Prof. Debasish Das at Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati in July, 2014. He successfully completed all the academic courses, examinations and presented his Synopsis seminar on 11th December, 2019 before the Doctoral Committee.