Improving Bio-Butanol Production from Lignocellulosic Feedstock by Tailoring Metabolic Perturbations

by

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Abstract

The objective of this study is to enhance bio-butanol production using lignocellulosic feedstock via supplements of metabolism perturbation. Metabolic perturbations are non-substrate-based chemical additives that can reinforce metabolic flux towards butanol formation, or increase tolerance to microbial inhibitors in the feedstock. Typical metabolic perturbations include $CaCO_3$, $ZnSO_4$, methyl red, and furan derivatives such as furfural and hydroxymethylfurfural (HMF). In this study, we stepwise tailored metabolic perturbations to maximize butanol production from pure sugar and lignocellulosic feedstock. Under optimized conditions of 4 g/L $CaCO_3$, 2 mg/L $ZnSO_4$, butanol production exceeded 10g/L in wheat straw hydrolysate, which was significantly higher than that obtained in the absent of $ZnSO_4$ and $CaCO_3$. As compared to traditional lignocellulosic feedstock post-treatment method, metabolic perturbations method shows advantages in terms of productivity and economics. Improved bio-butanol production is related to the overexpression of NAD(P)H dependent genes.

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Chapter 1 Introduction

Concerns over greenhouse gas emissions and global warming have raised a demand for renewable source of energy. "Energy Information Administration" has projected a rapid annual growth rate of 4.0% in renewable energy from 2012 to 2020 (U.S. Energy Information Administration, 2016), with production of transportation renewable energy biofuel to be doubled by 2040, reaching 5 million barrels per day. Biofuels, such as bio-ethanol, has been commercialized for years blended with gasoline in the USA (E10, 10% blending ratio by volume), Canada (E5, 5% blending ratio) and Brazil (E20-25, 20-25% blending ratio). Recently gas stations in Houston, USA, started to sell a fuel that is 12.5% blend of bio-butanol and gasoline (Gevo, 2017).

As compared to two-carbon alcohol ethanol, four-carbon butanol holds ~30% higher energy density. Moreover, stoichiometric air-to-fuel ratio of butanol is 11.1, which is higher than ethanol (has ratio of 9.0). Higher stoichiometric air-to-fuel ratio allows high butanol blend volume with gasoline (up to 16%v/v) without modifying the motor engine. Consequently, an engine fueled with butanol/gasoline would work much longer than it would with ethanol/gasoline. This advantage of butanol is amplified when it is used as aviation fuel. Wang et al. (2016) proposed butanol as a blend stock for commercial jet fuel. In December 2014, Naval Air System Command of US Navy announced its first successful supersonic flight fueled by renewable butanol blend produced by Gevo company. Later in November 2016, Alaska Airlines utilized 20% blend of butanol in the fuel in its flight from Seattle to Washington (Frangoul, 2016).

Most of conventional biofuels are produced using edible food as feedstock such as corn and sugarcanes. The excessive farming of corn and sugarcanes has caused local deforestation, soil fertility loss, and possible community's dislocation. Moreover, studies showed that the nitrogen element in fertilizers applied in cornfields is partially released in the form of N_2O and contributed to greenhouse emission (Wang et al., 1997).

These problems such as excessive farming and greenhouse gas emission in fertilizers raised concerns of the sustainability of conventional biofuels, which accelerated the use of new-generation biofuel. As

compared to conventional biofuels, new generation of biofuels use lignocellulosic feedstock, mainly agriculture residues, woody residues and municipal residues as raw materials (Wang et al., 2012). By using lignocellulosic waste instead of edible food as a material, less greenhouse gas is generated in the lifecycle. New generation biofuels were standardized clearly by USA government in Renewable Fuel Standard based on greenhouse gas emission reduction. Two type of new generation biofuels, advanced biofuel and cellulosic biofuel, must have emissions that are at least a 50% and 60% reduction respectively as compared to gasoline or diesel fuel that would be used in its place (Table 1.1). Actual used amount of cellulosic biofuel and advanced biofuel in 2015 increased by 4-folds and 8% respectively once compared to 2014, according to the newest announcement (U.S. Environmental Protection Agency, 2015). Therefore, an increasing usage of the new generation biofuels and reduction in greenhouse gas emissions due to these biofuels use could be expected.

Table 1.1 Specifications of new generation biofuel and the sale volumes in 2014 and 2015

Code	Biofuel Categories	GHG reduction	Sales Volume in 2014	Sales Volume in 2015
D6	Conventional biofuel	20%	-	-
D5	Advanced biofuel	50%	2.67b.g.	2.88b.g.
D3	Cellulosic biofuel	60%	33m.g.	123m.g.
D4	Biomass-derived diesel	50%	-	-
D7	Cellulosic diesel	60%	-	-

*b.g. : billion gallons m.g.: million gallons

However, development bottleneck of advanced biofuels, especially bio-butanol, is the high selling price of biobutanol ($3~3.5/gallon), which is uncompetitive to $1.5/gallon of conventional bio-ethanol (Quality, 2016). The spending on lignocellulosic feedstock is high, which includes cost of collection,

transportation to the gate of factory and storage in the factory (Kenney et al., 2014). Based on the 2012 model by National Renewable Energy Lab (Humbird et al., 2011), the manufacturing cost and handling cost of corn stove accounts for one third of the minimal selling price of biofuel. Unfortunately, the costs are barely deducible.

Therefore, improving efficiency of production with lignocellulosic feedstock became the prior feasible way to lower the overall cost of production. In addition to improving feedstock, lots of progress have been reported with utilizing pure sugars in the feedstock. This includes modifying genetic structure of producing strain (Papoutsakis, 2008) and developing affordable downstream separation processes to separate high concentration of butanol liquid from fermentation broth (Xue et al., 2012). However, these technologies have been severely limited when lignocellulosic feedstocks are used. Lignocellulosic feedstocks contain a large percentage of pentose, like xylose, which is not preferable for fermentation, and high levels of toxic substances that are detrimental to fermentative strains. These drawbacks of lignocellulosic feedstock have necessitated unconventional methods to mitigate inhibitory effects and improve xylose utilization.

Researchers are using an unconventional method of supplying metabolic perturbations in butanol-producing microorganisms to increase sugar utilization, cell growth, and butanol production. Metabolic perturbations mainly involve non-substrate-based supplements to the feedstock that may reinforce metabolic flux towards butanol formation, or increase tolerance to microbial inhibitors in the feedstock that impair cell growth and product formation (Ujor et al., 2016). Metabolic perturbations mainly include metallic compound ($CaCO_3$, $ZnSO_4$) and electron carriers/acceptors (furfural, HMF, methyl red). In this study, we stepwise tailored the use of metabolic perturbations to maximize butanol production using pure sugar and lignocellulosic feedstock. In preliminary investigations, we evaluated the impacts of $CaCO_3$, furfural and methyl red on cell growth, sugar utilization, acid production and butanol production in glucose and xylose feedstock, separately. Following the preliminary investigation, under the supplement of 4 g/L $CaCO_3$, we optimized the concentrations of furan derivatives (75% furfural and 25% HMF) and $ZnSO_4$ for maximal butanol production from glucose and xylose feedstocks respectively. Fixed ratio of 3:1 furfural and HMF concentration was mimicking the furan derivative composition in wheat straw hydrolysate. A

final experiment of butanol production was concluded using lignocellulosic feedstock hydrolysate normally containing 0.5~1.5 g/L furan derivatives under optimized conditions of 2 mg/L $ZnSO_4$ and 4 g/L $CaCO_3$.

Chapter 2 Literature Review

2.1 ABE Fermentation

Bio-butanol is mainly produced via acetone-butanol-ethanol (ABE) fermentation. ABE fermentation was first performed by French scientist Louis Pasteur in 1861. During prohibition in the United States in 1920s, there was a high demand of amyl alcohol, the precursor of amyl acetate, by the automobile industry (Dürre, 2007). Butanol was found to be a perfect alternative for amyl alcohol. Therefore, many ABE fermentation plants were opened. Eventually ABE fermentation process became the second largest biotechnological process in the world, and the plants contributed to two thirds of global butanol production in 1950. Since the robust development of petroleum industry, petroleum butanol production has led to a screeching halt of ABE fermentation industry. Nowadays, with complex global circumstances such as oil price fluctuation, global warming, and expediting biotechnologies, researchers and investors are revisiting bio-butanol production.

To run a ABE fermentation, microorganism like *clostridium acetobutylicum* and *clostridium beijerinckii* are typically used (Tomas et al., 2003). *Clostridium acetobutylicum* and *clostridium beijerinckii* are gram positive, rod shaped, mesophilic bacterium. Under strictly anaerobic conditions, these strains could use a wide range of substrates including pentoses, hexoses and starch to eventually produce a mixture solvent of acetone butanol and ethanol, with a typical ratio of 3:6:1 by mass concentration.

ABE fermentation is known as one of most complicated fermentations, not only because of multiples products like acetone, butanol, ethanol and gases including carbon dioxides and hydrogen gas, but also due to various metabolic stages in the metabolic pathways. Figure 2.1 is metabolism pathway of a typical ABE fermentation by clostridium. Principally, metabolic pathway of ABE fermentation could be divided into three stages: glycolysis, acidogenesis and solventogensis.

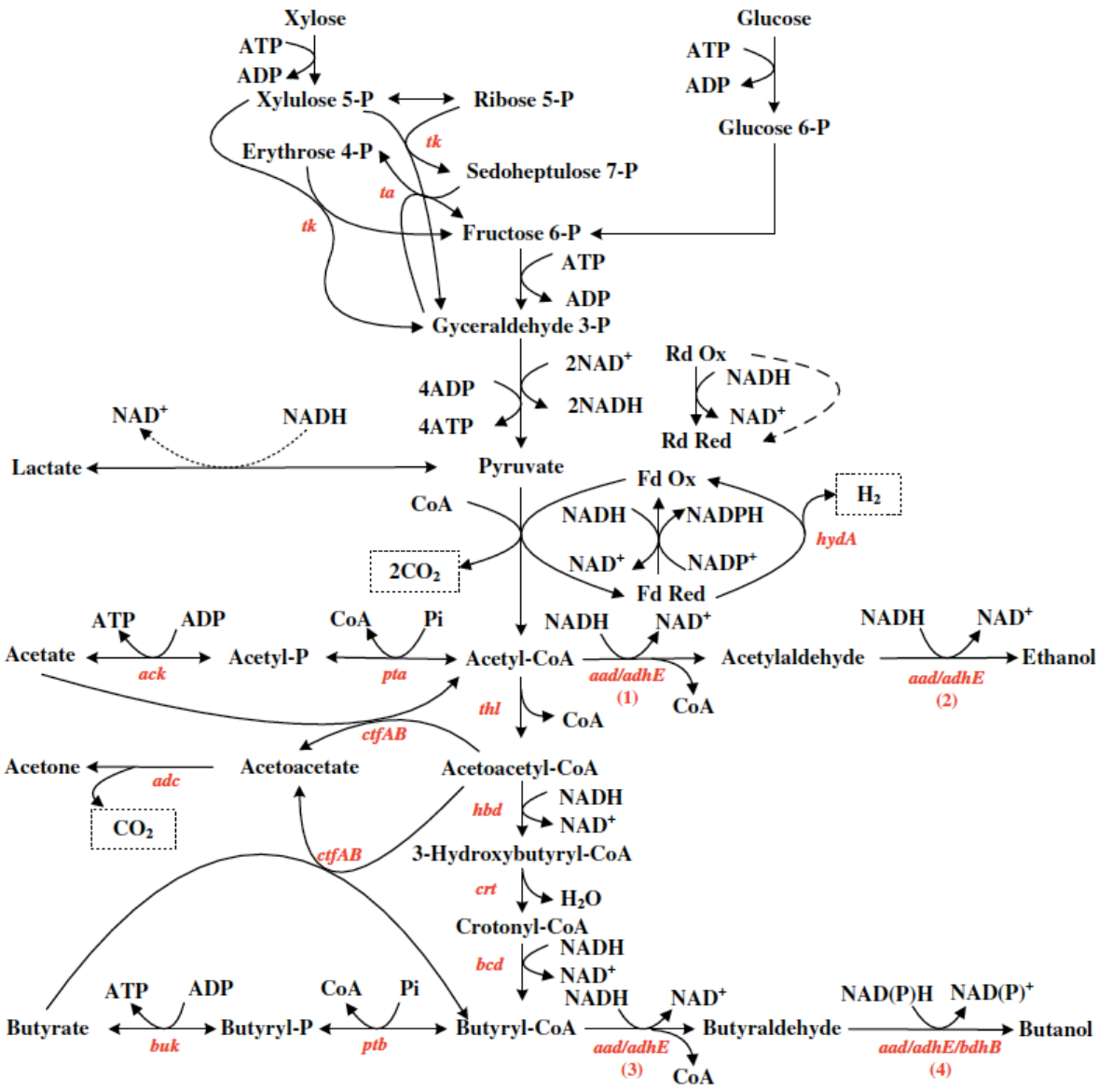


Figure 2.1 Metabolic pathways of ABE Fermentation in glucose and xylose feedstocks

tk, transketolase; ta, transaldolase; hydA, hydrogenase; pta, phosphotransacetylase; ack, acetate kinase; thl, thiolase; adc, acetoacetate decarboxylase; ctfAB, acetoacetyl-CoA: acetate/butyrate:CoA-transferase; hbd, 3-hydroxybutyryl-CoA dehydrogenase; crt, crotonase; bcd, butyryl-CoA dehydrogenase; buk, butyrate kinase; ptb, phosphotransbutyrylase; 1, acetaldehyde dehydrogenase; 2, ethanol dehydrogenase; 3, butryraldehyde dehydrogenase; 4, butanol dehydrogenase. (Zheng et al., 2015)

During the process of glycolysis, monosaccharides are consumed by clostridium. Most common monosaccharides are glucose and xylose. During glucose glycolysis, pyruvate, cofactor and ATP are generated following Embdene-Meyerhofe-Parnas (EMP) pathway (Jones and Woods, 1986). By contrast, xylose glycolysis basically follows Pentose-Phosphate Pathway (PPP) (Jeffries, 2006). One major difference between two glycolysis metabolic pathways are the cofactors: in EMP and PPP, cofactors nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are produced respectively. NADH and NADPH serve as reductive compounds of the catabolic pathways. After glycolysis, pyruvate is converted into acetyl-coenzyme A (acetyl- CoA), which is further degraded into acetate and butyrate with the assistance of key enzymes including phosphotransacetylase (pta)/acetate kinase (ack) and phosphotransbutyrylase (ptb)/butyrate kinase (buk). The stage of generating acid by consuming pyruvates, ATP, and cofactors, which have been generated in glycolysis, is called "acidogenesis". As transmitting phase of ABE fermentation, acidogenesis also plays significant roles in final solvent productions. Normally without any metabolic disturbers, accumulated butyrate and acetate would be further converted into final products, butanol and acetone. On the other hand, if metabolic disturber existed and excessive acid was accumulated, intracellular pH would be imbalanced, and eventually clostridium would fail to produce solvent. Therefore, pH control and inhibitors removal are often used in ABE fermentation if necessary (Lee et al., 2008). Following acidogenesis, acetate and butyrate are converted into final products butanol and acetone by butanol dehydrogenase (aad/aahE/bdhB) and acetoacetate decarboxylase (adc). This stage is called solventogensis. In solventogensis cofactors NAD(P)H are continuously consumed. All these stages are regulated by the expressions of corresponding enzymes/coenzymes, which have been partially studied through transcriptional analysis. These information could facilitate production improvement through metabolic regulation (Tashiro et al., 2013)(Papoutsakis, 2008).

2.2 Lignocellulosic Feedstocks for ABE Fermentation

Natural feedstocks containing sugars for ABE fermentation are mainly edible food like corn and sugarcane, and non-edible lignocellulosic feedstock including agricultural residues, woody residues and municipal residues. Agricultural and woody residuals are abundant in Canada and USA. There are 45~90 oven dry tons/year of agricultural residuals in Canada, consisting of energy content up to 1.44×10^{15} BTU/year (Wood et al., 2003). For woody residual, the study shows 370 million oven dry tons of woody biomass can be sustainably produced annually in USA accounting for 30% of the total biomass projected to be available for biofuel (Zhu et al., 2010). Among all the residuals, wheat straw, corncob and softwoods including pine and spruce are most studied due to their abundance in the natures.

Table 2.1 Compositions of representative lignocellulosic biomass (Zhu et al., 2010)

* Hemicellulose contributes to a part of glucan content in biomass

Lignocellulosic Biomass	Composition (%w)					
	Cellulose	Hemicellulose*				Lignin
	Glucan	Xylan	Mannan	Arabinan	Galactan	
Wheat straw	32.6	19.2	0.3	2.4	0.8	16.9
Corn stover	30.6	16.0	0.5	1.9	0.7	18.2
Switch grass	35.9	19.6	0.4	1.5	0.5	23.1
Spruce	43.2	5.7	11.5	1.4	2.7	28.3
Lodgepole pine	42.5	5.5	11.6	1.6	2.1	27.9
Loblolly pine	45.0	6.8	11.0	1.7	2.3	28.0
Red maple	42.0	9.3	7.4	2.4	1.8	29.0
Aspen	45.9	16.7	1.2	0.0	0.0	23.0

Table 2.1 are chemical components of typical lignocellulosic biomass for biofuel production. These lignocellulosic feedstocks are unambiguously composed of polymers cellulose, hemicellulose and lignin. Cellulose is a polysaccharide with the formula $(C_6H_{10}O_5)n$, consisting of a linear chain of thousands of β(1→4) linked D-glucose units (glucan) in chains. While cellulose is strong, resistant to hydrolysis, hemicellulose has amorphous structure with little strength. Hemicellulose is the branched hetero-polymer consisting of many sugar monomers like glucan, xylan, mannan, arabinan and galactan. Glucan and xylan are most abundant sugar monomers in biomasses, although softwoods contain more mannose than xylan (Table 2.1). At last, lignin is a complex framework that provides the biomass recalculation. Lignin doesn't contain any sugar monomer but phenolic compounds like p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol.

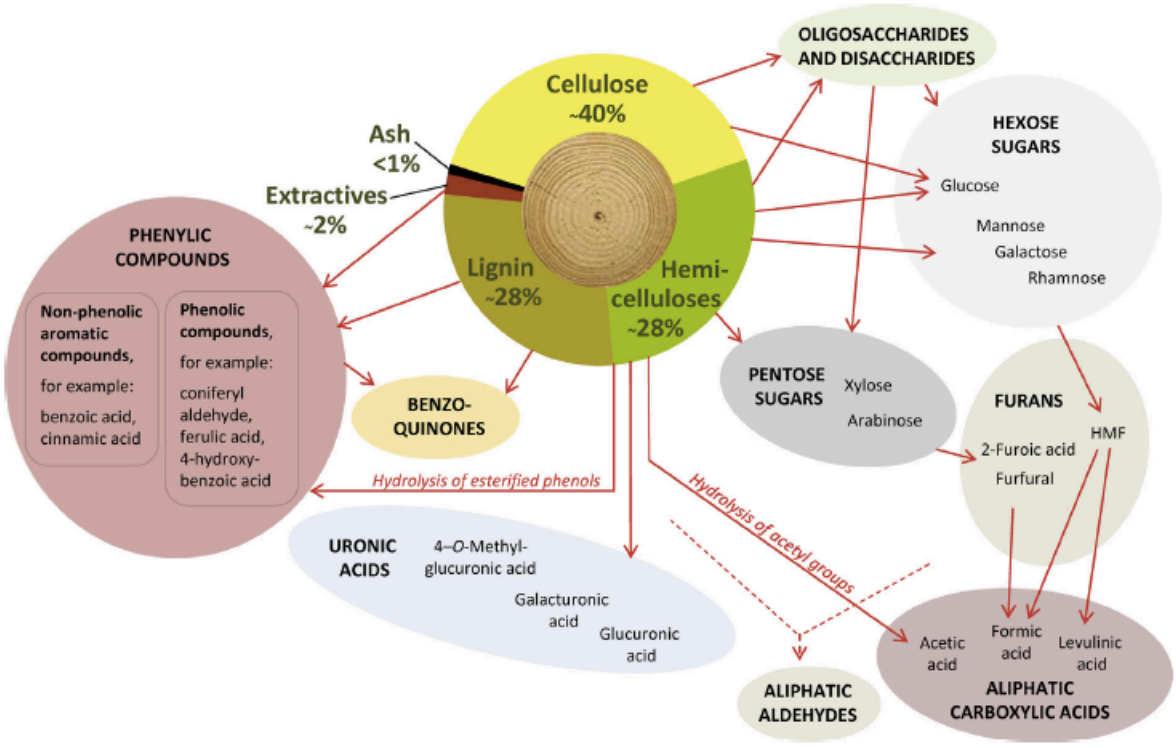


Figure 2.2 Degradation products from lignocellulose because of pretreatment under acidic conditions. Numbers indicate fractions of constituents of wood of Norway spruce (Jönsson and Martín, 2016)

Sugar monomers contained in lignocellulosic biomass are not fermentable for clostridium unless lignocellulosic biomass is pre-treated by acid, alkaline, oxidizer and heat. By doing pretreatments, sugar monomers would be released from decomposition of cellulose and hemicellulose. Beside sugars, a variety of side products are also generated during the pretreatments. Thermal treatment above 100ºC is necessary for the decomposition of cellulose and hemicellulose, at this temperature, monosaccharides are partially dehydrated, and converted into furan derivatives (Figure 2.2) (Guerra-Rodríguez et al., 2012): hexose like glucose and mannose are converted into 5-hydroxymethyl-2-furaldehyde (HMF), while pentose like xylose and arabinose are converted to furfural. These furan derivatives are further decomposed to aliphatic compounds like formic acid, and levulinic acid. At the same time, lignin which is consisted of phenolic molecules will release its phenolic monomers like pho-coumaric acid and ferulic acid. Contents of sugar and sides products were studied (Larsson et al., 1999 ;Qureshi et al., 2007 ;Jönsson et al., 2016).

2.3 Challanges of ABE Fermentation in Lignocellulosic Feedstock

In previous sections, through reviewing ABE fermentation metabolic pathways, we know pure sugars feedstocks like glucose and starch are suitable to supply clostridium for ABE fermentation. However, when lignocellulosic feedstock is used instead of pure sugar, ABE fermentation would be highly inefficient, which is due to unfavourable sugar and microbiological inhibitors in lignocellulosic feedstocks.

Glucose is long-established sugar for ABE fermentation (Formanek et al., 1997). Solventogenic ability of a clostridium is often measured in corresponding butanol production in glucose feedstock(Qureshi et al., 2007;Papoutsakis, 2008). In lignocellulosic feedstock, xylose is second abundant monosaccharide due to high xylan composition. However, xylose is not preferable sugar for clostridium. When xylose is used as feedstock, clostridium growth are weaker, and sugar utilization are lower than when glucose is used (Ezeji et.al., 2008; Xin et al., 2014). In feedstock of glucose and xylose mixture, xylose is only partially utilized whereas glucose is completed depleted (Kanouni et al., 1998). The phenomena which clostridium preferentially utilize glucose over xylose is due to carbon catabolite repression (Vinuselvi et al., 2012).

Another downside of xylose feedstock is related to butanol toxicity. The study showed butanol can inhibites membrane-bound ATPase enzyme activity, and disruptes the phospholipids of the cell membrane (Ezeji et al., 2010). Therefore, the maintaince of internal pH and electrochemical gradient is adversely impacted. When butanol concentration reaches 10 g/L in glucose feedstock, clostridium cells quickly decay and butanol production is halted partially or completely (Ellefson et al., 1985; Buehler et al., 2016). Clostridium is more affected by butanol toxicity to when xylose is used as feedstock: when butanol concentration is as low as 8 g/L, cell growth, sugar utilization and butanol production is severely compromised. This sensitivity of xylose fermentation was found to be related to lower tolerance of butanol by xylose permease than that of glucose permease (Petitdemange, 1985).

In summary, xylose feedstock has been the limiting factor for butanol production from ABE fermentation. Much work has been done to improve ABE fermentation in pure xylose feedstock. Gu et al., (2009) revealed that the insufficiency of transaldolase in the pentose phosphate pathway of *C. acetobutylicum* is the bottleneck for xylose metabolism. Through overexpressing the gene encoding transaldolase, xylose utilization and solvent production have been improved. Hu et al. (2011) illustrated that the over-expressions of sporulation regulator spo0A and aldehyde-alcohol dehydrogenase (adhEII) were the causes of xylose utilization enhancement. Butanol production in xylose fermentation is not highly improved until Xin's work (Xin et al., 2014). They successfully separated a strain *clostridium sp*. (BOH3) which could completely ferment 60g/L xylose to 14.9g/L butanol, as compared to 14.5g/L butanol produced from 60g/L glucose. It was found that 69% enhanced sugar consumption and 186% increased butanol production were relative to ultra-high activities of xylose-isomerase (0.97 U/mg protein) and xylulokinase (1.16 U/mg protein) compared to normal clostridium. These studies show sugar consumption and butanol production of xylose fermentation could be improved through over-expression of certain enzyme/coenzyme directly despite of complex metabolic pathways.

Except sugars, lignocellulosic feedstock after pretreatment contains various side products. Unfortunately, these side products like aldehydes, acids, and phenols are microbial inhibitors (Baral and Shah, 2014). Furan derivatives like furfural and HMF are most studied inhibitors in the feedstock. Furan

derivatives not only cause DNA damage, inhibition of glycolytic enzymes, and disruption of cell membranes (Almeida et al., 2007; Zhang et al., 2013), but also perturb redox balance: that is, furfural and HMF deplete cofactor NAD(P)H, which is the reducing power of catabolic metabolism. As a result of cofactor depletion, ABE fermentation encounters deceleration, solvent production loss, and cell death (Baral et al., 2014). For example, butanol productivity of *C. acetobutylicum* was 21% less when furfural (0.5g/L) was added, comparing to 89% less when furfural was increased to 3.0g/L (Zverlov et al., 2006). Other microbial inhibitors like phenolic compounds including coumaric acid, ferulic acid, vanilic acid (1g/L) inhibited cell growth by 64-74% (Cho et al., 2009) , and formic acid (1mM) lead to immediate collapse of ABE fermentation (Wang et al., 2011). Generally, these inhibitory effects are concentration-dependent.

Many studies have focused on limiting inhibitors generation during the pretreatment (Canettieri et al., 2007; Roberto et al., 2003). However, one could not limit the inhibitor to negligible level and getting enough sugar (~50g/L) in hydrolysate for ABE production. The activation energy of inhibitory substance generation is lower than that of sugar generation (Mosier et al., 2002). For instance, diluted sulfuric acid pretreatment, the most used pretreatment method for lignocellulosic feedstock, is most affordable method to recover sugars from lignocellulosic biomass (Hu et al., 2010). This method generates highest number of inhibitors like furfural, HMF and acetate among all pretreatment methods (Figure 2.3) (Baral and Shah, 2014). Due to high inhibitors concentration, the loading of biomass in dilute acid is normally limited to 8~10% w/v (Qureshi et al., 2007; Nanda et al., 2014), more importantly, post-treatment removal of inhibitors is needed.

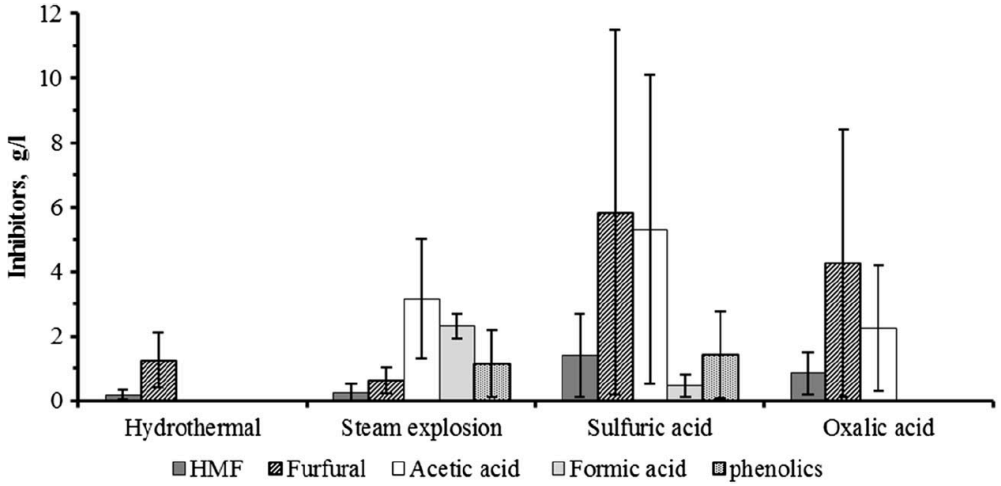


Figure 2.3 Inhibitors concentration in pretreated lignocellulosic feedstocks

Post-treatment, also called detoxification, could be implemented in ways of over-liming, physical adsorption, and in-situ detoxification. Over-liming is a widely used detoxification method on dilute sulfuric acid-pretreated lignocellulosic feedstock at industrial scale (Martinez et al., 2000). Over-liming is carried out by dosing lime $Ca(OH)_2$ into lignocellulosic feedstock until pH up to 10. Due to the high alkaline condition, part of organic compounds would be directly removed. This process can remove 42% of furfural and HMF, and 33% of phenolic in lignocellulosic hydrolysate (Anuj K. Chandel, 2011). Over-liming has its drawbacks: significant amount (as much as 13%) of sugar in the liquor could be lost due to reaction at high pH and pressed out with the wet gypsum generated during neutralization (Humbird et al., 2011). It also leads to high solid waste of gypsum. The controlling of overliming is complex too, since pH adjustments are needed twice after overliming, pH need to be adjusted to 6.5 for ABE fermentation. Therefore, in some engineering plant designs, there has been a switch from overliming to ammonia conditioning (Humbird et al., 2011). However, cost of ammonia has made this change unaffordable. On the other hand, physical adsorption has been widely used in ABE fermentation at research level. It includes ion-exchange resins (Nilvebrant et al., 2001), electrodialys (Jeong et al., 2014), membrane (Ghosh et al., 2010) etc., which are able to remove acid, furan, phenolic, aliphatic compounds from various feedstock

hydrolysate. Their downside is that these post-treatments lead to extra cost on equipment and maintenance, which is unacceptable for advanced biobutanol production.

The most promising method is in-situ microbial detoxification. In the case of in-situ microbial detoxification, microorganisms growing in lignocellulose feedstock are used to detoxify the inhibitors themselves. Schneider, (1996) used saccharomyces cerevisiae mutant to remove acetic acid from acid hydrolysate. López et al., (2004) reported that the fungi C. ligniaria (C8 NRRL 30616) eliminated 15mM (~1.5g/L) furfural and HMF from corn stove hydrolysate completely, as compared to 51% removal by over-liming. Similarly, *Clostridium beijerinckii* could detoxify moderate level of furfural and HMF (Almeida et al., 2009). In-situ detoxification are related to furan reductases and others reductases (Lewis Liu et al., 2008). During in-situ detoxification, furan derivatives would be reduced to ethanol. As Compared to over-liming and chemical adsorptions, in-situ detoxification shows great potential in terms of efficency and cost. Despite more transciptional studies is needed to explain the in-situ detoxification (Zhang and Ezeji, 2013), its application in ABE fermentation have attracted instant research interest (Jönsson et al., 2013).

Table 2.2 shows part of previous results of ABE fermentation in lignocellulosic feedstocks. Agricultural waste like rice straw, corncob and woody residues like palm bunches were used as feedstock for *clostridium acetobutylicum, beijerinckii, pasteurianum* etc. In despite of various pre-treatment and post-treatment technologies to the feedstock, in most cases butanol productions are less than 10g/L, the concentration at which clostridium growth is strongely inhibited. At current stage, efficient production is a huge challange where lignocellulosic feedstock is used.

Table 2.2 Butanol productions in lignocellulosic feedstocks in previous studies

Microorganism	Substrate	Butanol Production	Technology	Reference
C. beijerinckii (ATCC 55025)	wheat bran	8.8 g/L	Acid hydrolysis	(Liu et al., 2010)
C. acetobutylicum	Palm empty fruit bunches	0.78 g/L	Acid pretreatment/enzymatic hydrolysis	(Noomtim and Cheirsilp, 2011)
C. acetobutylicum (ATCC824)	Sugar maple	7.0 g/L	Alkali pretreatment/acid hydrolysis/overliming	(Sun and Liu, 2010)
C. saccharoperbutyl acetonicum (N1-4)	Rice straw	6.6 g/L	Absence of pretreatment/enzymatic hydrolysis/Non-sterile conditions	(Chen et al., 2013)
C. pasteurianum	Glycerol	8.8 g/L	Immobilized cells/Bath fermentation	(Khanna et al., 2013)
C. acetobutylicum (NCIM 2337)	Rice straw	13.5 g/L	Acid treatment with shear stress	(Ranjan et al., 2013)
C. acetobutylicum (MTCC 481)	Rice straw	2.1 g/L	Acid pre-treatment/enzymatic hydrolysis	(Ranjan and Moholkar, 2013)
C beijerinckii (NCIMB 8052)	Corncob	8.2 g/L	Alkali pre-treatment/enzymatic hydrolysis/overliming	(Zhang et al., 2012)
C. acetobutylicum (CICC 8008)	Corn straw	6.2 g/L	Enzymatic hydrolysis/bath reactor	(Lin et al., 2011)

2.4 Metabolic Perturbations Enhanced ABE Fermentation

In developing affordable technologies to improve butanol production in lignocellulosic feedstocks, the method using metabolic perturbations has attracted attentions (Ujor et al., 2016). Metabolic pertubations are chemicals that are able to adjust or regulate metabolism of fermentation. Metabolic perturbations are different from nutritions that necessary for strain growth or solvent production (Formanek et al., 1997).

These chemicals could improve cell growth, sugar consumption, acid accumulation and assimilation, most importantly, butanol production. Previous studies, which have been focused on metabolic perturbations improved monosaccharieds in ABE fermentation, are discussed as followed.

2.4.1 Metallic Compounds

El Kanouni (Kanouni et al., 1998) showed the supplement of $CaCO_3$ could increase sugar utilization and corresponding butanol production in either pure glucose or xylose feedstock. In the existence of 8 g/liter butanol mimicing butanol intolerance in xylose feedstock, xylose utilized was only 30 g/liter from a starting concentration of 60 g/liter. However, when $CaCO_3$ was supplied, up to 43 g/L xylose was utilized by clostridium. The study shows that $CaCO_3$ could increase butnaol tolerance, which resulted in an increase in xylose utilization. Lv et al., (2016) revealed effects of $CaCO_3$ on ABE fermentations are multiple, including pH buffering, overexpression of heat shock protein and solventogenesis enzymes, and increased DNA synthesis and replication. Moreover, Ca^{2+} ion was specailly beneficial to stablizing the clostridium membrane proteins, which neither Fe^{2} , Mn^{2+} nor Mg^{2+} was able to mimic (Han et al., 2013). Not only in pure sugar feedstock, $CaCO_3$ was also effective on ABE fermentation in lignocellulosic feedstock. Zhang and Ezeji, (2014) reported supplement of $CaCO_3$ enables growth of *C. beijerinckii* (NCIMB 8052) and production of butanol in miscanthus giganteus hydrolysate, wherase *C. beijerinckii* was not able to grow in this feedstock without $CaCO_3$. This study shows calcium carbonate can alleviate the toxic effects of lignocellulose-derived microbial inhibitory substances, and consequently enhanced bioconversion.

Another metallic compound, zinc sulphate, is found to be effective in increasing butanol production. It is well known that Zn^{2+} ion is a cofactor metal ion related to butanol dehydrogenase of clostridium (Walter et al., 1992). Very recently, Wu et al., (2016) reported that 1 mg/L of $ZnSO_4 \cdot 7H_2O$ supplement in xylose feedstock would improve butanol production by 10%. The improvement was ascribed to escalated butanol tolerance of clostridium due to zinc supplement. Transcriptional analysis showed overexpression of genes

including glycolysis (glcK and pykA), acidogenesis (thlA, crt, etfA, etfB and bcd) and solventogenesis (ctfB and bdhA) of C. acetobutylicum was observed when zinc was supplied.

2.4.2 Electron Acceptors/ Artifical Electron Carriers

Electron acceptors are oxidizing chemicals that acceptes electrons by consuming cofactors NAD(P)H. As we reviewed earlier, furfural and HMF are typical electron accetpor that consume cofactors NAD(P)H (Wahlbom and Hahn-Hägerdal, 2002). Normally the depletion of NAD(P)H inhibits cellular growth and butanol productivity, however, at certain case it could be stimulant to ABE fermentation. The study (Ezeji, 2007) showed when concentration of furan derivative was 0.5~1.0g/L in the feedstock, higher cell density and solvent production (~10% improvement) would be observed. Similarly, furfural present in lignocellulosic hydrolysate was beneficial for ethanolic fermentation of xylose (Wahlbom and Hahn-Hägerdal, 2002). Some other electron accetpors like acetoin, azo dye have also found to be effective in improving fermentative production. Nasser Al-Shorgani et al., (2015) found methyl red, a common azo dye, and an indicator dye that turns red in acidic solutions, is able to improve butanol solvent titer by 40% with very low supplement level (0.01mM). It is even better than traditional artifical electron carrier (netrual red or methyl viologen) at same supplement level, which are large conjugated organic moleculares mimicing NAD(P)H as both electron aceptor and donors (Kim and Zeikus, 1992). The electron acceptor at moderate concentration stimulated cofactor NAD(P)+ regeneration, consequently, more sugar was consumed and more solvent was produced (Ujor et al., 2016). In other word, this mechanism are related to in-situ detoxification of microbial inhibitors.

2.4.3 Metabolic Intermediates

Last type of metabolic perbutation is metabolic intermediates, especailly butyrate. Lee et al. (2008) ilustrated supplementary butyrate to the medium could not only prevented strain degeneration during continuous fermentation, but also improve butanol production greatly: 11.2 g/L butanol production versus 0.45 g/L with and without 36 mM butyrate. Ventura and Jahng, (2013) showed supplment of butyrate into glucose fermentation could increase butanol titer from 8.01 g/L to maximum 16.40 g/L. However, supplment of butyrate is not affordable, since butyrate ($5.4~6.5/gallon) is more expensive than butanol ($3~3.5/gallon).

Use of metalic compounds and electron acceptors appears affordable at the industrial level. $CaCO_3$ is one of the most abundant chemicals on earth. Futhermore, the cost of zinc sulphate is neglectable due to its micro dosage (~1mg/L). For electron acceptors, furan derivates are exsiting inhibitors in lignocellulosic hydrolysate, and methyl red is a common dye widely exsited in industrial waste. More importantly, these peturbative chemical additives could be highly effective in lignocellulosic feedstock. First, they have been proved benificial to sugar utilization and butanol production in pure monosaccharide feedstock. Second, some of them like $CaCO_3$ showed the ability to mitigate inhibition in lignocellulosic feedstock. Last, microbial inhibitors in lignocellulosic feedstock like furan and HMF could be stimulant to ABE fermentation if the concentration of furan and HMF was well controlled. Based on these findings in previous studies, we developed a relatively comprehensive study of these metabolic pertubations such as $CaCO_3$, $ZnSO_4$, furan derivatives, and methyl red. We derived a combination of chemical additives through preliminary investigation and optimization that could effctively improve butanol production in pure glucose and xylose feedstock seperately. This optimal chemical additives combination was tested using real lignocellulosic feestocks like wheat straw and spruce hydrolysates.

Chapter 3 Material and Methods

3.1 Materials and Chemicals

Cb ATCC (BA101) was purchased from American Type Culture Collections. All the chemicals were purchased from Sigma-Aldrich Canada, and were used as received without any further purification. Table 3.1 summarizes all chemicals that were used in the current study.

Table 3.1 List of chemicals that were utilized in the present study

Product	**Company**	**Catalogue No.**
Glucose (≥99.5%)	Sigma-Aldrich (Canada)	G8270
Xylose (≥99%)	Sigma-Aldrich (Canada)	X1500
Yeast extract (for use in microbial growth medium)	Sigma-Aldrich (Canada)	Y1625
Biotin (≥99%)	Sigma-Aldrich (Canada)	B4501
PABA (≥99%)	Sigma-Aldrich (Canada)	A9878
NaCl (≥99%)	Sigma-Aldrich (Canada)	S7653
Thiamine HCl (≥99%)	Sigma-Aldrich (Canada)	T4625
$FeSO_4.7H_2O$ (≥99%)	Sigma-Aldrich (Canada)	F8048
$MnSO_4.4H_20$ (≥99%)	Sigma-Aldrich (Canada)	M7634
$MgSO_4.7H_20$ (≥99%)	Sigma-Aldrich (Canada)	63138
$CH_3COO(NH_4)$ (≥99%)	Sigma-Aldrich (Canada)	A7730
Gelatin (Type B)	Sigma-Aldrich (Canada)	G9391
NaOH (≥99%)	Sigma-Aldrich (Canada)	S5881
H_2SO_4 (≥99%)	Sigma-Aldrich (Canada)	339741
KH_2PO_4 (≥99%)	Sigma-Aldrich (Canada)	322431
K_2HPO_4 (≥99%)	Sigma-Aldrich (Canada)	GO139
$CaCO_3$(≥99%)	Sigma-Aldrich (Canada)	C4830
$ZnSO_4.7H_2O$(≥99%)	Sigma-Aldrich (Canada)	Z0251
Furfural (≥99%)	Sigma-Aldrich (Canada)	185914
HMF(≥99%)	Sigma-Aldrich (Canada)	H40807
Methyl red (≥99%)	Sigma-Aldrich (Canada)	250198
Reinforced Clostridial Medium (RCM)	Oxoid Ltd. (Basingstoke, Hampshire, UK)	CM0149

3.2 Experimental Procedure and Methodology

3.2.1 Medium and Lignocellulosic Hydrolysate Preparation

P2 medium was prepared and used as the typical medium for ABE production and clostridium culture (Formanek et al., 1997). It contains sugar (glucose or xylose) 60 g/L, yeast extract 1 g/L, $CH_3COO(NH_4)$ 2.2 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4.H_2O$ 0.01 g/L, $FeSO_4.7H_2O$ 0.01 g/L, NaCl 0.01g/L, PABA 1 mg/L, thiamin hydrochloride 1 mg/L and biotin 0.01 mg/L. In this study, P2 medium was used on the pure sugar feedstock for ABE fermentation.

Reinforced Clostridium Medium (RCM) is a general medium for wild clostridium strain growth. In this experiment, RCM agar plates were used for strain recovery. RCM agar plate medium was prepared by adding 11.4 grams of RCM, 25 grams of gelatin and 7.5 grams of agar in 500 mL hot distilled water. This viscous medium was autoclaved at 121ºC for 20 min. Up cooling, the medium was poured into petri dishes and allowed to set until they have formed a smooth solid surface for the bacteria to grow. These are RCM agar plates and are an essential tool in microbiology. They allow clostridium to grow on a semi-solid surface to produce discrete colonies.

Lignocellulosic hydrolysates were wheat straw and spruce hydrolysate. Both were collected from Springridge Farm located in Milton, Ontario and St. James Square at Ryerson University, Toronto, Ontario. In order to produce lignocellulosic hydrolysates, wheat straw and spruce branch were grounded to reduce size using a hammer mill (Retsch GmbH Inc., USA), and then were filtered by a 1 mm Sieve screen. Then, the moisture content of feedstock was reduced through heating in a convention oven at 90°C for 2 days until no further weight loss was detected. To pursure acidic pretreatment, total 50 grams of dry feedstock was dispensed in 1-L flask containing 500 mL of 1% (v/v) H_2SO_4 followed by autoclave at 121 °C for 60 minutes (Guerra-Rodríguez et al., 2012). No separation of slurry from liquid was conducted. At last, pH was adjusted to 6.5 using 10 M NaOH.

3.2.2 Strain Recovery

Any anaerobic manipulations were carried out in an anaerobic chamber (i.e., Glove Box; Terra Universal, Canada) at a temperature of 25 ± 2°C. Anaerobic environment was created inside the glove box by initially purging any air out of the box using a vacuum pump for about 8-10 minutes and later was supplied with a constant flow of N_2 gas until all the containers were tightly sealed. The elements of aseptic technique were implemented throughout this study, including routine cleaning through wiping surface with 70% ethanol before and after work, in addition utilizating of ultraviolet light to sterilize the exposed work surfaces.

Clostridium Beijinrickii ATCC (BA101) was maintained as cell suspension in medium containing 30% (v/v) sterile glycerol and Cooked Meat Medium (CMM) and stored in Eppendorf tubes in -80ºC ultra low freezer (Thermo fisher Scientific, USA). The recovery of clostridium was conducted via RCM agar plate streaking protocol. Upon the preparation of RCM agar plates, 2 mL of clostridium spores from the freezer were heat shocked at 80 ºC for 5 minutes (Qureshi et al., 2007). Later, the spores were inoculated to five RCM agar plates by streaking. A sterile inoculating loop was used to streak the strains onto the agar RCM plates. The loop was sterilized by holding it under a flame until it is red-hot. Streaked agar plates were sealed in the anaerobic jar (HP011, Thermo Scientific) containing an anaerobic pack (AnaeroPack MGC). Later, the anaerobic jar was sealed and placed into incubator at 35ºC. White colonies were observed on agar plates two days later. The colonies were isolated, and inoculated into P2 medium containing 60 g/L glucose for ABE fermentation using sterile inoculating loop. Upon fermentation and following HPLC analysis, highest butanol productive strain (of 7.5 g/L butanol production) was determined (Appendix Table A.1). The corresponding strain was stored in Eppendorf tubes at 4ºC for the following study.

3.2.3 Bio-butanol Production

Fermentation was conducted in 100 mL Wheaton serum bottle containing 35 mL of P2 or lignocellulosic feedstock. P2 or lignocellulosic feedstock was autoclaved at 121ºC for 15 minutes and cooled to room temperature. Overnight pre-grown strains (OD600 0.6~1.0) were used for inoculation. Upon the inoculation, the medium was bubbled with N_2 gas for about 5-10 minutes. The blue neoprene rubber stopper along with a metallic cap were used to seal the bottles using a vial crimper (Cole Palmer Canada). The serum bottles were brought out of the glove box and transferred to the incubator. The temperature of the incubator was adjusted to 35ºC. Concentrations of glucose, xylose, butyrate, acetate and butanol in the liquid were analyzed using High Performance Liquid Chromatography (HPLC). The samples for HPLC were centrifuged at 4000 rpm for 15 min and double filtered through 0.2 μm PTFE- filters (Whatman, USA). HPLC testing vials was filed to a minimum headspace to reduce the loss of solvents in vapor phase. These samples were stored at 4ºC until analyzed.

3.2.4 Metabolic Perturbation

3.2.4.1 Preliminary Investigations

The purpose of the preliminary investigations was to reveal how ABE fermentation is affected by the existence of furfural, $CaCO_3$ and methyl red (MR) in glucose and xylose feedstocks, separately. Table 3.2 lists control variables and corresponding levels used in the preliminary investigation. Control variables were sugar type, furfural, $CaCO_3$, and MR. For sugar type, level "-1" encoded xylose 60 g/L in the feedstock while level "1" encoded glucose 60 g/L. For chemical additives, level " -1" represented an absence of furfural, $CaCO_3$ or MR, whereas level "1" stood for the supplement of furfural (1 g/L), $CaCO_3$ (2 g/L) or MR (0.01mM) in the feedstock, respectively. The concentration of furfural (1 g/L), $CaCO_3$ (2 g/L) and MR (0.01mM) were determined in previous studies (Ezeji, 2007; Han et al., 2013b; Nasser Al-Shorgani et al., 2015), respectively. The responses in the preliminary investigations included cell growth (OD600), sugar

consumption, butanol production, and acid concentration. Sugar consumption was calculated by dividing the concentration of consumed sugar with total initial sugar concentration. Moreover, in order to study whether effects of control variables were time-dependent, samplings were conducted at 24 and 48 hours after the start of incubation. Preliminary investigations were performed in an factorial design including 16 experimental runs in total.

Table 3.2 Control variables and their levels in preliminary investigations

Independent variable	Levels	
	-1	1
Sugar type (x_1)	Pure xylose (60 g/L)	Pure glucose (60 g/L)
Furfural (x_2)	0	Furfural (1 g/L)
$CaCO_3$ (x_3)	0	$CaCO_3$ (2 g/L)
MR (x_4)	0	MR (0.01M)

*Number of Runs = 16

P2 medium with two different sugars, glucose 60 g/L (coded 1) and xylose 60 g/L (coded -1), were prepared and autoclaved separately. 35mL of P2 medium was distributed in each serum bottle. The level "1" of furfural (x_2), $CaCO_3$ (x_3) or methyl red (x_4) was achieved by supplying 0.5 mL of furfural solution (72 g/L), solid $CaCO_3$ (0.072 g), or 0.5 mL of methyl red solution (0.54 g/L) in P2 medium, respectively. Overnight grown strain pre-grown in P2 containing glucose 60g/L was used for inoculation (1 mL/bottle). After anaerobiosis with N_2, the serum bottles were sealed and put into incubator at 35°C for 72-hour fermentation. Sample collection was performed inside the anaerobic biosafety hood, which was left in UV light for 10 min prior to sampling and was cleaned with ethanol. Syringes, needles, spatulas or any equipment that comes in contact with the bacteria was washed with ethanol sterilized under UV light for 10 minutes. Sampling was performed by inserting a sterilized syringe-needle combination through the

serum bottle's rubber stopper and collecting ~2 mL of liquid at 24th and 48th hour. All samples were analyzed for pH, cell growth, in addition to ABE, acid and sugar concentration using HPLC.

After measurement of all the responses, statistical analysis was performed (ANOVA) by Minitab 17.1 following the model of factorial design, where x_i are control variables, y is the response of interest, β is the constant coefficients, and ϵ is the random experimental error. The coefficients of control variables were main goals of our preliminary investigation.

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum \sum_{i<j} \beta_{ij} x_i x_j + \epsilon$$

3.2.4.2 Biobutanol Production Optimization

The purpose of optimization was to maximize butanol production in pure glucose or xylose feedstock (60g/L) separately by tailoring the concentrations of $ZnSO_4$ and furan derivatives (75% furfural and 25% HMF). 4 g/L $CaCO_3$ was supplied in every serum bottle as constant condition. Table 3.3 lists control variables and corresponding levels used in the optimization process. For each glucose and xylose feedstocks (60 g/L), butanol productions under 5-level concentrations of $ZnSO_4$ and 5-level concentrations of furan derivatives were examined. $ZnSO_4$ concentrations ranged from 0 to 2 mg/L (Wu et al., 2015), and furan derivatives concentrations varied from 0 to 3 g/L with a fixed furfural/HMF ratio (75% furfural and 25% HMF) mimicking the ratio of furfural/HMF in wheat straw hydrolysates (Almeida et al., 2009). The experiments were replicated twice. In each replication, center points were run three times for determination of internal estimators. There were totally 44 experimental runs in biobutanol production optimization.

Table 3.3 Control variables and their levels in biobutanol production optimization

Independent variable	Levels				
	-1.414	-1	0	1	1.414
$ZnSO_4 .7H_2O$ (x_1)	0.000 mg/L	0.293 mg/L	1.000 mg/L	1.707 mg/L	2.000 mg/L
Furfural/HMF (x_2)	0.000 g/L	0.439 g/L	1.500 g/L	2.561 g/L	3.000 g/L

*2-Replicates; Number of runs = 44

According to the design levels, 0, 0.146 mL, 0.5 mL, 0.853 mL and 1 mL of $ZnSO_4$ solution (72 mg/L) were added into serum bottles to make $ZnSO_4$ concentrations 0, 0.293 mg/L, 1.00 mg/L, 1.707 mg/L, and 2 mg/L, respectively. Similarly, 0, 0.146 mL, 0.5 mL, 0.853 mL and 1 mL of furan derivatives solution (75% furfural and 25% HMF, 83.3 g/L in total) were added in serum bottles to make concentration of furan derivatives 0, 0.439 g/L, 1.50 g/L, 2.531 g/L and 3 g/L, respectively. Furan derivatives solution (83.3 g/L) was prepared by adding solid HMF (1.041 g) and liquid furfural (3.124 g) in hot water to make a total liquid volume of 50 mL. Then, 0.144 g of solid $CaCO_3$ was added into every serum bottle. Overnight grown strain pre-grown in P2 containing glucose 60g/L was used for inoculation (1 mL/bottle). After anaerobiosis with N_2, the serum bottles were sealed and put into incubator at 35ºC for 72-hour fermentation. The strain with the highest butanol production in the first replication was used for inoculation in the second replication.

An experimental design called Response Surface Methodology (RSM) was used in the optimization. RSM has certain advantages comparing to classic experimental designs (Silva et al., 2011) (Canettieri et al., 2007). RSM allows researchers to derive an empirical model, from less experimental runs as compared to traditional experiments. More importantly, it also enables researchers to reveal the interactions between control variables. Weakness of the RSM is that the data has been fitted to a quadratic polynomial. Since no scientific systems are in quadratic principle exactly, RSM provides an approximate analysis of the results. In current study, Box-Wilson Central Composite Design (Khuri and Mukhopadhyay, 2010), the most used RSM design pattern, was used to designate levels of the control variables. The regression model of RSM is

in the form of quadratic model or even of higher orders as following, where x_i are control variables, y is the response of interest, β is the constant coefficients, and ϵ is random experimental error assumed to have a zero mean. By determination of all of coefficients including main effects and interaction effects, optimizations of chemical additives concentration were obtained.

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum\sum_{i<j} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ij} x_i^2 + \epsilon$$

3.2.4.3 Optimum Biobutanol Production Using Lignocellulosic Feedstock

Optimum metabolic perturbation of $CaCO_3$, $ZnSO_4$ and furan derivatives for maximizing butanol production were tested using lignocellulosic feedstock. Table 3.4 is the corresponding experimental design. The experiments were done in paralleled 2-replication. There were in 12 experimental runs in total.

Table 3.4 Experimental design of optimum biobutanol production using lignocellulosic feedstock

Order	Hydrolysate	Pre-grown medium for inoculated strain	Chemical additive in lignocellulosic hydrolysate
1	WSH	P2	0
2	WSH	P2+ $CaCO_3$ +$ZnSO_4$	0
3	WSH	P2+ $CaCO_3$ +$ZnSO_4$	P2+ $CaCO_3$ +$ZnSO_4$
4	SH	P2	0
5	SH	P2+ $CaCO_3$ +$ZnSO_4$	0
6	SH	P2+ CaCO3 +ZnSO4	P2+ CaCO3 +ZnSO4

* Two replicates; Number of Runs =12

For each batch of fermentation, approximately 50 mL lignocellulosic hydrolysate including slurry was transferred to a 100mL metric flask. A liquid volume of 35mL was determined after the slurry was precipitated. The slurry and the liquid of exact 35 mL were transferred to each serum bottle. The diluted acid pretreatment could not release enough sugar from lignocellulosic biomass for ABE fermentation: 12

g/L glucose, 35g/L total sugar and 6 g/L glucose, 25 g/L total sugar were achieved in the wheat straw hydrolysate and spruce hydrolysate, respectively. Enzymatic treatment is often further applied to derive more monosaccharides from lignocellulosic biomass (Qureshi et al., 2007). In our study, instead of using enzymatic treatment, solid glucose and xylose were directly added to make total sugar concentrations 60g/L and glucose concentration 27 g/L for lignocellulosic hydrolysates, which was comparable to the wheat straw hydrolysate after enzymatic treatment in previous study (Qureshi et al., 2007). Therefore, 15g/L glucose and 10g/L xylose were added into wheat straw hydrolysate, and 21g/L glucose and 14g/L xylose were added into spruce hydrolysate, seperately. Following sugar addition, the nutrition such as yeast, vitamins, metal ions for clostridium growth were added as described in P2 medium. $CaCO_3$ 4 g/L and $ZnSO_4$ 2 mg/L were supplied in experiment order 3 and 6. The strains pre-grown in two medias were used for inoculation: one was pre-grown in P2 medium (glucose/xylose; 30/30g/L), the other was pre-grown in the same P2 medium with $CaCO_3$ 4 g/L and $ZnSO_4$ 2 mg/L added. Inoculation volume was 2 mL. After anaerobiosis with N_2, the serum bottles were sealed and put into incubator at 35°C for 72-hour fermentation.

3.3 Measurements and Analysis

3.3.1 High Performance Liquid Chromatography (HPLC)

The HPLC (Agilent 1100) equipped with refractive index detector (RID) and (DAD) Diode array detector, was used for the analysis of glucose, xylose, butanol, acetone, ethanol, acetate and butyrate concentrations. The column HPX-87H (Aminex HPX-87H) was the column for the analysis of sugars, ABE and acids. The mobile phase (distilled water) was filter sterilized. The flow rate and column temperature were maintained at 1 mL/min and 60°C, respectively. Sample vials were arranged in a sequence and each 10 µl of sample was extracted by the automatic sample injector. The calibration of HPLC were performed by running solvents, acids, and sugars standards as Table C in appendix shows. Each sample was analyzed through the HPLC for 28 minutes.

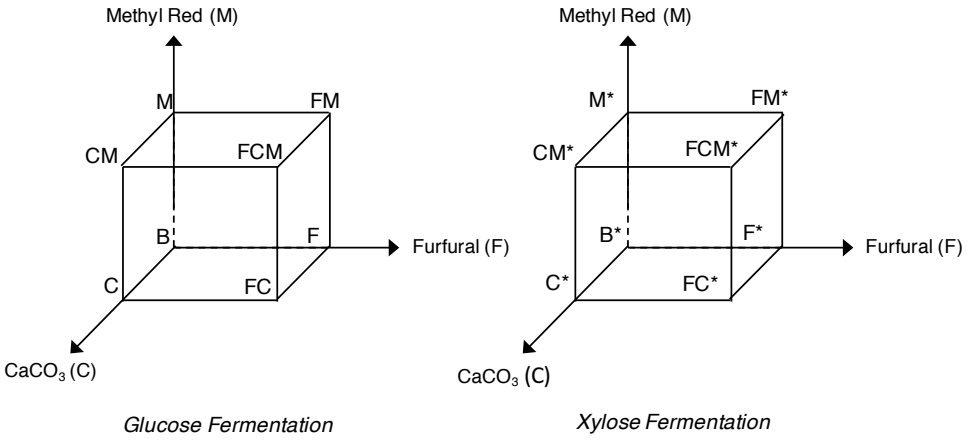
3.3.2 Cell Density Measurement

Optical density of a sample measured at the wavelength of 600 nm (OD600) was used to quantify clostridium cell density (Sreekumar et al., 2015). Genesys 10S UV-VIS (Thermo Scientific) was used for OD600 measurement. The relationship between observed OD600 and actual OD600 was determined as (Appendix Figure C.8).

Chapter 4 Results and Discussion

4.1 Preliminary Investigations

The preliminary investigations were to reveal how ABE fermentations were affected by the existence of furfural, $CaCO_3$ and methyl red in glucose and xylose feedstocks separately. The experimental design is shown in Figure 4.1. The origin B of Cartesian coordinate denoted the control run with no chemical additives supplied. Three axes represented three control variables: Furfural, $CaCO_3$, and Methyl Red. F, C, M denoted furfural (1.0g/L), $CaCO_3$ (2.0g/L), and Methyl red (0.01mM) was supplied in the feedstock, respectively, while FC, CM, FM, FCM meant multiple chemical additives were supplied simultaneously. In total, there were 16 experimental runs for glucose and xylose fermentation in the preliminary study. For each run, responses such as cell density, sugar consumption, butanol production, acid concentration (butyrate and acetate), and pH were measured individually on day 1 (24^{th} hour), day 2 (48^{th} hour) and day 3 (72^{nd} hour) via sampling and displayed in Table 4.1 and Figure 4.2.



Notation	B	F	C	FC	M	FM	CM	FCM	B*	F*	C*	FC*	M*	FM*	CM*	FCM*
Run#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 4.1 Experimental design of preliminary investigations

Table 4.1 Results of preliminary investigations

Run	No.	Responses*														
		Cell Density (OD600)			Sugar Consumption (%)			Butanol concentration (g/L)			Total acid concentration (mmol/L)			pH		
		Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3
B	1	1.9	2.3	2.3	21.7%	46.0%	55.9%	2.4	7.0	7.5	45.9	38.0	34.7	5.12	5.20	5.12
F	2	1.9	2.0	1.9	18.3%	38.5%	43.0%	2.1	5.3	6.0	59.1	56.5	76.4	5.04	5.03	4.63
C	3	2.7	3.6	2.9	26.9%	68.3%	71.6%	3.1	9.0	9.5	25.7	26.1	29.9	5.40	5.28	5.07
FC	4	2.2	3.6	2.9	32.1%	72.4%	74.5%	3.6	9.5	9.6	90.4	89.2	62.9	5.30	5.34	5.02
M	5	1.8	2.4	2.4	22.0%	54.7%	63.5%	2.5	6.3	6.8	52.1	42.9	42.3	5.21	5.12	5.18
FM	6	1.7	2.4	2.3	21.2%	46.5%	49.0%	2.2	6.1	6.5	54.8	50.5	68.5	5.20	4.99	4.76
CM	7	1.7	2.3	2.4	20.8%	53.1%	62.5%	1.9	6.5	7.3	30.2	29.1	28.3	5.16	5.43	5.07
FCM	8	2.0	2.9	2.7	29.3%	66.7%	71.3%	2.7	7.6	8.4	59.5	64.1	44.2	5.11	5.27	4.88
B*	9	1.4	1.8	1.4	17.4%	27.6%	33.8%	2.1	4.3	5.5	11.3	12.1	12.7	5.31	5.45	5.26
F*	10	1.5	1.6	1.5	17.7%	29.4%	36.3%	2.1	4.2	5.5	11.1	12.0	13.3	5.27	5.33	5.14
C*	11	2.3	2.5	2.7	27.9%	59.1%	61.1%	3.0	6.2	9.0	15.2	18.8	19.7	5.29	5.43	5.19
FC*	12	2.1	2.3	2.6	25.3%	51.2%	55.1%	2.9	7.1	8.2	52.7	64.6	64.8	5.52	5.38	5.22
M*	13	1.8	2.3	1.9	19.1%	34.6%	41.3%	2.0	5.4	6.8	44.1	44.5	50.2	5.29	5.38	5.22
FM*	14	1.5	1.9	1.8	15.5%	30.9%	40.5%	2.0	5.0	6.5	39.4	40.2	37.8	5.58	5.32	5.20
CM*	15	2.5	2.3	2.8	29.7%	59.0%	62.2%	3.5	8.7	9.3	13.1	17.9	19.1	5.26	5.38	5.16
FCM	16	1.4	1.9	1.9	18.0%	41.9%	48.5%	2.1	6.4	7.6	44.5	46.0	61.8	5.38	5.56	5.28

*Cell density was quantified in optical adsorption at 600 nm; sugar consumption was calculated by dividing utilized sugar with initial total sugar; total acid concentration was calculated by adding butyrate and acetate concentration, and total acid concentration was in the unit of "mmol/L" in stead of "g/L" by referring to the study (Maddox et al., 2000). Acetone, ethanol, butyrate and acetate concentrations are listed in appendix Table A.2 and A.3.

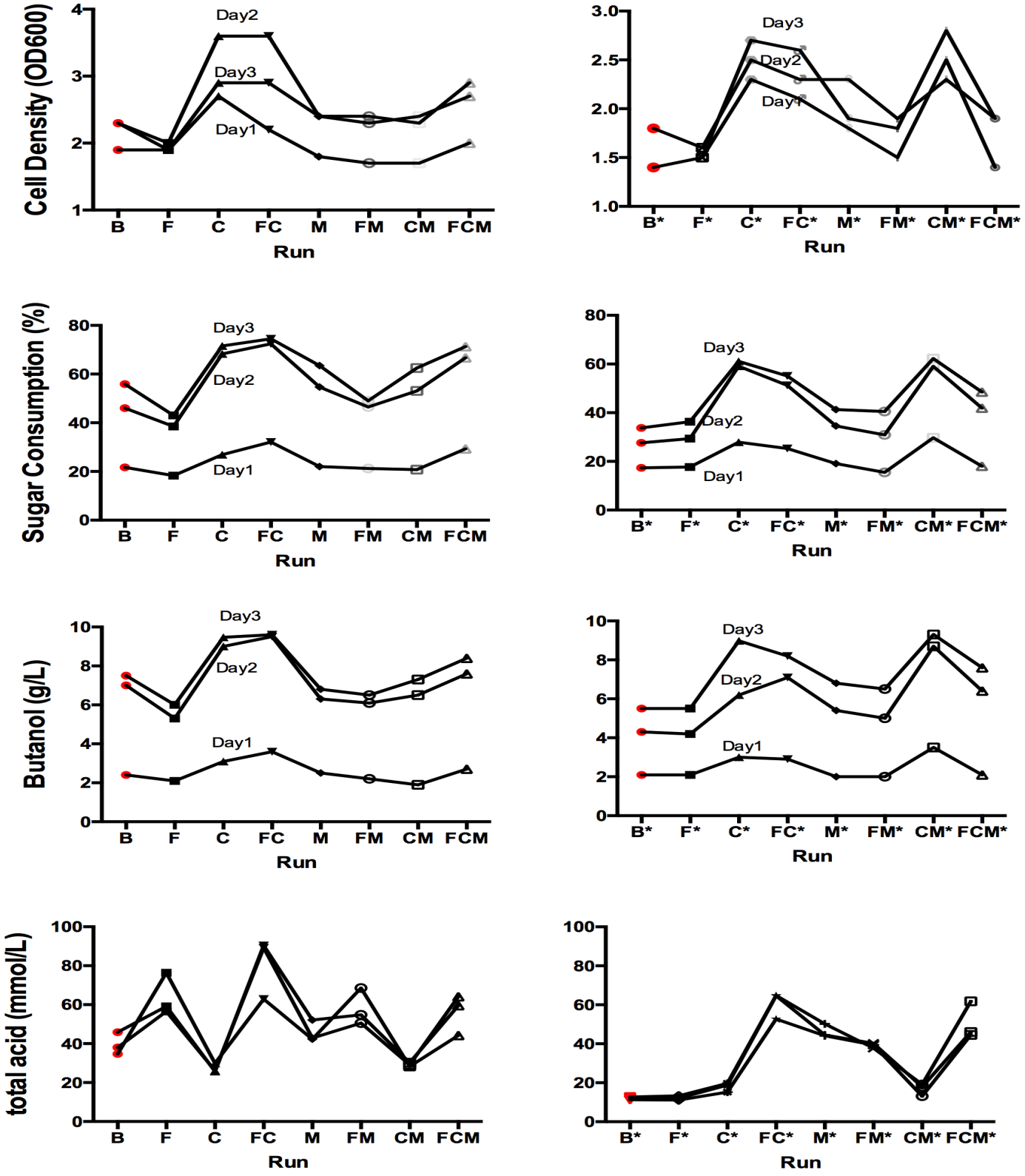


Figure 4.2 Time-dependent plots of responses in preliminary investigations

On the first day cell density of run C was soaring. Clostridium kept strong growth and reached the peak on the second day. Run C and FC held exceptionally high cell density of 3.6, which was 56% above that of glucose control B. Run C* and FC* in xylose feedstock also showed enhancement over control run B*. Later, the decay of cell indicated the end of the fermentation cycle. Highest cell density among all glucose fermentations dropped to 2.9. By contrast, cell densities of run C*, FC* and CM* were still on the rise and eventually reached 2.8. The commonplace of runs C, FC, C*, FC* and CM* were the supplement of $CaCO_3$. In M* and CM*, cell density was also escalated, which suggested methyl red may be beneficial to clostridium growth in xylose feedstock.

Sugar consumptions were related to cell density from the similarity in pattern. It is well described by Shinto's kinetics models that in ABE fermentation, sugar consumption is clostridium cell density dependent (Shinto et al., 2008). The depletion of sugar nearly stopped after the second day. Final sugar consumptions of glucose control B and xylose control B* were 55.9% and 33.8% respectively. Through a chemical additive supplements, maximum sugar consumptions in glucose and xylose feedstocks were escalated to 74.5% (of FC) and 62.2% (of CM*). M*, FM* and CM* also showed higher xylose depletion percentage than the control. Oppositely, weakened glucose utilization was observed in run F and FM.

Final butanol production of B and B* were 7.5 g/L and 5.5 g/L, respectively. As compared to 7.5g/L butanol production of glucose control B, 5.5 g/L butanol production of xylose control B* was reasonably lower. The weak solventogenic ability of the clostridium was improved by the supplement of chemical additives such as $CaCO_3$. Butanol productions in glucose and xylose fermentation were increased to 9.6 g/L (of run FC) and 9.3 g/L (of run CM*), which are 28% and 69% above the controls. However, butanol production below the control level, 6.0g/L (of run F) and 6.5g/L (of run FM) were observed, suggesting that in glucose feedstock, furfural was detrimental to butanol production, and sugar consumption as demonstrated earlier.

Furfural was found to improve acid production in glucose feedstock with exceptionally high acid concentrations in run F, FC, FM, FCM. Acids including butyrate and acetates, the precursor of butanol and acetone, are produced in acidogenesis phase then assimilated into butanol and acetone in solventogensis

phase. Clostridiums response to furfural by accelerating acid production to release inhibitory stress due to furfural (Zhang and Ezeji, 2013). Clostridium generated higher acid concentration in glucose fermentations than that in xylose fermentations. The disparity is due to higher metabolic flux in glucose fermentation (Shinto et al., 2008). Remarkably, FC and FC* held highest acid concentration in both glucose fermentation and xylose fermentation on the first day.

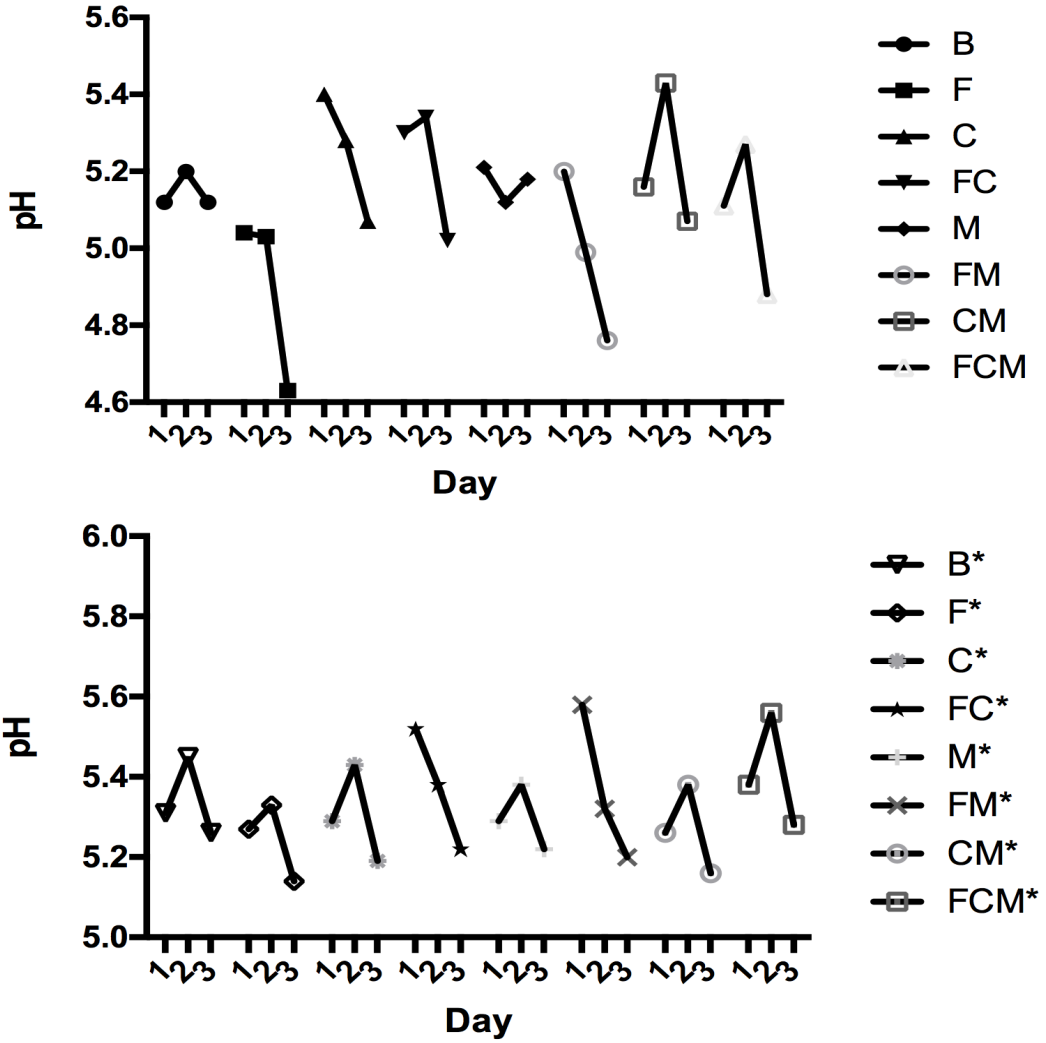


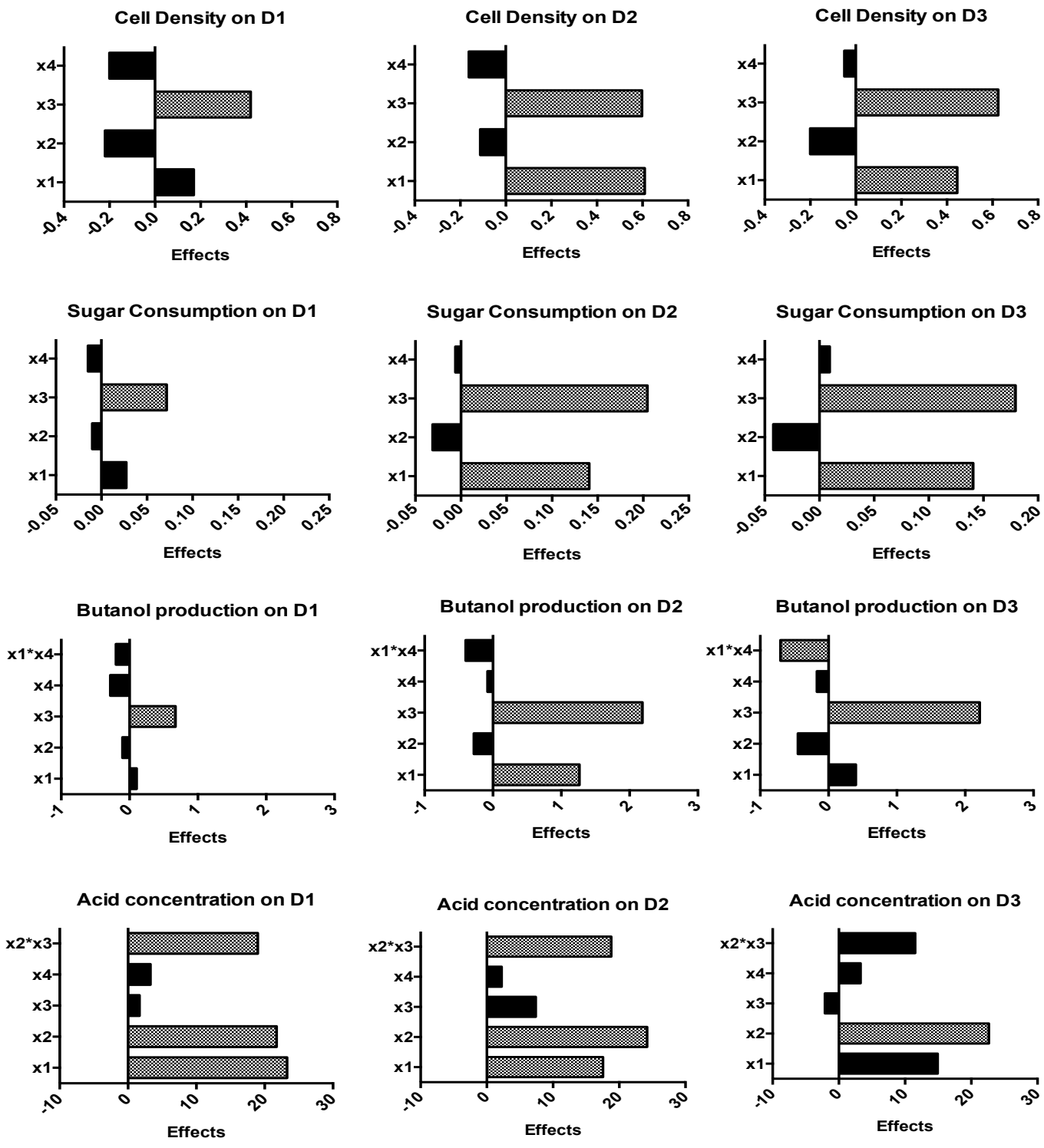
Figure 4.3 pH time-series plots in preliminary investigations

Acid concentration was linked to pH of the broth. pH responses are illustrated in Figure 4.3. The pH level dropped on the last day to below 4.8 in run F, FM. By comparing the values in Table 4.1, we concluded

that the pH level basically decreased with the increase of acid concentration, if no $CaCO_3$ was added. Extracellular pH is an indicator of whether solventogensis are performed normally in ABE fermentation (Millat et al., 2011). Higher acid concentration and pH < 4.8 in the final broth due to furfural supplement impaired the butanol production as F and FM showed. Despite high acid concentration was also observed in FC and FCM, the corresponding pH level were maintained at 5.02 and 4.88, and butanol production was 9.6g/L and 8.4 g/L respectively. This indicated that CaCO3 could mitigate furfural stress through pH buffering (Kanouni et al., 1998).

ANOVA was used to quantify effects and reveal potential impacts of chemical additives (Figure 4.4). Internal error estimate was determined by pooling high order ($n \geq 3$) interactions (Cuthbert and Daniels, 1959). $P<0.05$ was considered statistical significant. Significant effects were marked in shadow. x1, x2, x3, and x4 were corresponding to sugar type, furfural, $CaCO_3$, and methyl red respectively. Detailed analysis is listed in Table B.1~B.12.

$CaCO_3$ stimulated significantly the cell growth, with coefficient of 0.4, 0.6 and 0.6 on day 1, day 2 and day 3, respectively. Glucose was superior over xylose as feedstock to clostridium culture in terms of cell growth improvement. However, the change sugar from xylose to glucose was less effective than the supplement of $CaCO_3$. Furfural was constantly negative to cell density, in despite of the statistical insignificance. The effects on sugar consumption and butanol production agree with the effects on cell growths. $CaCO_3$ improved butanol production by cal. 2.2 g/L. Interaction Sugar type* Methyl Red was found to be significant to butanol production. As we mentioned in Figure 4.2, the xylose runs M*, CM*, FM* showed higher responses than the control B* in terms of cell density, xylose utilization and butanol production. This interaction meant that the supplement of methyl red stimulated significantly butanol production in xylose feedstock. This term is displayed in Figure 4.5. In xylose feedstock, methyl red increased butanol production. By contrast, butanol production was impaired by methyl red.



*x1=sugar type (glucose or xylose), x2=$CaCO_3$ (2g/L or 0), x3=Furfural (1g/L or 0), x4=Methyl Red (0.01mM or 0)

Figure 4.4 Effects of control variables on cell density, sugar consumption, butanol production and acid concentration in preliminary investigations (*D1: Day1; D2: Day2; D3: Day3)

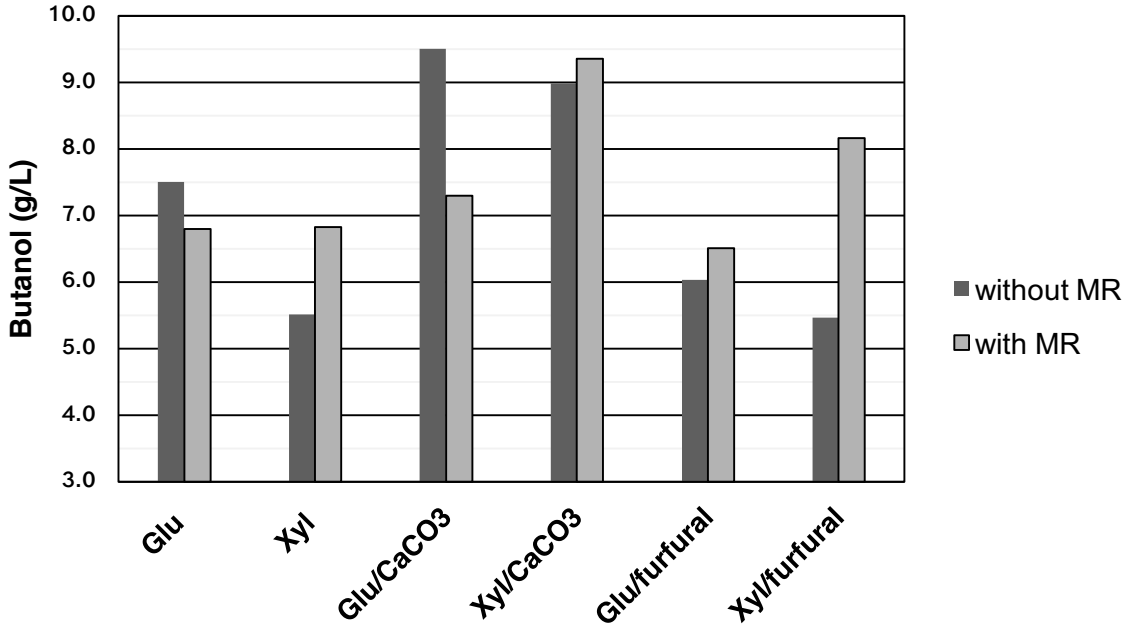


Figure 4.5 Butanol productions with and without methyl red supplement in preliminary investigations

Effects of control variables on acid concentration was furfural> Sugar type > furfural*$CaCO_3$ (on day 2 and 3). Furfural gave a constant rise to the acid in total by 22 mmol/L. The interaction of furfural*$CaCO_3$ was significant. Because $CaCO_3$ wasn't stimulant to acid production, we concluded that $CaCO_3$ serve as acid production catalyst when furfural was present. Further study on this interaction term may be beneficial to the understanding of $CaCO_3$ mitigated furfural stress.

To summarize, the supplement of $CaCO_3$ greatly improved the cell growth, the sugar utilization, and the butanol tolerance by clostridium. It is known that the upgradation of DnaK/GrpE took place when $CaCO_3$ was supplied for clostridium (Han et al., 2013a). The study (Zylicz et al., 1989) showed that DnaK/GrpE was critical to cell division and DNA replication. Butanol production improvement and furfural stress mitigation were also contributed to pH buffering ability of $CaCO_3$. In our study, when furfural exist in glucose feedstock, the pH level was lower than 4.8 and butanol production was impaired. Via the supplement of $CaCO_3$, FC showed well maintained pH level at ~5.0 and no impaired butanol production was observed.

Methyl red was the stimulant to xylose fermentation, however, the inhibitor to glucose fermentation. The possibility that methyl red served an electron carrier improving butanol production (Nasser Al-Shorgani et al., 2015) was excluded. In terms of molecular, artificial electron carrier is the molecular that could mimic the structure of C=N conjugated ring of NAD(P)H for quick electron red/ox transfer. Azo molecular of methyl red doesn't consist the conjugated C=N hetero-ring (Saratale et al., 2011) (Figure A.1) thus fails to function as artificial electron carrier. Moreover, final ratio of butanol: acetone (w/w) of fermentations with methyl red was approximately 2:1. It is a contradictory to the results (ratio>2) when traditional artificial electron carrier such as neutral red was used (Zheng et al., 2015). In contrast, studies (Adedayo et al., 2004) showed that methyl red may be degraded by reductive cleavage of the azo bond (N=N) when acting as an electron acceptor for the microbial electron transport chain. (Chen et al., 2005) showed that the enzyme of azo-dye detoxification in *Escherichia coli*, azoreductase (Azo1) , is xylose fermentation pathway dependent. Therefore, the xylose fermentation can be improved by detoxification of methyl red. In the current study, since methyl red was detrimental to glucose fermentation, it was not used in the following studies.

Furfural at 1g/L had negative effects on cell density, sugar consumption and butanol production in glucose fermentation. It is confirmed that furfural could substantially escalate acid production, and lead to pH drop. However, furfural 1g/L would not impair xylose fermentation.

The stimulants $CaCO_3$ and methyl red combatively enhanced cell density, sugar consumption and the butanol production in xylose fermentation CM*. This indicated that ABE fermentation could be more beneficial from multiple supplements of metabolic perturbations. Therefore, after the preliminary study, we need to reveal more chemical additives that could boost ABE fermentation together with $CaCO_3$. We used zinc sulphate in the following experiment since it is known for its ability on improving butanol tolerance (Wu et al., 2015). On the other hand, furan derivative was further studied, because it is most important inhibitor in typical lignocellulosic feedstock hydrolysate. HMF was added in a fixed ratio to furfural to mimic furan derivatives composition of wheat straw hydrolysate. Last, $CaCO_3$ at 4 g/L was used as constant condition in the following optimization design according to the study (Han et al., 2013a). By tailoring

concentrations of these chemical additives, we expected a combinative and syngeneic improvement on butanol production.

4.2 Biobutanol Production Optimization

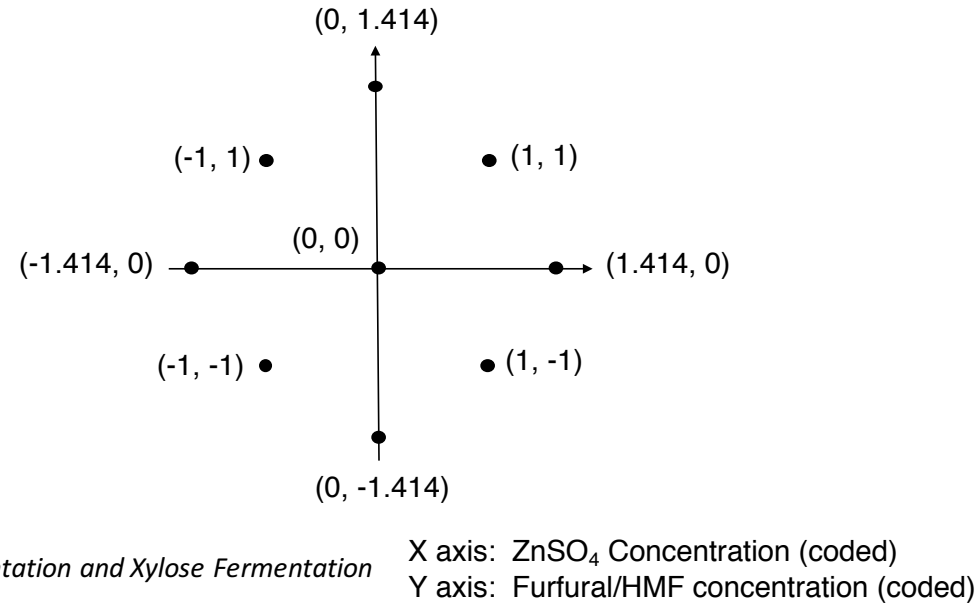


Figure 4.6 Experimental design of biobutanol production optimization

The purpose of optimization was to maximize butanol production in glucose and xylose feedstock separately by tailoring the initial concentrations of $ZnSO_4$ and furan derivatives (75% furfural and 25% HMF). $CaCO_3$ (4 g/L) was supplied in every experimental run as constant condition. The experiments were designed according to response surface methodology - central composite design (RSM-CCD) (Khuri and Mukhopadhyay, 2010). There were 2 blocks all of 44 experimental runs. Central points were replicated tripled in each block.

Table 4.2 Results of biobutanol production optimization in glucose feedstock

Run	Blocks	$ZnSO_4$		Furfural /HMF		Butanol	Prediction
		(g/L)	Coded	(g/L)	Coded	(g/L)	(g/L)
1	1	2.00	1.414	1.50	0	10.72	10.4990
2	1	1.7!	1	2.56	1	10.04	10.2571
3	1	1.71	1	0.44	-1	10.51	10.7596
4	1	1.00	0	1.50	0	9.42	9.3622
5	1	1.00	0	1.50	0	9.32	9.3622
6	1	1.00	0	1.50	0	9.29	9.3622
7	1	1.00	0	3.00	1.414	9.51	9.4394
8	1	1.00	0	0.00	-1.414	11.45	11.0937
9	1	0.29	-1	0.44	-1	10.69	10.5715
10	1	0.29	-1	2.56	1	8.73	8.7340
11	1	0.00	-1.414	1.50	0	9.05	9.2891
12	2	2.00	1.414	1.50	0	10.92	10.4881
13	2	1.71	1	2.56	1	10.07	10.2462
14	2	1.71	1	0.44	-1	10.30	10.7487
15	2	1.00	0	1.50	0	9.32	9.3513
16	2	1.00	0	1.50	0	9.67	9.3513
17	2	1.00	0	1.50	0	9.12	9.3513
18	2	1.00	0	3.00	1.414	9.43	9.4285
19	2	1.00	0	0.00	-1.414	11.23	11.0828
20	2	0.29	-1	0.44	-1	10.26	10.5606
21	2	0.29	-1	2.56	1	8.85	8.7231
22	2	0.00	-1.414	1.50	0	9.44	9.2782

Table 4.3 Results of biobutanol production optimization in xylose feedstock

Run	Blocks	$ZnSO_4$		Furfural /HMF		Butanol	Prediction
		(mg/L)	Coded	(g/L)	Coded	(g/L)	(g/L)
1	1	2.00	1.414	1.50	0	9.35	9.6835
2	1	1.71	1	2.56	1	0.00	-0.1773
3	1	1.71	1	0.44	-1	10.20	10.6152
4	1	1.00	0	1.50	0	10.86	9.9437
5	1	1.00	0	1.50	0	10.10	9.9437
6	1	1.00	0	1.50	0	9.38	9.9437
7	1	1.00	0	3.00	1.414	0.00	N/A
8	1	1.00	0	0.00	-1.414	9.19	9.2060
9	1	0.29	-1	0.44	-1	9.52	10.0802
10	1	0.29	-1	2.56	1	0.00	-0.7123
11	1	0.00	-1.414	1.50	0	10.25	10.3235
12	2	2.00	1.414	1.50	0	11.10	10.5985
13	2	1.71	1	2.56	1	0.00	0.7377
14	2	1.71	1	0.44	-1	11.82	11.5302
15	2	1.00	0	1.50	0	10.72	10.8587
16	2	1.00	0	1.50	0	11.04	10.8587
17	2	1.00	0	1.50	0	10.24	10.8587
18	2	1.00	0	3.00	1.414	0.00	N/A
19	2	1.00	0	0.00	-1.414	10.54	10.1210
20	2	0.29	-1	0.44	-1	10.71	10.9952
21	2	0.29	-1	2.56	1	0.00	0.2027
22	2	0.00	-1.414	1.50	0	11.48	11.2385

Table 4.2 and Table 4.3 are butanol productions in glucose and xylose feedstock, respectively. All glucose-fed clostridium produced solvent successfully, whereas in xylose feedstock, runs 2, 7, 10, 13, 18 and 21 failed to commence the fermentation (Table 4.3). Furan derivatives concentrations of these runs were no less than 2.56 g/L. This indicated that furan derivatives of 2.56 g/L are lethal for xylose-fed clostridium. Butanol productions in glucose and xylose feedstock ranged 8.73 ~ 11.45 g/L and 9.19 ~ 11.82 g/L respectively (except failed runs), which were improved as compared to 6.54~9.57 g/L and 5.51~9.35 g/L in glucose and xylose feedstock in preliminary investigations.

Figure.4.7 shows glucose and xylose consumption in the optimization study (block1). The range of sugar consumption in the optimization study (Opt) was 53~87% (except failed runs), which was higher than 34-74% in preliminary investigations (PI). Given the same level of butanol was produced, more glucose was consumed than xylose. Importantly, sugar consumption was highly correlated to butanol production ($y = 12.9x$, R^2=84.3% for glucose fermentation, $y = 15.5x$, R^2=94.8% for xylose fermentation). Therefore, it is not necessary to optimize the concentration on metabolic perturbation concentration on both butanol production and sugar consumption. Since no kinetics study was conducted, measurements of acid concentration and cell density in the final broth were not beneficial to the optimization studies. Consequently, acid concentration and cell density in the optimization study were not analyzed.

Table 4.4 is ANOVA and the regression model of butanol production in glucose feedstock. R-square of 91.48% and P-value of lack-of-fit (0.251) suggested that the proposed model fitted well with the experimental data. From the normal probability plot of standardized residual (Figure 4.8), neither skewness nor outlier were observed. The residuals were normally distributed from the histogram of residual. A removal of any term in this model led to substantial decrease of the adjusted R-squared, and skewness in the normal probability plot of residual. Upon failing to reveal potential violations of regression uniformity, we confirmed the regression model adequately described the relationship between control variables and the responses. Significant terms included first and second order terms of $ZnSO_4$ and furan derivatives, and also the interaction. Block was not significant.

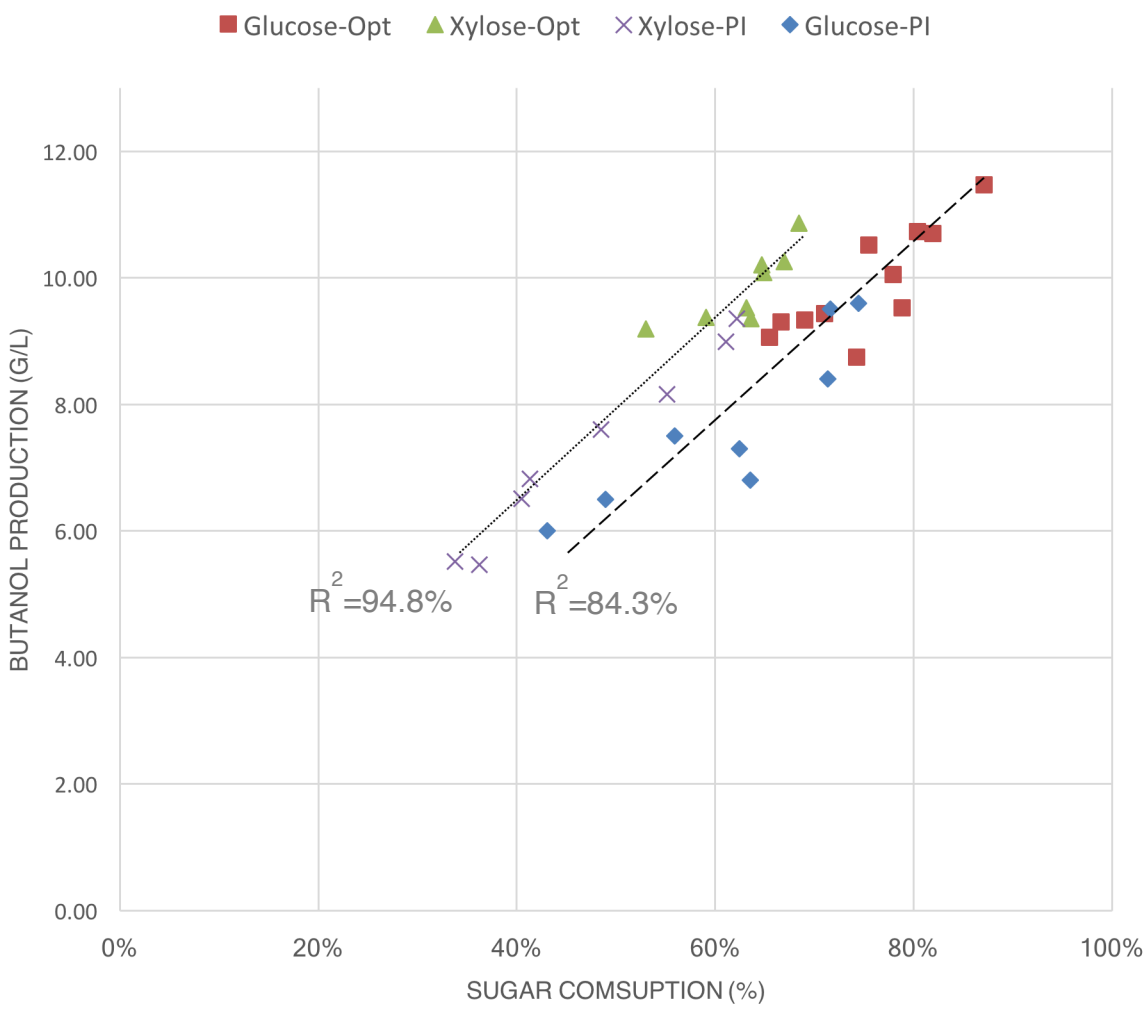


Figure 4.7 Relationship between butanol production and sugar consumption

Table 4.4 ANOVA and the regression model of butanol production in glucose feedstock

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	11.8229	1.97048	26.84	0.000
$ZnSO_4$	1	2.9282	2.92817	39.89	0.000
Furfural/HMF	1	5.4740	5.47403	74.57	0.000
Blocks	1	0.0007	0.00065	0.01	0.926
$(ZnSO_4)^2$	1	0.7989	0.79890	10.88	0.005
$(Furfural/HMF)^2$	1	2.3097	2.30975	31.46	0.000
$ZnSO_4$*Furfural/HMF	1	0.8911	0.89114	12.14	0.003
Error	15	1.1011	0.07341		
Lack-of-Fit	11	0.9369	0.08517	2.07	0.251
Pure Error	4	0.1643	0.04107		
Total	21	12.9240			

Regression Equation (Uncoded):

Block 1 $\text{Butanol (g/L)} = 11.688 - 1.126\ ZnSO_4 + 0.532\ (ZnSO_4)^2 - 2.202\ Furfural/HMF + 0.4019\ (Furfural/HMF)^2 + 0.445\ (ZnSO_4)*Furfural/HMF$

Block 2 $\text{Butanol (g/L)} = 11.677 - 1.126\ ZnSO_4 + 0.532\ (ZnSO_4)^2 - 2.202\ Furfural/HMF + 0.4019\ (Furfural/HMF)^2 + 0.445\ (ZnSO_4)*Furfural/HMF$

*DF: degree of freedom; SS: sum of squares; MS: the mean sum of squares

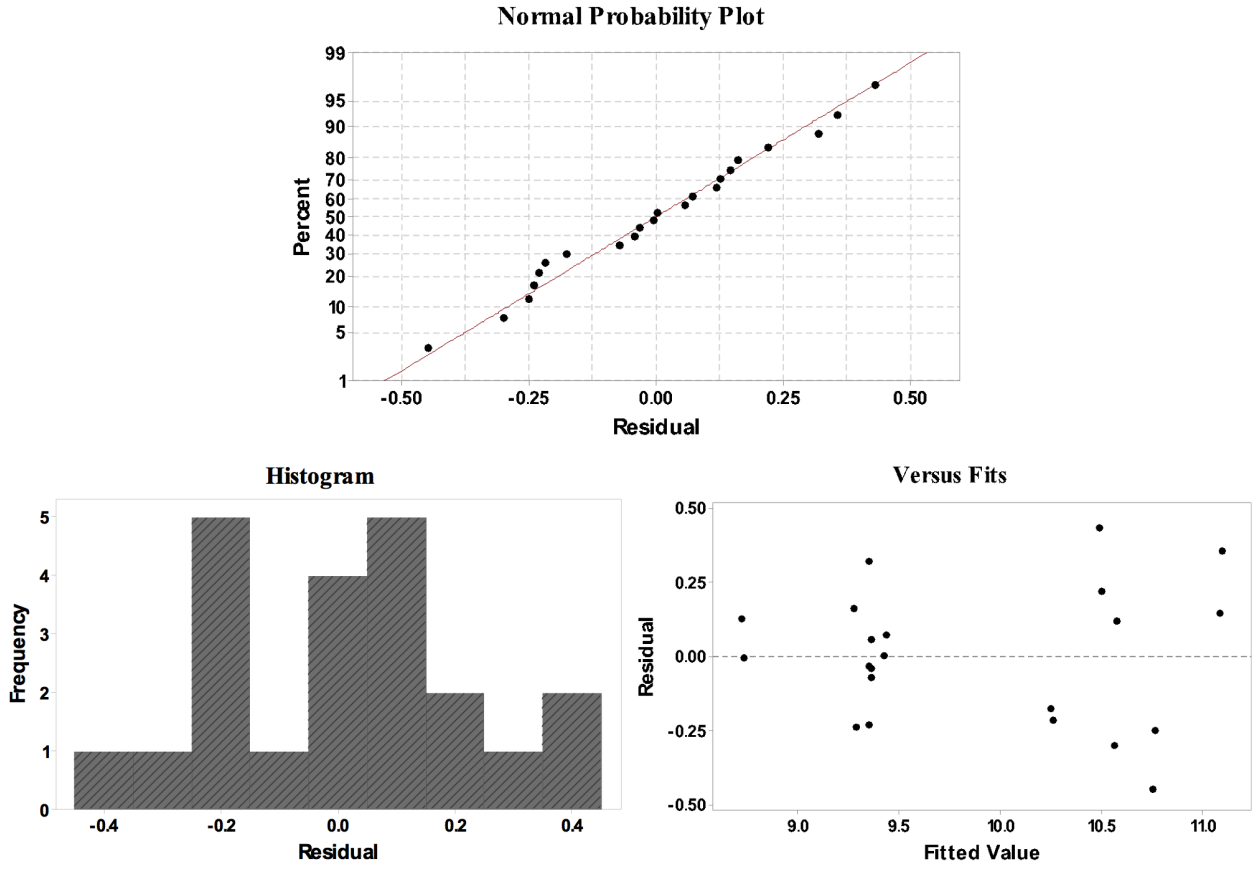


Figure 4.8 Residual plots of the regression model of biobutanol production in glucose feedstock (a) normal probability plot (b) histogram (c) residual versus fitted value

Table 4.5 ANOVA and the regression model of butanol production in xylose feedstock

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	351.642	58.607	192.55	0.000
$ZnSO_4$	1	0.734	0.734	2.41	0.144
Furfural/HMF	1	21.662	21.662	71.17	0.000
Blocks	1	3.872	3.872	12.72	0.003
$(ZnSO_4)^2$	1	0.808	0.808	2.66	0.127
$(ZnSO_4)^3$	1	0.810	0.810	2.66	0.127
$(Furfural/HMF)^3$	1	159.128	159.128	522.81	0.000
Error	13	3.957	0.304		
Lack-of-Fit	9	2.537	0.282	0.79	0.646
Pure Error	4	1.420	0.355		
Total	19				

Regression Equation (Uncoded):

Block 1 $\text{Butanol (g/L)} = 9.525 - 2.94\,(\text{ZnSO4}) + 2.766(\text{Furfural/HMF}) + 3.85\left(\text{ZnSO4}\right)^2 - 1.227(\text{ZnSO4}) - 1.273\,(\text{ZnSO4})^3 - 0.9942\,(\text{Furfural/HMF})^3$

Block 2 $\text{Butanol (g/L)} = 10.405 - 2.94\,(\text{ZnSO4}) + 2.766(\text{Furfural/HMF}) + 3.85\left(\text{ZnSO4}\right)^2 - 1.227(\text{ZnSO4}) - 1.273\,(\text{ZnSO4})^3 - 0.9942\,(\text{Furfural/HMF})^3$

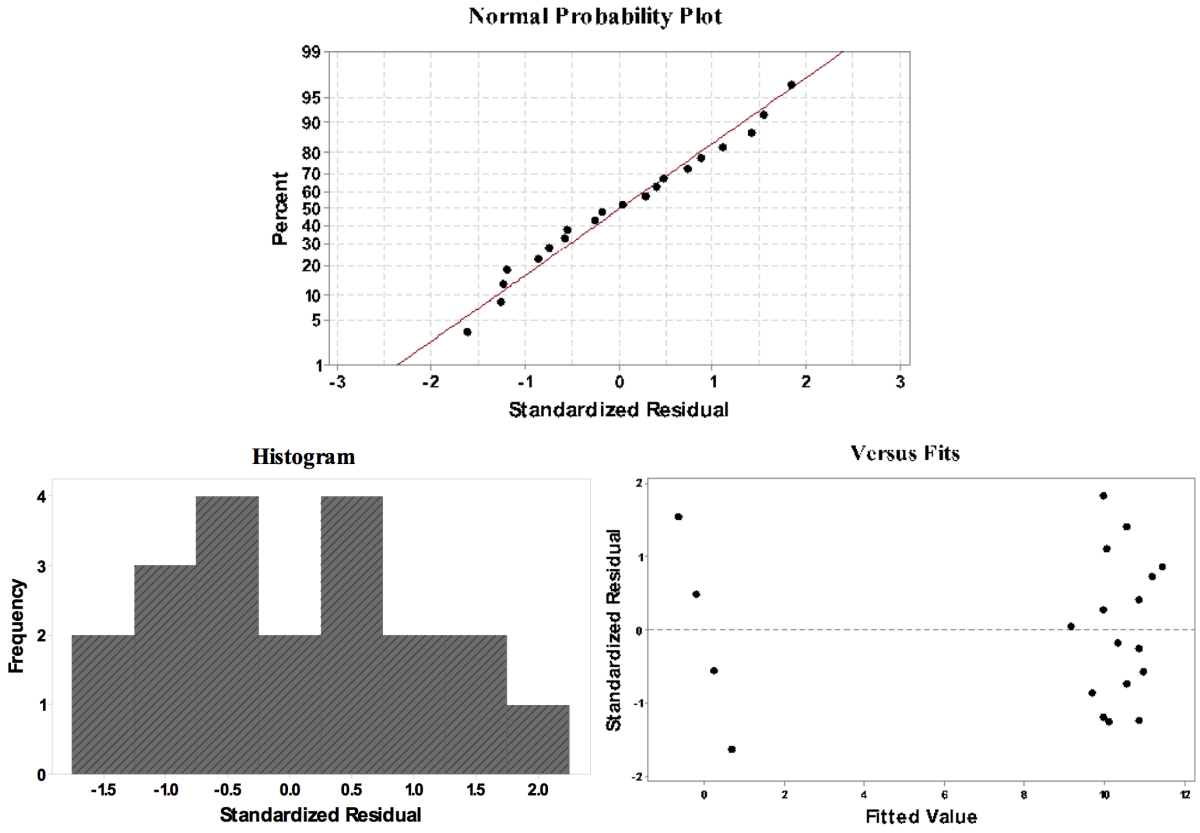


Figure 4.9 Residual plots of the regression model of biobutanol production in xylose feedstock (a) normal probability plot (b) histogram (c) residual versus fitted value.

In xylose fermentation, responses widely ranged 0 ~ 12 g/L due to zero butanol concentration at high furan derivatives concentration. Wide range of the response induced large variance in the quadratic regression, albeit the regression result held high R-square and insignificant lack-of-fit (data not shown). A higher order (n=3) regression was used instead. To use cubic order regression, it is required that m $\geq \frac{(n+1)(n+2)}{2} + n$ (m: number of data points; n: number of variables) (Davim, 2012). In current study, eight data points (except run 7 and 18) were enough to perform the regression model ($8 \geq \frac{(2+1)(2+2)}{2} + 2 = 8$). It is important to avoid overfitting in high order regression. Regression terms were included in the model stepwise, until the adjusted R-square was maximized, and skewness of the normal probability plot was eliminated. During the stepwise regression, all interaction terms such as $ZnSO_4$*Furfural/HMF, ($ZnSO_4$)

^{2}*Furfural/HMF, $ZnSO_4$*(Furfural/HMF)^2 and 2nd order term (furfural/HMF)^2 were eliminated. Table 4.5 is ANOVA and the regression model of butanol production in xylose feedstock. R-square and p-value of lack-of-fit were 98.89% and 0.646, respectively. Neither skewness nor outlier were observed in the normal probability plot and histogram of residual (Figure 4.9). Therefore, the reliability and uniformity of model were confirmed. The terms remained in the model include 1st and 3rd term of furfural/HMF, which was dominating terms in the model (with F-value of 71.17 and 522.81). None of zinc sulphate terms were significant, but removal of any of them led to a severe misfit of regression, a severe skewness in residual probability plot and a slump of adjusted r-square. Therefore, all zinc sulphate terms remained in the model. At last, the block was significant. Butanol production in block2 was ~0.9 g/L higher than that in block1 as regression equation shows.

The effects of control variables in glucose and xylose fermentation were examined. According to the model in Table 4.6, in glucose fermentation, coefficients of first order and second order terms of zinc sulphate were -1.126 and 0.532, respectively. Correspondingly, the effect of zinc sulphate is shown in Figure 4.10 (bottom, left). Butanol production improved with the increase of zinc sulphate concentration. In furfural/HMF concentration, the coefficient of first and second order terms were -2.202 and 0.4019, respectively. The corresponding effect is shown in Figure 4.10 (bottom, right). Butanol production was nearly monotonically decreasing with the increase of furfural/HMF concentrations. However, the decrease of butanol production plateaued at furfural/HMF concentration of more than 2.56 g/L. By averaging the experimental values under same levels of control variable, experimental effect plots are illustrated (Figure 4.10 bottom). The only inconformity, which was the convex at the lower zinc concentration in the regression model, was ascribed to different methods. To summarize, in glucose fermentation, the effects of $ZnSO_4$ and furan derivatives on butanol production were stimulant and inhibitory, respectively, and the effects strengthened with increasing concentrations of the supplements.

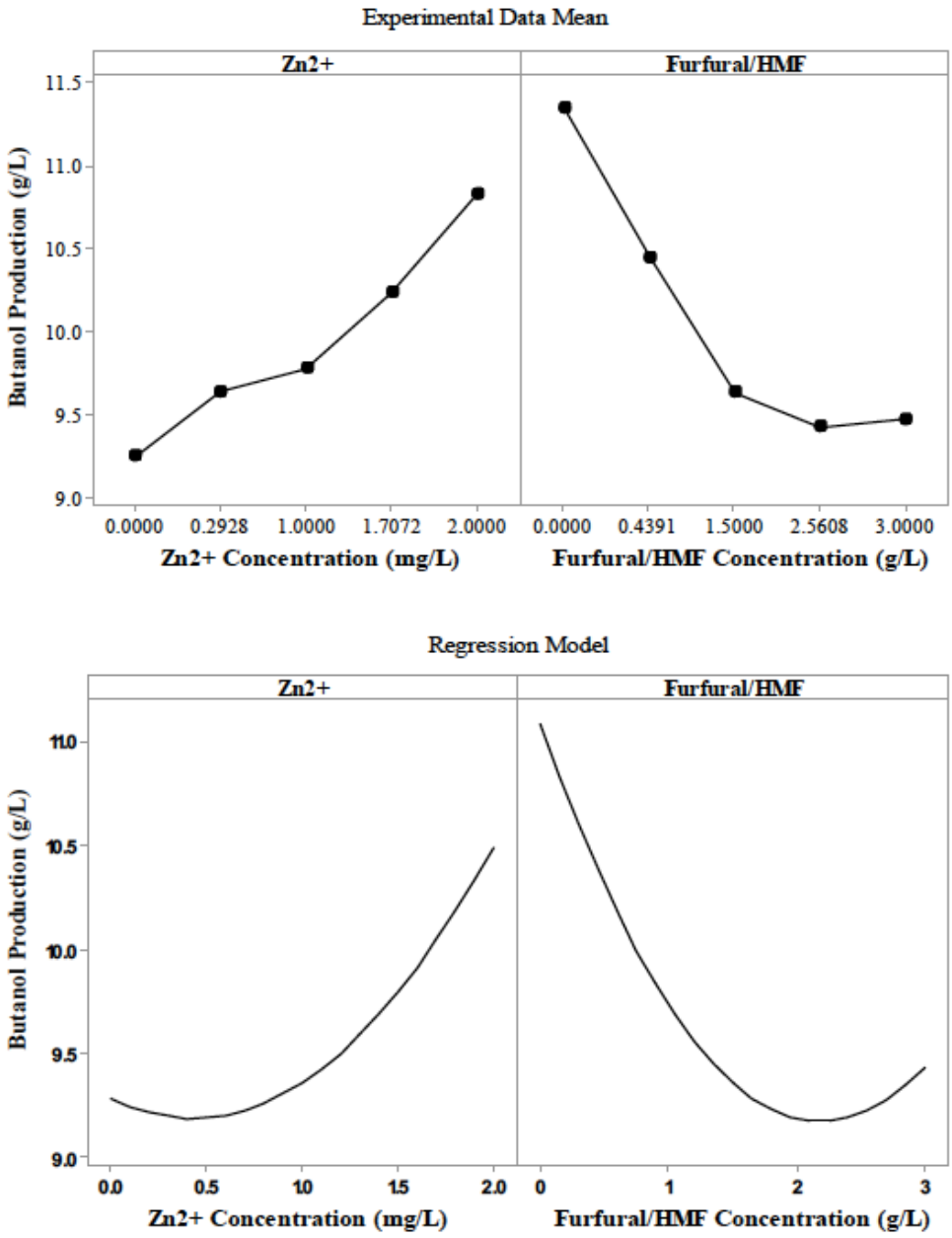


Figure 4.10 Individual effects of $ZnSO_4$ and furfural/HMF on butanol production in glucose feedstock

The interaction effect between zinc sulphate and furfural/HMF was significant according to the regression model. The coefficient of this interaction term was +0.445. In Figure 4.11, the blue line represented the butanol production without the supplement of furfural/HMF, and the corresponding butanol production ranged cal. 11.5~11.0g/L. The interaction effect was zero since furfural concentration was absent. When furfural dosage was 1.5g/L, butanol titer dropped to less than 9.4g/L from 11.0g/L. But with

the supplement of zinc sulphate, butanol production was partially recovered from 9.4 g/L to 10.5 g/L. Finally, when furfural concentration was increased to 3 g/L, butanol production was slump to 8.7g/L. Butanol production was recovered as cal. 11.0 g/L when zinc sulphate was supplied. The amount of recovery is the measurement of the interaction terms, which was calculated 2.19 g/L. Practically, the interaction term meant that the supplement of zinc sulphate was not only improved butanol production, but also recovered butanol production loss due to furan inhibitors. The recovered butanol production loss increased with the increasing of either zinc or furan derivatives concentration.

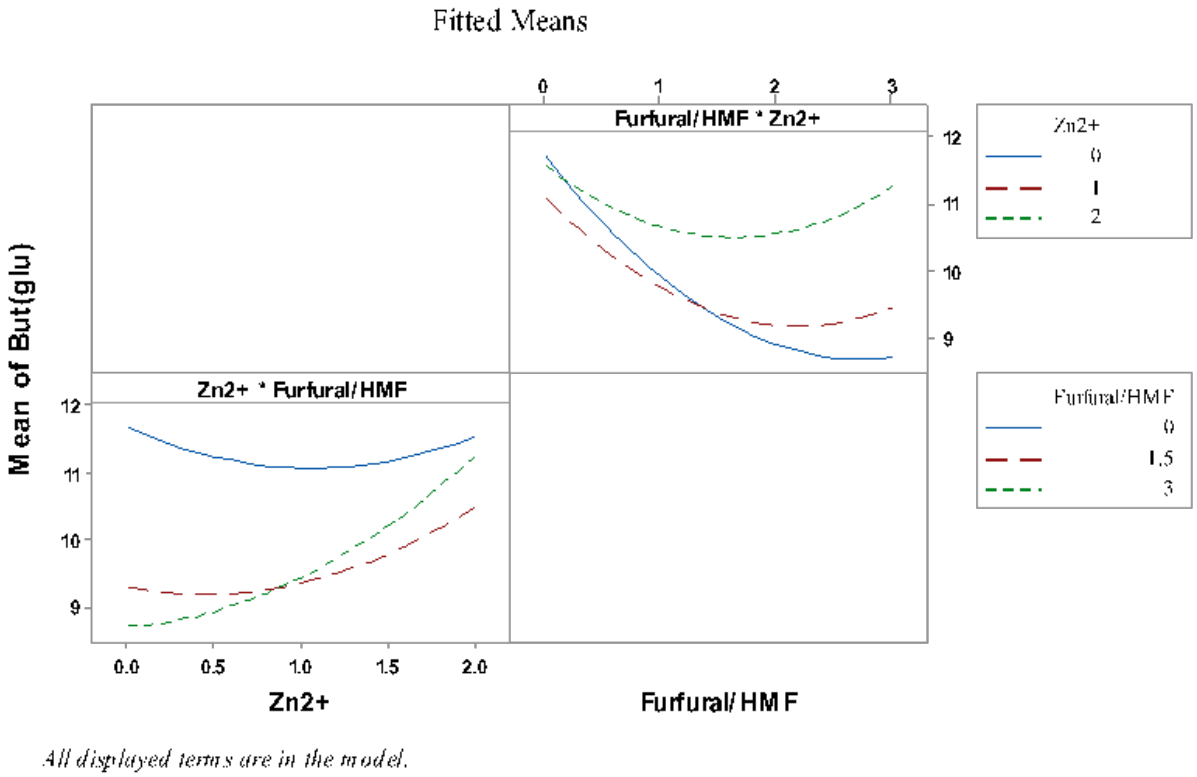


Figure 4.11 Interaction effect of $ZnSO_4$*furfural/HMF for butanol production in glucose feedstock

For butanol production in xylose feedstock, the effect of zinc sulphate was not evaluated since we cannot access it at high furan derivatives (butanol production = 0g/L). However, through point-to-point comparison, we found some tendencies on the zinc sulphate supplement: that is, zinc sulphate was a simulant to butanol

production when concentration of furan derivatives was as low as 0.44 g/L (coded -1). Zinc sulphate was unable to improve butanol production at 1.5g/L furan derivatives.

The function of butanol production furfural/HMF concentration is parabolic. As the furfural/HMF concentration increase, the stimulatory effect gradually faded and turned into inhibitory beyond 1.5 g/L of furfural/HMF. Experimental effect plot is in agreement with modeled main effect plot (Figure 4.12).

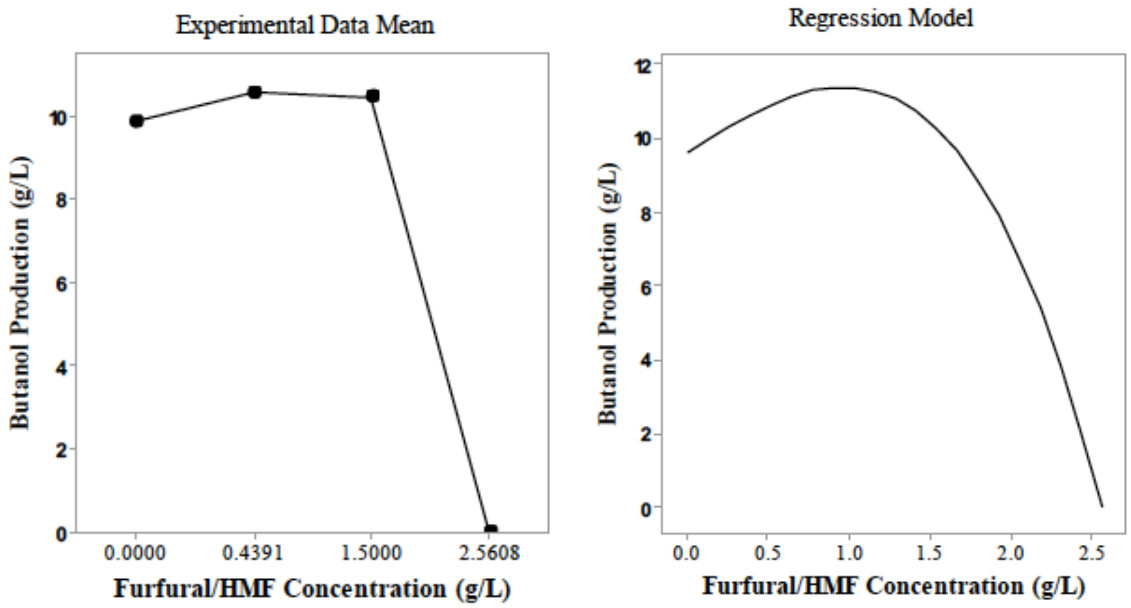


Figure 4.12 Individual effect of furfural/HMF on butanol production in xylose feedstock

In summary, furan derivatives were inhibitory to glucose fermentation whereas partially stimulated to xylose fermentation. Exclusively stimulant effect of furan derivatives on xylose fermentation wasn't reported in previous study. Gorsich et al., (2006) elucidated for *Saccharomyces cerevisiae*, furfural stress induced the overexpression of ZWF1, GND1, TKL1, RPEI genes. ZWFI is Pentose Phosphate Pathway activity dependent (or NADPH dependent). Therefore, the furfural detoxification was linked to NADPH regeneration in xylose glycolysis. For *clostridium beijerinckii,* the transcriptional analysis (Zhang and Ezeji, 2013) demonstrated in detoxification of furfural, key enzymes were the aldo/keto reducatase (AKR) and the short chain dehydrogenase (ADH). AKR family is solely NADPH-dependent protein (Bohren et al.,

1989). A large subset of short chain ADH are solely NADPH dependent in *Escherichia coli* (Miller et al., 2009), partially NADPH dependent in *saccharomyces cerevisiae* (Lewis Liu et al., 2008). Under furfural stress, NADPH dependent genes were overexpressed. Consequently, xylose glycolysis was stimulated, and solventogensis was observed (Gorsich et al., 2006). On the other hand, the cell used the extra NADH to produce more solvent like butanol (Wahlbom and Hahn-Hägerdal, 2002). Moreover, reductive cofactors preference of detoxification could be inhibitory concentration-dependent. Heer et al., (2009) showed at lower concentrations of furfural 6mM (≈0.6g/L), NADH-dependent ox-reductases are the main defense mechanism. At furfural concentrations above 15 mM (≈1.5g/L), transcript analysis demonstrated that the NADPH-generating flux through the pentose phosphate pathway increases and that NADPH-dependent ox-reductases become the major resistance mechanism. At higher concentration of furfural, NADH supply no longer suffice to detoxify furfural. As a result, PPP activity was enhanced as a second mechanism to deliver NADPH as extra reductive power. Reported stimulant furfural concentrations were ~1.1g/L furfural (Heer et al., 2009), and 0.5~1.0 g/L furfural (Ezeji, 2007), which were comparable to 0.5~1.5g/L furan derivative (0.3~1.12 g/L furfural) in our study.

In the current study, microgram dosage of zinc sulphate was beneficial to butanol production as interpreted in a previous study (Wu et al., 2015). It was due to zinc sulphate overexpressed certain coenzymes that related to enhanced glycolysis, solvent production and butanol tolerance. We found butanol production was proportional to zinc sulphate dosage up to 2mg/L, which would be further increased. More importantly, for the first time, the zinc sulphate supplement was found to mitigate the stress of furan derivatives and recover butanol production in glucose feedstock. Li et al., (2011) showed the Zn-dependent dehydrogenase quickly detoxified 18mM (1.73 g/L) furfural to 3mM (0.29 g/L) in 40 minutes. Kang et al., (2012) illustrated that furfural reducing enzyme, Zn-dependent alcohol dehydrogenase from Cuprivadiduas necator JMP134, was exclusively NADH dependent. The cofactor NADH is produced in glucose glycolysis, hence the zinc dependent furfural detoxification is expected to be strengthened in glucose feedstock. Consequently, furfural stress was alleviated and butanol production loss was recovered.

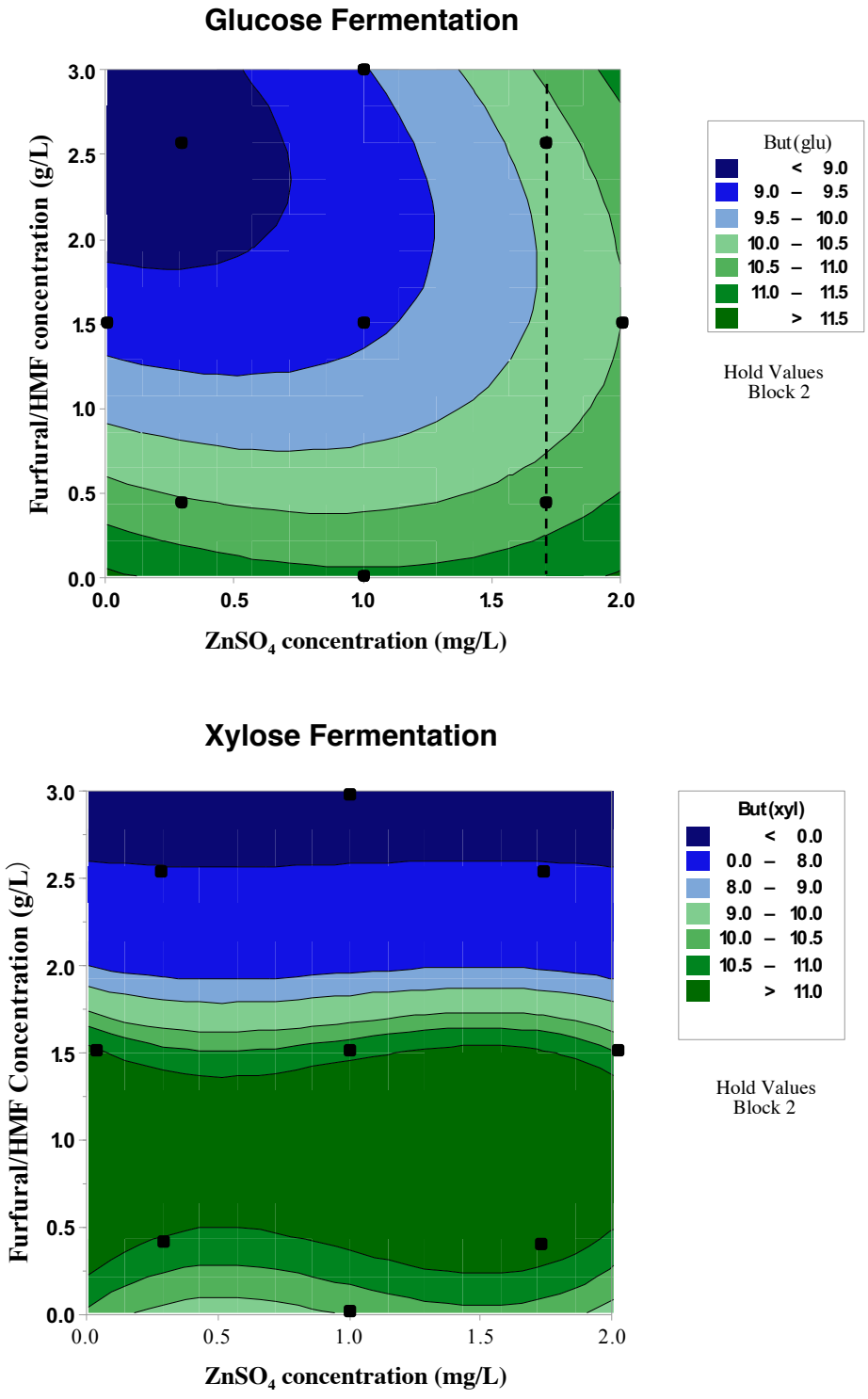


Figure 4.13 Contour plots of butanol production in glucose and xylose feedstock

Generally, $ZnSO_4$ and furan derivative were found effective in improving butanol production, which are related to cofactor generations. It's remarkable that $CaCO_3$ 4 g/L was a constant condition applied in all 44 experimental runs in optimization so the effects of $ZnSO_4$ and furfural we discussed above are based on the prerequisite of 4 g/L $CaCO_3$ supplement.

Figure 4.13 is the contour plots of regression models in glucose and xylose fermentation, separately. They facilitate straightforward optimization of perturbation concentrations and prediction of butanol production. For glucose fermentation, the butanol production raised with the increase of zinc sulphate concentration while decreased with the increase of furan derivative concentration. When furan derivative concentration exceeded 1.5 g/L, butanol production was down to 9.0 g/L. However, furan derivatives inhibition was not a concern when ≥1.7 mg/L $ZnSO_4$ was supplied – to the right of the reference line of $ZnSO_4$ concentration = 1.7 mg/L, butanol production was constantly more than 10g/L. For xylose fermentation, control of furan derivatives concentration was critical to butanol production. Increasing furan derivatives concentration to 2.5 g/L led to complete cell death, which should be avoid. When the concentration of furan derivatives was regulated to 0.5~1.5 g/L, butanol production was no less than 10.5 g/L. These findings were confirmed by the experimental data in Table 4.2 and Table 4.3.

Therefore, to perform a productive fermentation, $ZnSO_4$ supplement should be 2 mg/L (1.7 mg/L was the lower limit) in glucose fermentation. $ZnSO_4$ supplement was not a limiting factor to xylose fermentation. At 2 mg/L $ZnSO_4$ concentration, clostridium could maintain 10 g/L butanol production, regardless of the concentration of furan derivatives. The concentration of furan derivatives was to be controlled at 0.5~1.5 g/L in xylose fermentation and predicted butanol production was more than 10.5 g/L. Therefore, to an arbitrary feedstock containing 60 g/L total sugar regardless of the mixing ratio of glucose and xylose, a butanol production of no less than 10 g/L was expected, when 2 mg/L $ZnSO_4$ and 0.5~1.5 g/L furfural/HMF, and 4 g/L $CaCO_3$ were contained in the feedstock (Table 4.6).

Table 4.6 Optimal concentrations of chemical additives for butanol production in glucose, xylose, glucose/xylose feedstocks

Chemical Additives	Optimum Concentrations		
	Glucose (60 g/L)	Xylose (60 g/L)	Glucose/Xylose (60 g/L)
$ZnSO_4$ (mg/L)	2	not limiting	2
furfural/HMF (g/L)	not limiting	0.5~1.5	0.5~1.5
Predicted butanol production	≥10.0 g/L	≥10.5 g/L	≥10.0 g/L

* $CaCO_3$ of 4 g/L was supplied in all cases.

In preliminary investigations and butanol production optimization, we substantially improved butanol production in pure sugar feedstocks by supplying metabolic perturbations. Table 4.7 is the comparison of butanol productions in pure xylose and glucose feedstock in current studies. Without any chemical additives, *Clostridium Beijerinckii* (BA101) produced less butanol in xylose feedstock than in glucose feedstock. The ratio of butanol production in xylose feedstock to in glucose feedstock was 0.75 in the current study. Since $CaCO_3$, $ZnSO_4$ and furan additives were gradually introduced, butanol productions in both glucose and xylose feedstock were enhanced stepwise. Highest butanol productions in glucose and xylose feedstock were 57% and 109% above those of the controls, respectively. Xylose-fed clostridium produced more butanol, with a ratio of 1.15. In general, through tailoring metabolic perturbation, xylose was no longer an unfavorable sugar for clostridium.

Table 4.7 Comparisons of butanol productions in glucose and xylose feedstocks

Strain	Chemical Additive	Sugar (60g/L)	Butanol (g/L)	Ratio (xyl:glu)
C.beijerinckii (BA101)	0	glucose	7.50	0.74
		xylose	5.51	
	$CaCO_3$ 2g/L	glucose	9.47	0.95
		xylose	8.98	
	$ZnSO_4$ 1 mg/L $CaCO_3$ 4 g/L	glucose	11.09	0.91
		xylose	10.12	
	$ZnSO_4$ 1.7mg/L $CaCO_3$ 4g/L Furan 0.4g/L	glucose	10.30	1.15
		xylose	11.82	

*all data in present study were from block 2.

4.3 Optimum Biobutanol Production Using Lignocellulosic Feedstock

Finally, metabolic perturbation optimums ($CaCO_3$ 4g/L, $ZnSO_4$ 2mg/L, furan derivatives 0.5~1.5g/L) were tested in lignocellulosic feedstocks. In order to generate furan derivatives 0.5~1.5g/L in wheat straw hydrolysate (WSH), proper experimental parameters were used as described in experimental part. Furan derivatives yield of wheat straw are predictable and controllable based on the model of acid concentration, pretreatment time, and temperature etc (Guerra-Rodríguez et al., 2012). Spruce was treated at the same condition for spruce hydrolysate (SH).

Table 4.8 Results of optimum biobutanol production using lignocellulosic feedstock

Run Order	Hydrolysate	Pre-grown medium for inoculated strain	Chemical additive in lignocellulosic hydrolysate	Sugar consumption (%)	Butanol production (g/L)
1	WSH	P2	0	0	0
2	WSH	P2+ $CaCO_3$ +$ZnSO_4$	0	60.0±0.9	9.31±0.09
3	WSH	P2+ $CaCO_3$ +$ZnSO_4$	$CaCO_3$ +$ZnSO_4$	75.3±1.7	10.11±0.18
4	SH	P2	0	0	0
5	SH	P2+ $CaCO_3$ +$ZnSO_4$	0	54.6±1.6	7.45±0.43
6	SH	P2+ $CaCO_3$ +$ZnSO_4$	$CaCO_3$ +$ZnSO_4$	70.2±2.1	8.94±0.39

* $CaCO_3$ and $ZnSO_4$. concentration were 4g/L and 2mg/L, respectively

Table 4.8 lists sugar consumption, butanol production in the lignocellulosic hydrolysate fermentation. For both WSH and SH, total sugar and glucose concentration were adjusted to 60g/L and 27g/L. We found inoculated strains which cultured in P2 medium were still unable to commence the fermentation. By contrast, inoculated strains pre-grown in P2 with $CaCO_3$ 4g/L and $ZnSO_4$ 2mg/L supplements initiated the fermentation, and produced butanol of 9.31g/L and 7.45g/L in wheat straw hydrolysate and spruce hydrolysate, respectively. Butanol production and sugar consumption were further enhanced by suppling $CaCO_3$ and $ZnSO_4$ in lignocellulosic hydrolysates: 10.11g/L and 8.94g/L butanol were harvested in WSH and SH, respectively.

The result in WSH was consistent with this prediction in the optimization. According to optimization, in the presence of 0.5~1.5g/L furan derivative, 2 mg/L $ZnSO_4$ and 4g/L $CaCO_3$, the butanol production in 60g/L sugar feedstock would be no less than 10g/L, regardless of mixing ratio of glucose and xylose. The consistence of butanol productions between pure sugar and lignocellulosic feedstock suggested dominant stimulant and inhibitory effects in wheat straw hydrolysate could be ascribed to furan derivate, which was well illustrated and explained in the optimization. For SH, butanol production was significantly

lower than that of WSH. Furfural and HMF concentrations in WSH and SH are typically different. In WSH furfural is dominant form of furan derivative (furfural: HMF = 3:1). In SH, overall furan derivatives concentration is higher and HMF is dominant furan derivatives (Larsson et al., 1999) (Du et al., 2010). According to studies (Gorsich et al., 2006) (Ezeji, 2007), HMF is more toxic than furfural. Therefore, clostridium was more inhibited in SH than it was in WSH. Moreover, softwood like SH contained higher lignin than agricultural lignocellulosic. Therefore, more phenolic compound and aliphatic compound released from lignin were contained in SH than WSH (Larsson et al., 1999).

Failing to start a fermentation in lignocellulosic hydrolysate with a high feedstock loading (~10% w/v or more) is very common (Qureshi et al., 2014). Microbiological inhibitors including furan derivatives, weak acid (levulinic acid, formic acid), phenolic compound (ferulic acid) and high sodium content introduced at pH neutralization could severely hamper the cell growth (Zhao et al., 2016). In current study, *Cb* (BA101) cannot grow and commence ABE fermentation, unless $CaCO_3$ and $ZnSO_4$ were supplied in the pre-grown culture. This indicated that these metabolic perturbations strengthened the tolerance by the strains to microbial inhibitors in hydrolysate. Moreover, butanol production was further increased to more than 10 g/L in wheat straw hydrolysate, also exhibiting the abilities of metabolic perturbations to boost butanol production.

In Table 4.9, we compared ABE fermentation in lignocellulosic feedstocks in certain studies. The total sugar of different feedstock hydrolysate in these studies were mostly 50~60g/L and there was unconsumed sugar in the residuals after fermentation, therefore total sugar amount was not a limiting factor to butanol production in these studies. Without the inhibitor removal ("untreated"), butanol productions were 25~100% less than their controls (glucose 60g/L). For example, *Cb* (P260) was not able to generate solvents in corn stove hydrolysate when the hydrolysate was not post-treated (Qureshi et al., 2014). After post-treatments such as $Ca(OH)_2$ overliming, NaOH overliming, ion-exchanger and active carbon, butanol productions were better than that of untreated unanimously.

Table 4.9 Comparisons of butanol productions in lignocellulosic feedstock

Strains	Substrate	Post-treatment of lignocellulosic feedstock	Experiment	Total sugar/glucose (g/L)	Butanol (g/L)	Improvement over control	Ref.
C. beijerinckii (NCIMB 8052)	corncob	$Ca(OH)_2$ overliming	control	60/60	9.4		(Zhang et al., 2012)
			untreated	50/35	5.6	-40%	
			treated	60/45	8.2	-12.80%	
C. beijerinckii (BA101)	corn fiber	XAD-4 inhibitor resin remover	control	55/55	13.2		(Qureshi et al., 2008)
			untreated	29.8 /4.4	1.0	-92%	
			treated	54.3/22.4	6.4	-52%	
C. beijerinckii (CC101)	wood pulping hydrolysate	resin	control	-	10.6		(Lu et al., 2013)
			untreated	62/12.0	4.4	-58%	
			treated	65/23	9.1	-14%	
C. acetobutylicum (ATCC 824)	corn stove	alkaline twin-screw extrusion	control	42.2/26.7	7.0		(Zhang et al.,
			treated	42/42	7.1	1%	
C. beijerinckii (P260)	corn stove	NaOH overliming	control		13.2		(Qureshi et al., 2014)
			untreated		0	-100%	
			treated		9.0	-32%	
C. beijerinckii (IB4)	corn stove	active carbon	control	55/4.7	9.1		(Guo et al., 2012)
			untreated		6.8	-25%	
			treated		7.2	-21%	
C. beijerinckii (BA101)	Wheat straw/spruce hydrolysate	metabolic perturbation	control	60/60	7.5		(Present study)
			untreated	60/27	0	-100%	
			WSH	60/27	10.1	+37%	
			SH	60/27	8.9	+19%	

However, butanol productions in treated hydrolysate were still lower than the controls (60g/L glucose feedstock). Take $Ca(OH)_2$ overliming treated corncob hydrolysate (Zhang et al., 2012) for example, *C. beijerinckii* (NCIMB 8052) generated 12.80% less butanol than control. One exception was Zhang's work (Zhang et al., 2014). They showed after alkaline twin-screw extrusion treatment, butanol production in corn stove feedstock was 1% than that of control. By contrast, in current study, butanol production in wheat straw hydrolysate and spruce hydrolysate were 10.1 and 8.9 g/L respectively, which were 37% and 19% higher than the control. 10.1 g/L surpassed many of butanol production in previous studies. Results showed metabolic perturbation was highly effective in both pure sugar and lignocellulosic feedstock.

Chemical additives supplement is completely different from $Ca(OH)_2$ overliming in terms of mechanism. Over-liming directly removes microbial inhibitors like furan derivatives by chemical reactions at high pH (optimal 10). pH is still weak acidic after the supplement of $CaCO_3$ and $ZnSO_4$ thus a direct inhibitor removal like $Ca(OH)_2$ overliming is inaccessible. Granular adsorption is unable to remove any furan derivatives which has been proved in previous study (Martinez et al., 2000). Therefore, the supplement of metabolic perturbations would not affect furan derivatives concentration directly. Metabolic perturbations are influencing ABE fermentation in lignocellulosic feedstock hydrolysate through strengthening strain tolerance to microbial inhibitors. In terms of economics, chemical additives method showed superior over over-liming. To conduct a typical overliming, excess lime 25.9 g/L was added into feedstock hydrolysate to make medium pH = 10, followed by adding 1 g/L Na_2SO_3, and pH neutralization with H_2SO_4 (Qureshi et al., 2010). The chemical consumption and operation cost is high, and drawback like large amount of solid waste and sugar loss up to 13% were also concerned. Instead, to perform metabolic perturbations method, neutralization is only conducted once, and cheap chemicals like $CaCO_3$ of 4 g/L and $ZnSO_4$ of 2 mg/L barely requires extract spending.

The benefit of using metabolic perturbation method in lignocellulosic hydrolysate is not limited as above. In most studies listed in Table 4.9, insoluble solid (slurry) in lignocellulosic biomass hydrolysate were removed by centrifugation or/and Millipore filters before incubation, since the slurry contains plenty of inhibitors (Parreiras et al., 2014). In our lignocellulosic feedstock fermentation, we didn't remove the

slurry in the hydrolysates. This operation is related to a fast-developing subject in biofuel study called "Simultaneous saccharification fermentation" (SSF). In SSF, enzymatic hydrolysis of soluble and insoluble in hydrolysate are performed together with the fermentation. SSF avoids sugar loss due to the slurry separation, and decreases the number of vessels and steps by combinations of hydrolysis and fermentation simultaneously. The decrease in capital investment was estimated to be larger than 20% (Olofsson et al., 2008). The major variable to improve SSF efficiency is to increase solid loading ratio up to ~10%, which is limited by overall inhibitory levels. Interestingly, the loading ratio of our experiment was 10%. Our results suggested metabolic perturbations method could be applied in SSF.

Chapter 5 Conclusion

The objective of this study is to enhance bio-butanol production from lignocellulosic feedstock via supplements of metabolism perturbation. We developed a combination of metabolic perturbations that improves butanol productivity of *clostridium beijerinckii* in wheat straw hydrolysate and spruce hydrolysate. Zinc sulphate can increase butanol production and additionally alleviate butanol production loss due to furan derivatives. Furan derivatives affects butanol production differently in glucose and xylose feedstock, that is: furan derivatives at moderate concentration boost butanol production, while they are inhibitory to clostridium in glucose feedstock. Metabolic perturbations of $CaCO_3$ 4 g/L and 2 mg/L $ZnSO_4$ added in pre-grown culture can strengthen the tolerance of inhibitory by clostridium and facilitate the growth of clostridium in lignocellulosic feedstocks such as wheat straw hydrolysate and spruce hydrolysate. According to previous transcriptional studies, we related the effects of metabolic perturbations on butanol production to NAD(P)H dependent gene in response to these chemical additives. The stepwise studies by tailoring metabolic perturbation differentiates the fermentability of pure sugar and lignocellulosic feedstocks. Lower butanol production of ~9 g/L from spruce hydrolysate suggests more inhibitory effects in spruce hydrolysate are to be exploited and overcame.

Chapter 6 Recommendations For Further Studies

Combinations of metabolic perturbation can be enriched with more diversity. Metallic compounds containing cofactors ions such as cobalt, coppers at micrograms levels are potential metabolic perturbations. It is expected that these metallic compounds could not only improve butanol production but mitigate inhibitory effects originated from lignocellulosic feedstock. Like furan derivatives, the effects of other common microbial inhibitors such as aliphatic and phenolic compounds in lignocellulosic feedstock can be also quantified. Carefully control on their concentration under the supplement of metabolic perturbation can turn these inhibitors into stimulant.

Metabolic perturbation method is easy to apply and used cooperatively with any existing technologies. Recombinant strains of ultra-high butanol production supplied with metabolic perturbations might produce more butanol. Combined with gas stripping, which is an in-situ operation that remove butanol from reactor, metabolic perturbation is expected to increase butanol production additionally.

The transcriptional study on the reaction of clostridium to metabolic perturbations are important, especially on why metabolic perturbations impact differently to glucose and xylose fermentations. The transcriptional study should also emphasis on how the metallic compounds alleviate inhibitory effects stemmed from lignocellulosic feedstock.

Appendices

Appendix A. Experimental data (Raw data)

Table A. 1 Butanol productions and cell densities of recovered strains

No. of agar plates	Cell Density (OD600)	Butanol Production (g/L)
1	2.50	5.30
2	2.65	5.37
3	2.01	N.A
4	2.64	7.37
5	3.01	7.51

Table A. 2 Acid concentrations (preliminary investigations)

Run Order	Butyrate (g/L)			Acetate (g/L)		
	Day1	Day2	Day3	Day1	Day2	Day3
1	1.93	1.82	1.03	1.40	2.45	1.07
2	2.52	2.40	1.71	1.78	3.18	2.35
3	1.69	0.59	1.14	0.37	1.27	0.91
4	2.36	4.33	2.33	3.73	2.76	1.84
5	2.20	1.45	1.55	1.58	1.73	1.33
6	2.54	2.35	1.39	1.51	2.52	2.34
7	1.56	1.93	0.40	0.72	1.01	0.98
8	1.58	1.39	2.84	2.44	2.16	1.14
9	0.65	0.35	0.48	0.22	0.42	0.46
10	0.67	0.30	0.51	0.20	0.69	0.32
11	0.80	0.32	0.90	0.35	0.30	0.96
12	2.40	2.50	2.11	1.48	1.89	2.54
13	1.95	1.61	1.53	1.28	1.86	1.70
14	1.67	1.46	1.38	1.19	1.08	1.50
15	0.80	0.31	0.85	0.23	0.23	0.97
16	2.07	2.27	1.17	1.22	1.96	2.32

Table A. 3 Acetone, ethanol concentrations (preliminary investigations)

Run Order	Acetone (g/L)			Ethanol (g/L)		
	Day1	Day2	Day3	Day1	Day2	Day3
1	1.77	3.25	3.80	0.45	1.37	1.34
2	1.62	3.15	3.52	0.49	1.27	1.11
3	1.89	4.74	4.60	0.53	1.02	2.53
4	2.71	5.25	5.49	0.55	1.86	2.61
5	1.12	2.54	2.90	0.48	0.88	1.03
6	1.60	3.45	3.83	0.36	1.55	1.22
7	1.33	3.59	4.30	0.39	0.95	1.08
8	2.14	4.14	4.13	0.63	1.48	0.74
9	1.84	2.85	3.43	0.59	0.75	1.03
10	1.90	2.81	3.29	0.64	0.62	0.61
11	2.06	3.02	4.39	0.64	0.89	2.04
12	2.39	3.91	4.15	0.53	1.03	1.61
13	2.11	3.18	3.41	0.76	0.93	1.02
14	2.08	3.39	4.15	0.56	0.83	1.61
15	2.41	4.18	4.82	0.43	1.95	2.26
16	1.90	3.81	3.97	0.50	0.99	1.65

Table A. 4 Sugar consumption (butanol production optimization, block1)

Glucose fermentation			Xylose fermentation		
Run	Sugar Consumption (%)	butanol (g/L)	Run	Sugar Consumption (%)	butanol (g/L)
glucose run1	80.5%	10.72	xylose run1	63.6%	9.35
glucose run2	78.0%	10.04	xylose run2	0.0%	0.00
glucose run3	75.5%	10.51	xylose run3	64.7%	10.20
glucose run4	71.1%	9.42	xylose run4	68.5%	10.86
glucose run5	69.1%	9.32	xylose run5	64.9%	10.09
glucose run6	66.7%	9.29	xylose run6	59.0%	9.38
glucose run7	78.9%	9.51	xylose run7	0.0%	0.00
glucose run8	87.2%	11.45	xylose run8	53.0%	9.19
glucose run9	82.0%	10.69	xylose run9	63.2%	9.52
glucose run10	74.3%	8.73	xylose run10	0.0%	0.00
glucose run11	65.5%	9.05	xylose run11	67.0%	10.25

Table A. 5 Butanol production (butanol production optimization)

Control Variables		Butanol production (g/L)				
Glucose fermentation						
$ZnSO_4$(mg/L)	Furfural /HMF(g/L)	Block1	Block2	Average	Standard deviation	Standard error
2.000	1.500	10.72	10.92	10.82	0.14	0.100
1.707	2.561	10.04	10.07	10.06	0.02	0.015
1.707	0.439	10.51	10.30	10.41	0.15	0.105
1.000	3.000	9.51	9.43	9.47	0.06	0.040
1.000	0.000	11.45	11.23	11.34	0.16	0.110
0.293	0.439	10.69	10.26	10.48	0.30	0.215
0.293	2.561	8.73	8.85	8.79	0.08	0.060
0.000	1.500	9.05	9.44	9.25	0.28	0.195
1.000	1.500	9.42	9.32	9.37	0.07	0.050
1.000	1.500	9.32	9.67	9.50	0.25	0.175
1.000	1.500	9.29	9.12	9.21	0.12	0.085
Xylose fermentation						
$ZnSO_4$(mg/L)	Furfural /HMF(g/L)	Block1	Block2	Average	Standard deviation	Standard error
2.000	1.500	9.35	11.10	10.23	1.24	0.875
1.707	2.561	0.00	0.00	0.00	0.00	0.000
1.707	0.439	10.20	11.82	11.01	1.15	0.810
1.000	3.000	0.00	0.00	0.00	0.00	0.000
1.000	0.000	9.19	10.54	9.87	0.95	0.675
0.293	0.439	9.52	10.71	10.12	0.84	0.595
0.293	2.561	0.00	0.00	0.00	0.00	0.000
0.000	1.500	10.25	11.48	10.87	0.87	0.615
1.000	1.500	10.86	10.72	10.79	0.10	0.070
1.000	1.500	10.10	11.04	10.57	0.66	0.470
1.000	1.500	9.38	10.24	9.81	0.61	0.430

Table A. 6 Sugar consumption and butanol production (lignocellulosic hydrolysate fermentation)

Sugar Consumption (%)					
Run Order	Block1	Block2	Average	Standard deviation	Standard error
1	0	0	0	0	0
2	60.9	59.1	60.0	1.3	0.9
3	77.0	73.6	75.3	2.4	1.7
4	0	0	0	0	0
5	56.2	53.0	54.6	2.3	1.6
6	68.1	72.3	70.2	3.0	2.1
Butanol Production (g/L)					
Run Order	Block1	Block2	Average	Standard deviation	Standard error
1	0	0	0	0	0
2	9.22	9.41	9.32	0.13	0.09
3	10.29	9.93	10.11	0.25	0.18
4	0	0	0	0	0
5	7.88	7.01	7.45	0.62	0.44
6	8.56	9.33	8.95	0.54	0.39

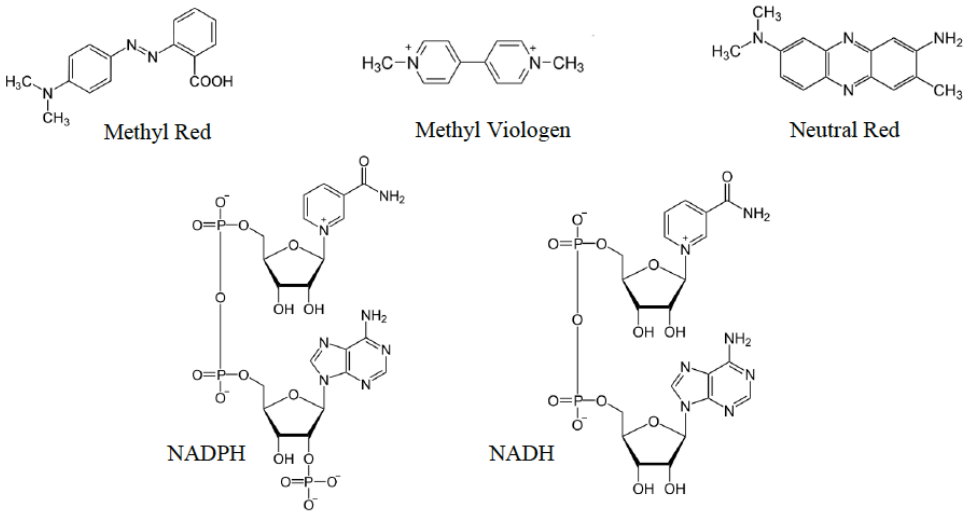


Figure A. 2 Molecular structures of typical electron carriers and azo-dye methyl red

Appendix B - ANOVA in Preliminary Investigation

Table B. 1 Effects of control variables in preliminary investigations (OD600, Day 1)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar	1	0.12250	0.12250	1.38	0.294
Furfural	1	0.20250	0.20250	2.28	0.192
CaCO3	1	0.72250	0.72250	8.12	0.036
MethylRed	1	0.16000	0.16000	1.80	0.238
Sugar*Furfural	1	0.09000	0.09000	1.01	0.361
Sugar*CaCO3	1	0.04000	0.04000	0.45	0.532
Sugar*MethylRed	1	0.12250	0.12250	1.38	0.294
Furfural*CaCO3	1	0.09000	0.09000	1.01	0.361
Furfural*MethylRed	1	0.02250	0.02250	0.25	0.636
CaCO3*MethylRed	1	0.20250	0.20250	2.28	0.192
Error	5	0.44500	0.08900		
Total	15	2.22000			

S	R-sq	R-sq(adj)
0.298329	79.95%	39.86%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	1.9000	0.0746	25.48	0.000
Sugar	0.0875	0.0746	1.17	0.294
Furfural	-0.1125	0.0746	-1.51	0.192
CaCO3	0.2125	0.0746	2.85	0.036
MethylRed	-0.1000	0.0746	-1.34	0.238
Sugar*Furfural	0.0750	0.0746	1.01	0.361
Sugar*CaCO3	-0.0500	0.0746	-0.67	0.532
Sugar*MethylRed	-0.0875	0.0746	-1.17	0.294
Furfural*CaCO3	-0.0750	0.0746	-1.01	0.361
Furfural*MethylRed	-0.0375	0.0746	-0.50	0.636
CaCO3*MethylRed	-0.1125	0.0746	-1.51	0.192

Table B. 2 Effects of control variables in preliminary investigations (OD600, Day 2)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sugar	1	1.50063	1.50063	30.86	0.003
Furfural	1	0.05063	0.05063	1.04	0.354
CaCO3	1	1.38062	1.38062	28.39	0.003
MethylRed	1	0.10563	0.10563	2.17	0.201
Sugar*Furfural	1	0.14062	0.14062	2.89	0.150
Sugar*CaCO3	1	0.22563	0.22563	4.64	0.084
Sugar*MethylRed	1	0.18062	0.18062	3.71	0.112
Furfural*CaCO3	1	0.05062	0.05062	1.04	0.354
Furfural*MethylRed	1	0.01563	0.01563	0.32	0.595
CaCO3*MethylRed	1	0.95062	0.95062	19.55	0.007
Error	5	0.24313	0.04863		
Total	15	4.84437			

S	R-sq	R-sq(adj)
0.220511	94.98%	84.94%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	2.3812	0.0551	43.20	0.000
Sugar	0.3063	0.0551	5.56	0.003
Furfural	-0.0563	0.0551	-1.02	0.354
CaCO3	0.2938	0.0551	5.33	0.003
MethylRed	-0.0813	0.0551	-1.47	0.201
Sugar*Furfural	0.0938	0.0551	1.70	0.150
Sugar*CaCO3	0.1187	0.0551	2.15	0.084
Sugar*MethylRed	-0.1062	0.0551	-1.93	0.112
Furfural*CaCO3	0.0562	0.0551	1.02	0.354
Furfural*MethylRed	0.0313	0.0551	0.57	0.595
CaCO3*MethylRed	-0.2437	0.0551	-4.42	0.007

Table B. 3 Effects of control variables in preliminary investigations (OD600, Day 3)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	3.16000	0.31600	3.85	0.075
Linear	4	2.55250	0.63812	7.78	0.022
Sugar	1	0.64000	0.64000	7.80	0.038
Furfural	1	0.09000	0.09000	1.10	0.343
CaCO3	1	1.82250	1.82250	22.23	0.005
MethylRed	1	0.00000	0.00000	0.00	1.000
Sugar*Furfural	1	0.04000	0.04000	0.49	0.516
Sugar*CaCO3	1	0.12250	0.12250	1.49	0.276
Sugar*MethylRed	1	0.01000	0.01000	0.12	0.741
Furfural*CaCO3	1	0.00250	0.00250	0.03	0.868
Furfural*MethylRed	1	0.01000	0.01000	0.12	0.741
CaCO3*MethylRed	1	0.42250	0.42250	5.15	0.072
Error	5	0.41000	0.08200		
Total	15	3.57000			

S	R-sq	R-sq(adj)
0.0575027	89.64%	80.57%

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value
Constant		2.2750	0.0716	31.78	0.000
Sugar	0.4000	0.2000	0.0716	2.79	0.038
Furfural	-0.1500	-0.0750	0.0716	-1.05	0.343
CaCO3	0.6750	0.3375	0.0716	4.71	0.005
MethylRed	0.0000	0.0000	0.0716	0.00	1.000
Sugar*Furfural	0.1000	0.0500	0.0716	0.70	0.516
Sugar*CaCO3	-0.1750	-0.0875	0.0716	-1.22	0.276
Sugar*MethylRed	-0.0500	-0.0250	0.0716	-0.35	0.741
Furfural*CaCO3	-0.0250	-0.0125	0.0716	-0.17	0.868
Furfural*MethylRed	-0.0500	-0.0250	0.0716	-0.35	0.741
CaCO3*MethylRed	-0.3250	-0.1625	0.0716	-2.27	0.072

Table B. 4 Effects of control variables in preliminary investigations (Sugar consumption, Day 1)

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	7	0.031354	0.031354	0.004479	4.15	0.032
Sugar	1	0.002954	0.002954	0.002954	2.74	0.137
Furfural	1	0.000403	0.000403	0.000403	0.37	0.558
CaCO3	1	0.020478	0.020478	0.020478	18.98	0.002
Methyl Red	1	0.000863	0.000863	0.000863	0.80	0.397
Sugar*Furfural	1	0.004558	0.004558	0.004558	4.22	0.074
Furfural*CaCO3	1	0.000287	0.000287	0.000287	0.27	0.620
CaCO3*Methyl Red	1	0.001812	0.001812	0.001812	1.68	0.231
Error	8	0.008633	0.008633	0.001079		
Total	15	0.039987				

S	R-sq	R-sq(adj)
0.0328495	78.41%	59.52%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	0.22679	0.00821	27.62	0.000
Sugar	0.01359	0.00821	1.65	0.137
Furfural	-0.00502	0.00821	-0.61	0.558
CaCO3	0.03577	0.00821	4.36	0.002
Methyl Red	-0.00734	0.00821	-0.89	0.397
Sugar*Furfural	0.01688	0.00821	2.06	0.074
Furfural*CaCO3	0.00424	0.00821	0.52	0.620
CaCO3*Methyl Red	-0.01064	0.00821	-1.30	0.231

Table B. 5 Effects of control variables in preliminary investigations (Sugar consumption, Day 2)

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	7	0.275239	0.275239	0.039320	10.75	0.002
Sugar	1	0.079325	0.079325	0.079325	21.68	0.002
Furfural	1	0.003865	0.003865	0.003865	1.06	0.334
CaCO3	1	0.167318	0.167318	0.167318	45.74	0.000
MethylRed	1	0.000164	0.000164	0.000164	0.04	0.838
Sugar*Furfural	1	0.005209	0.005209	0.005209	1.42	0.267
Sugar*MethylRed	1	0.000069	0.000069	0.000069	0.02	0.894
CaCO3*MethylRed	1	0.019289	0.019289	0.019289	5.27	0.051
Error	8	0.029267	0.029267	0.003658		
Total	15	0.304506				

S	R-sq	R-sq(adj)
0.0604842	90.39%	81.98%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	0.4874	0.0151	32.24	0.000
Sugar	0.0704	0.0151	4.66	0.002
Furfural	-0.0155	0.0151	-1.03	0.334
CaCO3	0.1023	0.0151	6.76	0.000
MethylRed	-0.0032	0.0151	-0.21	0.838
Sugar*Furfural	0.0180	0.0151	1.19	0.267
Sugar*MethylRed	-0.0021	0.0151	-0.14	0.894
CaCO3*MethylRed	-0.0347	0.0151	-2.30	0.051

Table B. 6 Effects of control variables in preliminary investigations (Sugar consumption, Day 3)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	3.16000	0.31600	3.85	0.075
Sugar	1	0.64000	0.64000	7.80	0.038
Furfural	1	0.09000	0.09000	1.10	0.343
CaCO3	1	1.82250	1.82250	22.23	0.005
MethylRed	1	0.00000	0.00000	0.00	1.000
Sugar*Furfural	1	0.04000	0.04000	0.49	0.516
Sugar*CaCO3	1	0.12250	0.12250	1.49	0.276
Sugar*MethylRed	1	0.01000	0.01000	0.12	0.741
Furfural*CaCO3	1	0.00250	0.00250	0.03	0.868
Furfural*MethylRed	1	0.01000	0.01000	0.12	0.741
CaCO3*MethylRed	1	0.42250	0.42250	5.15	0.072
Error	5	0.41000	0.08200		
Total	15	3.57000			

S	R-sq	R-sq(adj)
0.0575027	89.64%	80.57%

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value
Constant		2.2750	0.0716	31.78	0.000
Sugar	0.4000	0.2000	0.0716	2.79	0.038
Furfural	-0.1500	-0.0750	0.0716	-1.05	0.343
CaCO3	0.6750	0.3375	0.0716	4.71	0.005
MethylRed	0.0000	0.0000	0.0716	0.00	1.000
Sugar*Furfural	0.1000	0.0500	0.0716	0.70	0.516
Sugar*CaCO3	-0.1750	-0.0875	0.0716	-1.22	0.276
Sugar*MethylRed	-0.0500	-0.0250	0.0716	-0.35	0.741
Furfural*CaCO3	-0.0250	-0.0125	0.0716	-0.17	0.868
Furfural*MethylRed	-0.0500	-0.0250	0.0716	-0.35	0.741
CaCO3*MethylRed	-0.3250	-0.1625	0.0716	-2.27	0.072

Table B. 7 Effects of control variables in preliminary investigations (Butanol production, Day 1)

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	7	2.99111	2.99111	0.42730	2.29	0.135
Sugar	1	0.04372	0.04372	0.04372	0.23	0.641
Furfural	1	0.04307	0.04307	0.04307	0.23	0.644
CaCO3	1	1.80571	1.80571	1.80571	9.67	0.014
Methyl Red	1	0.32190	0.32190	0.32190	1.72	0.226
Sugar*Furfural	1	0.28418	0.28418	0.28418	1.52	0.252
Sugar*Methyl Red	1	0.15928	0.15928	0.15928	0.85	0.383
CaCO3*Methyl Red	1	0.33327	0.33327	0.33327	1.78	0.218
Error	8	1.49408	1.49408	0.18676		
Total	15	4.48519				

S	R-sq	R-sq(adj)
0.432157	66.69%	37.54%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	2.528	0.108	23.40	0.000
Sugar	0.052	0.108	0.48	0.641
Furfural	-0.052	0.108	-0.48	0.644
CaCO3	0.336	0.108	3.11	0.014
Methyl Red	-0.142	0.108	-1.31	0.226
Sugar*Furfural	0.133	0.108	1.23	0.252
Sugar*Methyl Red	-0.100	0.108	-0.92	0.383
CaCO3*Methyl Red	-0.144	0.108	-1.34	0.218

Table B. 8 Effects of control variables in preliminary investigations (Butanol production, Day 2)

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F-Value	P-Value
Regression	7	31.1979	31.1979	4.4568	5.72	0.013
Sugar	1	6.4141	6.4141	6.4141	8.24	0.021
Furfural	1	0.3133	0.3133	0.3133	0.40	0.544
CaCO3	1	19.1404	19.1404	19.1404	24.58	0.001
MethylRed	1	0.0251	0.0251	0.0251	0.03	0.862
Sugar*Furfural	1	0.1527	0.1527	0.1527	0.20	0.670
Sugar*MethylRed	1	3.9389	3.9389	3.9389	5.06	0.055
CaCO3*MethylRed	1	1.2135	1.2135	1.2135	1.56	0.247
Error	8	6.2286	6.2286	0.7786		
Total	15	37.4265				

S	R-sq	R-sq(adj)
0.882371	83.36%	68.80%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	6.557	0.221	29.72	0.000
Sugar	0.633	0.221	2.87	0.021
Furfural	-0.140	0.221	-0.63	0.544
CaCO3	1.094	0.221	4.96	0.001
MethylRed	-0.040	0.221	-0.18	0.862
Sugar*Furfural	0.098	0.221	0.44	0.670
Sugar*MethylRed	-0.496	0.221	-2.25	0.055
CaCO3*MethylRed	-0.275	0.221	-1.25	0.247

Table B. 9 Effects of control variables in preliminary investigations (Butanol production, Day 3)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	25.7960	2.5796	5.43	0.038
Sugar	1	0.6395	0.6395	1.35	0.298
Furfural	1	0.7990	0.7990	1.68	0.251
CaCO3	1	19.5901	19.5901	41.25	0.001
MethylRed	1	0.1167	0.1167	0.25	0.641
Sugar*Furfural	1	0.3306	0.3306	0.70	0.442
Sugar*CaCO3	1	0.2169	0.2169	0.46	0.529
Sugar*MethylRed	1	2.0380	2.0380	4.29	0.093
Furfural*CaCO3	1	0.0288	0.0288	0.06	0.815
Furfural*MethylRed	1	0.0541	0.0541	0.11	0.750
CaCO3*MethylRed	1	1.9823	1.9823	4.17	0.096
Error	5	2.3745	0.4749		
Total	15	28.1705			

S	R-sq	R-sq(adj)
0.689126	91.57%	74.71%

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value
Constant		7.500	0.172	43.53	0.000
Sugar	0.400	0.200	0.172	1.16	0.298
Furfural	-0.447	-0.223	0.172	-1.30	0.251
CaCO3	2.213	1.107	0.172	6.42	0.001
MethylRed	-0.171	-0.085	0.172	-0.50	0.641
Sugar*Furfural	0.287	0.144	0.172	0.83	0.442
Sugar*CaCO3	-0.233	-0.116	0.172	-0.68	0.529
Sugar*MethylRed	-0.714	-0.357	0.172	-2.07	0.093
Furfural*CaCO3	0.085	0.042	0.172	0.25	0.815
Furfural*MethylRed	0.116	0.058	0.172	0.34	0.750
CaCO3*MethylRed	-0.704	-0.352	0.172	-2.04	0.096

Table B. 10 Effects of control variables in preliminary investigations (Acid concentration, Day 1)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	10	6904.14	690.41	12.65	0.006
Sugar	1	2169.23	2169.23	39.74	0.001
Furfural	1	1890.08	1890.08	34.62	0.002
CaCO3	1	11.39	11.39	0.21	0.667
MethylRed	1	43.23	43.23	0.79	0.414
Sugar*Furfural	1	131.68	131.68	2.41	0.181
Sugar*CaCO3	1	41.28	41.28	0.76	0.424
Sugar*MethylRed	1	354.38	354.38	6.49	0.051
Furfural*CaCO3	1	1442.10	1442.10	26.42	0.004
Furfural*MethylRed	1	199.52	199.52	3.65	0.114
CaCO3*MethylRed	1	621.26	621.26	11.38	0.020
Error	5	272.96	54.59		
Total	15	7177.09			

S	R-sq	R-sq(adj)
7.38861	96.20%	88.59%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	40.57	1.85	21.96	0.000
Sugar	11.64	1.85	6.30	0.001
Furfural	10.87	1.85	5.88	0.002
CaCO3	0.84	1.85	0.46	0.667
MethylRed	1.64	1.85	0.89	0.414
Sugar*Furfural	2.87	1.85	1.55	0.181
Sugar*CaCO3	-1.61	1.85	-0.87	0.424
Sugar*MethylRed	-4.71	1.85	-2.55	0.051
Furfural*CaCO3	9.49	1.85	5.14	0.004
Furfural*MethylRed	-3.53	1.85	-1.91	0.114
CaCO3*MethylRed	-6.23	1.85	-3.37	0.020

Table B. 11 Effects of control variables in preliminary investigations (Acid concentration, Day 2)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	10	6561.51	656.15	10.98	0.008
Sugar	1	1230.26	1230.26	20.59	0.006
Furfural	1	2344.98	2344.98	39.24	0.002
CaCO3	1	218.30	218.30	3.65	0.114
MethylRed	1	20.03	20.03	0.34	0.588
Sugar*Furfural	1	187.01	187.01	3.13	0.137
Sugar*CaCO3	1	20.03	20.03	0.34	0.588
Sugar*MethylRed	1	258.41	258.41	4.32	0.092
Furfural*CaCO3	1	1411.88	1411.88	23.62	0.005
Furfural*MethylRed	1	231.80	231.80	3.88	0.106
CaCO3*MethylRed	1	638.83	638.83	10.69	0.022
Error	5	298.82	59.76		
Total	15	6860.32			

S	R-sq	R-sq(adj)
7.73069	95.64%	86.93%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	40.78	1.93	21.10	0.000
Sugar	8.77	1.93	4.54	0.006
Furfural	12.11	1.93	6.26	0.002
CaCO3	3.69	1.93	1.91	0.114
MethylRed	1.12	1.93	0.58	0.588
Sugar*Furfural	3.42	1.93	1.77	0.137
Sugar*CaCO3	-1.12	1.93	-0.58	0.588
Sugar*MethylRed	-4.02	1.93	-2.08	0.092
Furfural*CaCO3	9.39	1.93	4.86	0.005
Furfural*MethylRed	-3.81	1.93	-1.97	0.106
CaCO3*MethylRed	-6.32	1.93	-3.27	0.022

Table B. 12 Effects of control variables in preliminary investigations (Acid concentration, Day 3)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	10	5420.34	542.03	2.83	0.131
Sugar	1	891.02	891.02	4.65	0.084
Furfural	1	2052.09	2052.09	10.71	0.022
CaCO3	1	17.64	17.64	0.09	0.774
MethylRed	1	42.90	42.90	0.22	0.656
Sugar*Furfural	1	53.29	53.29	0.28	0.620
Sugar*CaCO3	1	894.01	894.01	4.67	0.083
Sugar*MethylRed	1	513.02	513.02	2.68	0.163
Furfural*CaCO3	1	531.30	531.30	2.77	0.157
Furfural*MethylRed	1	82.81	82.81	0.43	0.540
CaCO3*MethylRed	1	342.25	342.25	1.79	0.239
Error	5	957.84	191.57		
Total	15	6378.18			

S	R-sq	R-sq(adj)
13.8408	84.98%	54.95%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	42.39	3.46	12.25	0.000
Sugar	7.46	3.46	2.16	0.084
Furfural	11.33	3.46	3.27	0.022
CaCO3	-1.05	3.46	-0.30	0.774
MethylRed	1.64	3.46	0.47	0.656
Sugar*Furfural	1.83	3.46	0.53	0.620
Sugar*CaCO3	-7.47	3.46	-2.16	0.083
Sugar*MethylRed	-5.66	3.46	-1.64	0.163
Furfural*CaCO3	5.76	3.46	1.67	0.157
Furfural*MethylRed	-2.28	3.46	-0.66	0.540
CaCO3*MethylRed	4.62	3.46	-1.34	0.239

Appendix C - HPLC and Cell Density Calibration

Table C. 1 Summary of methods of chemical concentration measurement in HPLC

	Detector	Wavelength (nm)	Concentration range (g/L)	Retention Time (min)
Glucose	RID	na	0-60	6.8
Xylose	RID	na	0-60	7.1
Butanol	RID	na	0-20	23.0
Acetone	DAD	280	0-10	13.3
Ethanol	RID	na	0-5	13.0
Acetate	DAD	210	0-5	9.3
Butyrate	DAD	210	0-5	12.7

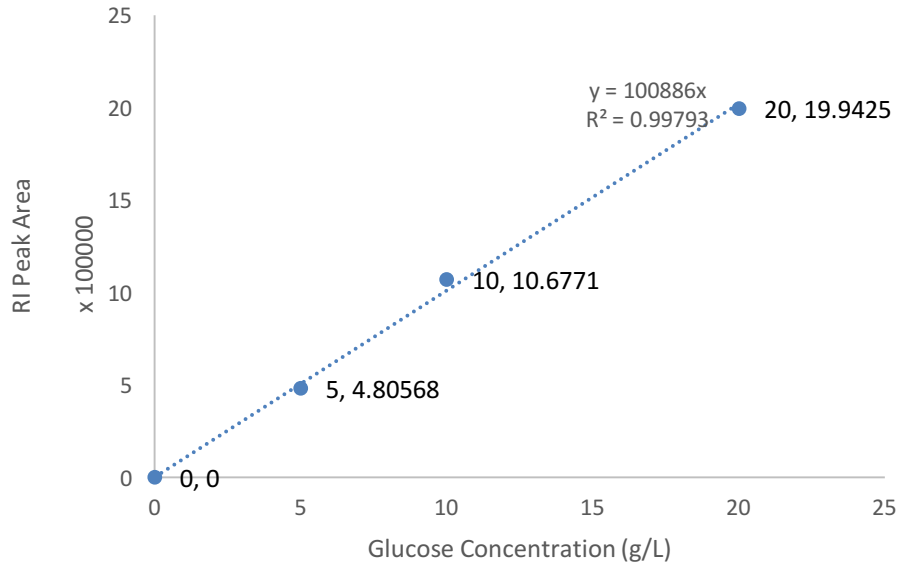


Figure C. 2 HPLC calibration of Glucose (Injection volume =10 μL; Retention time = 6.7min)

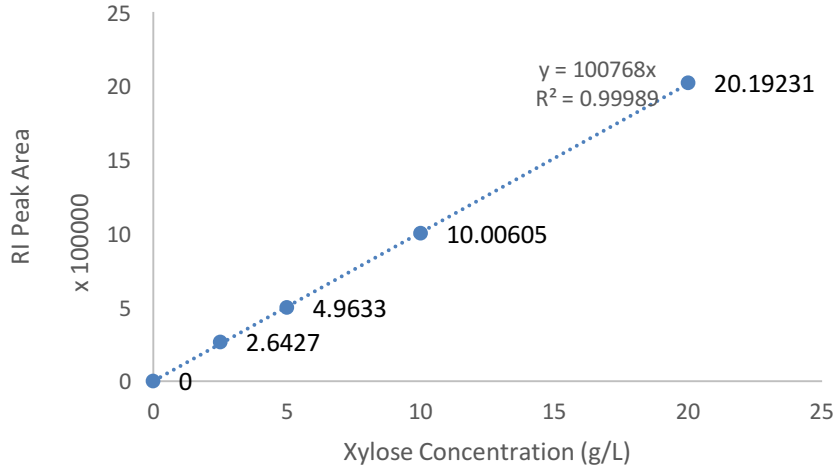


Figure C. 2 HPLC calibration of Xylose (Injection volume =10 μL; Retention time =7.2 min)

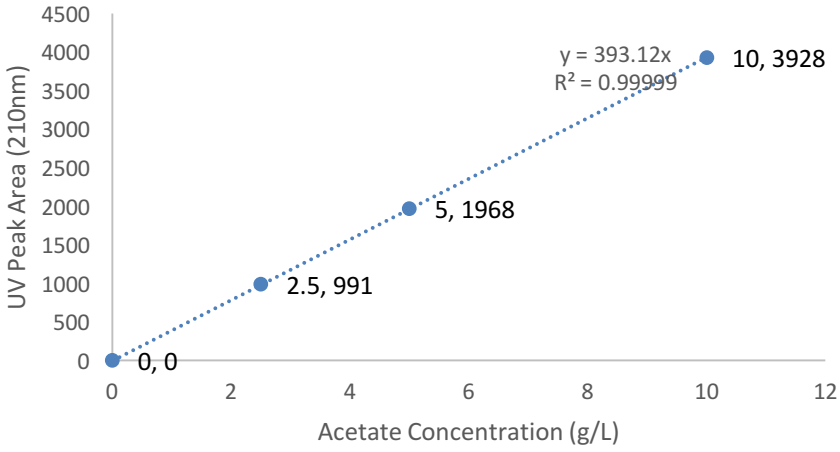


Figure C. 3 HPLC calibration of Acetate (Injection volume =10 μL; Retention time =8.9 min)

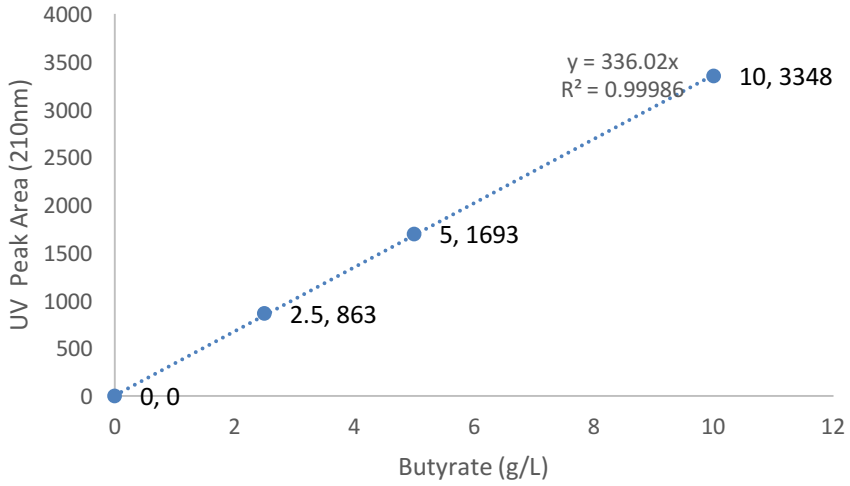


Figure C. 4 HPLC Calibration of Butyrate (Injection volume =10 μL; Retention time =12.7 min)

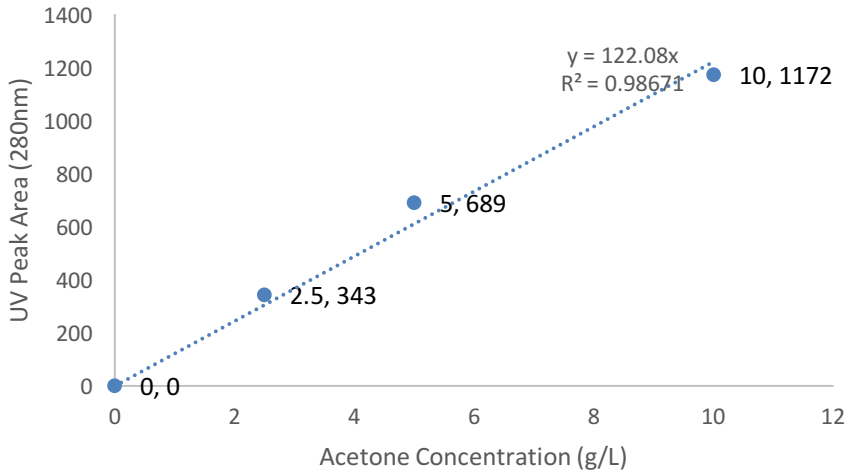


Figure C. 5 HPLC Calibration of Acetone (Injection volume =10 μL; Retention time =13.3 min)

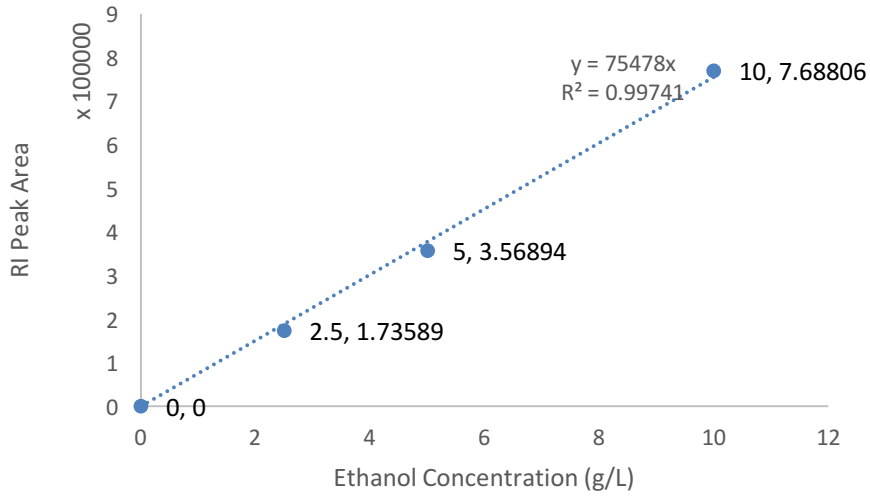


Figure C. 6 HPLC Calibration of Ethanol (Injection volume =10 μL; Retention time =14.2 min)

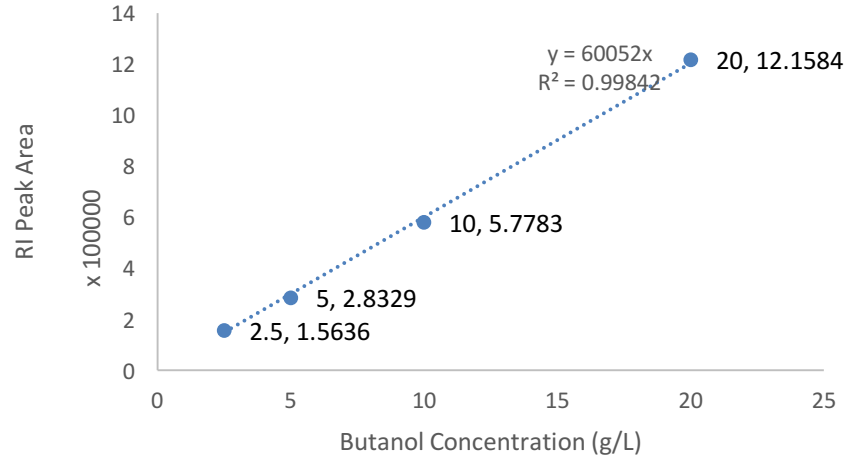


Figure C. 7 HPLC Calibration of Butanol (Injection volume =10 μL; Retention time =22.4 min)

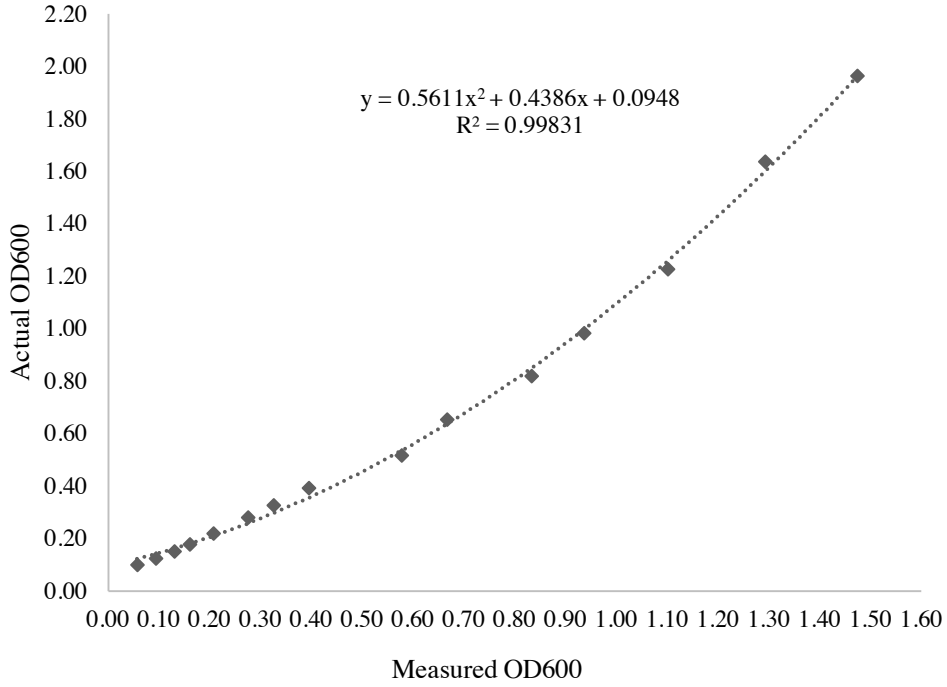


Figure C. 8 Calibration of cell density (OD600)

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