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Strategies for Improving Lignocellulosic Butanol Production and Recovery in ABE Fermentation by Tailoring Clostridia Metabolic Perturbations

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Abstract: The present study investigates approaches to enhance bio-butanol production using lignocellulosic feedstock via supplements of metabolism perturbation. Traditionally, bio-butanol has been produced through chemical synthesis in a process known as acetone–butanol–ethanol (ABE) fermentation. Today, biochemical techniques involving bacterial strains capable of producing butanol are used with renewable sources of biomass. In this study, a stepwise approach was tailored for metabolic perturbations to maximize butanol production from pure sugar and lignocellulosic feedstock as a reference model fermentation. In preliminary investigations, impacts of $CaCO_3$, furfural and methyl red on cell growth, sugar utilization, acid production and butanol production were evaluated in glucose feedstock and xylose feedstock. Following the preliminary investigation, with supplementation of 4 g/L $CaCO_3$, the concentrations of furan derivatives (75% furfural and 25% HMF) and $ZnSO_4$ were optimized for maximal butanol production from glucose and xylose feedstocks, respectively. 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 $CaCO_3$. Under optimized conditions, butanol production exceeded 10 g/L in wheat straw hydrolysate, which was significantly higher than that obtained in the absence of $ZnSO_4$ and $CaCO_3$. As compared to the traditional lignocellulosic feedstock post-treatment method, the metabolic perturbations method shows advantages in terms of productivity and economics.

Keywords: acetone–butanol–ethanol fermentation; biofuels; bio-butanol; lignocellulosic; simultaneous saccharification; fermentation

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1. Introduction

Due to rising concerns over greenhouse gas (GHG) emissions and global warming, the production of transportation renewable energy biofuel is expected to double by 2040, reaching 5 million barrels per day [1]. Biofuels, such as bio-ethanol, have been commercialized for years in the USA, Canada and Brazil. The challenge with most conventional biofuels is sustainability and GHG emissions as they are produced using foods such as corn and sugarcane as feedstock [2]. As compared to conventional biofuels, new-generation biofuels use lignocellulosic feedstock (comprised of agriculture residues, woody residues and municipal residues as raw materials), which produces less GHG emissions [3]. The two types of new-generation biofuels are advanced biofuel and cellulosic biofuel, which are standardized based on GHG emission reduction by the USA government in the Renewable Fuels Standard. Advanced and cellulosic biofuels must have emissions that are at least 50% and 60% lower, respectively, as compared to gasoline or diesel fuel.

Butanol has several advantages over ethanol as a biofuel as it has higher energy density, lower hygroscopicity, compatibility with existing infrastructure, lower vapor pressure, reduced corrosivity, and a wider temperature range. Despite these advantages, butanol production has faced challenges related to cost, scalability and the development of efficient fermentation processes. Ethanol, on the other hand, has been more widely

adopted as a biofuel due to its established production processes and lower production costs. However, ongoing research and development in the field of biofuels may lead to increased use of butanol in the future, especially as technologies improve and economies of scale are achieved [4,5]. In spite of the better characteristics of biobutanol, its yield and titer through fermentation in addition to production cost are lower compared with ethanol. Therefore, the improvement of substrates, microbial strains and processes for its cost-competitive production became a research priority [6]. Another issue is the dilute butanol–water solution produced through fermentation processes, which requires further processing and thus energy consumption to produce anhydrous biobutanol with minimal water content [7]. To produce bio-butanol, an ABE fermentation process needs to be conducted, in which microorganisms like *Clostridium acetobutylicum* and *Clostridium beijerinckii* are typically used [5]. ABE fermentation is complicated as it produces multiple products and has various metabolic stages. These stages are regulated by the expressions of corresponding enzymes/coenzymes, which have been partially studied through transcriptional analysis. This information could facilitate production improvement through metabolic regulation [5]. However, the development bottleneck of bio-butanol is its high selling price (3~3.5 USD/gallon), which is uncompetitive in comparison to the $1.5/gallon of conventional bio-ethanol [4]. Therefore, improving the efficiency of production with lignocellulosic feedstock became the primary feasible way to lower the overall cost of production. However, sugar monomers contained in lignocellulosic biomass are not fermentable for clostridium unless the lignocellulosic biomass is pre-treated using acid, alkaline, oxidizer and heat. By performing pretreatments, sugar monomers are released from the decomposition of cellulose and hemicellulose.

Thermal treatment above 100 °C is necessary for the decomposition of cellulose and hemicellulose. At this temperature, monosaccharides are partially dehydrated and converted into furan derivatives [8]. Common inhibitors like furan derivatives, which include furfural and HMF, not only cause DNA damage, inhibition of glycolytic enzymes and disruption of cell membranes [9], but also perturb the redox balance (deplete cofactor NAD(P)H). As a result of cofactor depletion, ABE fermentation encounters deceleration, solvent production loss and cell death [10].

Many studies have focused on limiting inhibitor generation during pre-treatment [9]. Due to high inhibitors concentration, the loading of biomass in dilute acid is normally limited to 8~10% w/v [11]. More importantly, post-treatment removal of inhibitors is needed. The most promising method of post-treatment is in situ microbial detoxification. During in situ detoxification, furan derivatives are reduced to ethanol. As compared to overliming and chemical adsorptions, in situ detoxification shows great potential in terms of efficiency and cost.

Several studies have been conducted in the area of ABE fermentation in lignocellulosic feedstock [12]. In one study, agricultural waste like rice straw, corncob and woody residues like palm bunches were used as feedstock for *Clostridium acetobutylicum, Beijerinckii, Pasteurianum*, etc. Maximum butanol production was achieved in rice straw feed stock with acid treatment and the application of sheer stress using *C. acetobutylicum* (NCIM 2337) [13]. Despite various pre-treatment and post-treatment technologies used on the feedstock, in most cases, butanol production was less than 10 g/L, the concentration at which clostridium growth is strongly inhibited. Currently, efficient production is a substantial challenge where lignocellulosic feedstock is used.

In lignocellulosic feedstock, xylose is the second most abundant monosaccharide. It is also the limiting factor for butanol production from ABE fermentation. When xylose is used as feedstock, clostridium growth is weaker, and sugar utilization is lower than when glucose is used [14]. Another downside of xylose feedstock is related to butanol toxicity. Butanol can inhibit membrane-bound ATPase enzyme activity and disrupts the phospholipids of the cell membrane [15]. However, through overexpressing the gene encoding transaldolase, xylose utilization and solvent production have been improved [14].

These drawbacks of lignocellulosic feedstock have necessitated unconventional methods such as the addition of perturbative chemicals to mitigate inhibitory effects and improve xylose utilization. Several studies, such as [16], show that $CaCO_3$ can alleviate the toxic effects of lignocellulose-derived microbial inhibitory substances and consequently enhance bioconversion. Furthermore, $ZnSO_4$ is found to be effective in increasing butanol production [17]. Electron acceptors such as furan derivate (furfural and HMF) are existing inhibitors in lignocellulosic hydrolysate. Furfural and HMF consume cofactors NAD(P)H [18], which inhibits cellular growth and butanol productivity. However, in certain cases this could be a stimulant to ABE fermentation [15]. Other electron acceptors like methyl red have also been found to be effective in improving fermentative production [19].

Based on these findings in previous studies, this comprehensive stepwise study was developed to maximize butanol production from pure sugar and lignocellulosic feedstock using metabolic perturbations. In preliminary investigations, the impacts of $CaCO_3$, furfural and methyl red on cell growth, sugar utilization, acid production and butanol production were evaluated in glucose and xylose feedstock, separately. Following the preliminary investigation, with supplementation of 4 g/L $CaCO_3$, concentrations of furan derivatives (75% furfural and 25% HMF) and $ZnSO_4$ were optimized for glucose and xylose feedstocks, respectively. 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 $CaCO_3$.

2. Materials and Methods

2.1. Materials and Chemicals

Clostridium beijinrickii ATCC (BA101) was purchased from the American Type Culture Collection. All chemicals were purchased from Sigma-Aldrich Canada and were used as received without any further purification. Wheat straws were collected from Springridge farm located in Milton, while spruces were collected from St. James Square at Toronto Metropolitan University (both located in Ontario, Canada).

2.2. Experimental Procedure and Methodology

2.2.1. Bio-Butanol Production

Fermentation experiments were conducted in 100 mL Wheaton serum bottles containing 35 mL of P2 or lignocellulosic feedstock. A semi-synthetic P2 medium is usually comprised of a buffer pH, vitamins, yeast extract and vitamins that support bacterial growth [20]. The P2 or lignocellulosic feedstock was autoclaved at 121 °C for 15 min and cooled to room temperature. *C. 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 a −80 °C ultra-low freezer (Thermo Fisher Scientific, Waltham, MA, 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 was heat-shocked at 80 °C for 5 min [11]. Later, the spores were inoculated onto 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 was red-hot. Streaked agar plates were sealed in an anaerobic jar (HP011, Thermo Scientific) containing an anaerobic pack (AnaeroPack MGC). Later, the anaerobic jar was sealed and placed in an incubator at 35 °C. White colonies were observed on agar plates two days later.

The colonies were isolated (OD600 0.6~1.0) and inoculated into pre-grown P2 medium overnight containing 60 g/L glucose for ABE fermentation using a sterile inoculating loop. Upon inoculation, the medium was bubbled with nitrogen gas for about 5–10 min. A blue neoprene rubber stopper and a metallic cap were used to seal the bottles using a vial crimper (Cole Palmer, Québec City, QC, Canada). The serum bottles were brought out of the glove box and transferred to the incubator. The temperature of the incubator was adjusted to 35 °C. The 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, Stockbridge, GA, USA). HPLC testing vials were filled to a minimum headspace to reduce the loss of solvents in vapor phase. These samples were stored at 4 °C until analysis. Upon fermentation and following HPLC analysis, the highest butanol production strains (i.e., 7.5 g/L butanol production) were stored in Eppendorf tubes at 4 °C to be used further in all fermentation experiments.

For HPLC, an Agilent 1100 (Santa Clara, CA, USA) equipped with a refractive index detector (RID) and (DAD) diode array detector was used to quantify the concentrations of ABE solvents, sugars and acids. An Aminex HPX-87H (Mississauga, ON, Canada) was the column used for the analysis of sugars, ABE and acids. It was operated in filter-sterilized mobile phase (i.e., distilled water) at 1 mL/min, while the column temperature was 60 °C. The calibration of HPLC was performed for all ABE solvents, acids and sugars.

Optical Density

The optical density of a sample measured at the wavelength of 600 nm (OD600) was used in all clostridium cell density measurements [21]. Genesys 10S UV-VIS (Thermo Scientific) was used for OD600 measurement.

Medium and Lignocellulosic Hydrolysate Preparation

P2 medium was prepared and used as the typical medium for ABE production and clostridium culture [22]. It contains 60 g/L sugar (glucose or xylose), 1 g/L yeast extract, 2.2 g/L $CH_3COO(NH_4)$, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4{\bullet}7H_2O$, 0.01 g/L $MnSO_4{\bullet}H_2O$, 0.01 g/L $FeSO_4{\bullet}7H_2O$, 0.01 g/L NaCl, 1 mg/L PABA, 1 mg/L thiamin hydrochloride and 0.01 mg/L biotin. 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 g of RCM, 25 g of gelatin and 7.5 g of agar to 500 mL of hot distilled water. This viscous medium was autoclaved at 121 °C for 20 min. Up cooling, the medium was poured into Petri dishes and allowed to set until a smooth solid surface had formed for the bacteria to grow. RCM agar plates are an essential tool in microbiology. They allow clostridium to grow on a semi-solid surface to produce discrete colonies.

In order to produce lignocellulosic hydrolysates, wheat straw and spruce branch were ground to reduce size using a hammer mill (Retsch GmbH Inc., Newtown, PA, USA), and then they were filtered using a 1 mm sieve screen. Then, the moisture content of the feedstock was reduced through heating in a conventional oven at 90 °C for 2 days until no further weight loss was detected. To pursue acidic pretreatment, a total of 50 g of dry feedstock was dispensed into a 1 L flask containing 500 mL of 1% (*v*/*v*) H_2SO_4, followed by autoclaving at 121 °C for 60 min [8,23]. No separation of slurry from liquid was conducted. Finally, pH was adjusted to 6.5 using 10 M NaOH [20,24].

Any anaerobic manipulations were carried out in an anaerobic chamber (i.e., Glove Box; Terra Universal, Fullerton, CA, USA) at a temperature of 25 ± 2 °C. An 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 min, and later, it 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 surfaces with 70% ethanol before and after work, in addition to utilization of ultraviolet light to sterilize the exposed work surfaces.

2.2.2. Metabolic Perturbations

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 in glucose and xylose feed-

stocks, separately. Table 1 lists the control variables and corresponding levels used in the preliminary investigations. The control variables were sugar type, furfural, $CaCO_3$ and methyl red. The concentration of furfural (1 g/L), $CaCO_3$ (2 g/L) and MR (0.01mM) were determined in previous studies [14,25,26]. 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 by the total initial sugar concentration. Moreover, in order to study whether the effects of control variables were time-dependent, samplings were conducted at 24 and 48 h after the start of incubation. Preliminary investigations were performed in a factorial design including 16 experimental runs in total.

Table 1. Control variables and their levels in preliminary investigations and butanol production optimization.

Preliminary Investigations Number of Runs = 16					
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 (0.01 M)	
Butanol Production Optimization Two Replicates; Number of Runs = 44					
Independent variable	Levels				
	−1.414	−1	0	1	1.414
$ZnSO_4 \bullet 7H_2O$ (x_1)	0 mg/L	0.293 mg/L	1.000 mg/L	1.707 mg/L	2.000 mg/L
Furfural/HMF (x_2)	0 g/L	0.439 g/L	1.500 g/L	2.561 g/L	3.000 g/L

P2 media with two different sugars, glucose 60 g/L (coded 1) and xylose 60 g/L (coded-1), were prepared and autoclaved separately, then 35mL of P2 medium was distributed into 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. An overnight-grown strain pre-grown in P2 containing 60 g/L glucose was used for inoculation (1 mL/bottle). After anaerobiosis with N_2, the serum bottles were sealed and put into the incubator at 35 °C for 72 h 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 and any equipment that came in contact with the bacteria were washed with ethanol sterilized under UV light for 10 min. Sampling was performed by inserting a sterilized syringe–needle combination through the serum bottle's rubber stopper and collecting ~2 mL of liquid after 24 and 48 h. All samples were analyzed for pH and cell growth, in addition to ABE, acid and sugar concentration using HPLC.

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

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

Bio-Butanol Production Optimization

The purpose of optimization was to maximize butanol production in pure glucose or xylose feedstock (60 g/L) separately by tailoring the concentrations of $ZnSO_4$ and furan derivatives (75% furfural and 25% HMF). To do so, 4 g/L $CaCO_3$ was supplied in every serum bottle as a constant condition. Table 1 lists control variables and corresponding levels used in the optimization process. For each glucose and xylose feedstock (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 [21], 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 [25]. The experiments were replicated twice. In each replication, center points were run three times for determination of internal estimators. There were 44 experimental runs in total in bio-butanol production optimization.

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 to serum bottles to make $ZnSO_4$ concentrations of 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 derivative solution (75% furfural and 25% HMF, 83.3 g/L in total) were added to serum bottles to make concentrations of furan derivatives of 0, 0.439 g/L, 1.50 g/L, 2.531 g/L and 3 g/L, respectively. Furan derivative solution (83.3 g/L) was prepared by adding solid HMF (1.041 g) and liquid furfural (3.124 g) to hot water to make a total liquid volume of 50 mL. Then, 0.144 g of solid $CaCO_3$ was added to every serum bottle. An overnight-grown strain pre-grown in P2 containing glucose 60 g/L was used for inoculation (1 mL/bottle). After anaerobiosis with N_2, the serum bottles were sealed and put into the incubator at 35 °C for 72 h 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. In the current study, Box–Wilson Central Composite Design [26], 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 a quadratic model or even of higher orders as following, where x_i are control variables, y is the response of interest, β are the constant coefficients and ϵ is random experimental error, assumed to have a zero mean. By determining all of the coefficients, including main effects and interaction effects, optimizations of chemical additives' concentrations were obtained.

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

Optimum Bio-Butanol Production Using Lignocellulosic Feedstock Results and Discussion

Optimum metabolic perturbations of $CaCO_3$, $ZnSO_4$ and furan derivatives for maximizing butanol production were tested using lignocellulosic feedstock. Table 2 shows the corresponding experimental design. The experiments were conducted in paralleled double replication. There were 12 experimental runs in total.

For each batch of fermentation, approximately 50 mL lignocellulosic hydrolysate including slurry was transferred into a 100 mL metric flask. A liquid volume of 35 mL was determined after the slurry was precipitated. The slurry and exactly 35 mL of the liquid were transferred into each serum bottle. The diluted acid pretreatment could not release enough sugar from lignocellulosic biomass for ABE fermentation: 12 g/L glucose, 35 g/L total sugar and 6 g/L glucose, 25 g/L total sugar were achieved in the wheat straw hydrolysate and spruce hydrolysate, respectively. In this study, instead of using enzymatic treatment, solid glucose and xylose were directly added to make total sugar concentration 60 g/L and glucose concentration 27 g/L for lignocellulosic hydrolysates, which was comparable to the wheat straw hydrolysate after enzymatic treatment in a previous study [11]. Therefore, 15 g/L glucose and 10 g/L xylose were added into the

wheat straw hydrolysate, and 21 g/L glucose and 14 g/L xylose were added into the spruce hydrolysate. Following sugar addition, nutrition sources such as yeast, vitamins and metal ions for clostridium growth were added as described into the P2 medium. The following amounts, $CaCO_3$ 4 g/L and $ZnSO_4$ 2 mg/L, were supplied in experiment runs 3 and 6. The strains pre-grown in two media were used for inoculation: one was pre-grown in P2 medium (glucose/xylose: 30/30 g/L), the other was pre-grown in the same P2 medium with $CaCO_3$ 4 g/L and $ZnSO_4$ 2 mg/L added. The inoculation volume was 2 mL. After anaerobiosis with N_2, the serum bottles were sealed and put into the incubator at 35 °C for 72 h fermentation.

Table 2. Experimental design and results of optimum bio-butanol 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

Two replicates; number of runs = 12; $CaCO_3$ and $ZnSO_4$ concentration were 4 g/L and 2 mg/L, respectively.

3. Results and Discussion

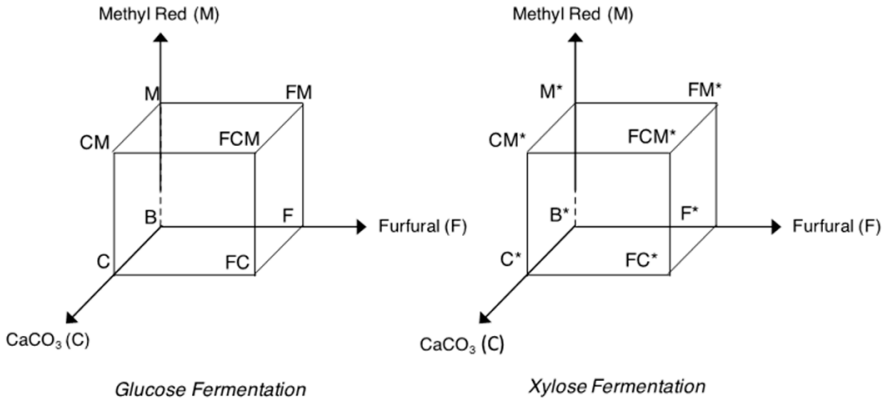
3.1. Preliminary Investigations

In the preliminary investigations, F, C, and M denoted that furfural (1.0g/L), $CaCO_3$ (2.0 g/L) and methyl red (0.01 mM), respectively, were supplied in the feedstock, while FC, CM, FM and FCM meant multiple chemical additives were supplied simultaneously. ANOVA was used to quantify the effects and reveal the potential impacts of chemical additives. The experimental design is shown in Figure 1. In total, there were 16 experimental runs for glucose and xylose (denoted by *) 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 (24th hour), day 2 (48th hour) and day 3 (72nd hour) via sampling. The results are shown in Table 3 and Figure 2. Butyrate, acetate, acetone and ethanol concentrations are listed in Table 4.

Table 3. Responses from preliminary investigations.

Run	No.	Cell Density * (OD600)			Sugar Consumption (%)			Butanol Concentration (g/L)			Total Acid Concentration (mmol/L)			pH		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
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 using optical adsorption at 600 nm; sugar consumption was calculated by dividing utilized sugar by initial total sugar; total acid concentration was calculated by adding butyrate and acetate concentration, and total acid concentration is in units of "mmol/L" instead of "g/L" after referring to the study in [27].



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

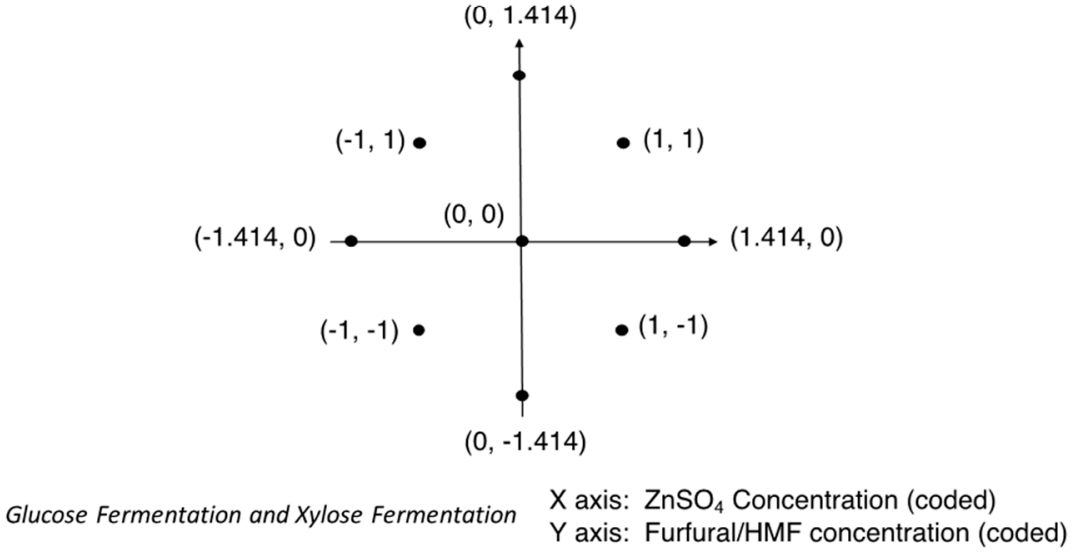


Figure 1. Experimental design of preliminary investigations and bio-butanol optimization.

According to Table 3, the highest cell density was observed in runs C, FC, C*, FC* and CM*, which were supplemented by $CaCO_3$. 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 as described by Shinto's kinetics models [28]. The final sugar consumptions of glucose control B and xylose control B* were 55.9% and 33.8%, respectively. Through chemical additive supplementation, the maximum sugar consumptions in glucose and xylose feedstock were escalated to 74.5% (of FC) and 62.2% (of CM*). M*, FM* and CM* also showed a higher xylose depletion percentage than the control. In contrast, weakened glucose utilization was observed in runs F and FM.

The final butanol production of B and B* was 7.5 g/L and 5.5 g/L, respectively. The weak solventogenic ability of the clostridium was improved by the supplementation 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. Therefore, the supplementation of $CaCO_3$ greatly improved the cell growth, the sugar utilization and the butanol tolerance by clostridium. However, butanol productions below the control level, 6.0 g/L (of run F) and 6.5 g/L (of run FM), were observed, suggesting that in glucose feedstock, furfural was detrimental to butanol production and sugar consumption, as demonstrated earlier.

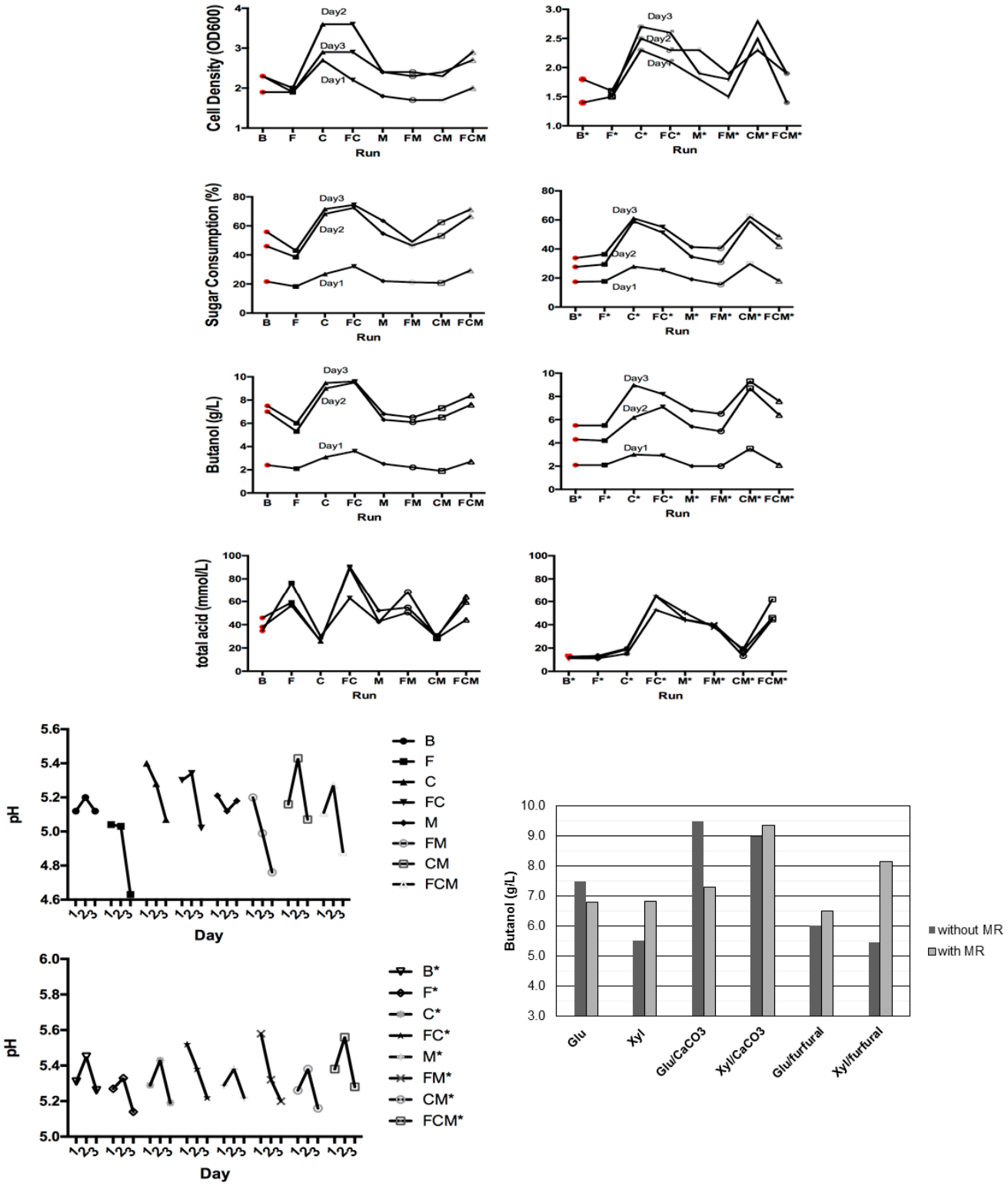


Figure 2. Time-dependent plots of responses in preliminary investigations.

Table 4. Acid concentrations (preliminary investigations).

Run Order	Butyrate (g/L)			Acetate (g/L)			Acetone (g/L)			Ethanol (g/L)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
1	1.93	1.82	1.03	1.40	2.45	1.07	1.77	3.25	3.80	0.45	1.37	1.34
2	2.52	2.40	1.71	1.78	3.18	2.35	1.62	3.15	3.52	0.49	1.27	1.11
3	1.69	0.59	1.14	0.37	1.27	0.91	1.89	4.74	4.60	0.53	1.02	2.53
4	2.36	4.33	2.33	3.73	2.76	1.84	2.71	5.25	5.49	0.55	1.86	2.61
5	2.20	1.45	1.55	1.58	1.73	1.33	1.12	2.54	2.90	0.48	0.88	1.03
6	2.54	2.35	1.39	1.51	2.52	2.34	1.60	3.45	3.83	0.36	1.55	1.22
7	1.56	1.93	0.40	0.72	1.01	0.98	1.33	3.59	4.30	0.39	0.95	1.08
8	1.58	1.39	2.84	2.44	2.16	1.14	2.14	4.14	4.13	0.63	1.48	0.74
9	0.65	0.35	0.48	0.22	0.42	0.46	1.84	2.85	3.43	0.59	0.75	1.03
10	0.67	0.30	0.51	0.20	0.69	0.32	1.90	2.81	3.29	0.64	0.62	0.61
11	0.80	0.32	0.90	0.35	0.30	0.96	2.06	3.02	4.39	0.64	0.89	2.04
12	2.40	2.50	2.11	1.48	1.89	2.54	2.39	3.91	4.15	0.53	1.03	1.61
13	1.95	1.61	1.53	1.28	1.86	1.70	2.11	3.18	3.41	0.76	0.93	1.02
14	1.67	1.46	1.38	1.19	1.08	1.50	2.08	3.39	4.15	0.56	0.83	1.61
15	0.80	0.31	0.85	0.23	0.23	0.97	2.41	4.18	4.82	0.43	1.95	2.26
16	2.07	2.27	1.17	1.22	1.96	2.32	1.90	3.81	3.97	0.50	0.99	1.65

Furfural was found to improve acid production in glucose feedstock with exceptionally high acid concentrations in runs F, FC, FM and FCM as a result of higher metabolic flux in glucose fermentation [28]. Clostridium responded to furfural by accelerating acid production to release inhibitory stress [9]. The acid concentration was linked to the pH of the broth. pH responses are illustrated in Figure 2. It was concluded that the pH level essentially decreased with the increase in acid concentration, if no $CaCO_3$ was added. Higher acid concentration and pH < 4.8 in the final broth due to furfural supplement impaired the butanol production, as runs F and FM showed. Despite high acid concentration in FC and FCM, the corresponding pH level was maintained at 5.02 and 4.88, and butanol production was 9.6 g/L and 8.4 g/L, respectively. This indicated that $CaCO_3$ could mitigate furfural stress through pH buffering [29].

ANOVA was used to quantify the effects and reveal the potential impacts of chemical additives (Figure 3). The internal error estimate was determined by pooling high-order ($n \geq 3$) interactions. $p < 0.05$ was considered statistically significant. In Figure 3, significant effects are marked with dark shading. x_1, x_2, x_3 and x_4 correspond to sugar type, furfural, $CaCO_3$ and methyl red, respectively.

Glucose was superior to xylose as a feedstock for clostridium culture in terms of cell growth improvement. However, the change of sugar from xylose to glucose was less effective than the supplementation of $CaCO_3$. The effects on sugar consumption and butanol production agree with the effects on cell growth. $CaCO_3$ improved butanol production by cal. 2.2 g/L. As shown in Figure 2, the xylose runs M*, CM* and FM* showed higher responses than the control B* in terms of cell density, xylose utilization and butanol production. This interaction meant that the supplementation of methyl red significantly stimulated butanol production in xylose feedstock. By contrast, in glucose feedstock, butanol production was impaired by methyl red, as displayed in Figure 2.

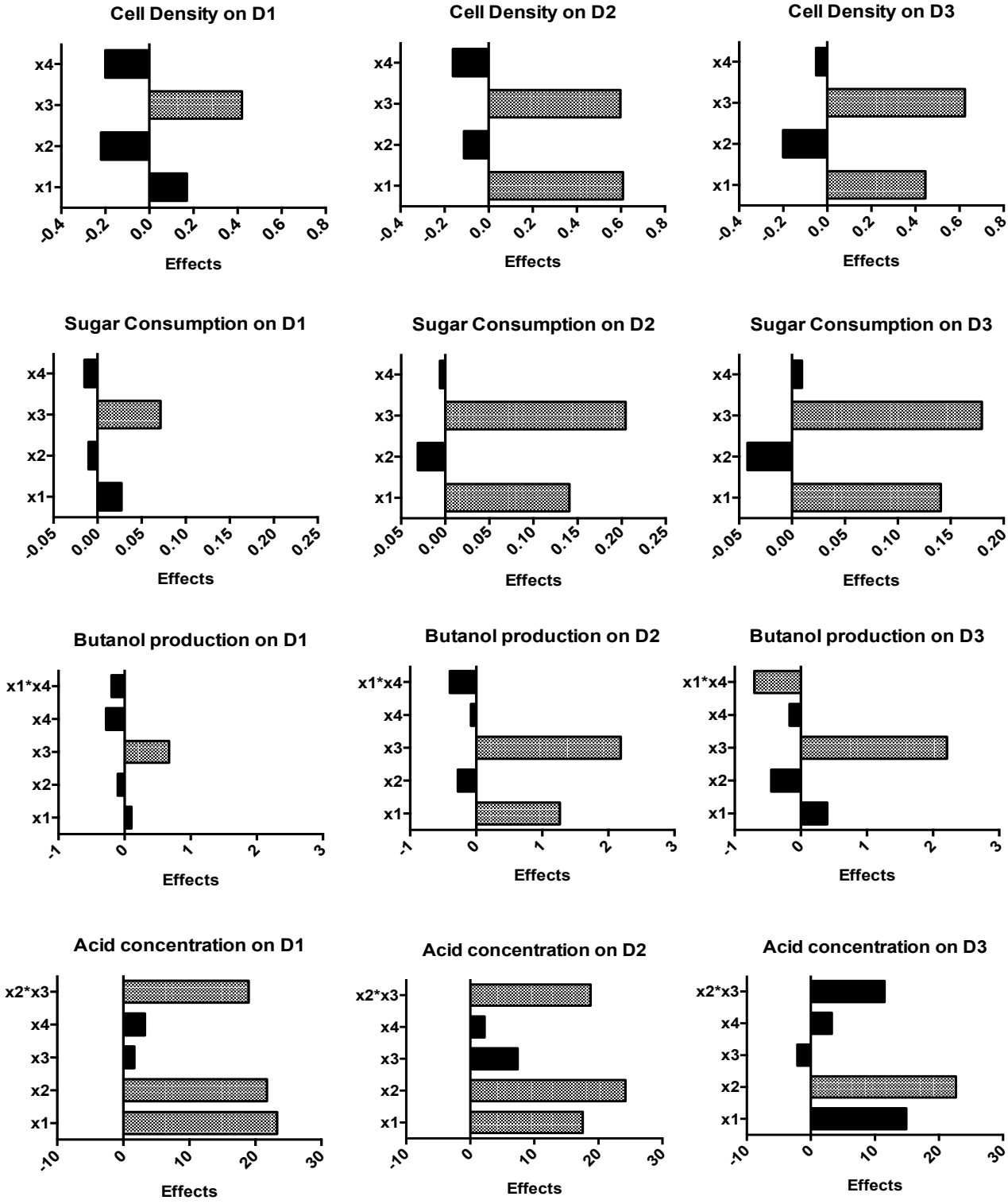


Figure 3. Effects of control variables on cell density, sugar consumption, butanol production and acid concentration in preliminary investigations (* D1: day 1; D2: day 2; D3: day 3). * x_1 = sugar type (glucose or xylose), x_2 = $CaCO_3$ (2 g/L or 0), x_3 = furfural (1 g/L or 0), x_4 = methyl red (0.01 mM or 0).

The effects of the control variables on acid concentration were 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 $CaCO_3$ was not a stimulant of acid production, we concluded that $CaCO_3$ served as an acid production

catalyst when furfural was present. Further study on this interaction may be beneficial to the understanding of $CaCO_3$-mitigated furfural stress.

Since methyl red was detrimental to glucose fermentation in the first study, it was not used in the following studies. Furfural at 1 g/L had negative effects on cell density, sugar consumption and butanol production in glucose fermentation. However, furfural 1 g/L did not impair xylose fermentation.

The results from the preliminary investigation indicated that ABE fermentation could benefit from multiple supplementations of metabolic perturbations. Zinc sulphate was used in the following experiment since it is known for its ability to improve butanol tolerance [24]. Furthermore, the furan derivative HMF was added in a fixed ratio to furfural to mimic the furan derivative composition of wheat straw hydrolysate. Lastly, $CaCO_3$ at 4 g/L was used as a constant condition in the following optimization design, as suggested by a previous study [30].

3.2. Bio-Butanol Production Optimization

The purpose of optimization was to maximize butanol production in glucose and xylose feedstock, respectively, 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 a constant condition. The experiments were designed according to response surface methodology–central composite design (RSM-CCD) [26]. There were two blocks, each consisting of 44 experimental runs. Central points were replicated and tripled in each block.

Table 5 displays the results of butanol production in glucose and xylose feedstock, respectively. All glucose-fed clostridium produced solvent successfully, whereas in xylose feedstock, fermentation failed to commence in runs 2, 7, 10, 13, 18 and 21. The furan derivative concentrations in these runs were all above 2.56 g/L. This indicated that furan derivatives at 2.56 g/L are lethal for xylose-fed clostridium. The butanol production ranges in glucose and xylose feedstock were 8.73~11.45 g/L and 9.19~11.82 g/L, respectively (except failed runs), which were an improvement as compared to 6.54~9.57 g/L and 5.51~9.35 g/L in glucose and xylose feedstock in the preliminary investigations.

Table 5. Results of bio-butanol production optimization in (a) glucose and (b) xylose feedstock.

(a) 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	10.50
2	1	1.71	1	2.56	1	10.04	10.26
3	1	1.71	1	0.44	−1	10.51	10.76
4	1	1.00	0	1.50	0	9.42	9.36
5	1	1.00	0	1.50	0	9.32	9.36
6	1	1.00	0	1.50	0	9.29	9.36
7	1	1.00	0	3.00	1.414	9.51	9.44
8	1	1.00	0	0.00	−1.414	11.45	11.09
9	1	0.29	−1	0.44	−1	10.69	10.57
10	1	0.29	−1	2.56	1	8.73	8.73
11	1	0.00	−1.414	1.50	0	9.05	9.29
12	2	2.00	1.414	1.50	0	10.92	10.49
13	2	1.71	1	2.56	1	10.07	10.25
14	2	1.71	1	0.44	−1	10.30	10.75

Table 5. *Cont.*

(a) Glucose feedstock							
Run	**Blocks**	**$ZnSO_4$**		**Furfural/HMF**		**Butanol**	**Prediction**
		(g/L)	**Coded**	**(g/L)**	**Coded**	**(g/L)**	**(g/L)**
15	2	1.00	0	1.50	0	9.32	9.35
16	2	1.00	0	1.50	0	9.67	9.35
17	2	1.00	0	1.50	0	9.12	9.35
18	2	1.00	0	3.00	1.414	9.43	9.43
19	2	1.00	0	0.00	−1.414	11.23	11.08
20	2	0.29	−1	0.44	−1	10.26	10.56
21	2	0.29	−1	2.56	1	8.85	8.72
22	2	0.00	−1.414	1.50	0	9.44	9.28
(b) Xylose Feedstock							
Run	**Block**	**$ZnSO_4$**		**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	9.68
2	1	1.71	1	2.56	1	0.00	−0.18
3	1	1.71	1	0.44	−1	10.20	10.62
4	1	1.00	0	1.50	0	10.86	9.94
5	1	1.00	0	1.50	0	10.10	9.94
6	1	1.00	0	1.50	0	9.38	9.94
7	1	1.00	0	3.00	1.414	0.00	N/A
8	1	1.00	0	0.00	−1.414	9.19	9.21
9	1	0.29	−1	0.44	−1	9.52	10.08
10	1	0.29	−1	2.56	1	0.00	−0.71
11	1	0.00	−1.414	1.50	0	10.25	10.32
12	2	2.00	1.414	1.50	0	11.10	10.60
13	2	1.71	1	2.56	1	0.00	0.74
14	2	1.71	1	0.44	−1	11.82	11.53
15	2	1.00	0	1.50	0	10.72	10.86
16	2	1.00	0	1.50	0	11.04	10.86
17	2	1.00	0	1.50	0	10.24	10.86
18	2	1.00	0	3.00	1.414	0.00	N/A
19	2	1.00	0	0.00	−1.414	10.54	10.12
20	2	0.29	−1	0.44	−1	10.71	11.00
21	2	0.29	−1	2.56	1	0.00	0.20
22	2	0.00	−1.414	1.50	0	11.48	11.24

The range of sugar consumption in the optimization study was 53~87% (except failed runs), which was higher than the 34–74% in the preliminary investigations. Importantly, sugar consumption was highly correlated with 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 metabolic perturbation concentration for 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 6 shows the ANOVA and the regression model of butanol production in glucose feedstock. The lack-of-fit F-test is used as a support test for the adequacy of the fitted model. In the error probability used by ANOVA statistics, if the *p*-value of the lack-of-fit is small compared to the significance level (i.e., F-statistic is large), then the lack-of-fit is significant and the model does not adequately explain data in the region of experimentation (there is lack of fit in the simple linear regression model). Accordingly, the lower the *p*-value (i.e., the higher the F-statistic), the more likely the rejection of the null hypothesis in favor of the alternative [31]. The R-square and *p*-value of the lack of fit were 91.48% and 0.251, respectively. These results suggested that the proposed model fitted well with experimental data. Through analysis of residual plots, the requirement of normal error distribution was satisfied. In addition, upon failing to reveal potential violations of regression uniformity, it was confirmed that 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. The block was not significant.

Table 6. 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
$ZnSO_4$	1	2.9282	2.92817	39.89	0
Furfural/HMF	1	5.4740	5.47403	74.57	0
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
$ZnSO_4$ × 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 $\text{Butanol (g/L)} = 11.688 - 1.126\ ZnSO_4 + 0.532\ (ZnSO_4)^2 - 2.202\ \text{Furfural/HMF} + 0.4019\ (\text{Furfural/HMF})^2 + 0.445\ (ZnSO_4)\text{*Furfural/HMF}$

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

In xylose fermentation, the response range was wide, 0~12 g/L, due to zero butanol concentration at high furan derivative concentration. The wide range of the responses induced large variance in the quadratic regression, albeit the regression result showed a high R-square and insignificant lack of fit. 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) [32]. In the current study, eight data points (except run 7 and 18) were enough to perform the regression ($8 \geq \frac{(2+1)(2+2)}{2} + 2 = 8$). 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 second-order terms $(furfural/HMF)^2$ were eliminated.

Table 7 shows the ANOVA and the regression model of butanol production in xylose feedstock. The R-square and *p*-value of the lack of fit were 98.89% and 0.646, respectively. Through analysis of residuals plots, requirement of normal error distribution was satisfied. Therefore, the reliability and uniformity of the model were confirmed. The terms remaining in the model include the first and third term of furfural/HMF, which were dominating terms in the model (with F-values of 71.17 and 522.81). None of the zinc sulphate terms were significant, but removal of any of them led to a severe misfit of regression, a severe skewness in the residual probability plot and a slump of the adjusted R-square. Therefore, all zinc sulphate terms remained in the model. At last, the block was significant. Butanol production in block 2 was ~0.9 g/L higher than that in block 1, as the regression equation shows.

Table 7. 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
$ZnSO_4$	1	0.734	0.734	2.41	0.144
Furfural/HMF	1	21.662	21.662	71.17	0
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
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 $\text{Butanol (g/L)} = 10.405 - 2.94\,(\text{ZnSO4}) + 2.766(\text{Furfural/HMF}) + 3.85\left(\text{ZnSO4}\right)^2 - 1.227(\text{ZnSO4}) - 1.273\,(\text{ZnSO4})^3 - 0.9942\,(\text{Furfural/HMF})^3$

The effects of control variables in glucose and xylose fermentation were examined. Correspondingly, the effects of zinc sulphate and furfural/HMF concentration are shown in Figure 4. 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. However, the decrease in butanol production plateaued at a furfural/HMF concentration of more than 2.56 g/L.

For butanol production in xylose feedstock, the effect of zinc sulphate was not evaluated since it was not accessible at high furan derivative concentrations (butanol production = 0 g/L). However, through point-to-point comparison, it was observed that zinc sulphate was a simulant to butanol production when the concentration of furan derivatives was as low as 0.44 g/L (coded-1). As the furfural/HMF concentration increased, the stimulatory effect gradually faded and became inhibitory beyond 1.5 g/L of furfural/HMF. The reported stimulant furfural concentrations were ~1.1 g/L furfural [33] and 0.5~1.0 g/L furfural [15], which were comparable to the 0.5~1.5g/L furan derivative (0.3~1.12 g/L furfural) in this study. In the current study, it was found that butanol production was proportional to zinc sulphate dosage up to 2 mg/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.

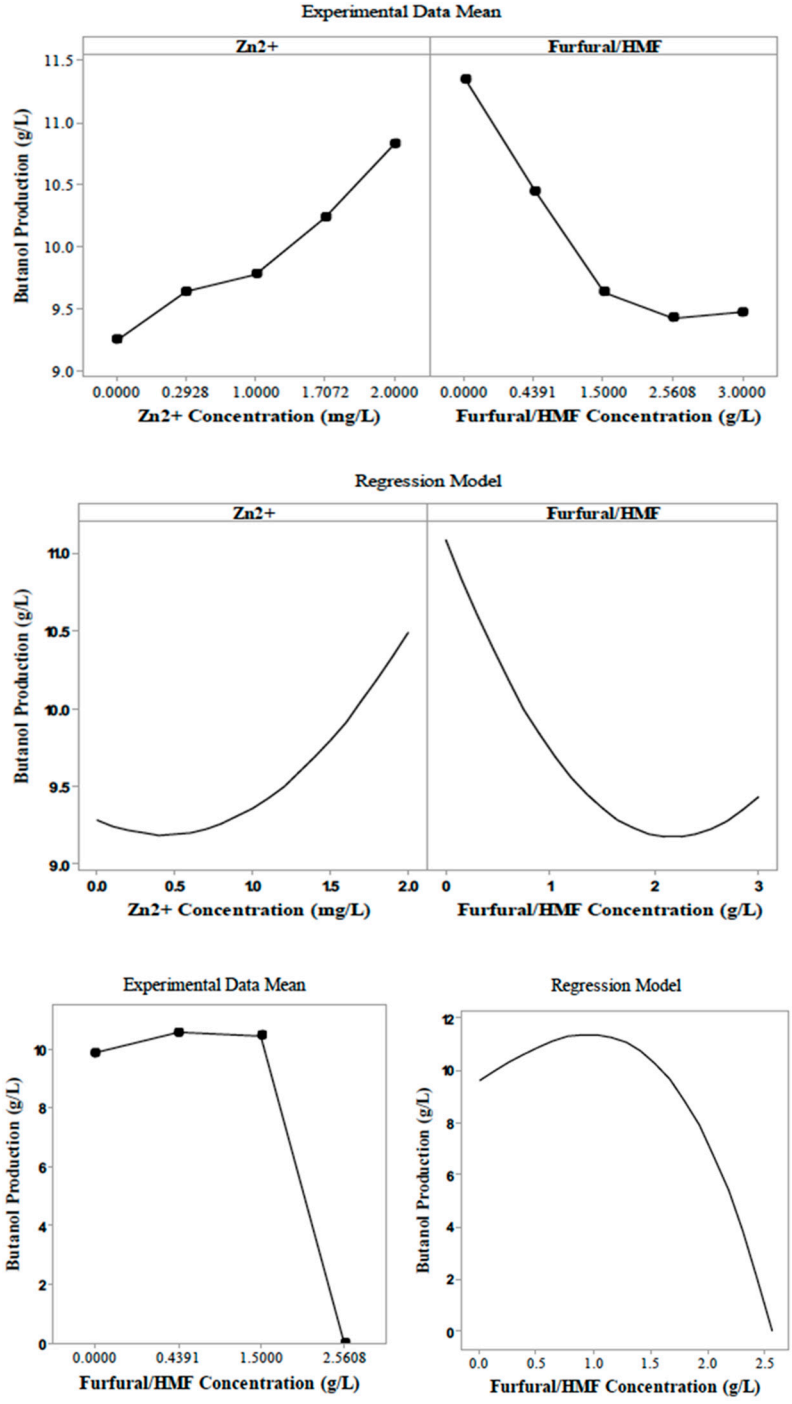


Figure 4. Individual effects of $ZnSO_4$ and furfural/HMF on butanol production in glucose feedstock and individual effect of furfural/HMF on butanol production in xylose feedstock.

Therefore, to perform a productive fermentation, $ZnSO_4$ supplementation should be 2 mg/L (1.7 mg/L was the lower limit) in glucose fermentation. $ZnSO_4$ supplementation was not a limiting factor in xylose fermentation. At a 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, for 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 $ZnSO_4$, 0.5~1.5 g/L furfural/HMF and 4 g/L $CaCO_3$ were contained in the feedstock.

Through tailoring metabolic perturbation, xylose was no longer an unfavorable sugar for clostridium, and butanol production in pure sugar feedstock was substantially improved. The ratio of butanol production in xylose feedstock to that in glucose feedstock was 0.74 in the current study. Since $CaCO_3$, $ZnSO_4$ and furan additives were gradually introduced, butanol productions in both the glucose and xylose feedstock were enhanced stepwise. The highest butanol productions in the 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. Table 8 shows the comparison of butanol production in the pure xylose and glucose feedstock in the current study.

Table 8. Comparisons of butanol production in glucose and xylose feedstock.

Chemical Additive	Sugar (60 g/L)	Butanol (g/L)	Ratio (Xylose/Glucose)
Control	glucose	7.50	0.74
	xylose	5.51	
$CaCO_3$ 2 g/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	
$ZnSO_4$ 1.7 mg/L $CaCO_3$ 4 g/L Furan 0.4 g/L	glucose	10.30	1.15
	xylose	11.82	

All data in the present study are from block 2.

3.3. Optimum Bio-Butanol Production Using Lignocellulosic Feedstock

Finally, metabolic perturbation optimums ($CaCO_3$ 4 g/L, $ZnSO_4$ 2 mg/L, furan derivatives 0.5~1.5 g/L) were tested in lignocellulosic feedstock. Furan derivative yields of wheat straw (WSH) are predictable and controllable based on the model of acid concentration, pretreatment time and temperature [8]. Spruce was treated in the same conditions for spruce hydrolysate (SH).

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

For SH, butanol production was significantly lower than that of WSH. The furfural and HMF concentrations in WSH and SH are typically different. In WSH, furfural is the dominant form of furan derivative (furfural: HMF = 3:1). In SH, overall furan derivative concentration is higher and HMF is the dominant furan derivative [34,35]. Furthermore, HMF is more toxic than furfural [15]. Therefore, clostridium was more inhibited in SH than it was in WSH. Moreover, softwood like SH contained higher lignin than agricultural lignocellulosic feedstock. Therefore, more phenolic compounds and aliphatic compounds released from lignin were contained in SH than WSH [34].

In Table 9, the ABE fermentation in lignocellulosic feedstock in certain studies is compared. In the current study, the 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, and 10.1 g/L surpassed the butanol production in many previous studies. The results showed metabolic perturbation was highly effective in both pure sugar and lignocellulosic feedstock.

Table 9. Comparisons of butanol production 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		[35]
			untreated	50/35	5.6	−40	
			treated	60/45	8.2	−13	
C. beijerinckii (BA101)	corn fiber	XAD−4 inhibitor resin remover	control	55/55	13.2		[36]
			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		[37]
			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		[38]
			treated	42/42	7.1	1	
C. beijerinckii (P260)	corn stove	NaOH overliming	control		13.2		[39]
			untreated		0	−100	
			treated		9.0	−32	
C. beijerinckii (IB4)	corn stove	active carbon	control	55/4.7	9.1		[40]
			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	

Metabolic perturbations influence ABE fermentation in lignocellulosic feedstock hydrolysate through strengthening strain tolerance to microbial inhibitors. In terms of economics, the chemical additives method was shown to be superior to over liming. To conduct a typical over liming, 25.9 g/L excess lime was added into feedstock hydrolysate to make the medium pH = 10, followed by adding 1 g/L Na_2SO_3 and pH neutralization with H_2SO_4 [41]. The chemical consumption and operation costs are high, and drawbacks like the large amount of solid waste and sugar loss up to 13% are all concerns that should be considered. Instead, to perform the metabolic perturbations method, neutralization is only conducted once, and chemicals like 4 g/L $CaCO_3$ and 2 mg/L $ZnSO_4$ can reduce the cost of extraction of butanol. In the lignocellulosic feedstock fermentation, slurry in the hydrolysates was not removed. This operation is related to a fast-developing subject in biofuel study called "Simultaneous saccharification fermentation" (SSF) [20,24,37]. In SSF, enzymatic hydrolysis of soluble and insoluble hydrolysate are performed together with the fermentation. SSF avoids sugar loss due to slurry separation and decreases the number of vessels and steps by combining hydrolysis and fermentation simultaneously. The decrease in capital investment has been estimated to be larger than 20% [42]. The major variable to improve SSF efficiency is to increase the 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 that the metabolic perturbations method could be applied in SSF.

4. Conclusions

In this study, a combination of stepwise metabolic perturbations was developed to improve the butanol productivity of *Clostridium beijerinckii* in wheat straw hydrolysate and spruce hydrolysate. The results from the preliminary investigation indicated that ABE fermentation could benefit more from multiple supplements of metabolic perturbations.

Through optimization, it was determined that for 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 $ZnSO_4$, 0.5~1.5 g/L furfural/HMF and 4 g/L $CaCO_3$ were contained in the feedstock. Finally, metabolic perturbation optimums ($CaCO_3$ 4 g/L, $ZnSO_4$ 2 mg/L, furan derivatives 0.5~1.5 g/L) were tested in lignocellulosic feedstock. Furthermore, metabolic perturbations of $CaCO_3$ 4 g/L and 2 mg/L $ZnSO_4$ were added to pre-grown culture to strengthen the tolerance to inhibitory effects and facilitate the growth of clostridium in lignocellulosic feedstock. As a result, the 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.

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