國立交通大學

環境工程研究所

碩士論文

利用農業廢棄物稻稈生產丁醇生質能源之研究

Biobutanol production from rice straw

研究生: 陳怡君

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摘要

此研究致力於探討最具經濟效益之稻稈醣化及 Acetone-Butanol-Ethanol (ABE) 發酵產醇之操作流程，並以中央合成設計及反應曲面法 (CCD-RSM) 找出於滅菌及無滅菌狀態下發酵分別之最佳化細胞植種濃度及培養溫度兩操作條件，以獲得最大之丁醇產率、丁醇產值、與 Gompertz 模擬之丁醇生產速率。未前處理之稻稈 (NPRS)、前處理之稻稈 (PRS) 及前處理稻稈與酸水解液之混合物 (MPRSH) 分別於一系列之批次反應瓶中進行酵素醣化實驗，結果顯示葡萄糖為主要之醣化產物，NPRS 的葡萄糖產值為每克之 NPRS 稻稈產出 0.52 g 之葡萄糖，與每克 PRS 及 MPRSH 的葡萄糖產量不相上下，PRS 及 MPRSH 的葡萄糖產值分別為 0.50 及 0.58 g/g。然而以操作成本及時間為考量，只經研磨之無化學性前處理稻稈 NPRS 為最具效益之醣化及發酵原料。模擬醣化結果所合成之 NPRS 水解液中含有 2.73 g/L 阿拉伯糖、28.10 g/L 葡萄糖、10.00 g/L 半乳糖與 5.00 g/L 之乙酸則用於 ABE 發酵批次實驗中。傳統 ABE 發酵皆於滅菌的環境下進行實驗，而滅菌過程中所損耗的能量及時間為成本來源之一，有鑑於此，本研究之發酵實驗分別於滅菌與無滅菌環境下進行，以探討 ABE 發酵於無滅菌條件下之可行性。各個批次反應皆於 pH 5.42±0.03 及 100 rpm 震盪之條件下進行。批次發酵結果用以計算丁醇產率、丁醇產值、與 Gompertz 方程式推估之丁醇生產速率。發酵反應期間，葡萄糖最容易被 *Clostridium saccharoperbutylacetonicum* N1-4 所利用，半乳糖次之，而阿拉伯糖則幾乎沒有被利用，乙酸則被微生物再利用轉換為丁醇、丙酮或乙醇。高濃度之初始細胞植種濃度，可抑制無滅菌操作實驗中污染之落菌或其他微生物，使丁醇生產之效率不受影響。低濃度之初始細胞濃度 (< 800 mg/L) 及過高之溫度 (> 42℃) 則

使產醇量下降，甚至造成細胞無活性或死亡。經表面曲面分析，滅菌組 ABE 發酵實驗之最大的丁醇產率 (1.45 g/L/d)、丁醇產值 (0.22 g/g)、及丁醇生產速率 (4.05 g/L/d) 可分別於初始細胞濃度 1.96 g/L、2.01 g/L 及 2.33 g/L 結合相對應之培養溫度 32.3℃、26.3℃及 30.5℃之操作條件下獲得; 而無滅菌組發酵實驗之最大丁醇產率 (1.45 g/L/d)、丁醇產值 (0.32 g/g) 及丁醇生產速率 (3.74 g/L/d) 則是分別於 26.4℃、25.0℃及 25.0℃之培養溫度結合 2.33 g/L 之初始細胞濃度的操作條件下獲得。於分別所適當的條件下進行 ABE 發酵，滅菌與無滅菌環境下之反應可達到相近的丁醇產率、產值與生產速率。總括本實驗的結果，可知以無滅菌方式進行 ABE 發酵未化學性前處理稻稈進行醣化後之水解液為一經濟且可行的生物產生質能源之方法。

關鍵字: ABE 發酵、生質能源、醣化、木質纖維生質量、丁醇生產

Biobutanol production from rice straw

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Abstract

This study aimed to integrate a cost-effective approach on the conversion of rice straw into fermentable sugars and biobutanol production through Acetone-Butanol-Ethanol (ABE) fermentation. The optimal initial cell concentration and incubation temperature for ABE fermentation under both sterile and non-sterile conditions were resolved by central composite design and response surface methodology (CCD-RSM). Saccharification experiments of non-pretreated rice straw (NPRS), pretreated rice straw (PRS), and mixture of pretreated rice straw and acid hydrolysate (MPRSH) were conducted in a series of batch reactors. Glucose was the major product. The results show that the glucose yield of 0.52 g glucose/g rice straw for NPRS was compatible to those of 0.50 and 0.58 g glucose/g rice straw for PRS and MPRSH, respectively. Thus, the saccharification of the rice straw grinded only without other pretreatment is more cost-effective if concerning to save operating time, energy and chemical cost. Simulated NPRS hydrolysate contained 2.73 g/L arabinose, 28.10 g/L glucose, 10.00 g/L galactose, and 5.00 g/L acetic acid was then used as the medium for ABE fermentation batch experiments with pH 5.42±0.03 and 100 rpm agitation. Conventional ABE fermentations are conducted under sterile condition to avoid contaminations from other microbes. However, sterilization is one of the costly steps in conventional ABE fermentation. To evaluate the feasibility of non-sterile ABE fermentation, the fermentation experiments in this study were performed under sterile and non-sterile environmental conditions. The results from the batch experiments were used

for determine the maximum butanol productivity, butanol yield, and butanol production rate estimated by the modified Gompertz equation. During the fermentation, glucose was easily and sharply utilized by *Clostridium saccharoperbutylacetonicum* N1-4 while arabinose was hardly utilized. Acetic acid was reutilized by cell to form butanol, acetone or ethanol. When batch experiments conducted under non-sterile condition, high initial cell concentration of *C. saccharoperbutylacetonicum* N1-4 can constrain the contaminations from other microbes and ensure the biobutanol production compatible with those under sterile condition. Low initial cell concentration (< 800 mg/L) or high incubation temperature (> 42 ℃) cause low biobutanol production. As results from the statistical approach by RSM, the maximum butanol productivity (1.45 g/L/d), butanol yield (0.22 g/g), and butanol production rate (4.05 g/L/d) were obtained at the initial cell concentrations and incubation temperatures of 1.96 g/L and 32.3℃, 2.01 g/L and 26.3℃, and 2.33 g/L and 30.5 ℃, respectively, under sterile condition. Meanwhile, under non-sterile condition, similar butanol productivity (1.45 g/L/d), butanol yield (0.32 g/g), and butanol production rate (3.74 g/L/d) could be achieved when the initial cell concentrations and incubation temperatures were controlled at 2.33 g/L and 26.4℃, 2.33 g/L and 25.0℃, and 2.33 g/L and 25.0℃, respectively. To overlook this study, the biobutanol production from non-pretreated rice straw powder can be achieved feasibly and economically under non-sterile environmental condition.

Keywords: ABE fermentation, Biofuel, Saccharification, Lignocellulosic biomass, Butanol production

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中華民國一百年六月

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Chapter 1 Introduction

1.1 Background

Since 1960s, fossil fuels have brought the convenience, economy improvement, and industrial development. Nowadays, almost all artificial production processes involve in petrochemical processes. For example, nearly all butanol is produced by petrochemical processes in current industry. Transportation system also uses petroleum as fuel. However, the by-products of fossil fuel combustion are one of the most important factors that caused global warming and pollution. According to the climate change report which published by Intergovernmental Panel on Climate Change (IPCC), the linear warming trend from 1956 to 2005 was 0.13℃ in average, which is nearly twice that for the past 100 years from 1906 to 2005. And the global green house gas emissions due to human activities have increased 70% between 1970 and 2004 (Pachauri and Reisinger, 2007). These data indicated that the global warming is getting serious. On the other hand, the oil crisis happened in 1970s emerged the problem of shortage. In current world, the issue of fossil fuel shortage still exists. In the future, the situation will not be better but worse. Therefore, it is important to develop renewable energy resources to decrease the emission of pollutants by replacing fossil fuel and to solve the energy shortage.

Biofuels have been known as clean energy carriers. Currently, fermenting carbohydrates for ethanol production has been a commercial biotechnology in industry (Abedinifar et al., 2009; Guo et al., 2009; Huang et al., 2009; Karimi et al., 2006). In addition to ethanol fermentation, Aceton-Butanol-Ethanol (ABE) fermentaion regains lots of attention albeit this biotechnology had already been developed in the early of twentieth century (Liu et al., 2005; Liu et al., 2009; Qureshi et al., 2010a; Qureshi et al., 2010b). However, there were number of main limitations of traditional ABE

fermentation processes (Jones and Woods, 1986): (1) High substrates cost by using carbohydrate substrates such as maize and molasses. (2) Economic viability to compete with fossil fuel. (3) The fermentation process was quite complex and needed to be run under sterile conditions. Contaminations, particularly due to phage infections, caused problems. Fermentation substrates are the most important factor influencing the cost of butanol production which made up about 60% of the overall cost (Qureshi and Blaschek, 2000). Rice is one of the main crops in Taiwan. According to Concil of Agriculture, Executive Yuan "Agricultural Statistics Yearbook 2009", the crop area planting rice in Taiwan was 254,590 ha and every hectare can harvest 6.2 tons of rice. The harvest of rice is accompanied with the significant production of rice straw. Rice straw mainly composed by carbohydrates offers a tremendous opportunity to be used as an economical and environmental friendly renewable resource. This renewable resource can be used for biofuels production through biological processes, providing a sustainable energy alternative.

1.2 Objectives

Considering all the aspects stated above, the overall objectives of this study are presented as below.

1. Production of biobutanol through Acetone-Butanol-Ethanol (ABE) fermentation using lignocellulosic biomass, rice straw, to lower the biofuel production cost.
2. To investigate a cost-effective approach on the conversion of rice straw into fermentable sugars.
3. Optimization of operating condition, initial cell concentration and incubation temperature, by central composite design and response surface methodology to further improve the economic viability of biobutanol production.
4. To study the feasibility of ABE fermentation operating under non-sterile condition.

Chapter 2 Literature Review

2.1 History of Acetone-Butanol-Ethanol fermentation

ABE fermentation was carried out industrially throughout the United States during the first half of last century. During World War I and World War II, ABE fermentation mostly used to produce acetone which is the raw material of Cordite (Dürre, 1998). Butanol was used as a replacement of amyl acetate for automobile coating, since United States government prohibited the usage of amyl acetate in 1920. The ABE fermentation process became the 2nd-largest industrial fermentation in the world (after ethanol fermentation). In 1950s, ABE fermentation process also developed in China peaked in the 1980s. However, the thriving development of ABE fermentation was discontinued in the early 1960s due to unfavorable economic conditions brought about by competition with the petrochemical industry (Ezeji et al., 2005). At the end of last century, ABE fermentation process still could not compete with the expanding petrochemical industry and be replaced (Ni and Sun, 2009).

2.2 Current developments of biofuel technology

Because of the environmental and energy requirement issues arise, technologies of biofuel production which produce clean energy has been an increasing worldwide interest. Nowadays, bioethanol and biobutanol are representative and feasible non-petroleum-based fuels. The following paragraphs and Table 2-1 are the current development status of these two bioconversion energy.

Bioethanol is fermentation-derived by ethanol fermentation process. The ethanol fermentation technology has been well-established under Governments support over the past 20 years. Today, ethanol is a commercialization biofuel. Since 2001, the first large-scale plant for conversion of waste biomass to bioethanol is planned to establish in

United State (Mielenz, 2001). At 2010, there are 137 U.S. plants with capacity to produce 7.6 billion gallons of ethanol, 62 plants being built, and 8 under expansion. The United States Congress mandated that 7.5 billion gallons of ethanol and biodiesel be produced per year by 2012, and 36 million gallons of ethanol be produced with 44% of it from cellulosic biomass by 2022 (Demain, 2009). China government enforce all area of Heilongjiang, Jilin, Liaoning, Henan, Anhui, Guangxi, and selected areas of Hebei, Shandong, Jiangsu, and Hubei provinces use 10% ethanol containing gasoline for motor vehicles. China claimed to reach 10 million metric tons (MMT) of total utilization of fuel ethanol (based on grain or non-grain) (Fang et al., 2010). Taiwan governmental organization, the Institute of Nuclear Energy research, also provide fund to support the development of ethanol production technologies (Guo et al., 2009).

ABE fermentation, on the other hand, has not been as well-established as ethanol fermentation. Although ABE fermentation had been carried out industrially throughout the United States during the first half of last century, it is keep in lab-scale research presently. Researchers dedicated in modifying the defects that hamper the economic viability of traditional ABE fermentation, like development of cellulosic-based ABE fermentation (Ezeji et al., 2007a; Qureshi et al., 2007; Qureshi et al., 2010a; Qureshi et al., 2010b), identification of energy-saving recovery technologies (Ishizaki et al., 1999; Qureshi et al., 2008a; Tashiro et al., 2005), improvement of productivities (Gu et al., 2009), modification of strains to resist inhibitors and to increase products, and so on. In 2005, Dr. David Ramey drove car solely use butanol as fuel and successfully traveled across the United States without causing the damage of the car. It demonstrated the feasibility of using biobutanol to replace petroleum in current system.

Table 2-1 Current development of Acetone-Butanol-Ethanol (ABE) fermentation and ethanol fermentation.

Ethanol fermentation	
Research Field	Well-established (Abedinifar et al., 2009; Huang et al., 2009; Karimi et al., 2006; Ko et al., 2009; Park et al., 2009)
Governments support	Over the past 20 years • The United States Congress mandated to develop ethanol especially from cellulosic biomass (Demain, 2009). • Taiwan governmental organization, the Institute of Nuclear Energy research, provide fund to support the development of ethanol production technology (Guo et al., 2009). • "A Long and Mid-Term Planning for Renewable Energy Plan" was planned by the National Development and Reform Commission, China Government (Fang et al., 2010).
Commercialization	• 137 U.S. plants with capacity to produce 7.6 billion gallons of ethanol, 62 plants being built, and 8 under expansion. • Pilot scale around the world, including Canada, Brazil, America, Japan, Denmark, and Sweden.
ABE fermentation	
Research Field	• Modifying the defects that hamper the economic viability of traditional ABE fermentation in lab-scale. • In 2005, Dr. David Ramey drove car solely use butanol as fuel.

2.2 Biofuels and fossil fuel

Fossil fuel, gasoline or petrol, is a liquid mixture derived from petroleum. It is used in internal combustion engines of vehicles. Gasoline consists of paraffins, olefins, naphthenes, aromatics, and O, N, S, and trace metals. The emissions of gasoline combustion are CO_x, NO_x, SO_x, etc., which pollute the air and harm human health. Ethanol produced from ethanol fermentation and butanol produced from ABE fermentation are two main sources of biofuels. As alternatives, ethanol and butanol biofuels have many advantages comparing to gasoline (Demain, 2009). First, ethanol

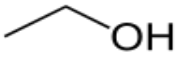
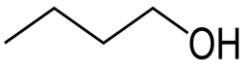
contains 35% oxygen while butanol contains 22% oxygen making them excellent fuel extenders and cleaner burning fuels. Combusted of biofuels produce CO_2 and H_2O without emission of particles and toxics. Second, ethanol and butanol has less smog formation because of their low volatility. Third, the production of biofuels is a sustainable process, since the production involves growing plants and converting plants into fuels. It is also decreases green house gas emission, because the growing plants recycle the green house gases. Fourth, biofuels enhance world energy security.

Between ethanol and butanol, butanol is superior to ethanol; even ethanol gets more attention in present days. Butanol has lower volatility, which decrease smog formation. Table 2-2 shows characteristics of gasoline, ethanol, and butanol. Energy content of ethanol is only 2/3 the energy content of gasoline. Ethanol requires engine modification when mixed with gasoline at over 15% of total fuel and cannot be shipped via pipelines. In contrast, butanol has 1/3 higher energy content than ethanol; less corrosive, and less hydroscopic (Dürre, 2007). Besides, butanol has sufficiently similar characteristics to gasoline can be used directly in any gasoline engine without modification and/or substitution.

2.3 Acetone-Butanol-Ethanol fermentation

Fermentation converts carbohydrates into cellular biomass and produces liquid energy carriers, i.e. acetone, butanol, and ethanol. In fermentation bioreactions, an organic compound serves as electron donor and electron acceptor (Rittmann and McCarty, 2001) in the absence of oxygen. In other words, the production of energy from carbohydrates or other organic substrates without using O_2 as an electron acceptor is called fermentation. Acetone-Butanol-Ethanol fermentation can abbreviate to ABE fermentation. The reaction which involved electron donor, acceptor and produced energy and biomass is displayed in Figure 2-1.

Table 2-2 The characteristics of biofuels and fossil fuel.

	Fossil fuel	Biofuels	
	Gasoline	Ethanol	Butanol
Composition	1.Paraffins (C_nH_{2n+2}) 2.Olefins (C_nH_{2n}) 3.Naphthenes (C_nH_{2n}) 4.Aromatic 5. O, N, S elements and trace metals	C_2H_6O 	$C_4H_{10}O$ 
Emission and products	CO_x, NO_x, SO_x……..	$C_2H_6O + 3.5\ O_2 \rightarrow 2\ CO_2 + 3\ H_2O$ (If complete combustion)	$C_4H_{10}O + 6\ O_2 \rightarrow 4\ CO_2 + 5\ H_2O$ (If complete combustion)
Density Mg/m^3 at 20℃	0.72-0.78	0.79	0.81
Energy density, MJ/L	32	19.6	29.2
LHV [a], kJ/g	43.3	27.0	33.4
Air fuel ratio [b]	14.6	9.0	11.2
Motor octane number	81-89	102	78

[a] LHV = Lower Heating Value = (Heat combustion) – (enthalpy of evaporation of water formed during combustion, at 100 kpa)

[b] Air fuel ratio = m_{air}/m_{fuel}

The data were from (Lee et al., 2008b; Pfromm et al., 2010).

Three major classes of ABE fermentation products are solvents (acetone, butanol, and ethanol), organic acids (acetic acid, butyric acid, and lactic acid), and gases (CO_2 and H_2) (Zheng et al., 2009). In theory, the production ratio of acetone, butanol, and ethanol is 3:6:1. Total solvent concentration is around 20-30 g/L when using traditional stains, *Clostridium acetobutylicum* with traditional batch fermentation processes (Karakashev et al., 2007; Qureshi and Blaschek, 2001). The metabolism pathway is described in detail in following sections.

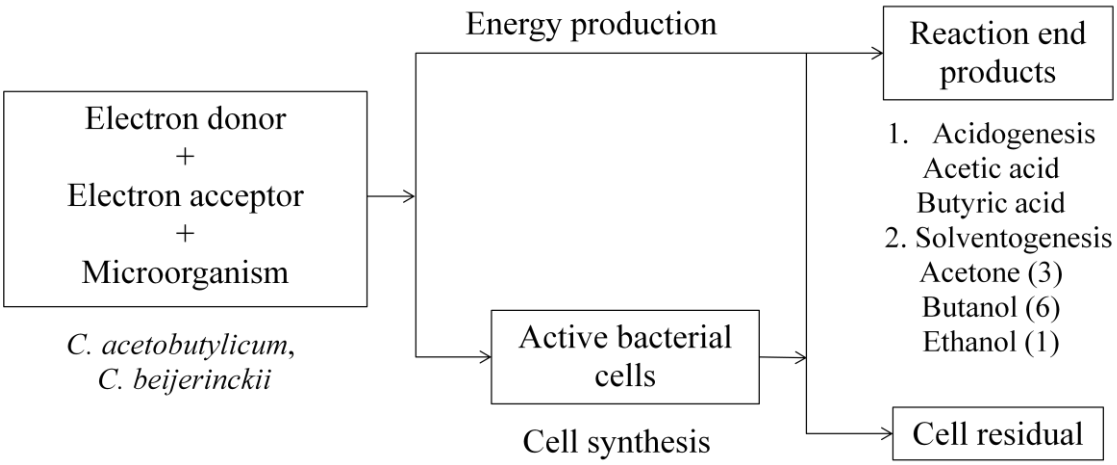


Figure 2-1 The scheme of ABE fermentation.

(Rittmann and McCarty, 2001)

2.3.1 Fermentation microorganisms and metabolic pathway

ABE fermentation microorganisms belong to genus *Clostridia*. *Clostridia* are rod-shaped, spore-forming gram-positive bacteria, and typically strict anaerobic. Solventogenic clostridia (*C. acetobutylicum* and/or *C. beijerinckii*) have an added advantage over natural ethanol producing strains as they can utilize both hexose and pentose sugars released from agricultural residues. All solvent-producing clostridia metabolize hexose sugars through fructose biphosphate pathway (Embdem-Myerhof pathway), as shown in Figure 2-2. One mole of hexose produces 2 moles of pyruvate with the net production of 2 ATP and 2 NADH. The species able to utilize pentose is via the hexose monophosphate pathway (Warburg-Dickens pathway). Fermentation of one mole pentose results in the production of 2/3 mole of fructose-6-phosphate and 1/3 mole of glyceraldehydes-3-phosphate with the net production of 5/3 moles of ATP and 5/3 moles of NADH (Jones and Woods, 1989). It shares the same metabolic pathways from sugars to acetyl-CoA but braches into different pathways thereafter. Following ATP and NADH, and pyruvate produced, the mechanism is typically a

biphasic involving process, including acidogenesis and solventogenesis, as shown in Figure 2-3.

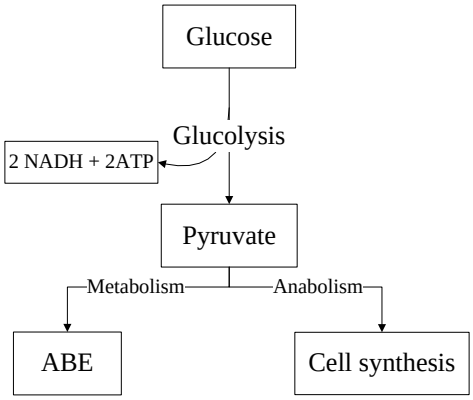


Figure 2-2 The scheme of hexose sugars metabolism in *Clostridia*.

Acidogenesis usually occurs during the exponential growth phase. Products in acidogenic phase are acetic acid and butyric acid, which cause pH decrease in broth. In solventogenesis, the organic acids produced in acidogenesis are reutilized and acetone, ethanol, and butanol are produced. As a result, pH value in broth increased. Solventogenic phase usually occurred at the end of exponential growth phase and cell stationary phase (Lee et al., 2008b). ABE fermentation first undergoes acidogenic phase. When the concentrations of undissociated acids exceed some threshold value, it switches to a solventogenic phase. For the metabolic pathway inside solvent-producing clostridia, five enzymes, acetoacetyl-CoA thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase and aldehyde/alcohol dehydrogenase, which are encoded by *thl*, *hbd*, *crt*, *bcd* and *adhE/adhE2*, respectively, are needed to complete the conversion of acetyl-CoA to butanol (Zheng et al., 2009).

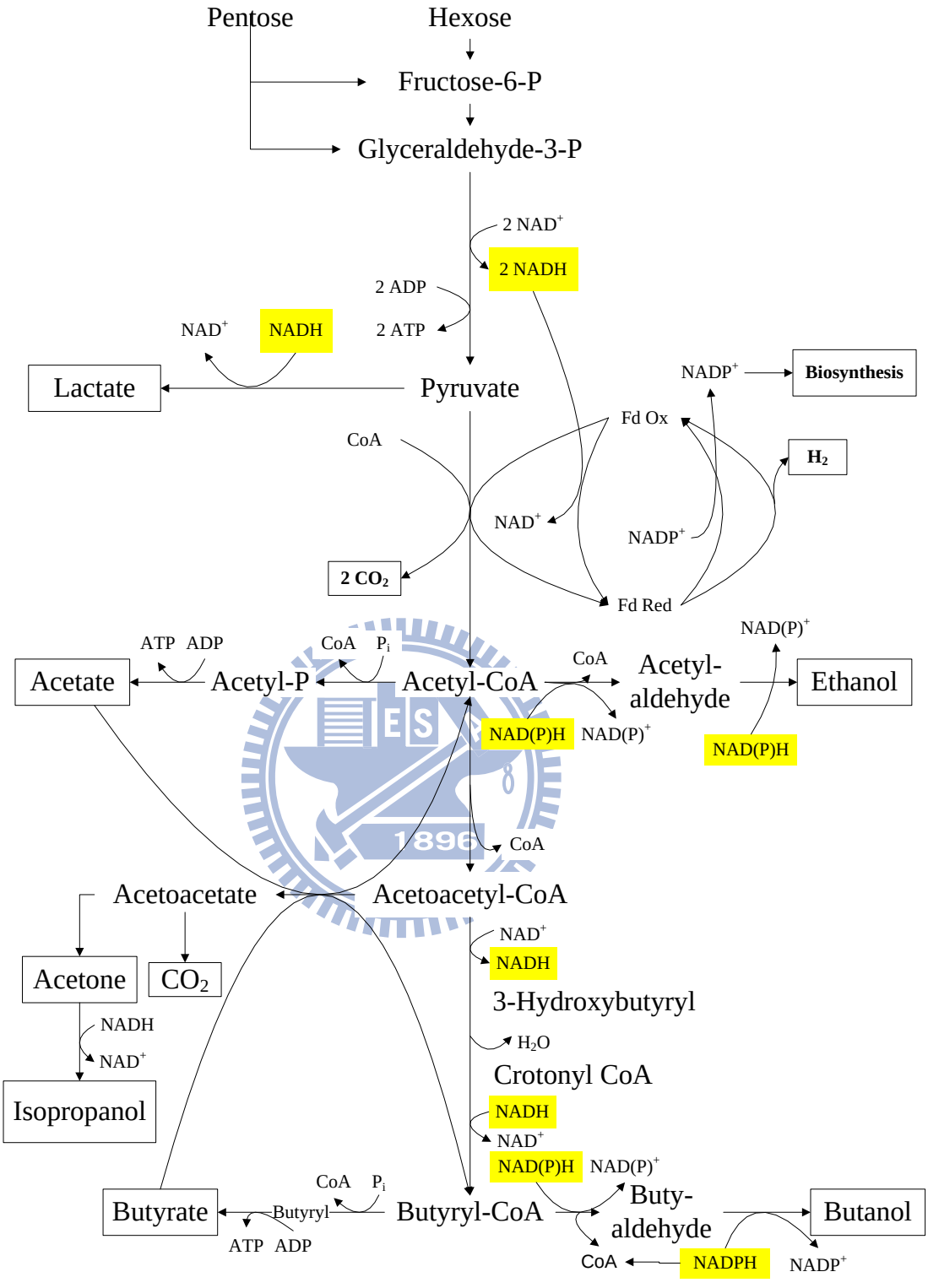


Figure 2-3 Metabolic pathway used by solvent-producing clostridia.

(Jones and Woods, 1989; Lee et al., 2008b)

Different *Clostridia* have different performances and productivities. Broad substrates utilization, high solvent productivities and yield, being highly tolerant to inhibitors and fermentation products, and thereby easy to cultivate are criteria that identify ideal solvent-producing microorganisms.

C. acetobutylicum, *C. beijerinkii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* are commonly regarded as productive species. *C. acetobutylicum* is the most extensively used and studied strain. The theoretical ratio of acetone, butanol, and ethanol, 3:6:1 is according to fermented by *C. acetobutylicum* in batch system. Different species of *Clostridia* are varying their products ratio based on their metabolism (Andreesen et al., 1989). In an attempt to compare performance of different *Clostridia*, Table 2-3 collected several batch studies with similar experimental conditions using glucose a carbon source and temperature in all studies was controlled at 30℃. *C. beijerinckii* and *C. saccharoperbutylacetonicum* are high productivity and yield strains. Recent studies are extensively used these two strains to produce ABE. On the other hand, *C. puniceum* and *C. aurantubutyrium* are not as efficient as *C. beijerinckii* and *C. saccharoperbutylacetonicum.*

Table 2-3 ABE fermentation of various *Clostridia* by using glucose as carbon source in batch system.

Microorganism	Initial sugar conc. (g/L)	ABE conc. (g/L)	Yield	Productivity (g/L/h)	Reference
Clostridium beijerinckii P260	62	20	0.41	0.28	(Qureshi et al., 2007)
Clostridium beijerinckii BA101	55-60	18	0.4	0.27-0.30	(Ezeji et al., 2007a; Ezeji et al., 2005; Ezeji et al., 2007d)
Clostridium saccharoperbutylacetonicum N1-4	50	19.2	0.43	-	(Tashiro et al., 2004)
Clostridium puniceum	-	-	0.37	-	(Andreesen et al., 1989)
Clostridium aurantubutyrium	-	-	0.23	-	(Andreesen et al., 1989)

2.3.2 Factors affecting the solvent production

Based on the mechanism of ABE fermentation, there are several conditions that can trigger a metabolic shift from acidogenesis to solventogenesis and thereby increase solvents production.

2.3.2.1 Intracellular status of *Clostridia*

The intracellular status is an important key to trigger solvent producing phase. As shown in Figure 2-3, accumulation of ferredoxin (Rd) and NAD(P)H or depletion of ferredoxin (Ox) and NAD (P); accumulation of acetyl-CoA/butyryl-CoA and depletion of phosphate pool; and accumulation of dissociated acetate and butyrate, induced the microorganisms to produce butanol and ethanol. Among all, a high concentration of acetyl-CoA plays a relatively important role in butanol production. Acetyl-CoA is mostly converted into butyryl-CoA since this reaction is thermodynamically favorable with $\Delta_r G_m^{\ominus}$ = -14.2 kcal/mol. However, the conversion of acetyl-CoA to acetoacetyl-CoA, which is the intermediate product in the reaction of acetyl/butyryl-CoA conversion, is considered to be the rate-limiting step ($\Delta_r G_m^{\ominus}$ = 5.3 kcal/mol) (Mavrovouniotis, 1990; Zheng et al., 2009), thus high concentration of acetyl-CoA is needed to overcome the barrier.

2.3.2.2 Medium conditions

The conditions of cell growth medium, including pH, buffering capacity, organic acids addition, and nutrient condition, are critical factors that decide the metabolic of microorganisms. pH value of the medium is very important for the biphasic mechanism of ABE fermentation. Acetic acids and butyric acids formed during acidogenic phase lower the pH of medium to a critical point and trigger the solventogenic phase. Thus, low pH is essential for solvent production (Lee et al., 2008b). It is reported that *Clostridia* tend to produce organic acids under higher pH while tend to produce solvents under lower pH.

Nevertheless, the pH range over which solvent formation may occur appears to vary quite widely depending on the particular strain. For instance, solvents production by *C. saccharoperbutylacetonicum* N1-4 and *C. beijerinckii* P260 are enhanced at pH 5 (Tashiro et al., 2004) and pH 4.5 (Li et al., 2011), respectively. In addition, butanol could be produce effectively by feeding organic acids such as acetic acid or butyric acid. Gu research group found that the addition of 30 mM ammonium acetate to cassava medium significantly increase solvent production by *C. acetobutylicum* EA 2018. They found 13 g/L of butanol within total solvent concentration of 19.4 g/L (Gu et al., 2009). It has been reported that the addition of 36 mM butyrate induced 24-folds higher of butanol production than without butyrate addition. Butanol yield of 0.32 was obtained when 36 mM acetate was added into the medium while the yield was reduced to 0 in absence of acetate into the medium (Lee et al., 2008a). The other researchers reported that specific butanol production rate (g/g/h) increased from 0.1 to 0.42 when 57 mM butyrate was added into the medium (Tashiro et al., 2004). In other words, organic acids, acetic acid and butyric acid, are important factors that induce solventogenesis. However, once the concentration of undissociated acids exceeds some threshold value, "acid crash" may occur in pH-uncontrolled batch ABE fermentation experiment. A threshold value of 57-60 mM for *C. beijerinckii* NRRL B592 was reported (Maddox et al., 2000). Also, if the pH decreases below 4.5 before enough acids are formed, solventogenesis will be brief and unproductive for some stains. Buffering, pH control and low temperature fermentation can be performed to effectively avoid the "acid crash" (Li et al., 2011; Maddox et al., 2000; Zheng et al., 2009).

Carbon source acts both electron acceptor and donor in ABE fermentation process. From metabolic aspect, high concentration of carbon sources, for example glucose, can provide energy for fermentation. However, it is reported that total sugar concentration higher than 250 g/L caused no growth of *C. beijerinckii* and therefore no ABE production. And mixture of wheat straw hydrolysate plus 140 g/L glucose where total sugar concentration was around

200 g/L used as medium showed poor growth and poor ABE production (Qureshi et al., 2007). Shaheen et al. investigated the fermentation solvent yield of several *Clostridia* in Tryptone-Yeast-Acetate (TYA) medium content 40, 50, 60, and 70 g/L of glucose concentration. Results indicated that 70 g/L glucose medium had the lowest solvent yield in all tests and the maximum solvent yields for *C. acetobutylicum* and *C. saccharoperbutylacetonicum* were 0.32 at 40 g/L sugar concentration (Shaheen et al., 2000). When the sugar concentration is too high, the residual sugar is also high. A high substrate concentration lead high concentration of ABE accumulated, which is toxic to culture (Qureshi et al., 2007).

Iron is an important mineral supplement since the conversion of pyruvate to acetyl-CoA involves ferredoxin oxidoreductase iron-sulfur protein. When cell was grown in batch culture under iron limitation (0.2 g/L) at pH 4.8, butanol is the major fermentation product due to the decrease of hydrogenease specific activity (Junelles et al., 1988). It was also reported that the ratio of butanol/acetone was dramatically increased from 2 to 8 under iron-limited condition (Bahl et al., 1986). In addition, solventogenesis was dominant in phosphate limited medium. ABE fermentation in continuous culture under phosphate limitation was revealed to produce 0.3, 0.2, and 0.01 of ethanol, butanol, and acetone yield, respectively, with only trace amount of acetic acid and butyric acid (Dabrock et al., 1992). Another research group use low-phosphate synthetic medium cofermented with L-lactate found that butanol/acetone ratio could be increased from 2:1 to 3.8:1 (Bahl et al., 1986).

2.3.2.3 Solvent toxicity

During the solvent-producing phase, cell metabolism is continuous and thereby accumulates solvents (butanol, acetone, and ethanol) in fermentation system. When the total concentration of solvent reaches 20 g/L, the inhibitory effects occur, after which cell metabolism ceases. Among all, butanol was happened to be the most toxic to *Clostridia*.

Solvent production and cellular growth were inhibited at 8 g/L of butanol, and another found that at 11 g/L of butanol inhibit 50% growth of *C. acetobutylicum* at pH 4 (Ladisch, 1991). It has been proven that butanol had numerous harmful effects on *C. acetobutylicum*: the cells lost the ability to maintain internal pH, the membrane ATPase was partially inhibited, intracellular ATP levels collapsed, glucose uptake was decreased, and membrane fluidity disrupted, and inhibited membrane-linked functions (Bowles and Ellefson, 1985; Jones and Woods, 1986). Researchers has been dedicated for years to reduce the effect of butanol toxicity by developing butanol tolerance strains, genetically modified strains and developed alternative fermentation and product recovery technologies which are discussed in section 2.5.

2.4 Substrates

2.4.1 Monosaccharide

The conversion efficiency of different substrates into ABE by *Clostridia* is various significantly. The simplest substrate is monosacharide, such as glucose. Generally, the productivity is about 0.28-0.30 g/L/h. Ezeji et al. used *C. beijerinckii* BA101 to ferment glucose. As results, *C. beijerinckii* BA101 converted 44.6 g/L sugar into 17.7 g/L ABE within 60 h. The productivity was 0.29 g/L/h (Ezeji et al., 2005). Qureshi et al. revealed that the productivity was 0.28 g/L/h with 48.9 g/L sugar fermented by *C. beijerinckii* P260 (Qureshi et al., 2007).

Fermentation substrates are the most important factor influencing the cost of butanol production. About 60-70% of the total production cost in ABE fermentation comes from fermentation raw materials (Madihah et al., 2001; Qureshi and Blaschek, 2000). In conventional ABE fermentation, the substrate was usually molasses, which is one of the factors that hamper the economic viability. For economic reason, series of studies started to use plant directly as substrates. Following we discuss two types of plants, starch/sugar-based crop and lignocellulosic biomass, in details. Starch/sugar-based crops

are mostly edible and easier to utilize by organisms than the other one. Table 2-4 compare the characteristics of different kinds of substrate, monosaccharide, starch-based crop, and lignocellulosic biomass used for butanol production.

2.4.2 Starch/sugar-based crop

Starch/sugar-based crops can be utilized by organisms directly or after liquification or gelatinization, such as sugarcane and corn. The technologies for converting starch/sugar containing energy crops into ABE products are well-established. Table 2-5 shows several researches that focus on starch-based crops as ABE fermentation substrates. The productivity of *C. acetobutylicum* was 0.26 g/L/h when cassava used as substrate (Gu et al., 2009) and sago starch (Madihah et al., 2001). The addition of ammonia acetate elevates the solvent productivity of *C. acetobutylicum* to 0.4 g/L/h in cassava medium. Ezeji research group made a series study of using corn starch as a medium, the productivity were 0.15-0.29 g/L/h for *C. beijerinckii* (Ezeji et al., 2007b; Ezeji et al., 2005; Ezeji et al., 2007d).

Table 2-4 Comparisons of three different kinds of fermentation substrates.

Type	Monosaccharide	Starch/sugar-based crops	Lignocellulosic biomass
Example	Glucose	Corn Starch Sugarcane Cassava	Agricultural residues (Rice straw, Corn cob, Wheat straw)
Advantages	Easily and directly utilize by organisms. (no further treatment needed)	Can utilize by organisms directly or after liquification or gelatinized.	Most abundant renewable resource on the planet.
Disadvantages	Costly	Costly Edible parts of plants, competition with the food and feed supplies.	Difficult to utilize directly by microorganisms due to the complex structure.
ABE Productivity	0.28-0.30 g/L/h	0.15-0.29 g/L/h	0.10-0.31 g/L/h
Reference	(Ezeji et al., 2005; Qureshi et al., 2007)	(Ezeji et al., 2007a; Guo et al., 2009)	(Qureshi et al., 2007; Qureshi et al., 2010a)

Table 2-5 Performances of different Starch/sugar-based crops being medium for ABE fermentation.

Substrate (Initial conc. [a])	**Microorganism**	**Temp. (℃) /pH**	**Substrate used [b] (g/L)**	**Reactor**	**ABE conc. (g/L)**	**Productivity [c] (g/L/h)**	**Yield [d]**	**Butanol conc. (g/L)**	**Acid [e] (g/L)**	**Reference**
Cassava (60 g/L)	*C. acetobutylicum* EA 2018	37/ND	48	Batch	15.4 (60 h)	0.26	-	9.9	0.7	(Gu et al., 2009)
			-	Batch (+ 30 mM ammonia acetate)	19.4 (48 h)	0.40	-	13.0	1.6	
Gelatinized sago starch (60 g/L)	*C. acetobutylicum* P262	35/ pH 6	-	Batch	11.0 (42 h)	0.26	0.33		1.5	(Madihah et al., 2001)
Corn starch (40.8 g/L)	*C. beijerinckii* BA101	36/ND	37.2	Batch	20.0 (72 h)	0.28	-	14.3	1.7	(Ezeji et al., 2005)

[a] Initial concentration indicate starch concentration at t=0 if there is no further explanation.

[b] The concentration of starch consumed by microorganism during ABE fermentation if no specific explanation.

[c] Productivity = Total ABE concentration/Fermentation time

[d] Yield = The weight of ABE solvent/The weight of sugar utilized by microorganism

[e] Acetic acid and butyric acid

Table 2-5 Different Starch/sugar-based crops for ABE fermentation (continuous).

Substrate (Initial conc. [a])	**Microorganism**	**Temp. (℃) /pH**	**Substrate used [b] (g/L)**	**Reactor**	**ABE conc. (g/L)**	**Productivity [c] (g/L/h)**	**Yield [d]**	**Butanol conc. (g/L)**	**Acid [e] (g/L)**	**Reference**
Liquefied corn starch (60 g/L sugars)	*C. beijerinckii* BA101	35/ND	45 sugars	Batch	18.4 (120 h)	0.15	0.41	13.4	-	(Ezeji et al., 2007d)
Degermed Corn (40-45 g/L)	*C. beijerinckii* BA 101	35/ND	32-37	Continuous (dilution rate 0.03 h^{-1})	8.98 (60 h)	-	-	5.9	3.8	(Ezeji et al., 2007b)
Saccharified degermed corn (55-60 g/L)			-		9.70 (504 h)	0.29	-	6.33	2.7	

[a] Initial concentration indicate starch concentration at t=0 if there is no further explanation.

[b] The concentration of starch consumed by microorganism during ABE fermentation if no specific explanation.

[c] Productivity = Total ABE concentration/ fermentation time

[d] Yield = The weight of ABE solvent/ The weight of sugar utilized by microorganism

[e] Acetic acid and butyric acid

2.4.3 Lignocellulosic biomass

The production of biofuel from edible parts of plants has been increasing dramatically, which results in competition with the food and feed supplies. Lignocellulosic biomass is the most abundant renewable resource on the planet which offers an attractive alternative as ABE fermentation substrate. Wheat straw (Qureshi et al., 2007; Qureshi et al., 2008b), corn stover, switchgrass (Qureshi et al., 2010b), barley straw (Qureshi et al., 2010a), corn fiber xylan (Ezeji et al., 2007a; Qureshi et al., 2006), bagasse, silvergrass (Guo et al., 2009), and rice straw (Ko et al., 2009) are commonly used lignocellulosic biomass for the production of biofuels through ABE fermentation or ethanol fermentation in recent studies.

Rice straw is considered to account for the largest portion of available biomass feedstock in the world and Asia is responsible for 90% of the annual global production (Kim and Dale, 2003). In Taiwan, rice is one of the main food crop. According to Concil of Agriculture, Executive Yuan of Taiwan "Agricultural Statistics Yearbook 2009", the crop area of rice in Taiwan was 254590 ha and every hectare of crop land could produce 6199 kilograms of rice in 2009. The great amount of rice straw residues left over after cropping would be excellent substrates for boifuel production. Currently, half of the agricultural residues of the world is burned, which cause health and environmental problems (Demain, 2009).

The main components of lignocellulose are cellulose, hemicellulose, and lignin. Depends on the sorts of plant and material, the compositions are different in proportion. Lignin and hemicellulose formed matrix and covered cellulose, which is naturally resistant to enzymatic attack (Sheehan John, 1994). Among the three main compositions of lignocelluloses, cellulose and hemicellulose are belongs to polysaccharides. Cellulose is the major components of plant's cell wall, which plays a role of structural support. It consists of a linear chain of β (1-4) linked glucose monomers. Cellulose is tightly packed and highly crystalline structures make it water insoluble and resistant to depolymerization.

The structure of cellulose is shown in Figure 2-4. Unlike cellulose, hemicelulose is a heterogeneous compound that contains not only hexose (glucose and/or galactose) but also pentose (xylose, arabinose, mannose, etc.) monomers. Because of the branched structure, hemicellulose can be attached and broken easier by enzyme than cellulose. In addition, hemicellulose is soluble in acid solution. The structure of hemicellulose is shown in Figure 2-5. On the other hand, lignin is a three dimensional, net structural and non-crystalline polymer, which mainly consist of aromatic compound. However, the actual structure is still unclear. Overall, hemicellulose hydrogen-bonds to cellulose microfibrils and form a network that provides the structural backbones of cell wall. And lignin further strengthens the cell walls and provides resistance against diseases and pests (Mosier et al., 2005). As a result, lignocellulosic biomass is difficult to utilize directly by fermenting microorganisms due to their complex structure. To utilize the valuable resources, potential sugar monomers, contained in lignocellulose for fermentation processes, appropriate pretreatment and hydrolysis steps are required for lignocellulosic materials before fermentation by microorganisms. Figure 2-6 shows the general scheme for lignocellulosic biomass to produce ABE biofuels.

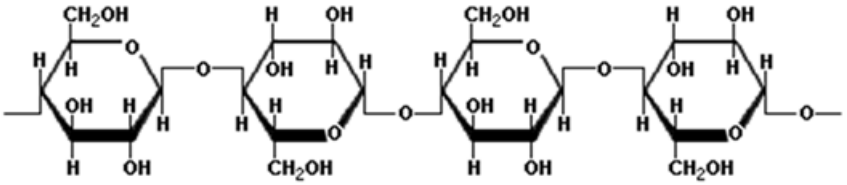


Figure 2-4 The structure of cellulose.

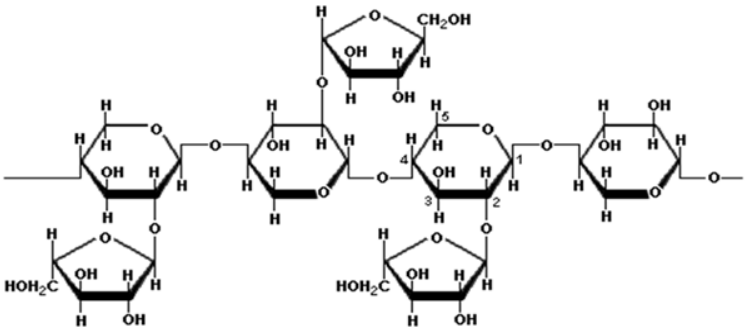


Figure 2-5 The structure of hemicellulose (arabinoxylan).

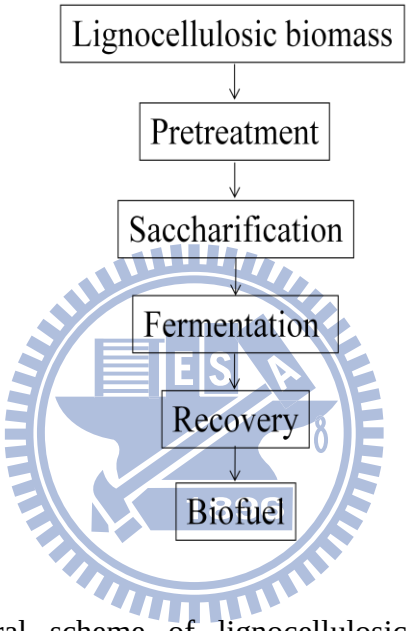


Figure 2-6 The general scheme of lignocellulosic biomass used for ABE fermentation.

2.4.2.1 Pretreatments

The main functions of pretreatments are to reduce the size of feedstock, open up the hemicelluloses-lignin matrix surrounds cellulose, and to break cellulose crystal structure (Sheehan John, 1994). The structure of lignocelluloses is altered to make cellulose and hemicellulose more accessible to the enzymes that saccharified the carbohydrate polymer into fermentable sugars (Mosier et al., 2005). A variety of pretreatment technologies with different characteristics have been developed. Pretreatments are mostly carried out under high temperature and pressure. However, a good pretreatment process is the one with a

high yield of carbohydrates combined with a low production of fermentation inhibitors. Also good pretreatments need to minimize energy demand and limit cost.

There are four common categories of pretreatment technologies, biological, physical, chemical, and physio-chemical pretreatments. Biological pretreatments utilize wood degrading fungi, brown-, white-, and soft-fungi, to modify the chemical composition of lignocellulosic biomass. Brown rots mainly attack cellulose, while white and soft fungi attack both cellulose and lignin. The advantages of biological pretreatments are low energy requirement and mild environmental conditions. However, biological pretreatment processes need careful control of growth conditions control, large operation space, and long residence time (10-14 days) (Chandra et al., 2007), and throughout biological pretreatments are considered to be less attractive commercially.

Physical pretreatments include comminution, and pyrolysis. Comminution is a method to mechanically reduce biomass into particulate size by chipping, grinding, and milling. The enzymatic conversion yield of wet disk milling and ball milling pretreated rice straw were reported to be 0.79 and 0.89 of glucose, and 0.42 and 0.54 of xylose, respectively (Hideno et al., 2009). Pyrolysis decomposes the lignocelluloses through high temperature. Overall, physical methods break the crystaline structure, decrease the size, and increase the surface area of lignocellulosic biomass through mechanical power or heat.

Chemical methods, on the other hand, mainly break structure by chemical reactions, such as bond breaking. The example of chemical pretreatment methods are ozonolysis, acid or base hydrolysis, oxidative delignification, organosolv process, etc. In ozonolysis, ozone mainly attack lignin. Hemicellulose is slightly attacked and cellulose is hardly affected. Ozonlysis pretreatment does not produce toxic residues. In addition, it performs under room temperature and pressure. However, this process is expensive because large amount of ozone is needed. H_2SO_4 and NaOH are commonly used reagents in acid and alkaline pretreatment, respectively. High concentration of acid and base

solution gets high treatment efficiency in pretreatment process. However, it also gets corrosive feature and safety issues. Many researchers use dilute acid and dilute base solution instead of concentrated acid and base for pretreatment, which is efficient, safe, and economical (Cara et al., 2008). Dilute acid pretreatment can significantly improve cellulose hydrolysis (Guo et al., 2009; Karimi et al., 2006). Dilute base pretreatment of lignocelluloses caused swelling, leading to an increase in internal surface area and a decrease of polymerization and crystallinity, and disruption of the lignin structure (Mosier et al., 2005). Both acid and base pretreatments need to neutralize pH for the following enzymatic saccharification or fermentation processes. Soaking in aqueous-ammonia (SAA) is a new method of alkaline pretreatments which is highly selective for lignin removal and shows significant swelling effect on lignocelluloses. And ammonia is easily recoverable due to its high volatility. Ko et al. reported that rice straw pretreated by SAA could reach the maximum enzymatic digestibility of 71.1% at 69℃ for 10 h with an ammonia concentration of 21% (w/w) (Ko et al., 2009). Organosolv process use mix solution of inorganic acid (HCl or H_2SO_4) and organic solvent (methanol, ethanol, acetone, ethylene glycol, etc.) as reagent to break lignocellulose structure. The inorganic acids play a catalyst role in organosolv process. It is necessary to remove the solvent from the system after pretreatment because the solvent may inhibit microorganisms in enzymatic and fermentation processes.

Physio-chemical pretreatment methods are combination of both chemical and physical processes. Steam explosion, ammonia fiber explosion (AFEX) (Dale et al., 1996), and CO_2 steam explosion are the most well-kown and common methods. Steam explosion process is tipically treated with high pressure (0.69-4.83 Mpa) and high temperature (160-260℃), and then reduce pressure in a few seconds or munites. The materials undergo an explosive decompression (Sun and Cheng, 2002). The major effect is attributed to the removal of hemicellulose which improve the acessibility of enzymes to

cellulose fibrils. Steam explosion is a cost-effective methods compared to mechanical comminution. However, degradation products formed in this process are kown to inhibit the microorganism activity in the following processes, and therefore water washing step needs to be performed after pretreatment. The washing step remove not only inhibitors but also soluble sugars which cause the decrease of overall saccharification yields. The concept of AFEX and CO_2 explosion is similar to steam explosion. They are performed at high temperature and pressure for a period of time, and then the pressure reduced swiftly. The major difference is that the materials are exposed in water, ammonia, and CO_2 for steam explosion, AFEX, and CO_2 explosion, respectively. Unlike steam explosion, both AFEX and CO_2 explosion processes do not produce inhibitors (Sun and Cheng, 2002).

Still there are other novel technologies, such as Teramoto research group examined a sulfuric acid-free ethanol cooking pretreatment (SFEC) to pretreat lignocellulosic biomass. This process exposes cut-milled lignocellulosic flours to an ethanol/water/acetic acid mixture in an autoclave. SFEC does not intensively delignified, instead it improves the accessibility of enzyme to cellulosic component (Teramoto et al., 2009).

Table 2-7 indicates the composition of lignocellulosic biomass before and after various pretreatment methods when rice straw represent as lignocellulosic biomass. Compared to other agriculture residues, rice straw primarily consists of cellulose, hemicellulose and lignin (Chandra et al., 2007), and 10-28% soft carbohydrates (starch, sucrose, glucose, fructose, and β-1,3-1,4-glucan.) (Park et al., 2009). It contains significantly larger amounts of starch than other cereal straws, and in some cases, the amount of starch in the rice straw reaches over 20% of the dry weight. Unlike wheat straw, high silica content and low digestibility prevents rice straw from being suitable cattle feed.

Table 2-6 Comparison of rice straw composition.

Cultivar	Crop time	Original composition of rice straw	Pretreatment method	Composition of rice straw after pretreatment	Carbohydrates of the hydrolysate solution	Reference
Sazandegi (Lenjan fields in Esfahan province of Iran (32°34'N, 51°32E')	Sep., 2003	Hemicellulose 24% Cellulose 38% Lignin 8% Ash 15%	Acid pretreatment (0.5% H_2SO_4)	Hemicellulose 1% Cellulose 55% Lignin 5% Ash 13%	-	(Abedinifar et al., 2009)
			Steam pretreatment	Hemicellulose 2% Cellulose 51% Lignin 7% Ash 13%		
Korea University Farm	2006	Glucan 36% Xylan 15% Galactan 3% Arabinan 3% Mannan 4% Lignin 20% Water 5%	Ammonia soaking	Glucan 48% Xylan 16% Lignin 11% Water 3%	Glucose 38% Xylose 9%	(Ko et al., 2009)

Table 2-6 Comparison of rice straw composition (continuous).

Cultivar	Crop time	Original composition of rice straw	Pretreatment method	Composition of rice straw after pretreatment	Carbohydrates of the hydrolysate solution	Reference
Longtan (Taoyan, Taiwan)	-	Glucose 35% Xylose 21% Arabinose 4% Acetyl group 1% Lignin (acid soluble) 3% Lignin (acid insoluble) 13% Ash 15%	Acid pretreatment (1.0% H_2SO_4)	Glucose 46% Xylose 8% Arabinose 0.1% Lignin (acid soluble) 14% Lignin (acid insoluble) 5% Ash 13%	-	(Guo et al., 2009)
Lenjan field (Isfahan, Iran)	-	Hemicellulose 27% Cellulose 39% Lignin12% Ash 11%	Acid pretreatment (0.5% H_2SO_4)	-	-	(Karimi et al., 2006)
The specified nonprofit corporation Shimane Bioethanol Workshop (Shimane, Japan)	-	Holocellulose 57% α-Cellulose 27% Hemicellulose 30% Klason lignin 26% Extractives 4% Ash 15%	Cooking	-	-	(Teramoto et al., 2009)

2.4.2.2 Enzymatic saccharification

After pretreatment, it is followed by an enzymatic saccharification step for conversion of cellulose and hemicellulose polysaccharides to fermentable monosaccharides (hexoses and pentoses). Enzymatic saccharification needs three categories of enzyme, cellulase, hemicellulase, and cellobiase. Commercial cellulase usually contains at least three different enzymes, which are endoglucanase, exoglucanase, and β-glucosidase. Three types of reaction are involved in the reaction of cellulase. First, breakage of the non-covalent interactions present in the crystalline structure of cellulose by endoglucanase. Second, hydrolysis of the individual cellulose fibers to break it into smaller sugar compounds by endoglucanase. Third, hydrolysis of disaccharides and tetrasaccharides to break them into glucose by β-glucosidase. Hemicellulase hydrolyzes hemicellulose, which releases pentose (xylose, arabinose, mannose, etc.) and hexose (glucose and galactose). Cellobiase has same function as β-glucosidase in cellulase, which adds to assist the enzyme activity. Enzymes reach their maximum activity when pH is 5 at 50℃ (Abedinifar et al., 2009).

Many studies reported that hydrolysates produced by dilute acid pretreatment coupled with enzymatic saccharification of lignocellulosic biomass are potential feedstocks for ABE fermentation. Table 2-7 shows the results of dilute acid pretreatment and enzymatic saccharification of lignocellulose in several previous studies.

Table 2-7 Dilute acid pretreatment and enzymatic hydrolysis of lignocellulosic biomass.

Material (g)	Pretreatment			Saccharification			Reference
	Grind	Acid	Time and Temp	pH / Buffer	Enzymes	Incubation condition	
Wheat straw (86 g)	0.13 cm sieve screen	1% (v/v) H_2SO_4	Autoclave 121℃, 1 h	pH 5	Celluclast 1.5 L, (cellulase) Novozyme 188, (β-glucosidase) Viscostar 150 L (xylanase)	45℃, 80 rpm, 72 h	(Qureshi et al., 2007)
Rice Straw (10 g)	0.5 cm	2% (w/w) H_2SO_4	Autoclave 130℃, 15 min	pH5/ 50 mM sodium acetate	Cellulase	50℃, 72 h	(Guo et al., 2009)
Rice straw (900 g)	0.3-0.8 cm (20-48 mesh)	0.5% H_2SO_4	High pressure (steam) 20 h	pH 5/ 50 mM citric acid	Commercial cellulase enzyme (BTXL) from *Trichoderma reesei*, β-glucosidase	45℃, 150 rpm, 48 h	(Abedinifar et al., 2009)

2.5 Alternative operation strategies of ABE fermentation to reduce the effect of solvent toxicity

Modified strain is one of the methods to reduce the solvent inhibitory effect on ABE production. *C. beijerinckii* P260, *C. beijerinckii* BA 101, and *E. coli* W3110 are developed through chemical mutagenesis to be hyper-butanogenic strains, while *S. cerevisiae* and *E. coli* JCL17 are genetically modified to be butanol tolerant strains (Ezeji et al., 2007c). Traditionally, batch system is commonly used for ABE fermentation, which is easily occurred inhibitory by accumulative butanol. The alternative fermentation systems are fed-batch/fed-batch coupled with recovery, and continuous/continuous coupled with cell recycling or cell immobilized fermentation system. The advantages and disadvantages of batch, fed-batch, and continuous fermentation system are summarized in Table 2-8. Fed-batch process can avoid exceeding the detrimental substrate level by starting with low substrate concentration, and subsequently adds substrates into bioreactor to maintained fermentation. In the mean time, the problem of butanol toxicity could be solved through the dilution effect during the addition of substrate solution. As a result of supplemental sugar feed to the reactor, ABE productivity was reported to be improved by 16% as compared with batch mode fermentation system (Qureshi et al., 2008a). Other studies found that cell mass and glucose utilization through ABE fermentation were 54% and 72% higher in pH-stat fed-batch culture with butyric acid than that of conventional batch culture, respectively (Tashiro et al., 2004). However, fed-batch fermentation is not suggested for solvent production by some researches because of long duration time of acidogenesis, dead and inactive cell presence (Li et al., 2011). Continuous fermentation not only eases the inhibitory, but also could reduce the time and energy necessary for cleaning and sterilization, and reduce volume of fermentor. Nevertheless, it should be noticed that dilution time should control at low level of 0.03 h^{-1} (Ezeji et al., 2007b) or even lower

than 0.01 h^{-1} (Li et al., 2011) to avoid cell wash out. To overcome low cell concentration due to cell wash out, numerous studies have been carried on bioreactors with cell immobilization (Lee et al., 2008a) or cell recycling (Tashiro et al., 2005).

Table 2-8 The advantages and disadvantages of batch, fed-batch, and continuous fermentation systems.

System	Advantages	Disadvantages	Improvement method
Batch	1. Most commonly used operation mode 2. High efficiency 3. Easy control	1. Butanol toxicity 2. Substrate inhibition	Fed-batch and continuous fermentation system
Fed batch	1. Solve butanol toxicity and inhibitions 2. Longer fermentation time	1. Presence of dead and inactive cells 2. Deficiency of nutrients may cause low solvent yield 3. Long duration of acidogenesis	Coupled with recovery technologies
Continuous	1. Minimizing equipment downtime 2. Reduce time loss due to the lag phase of the microbial culture 3. Volume of fermentor could reduced 4. Also solve the problem of inhibitory cause by butanol and substrate	Low cell concentration due to cell wash out may cause low solvent yield	Coupled with cell recycling and cell immobilization

Product recovery technologies could avoid the accumulation of butanol in bioreactor. Distillation is the traditional recovery process. However, it suffers from a high operation cost due to low concentration of butanol concentration in broth (Ezeji et al., 2007c; Lee et al., 2008b). Alternative recovery technologies are gas stripping,

pervaporation, reverse osmosis, liquid-liquid extraction, and adsorption. Gas stripping was conducted by bubbling gases through fermentation broth to capture ABE. Sequently, the gases cooled in a condenser to collect ABE. Then, the gases are recycled back to bioreactor to capture more ABE. Gas stripping is an easy and efficient *in situ* method to conduct in both batch and fed-batch fermentation systems. Ezeji research group applied gas stripping to recover ABE during fed-batch fermentation of liquefied corn starch resulting in 4-folds higher of ABE production than without conducting recovery (Ezeji et al., 2007d). Applied gas stripping to recovered ABE during batch fermentation of glucose, both ABE productivity and yield were elevated up to 200% and 118%, respectively, as compared to control batch fermentation data (Ezeji et al., 2003). Pervaporation is a membrane-based process, which placed membrane in contact with fermentation broth and ABE selectively diffuses through the membrane as vapor (Liu et al., 2005). Another membrane-based process is reverse osmosis, which is the most preferable from an economic point of view. However, it is suffered from membrane clogging or fouling. As for liquid-liquid extraction, water-insoluble extraction is mixed with fermentation broth. Butanol selectively concentrates in the organic phase and separates from broth. This recovery technology has high capacity but the extractant may be toxic to cells. Decanol and oleyl alcohol are commonly used extractant, where oleyl alcohol is less toxic for cells. A cheaper extractant, methylated crude oil was used to extract ABE from fermentation broth resulting in increase of total solvents and yield from 23.2 g/L and 0.38 in conventional fermentation to 29.8 g/L and 0.40, respectively (Ishizaki et al., 1999). Adsorbents such as silicalite, resins (XAD-2, XAD-4, XAD-7, XAD-8, XAD-16), bone charcoal, activated charcoal, bonopore, and olyvinylpyridine have been studied to use in adsorption recovery technologies. Silicalite appears to be the more attractive as it can be used to concentrate butanol from dilute solutions (5 to 790–810 g/L) and results in

complete desorption of butanol (or ABE) (Qureshi et al., 2005).

Chapter 3 Materials and Methods

The experimental flowchart to study the biobutanol production from rice straw is shown in Figure 3-1.

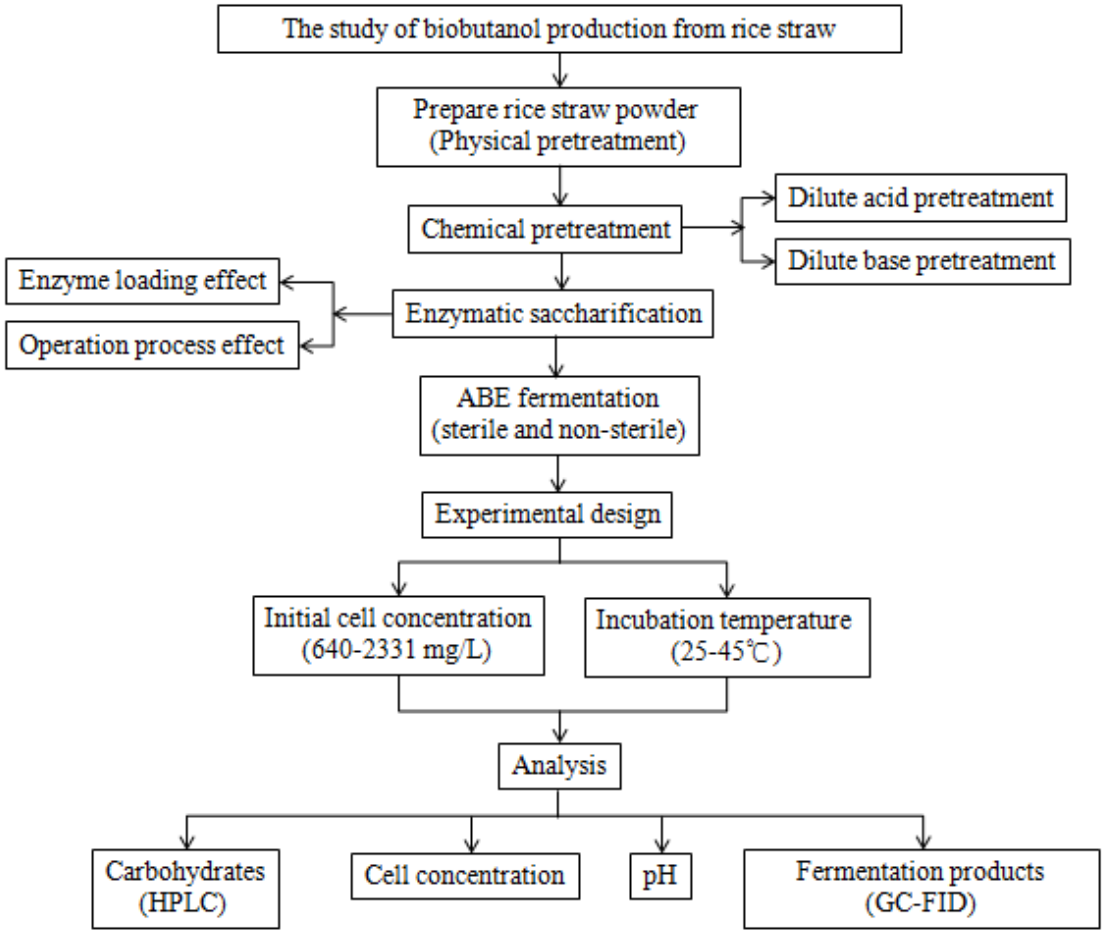


Figure 3-1 The experimental flowchart of this study.

3.1 Instruments and chemicals

Table 3-1 and 3-2 show the instruments and chemicals used in this study.

Table 3-1 Instruments used in this study.

Instrument	Model Number	Brand	Application
Autoclave	EA-635	Estern Medical	Acid pretreatment and sterilization
Blender	JF-102-2	Cook Pot	Cut and ground rice straw
GC-FID	7890A	Agilent Technologies	Fermentation products (Solvents and acids) analysis
HPLC-RI detector	410	Waters	
HPLC-pump	L-2130	Hitachi	
HPLC column heater module	-	Waters	Carbohydrates analysis
Carbohydrate analysis column	PWAT084038	Waters	
Shaking incubator	LE-509RD	Yih-Der	Incubation
Hot Plate Stirrer	HMS-212	Fargo Instruments Co.	Sample heating and mixing
pH meter	SP-2200	Suntex	pH detection
Oven	DV 602	Channel	Rice straw drying and MLVSS procedure
Laminar Flow horizontal type hood	VCM-420	Tsai Hsin	Sterile operation
Centrifuge	-	Gemmy Industrial corp.	Separation of strain and broth
4℃ Refrigerator	KS-103-30N	Mini Kingon	Preservation of samples
-80℃ Freezer	-	-	Preservation of strain

Table 3-2 Chemicals used in this study.

Chemical	Molecular Formula	Properties	Brand/ Country
D(+)-Glucose	$C_6H_{12}O_6$ (Hexose)	White powder; M.W. = 180.16 g/mol	Wako/ Japen
D(+)-Galactose	$C_6H_{12}O_6$ (Hexose)	White powder; M.W. = 180.16 g/mol Purity, >99%	Acros Organics/ USA
D(+)-Mannose	$C_6H_{12}O_6$ (Hexose)	White powder; M.W. = 180.1 g/mol	Sigma/ USA
D(+)-Xylose	$C_5H_{10}O_5$ (Pentose)	White crystal; M.W. = 150.13 g/mol; Purity, >98%	Alfa Aesar/ USA
L(+)-Arabinose	$C_5H_{10}O_5$ (Pentose)	White powder; M.W. = 150.13 g/mol; Purity, 98%	Panreac/ E.U.
Cellobiose	$C_{12}H_{22}O_{11}$	White powder; M.W. = 342.3 g/mol; Purity, ≧98%	Sigma/ USA
Sulfuric acid	H_2SO_4	Colorless, odorless liquid; M.W. = 98.08 g/mol; ACS reagent, 95%-98%	J.T. Baker/ USA
Hydrochloric acid	HCl	Clear colorless to light-yellow liquid, >35%; M.W. = 36.46% g/mol	Shimakyu/ Japan
Sodium hydroxide	NaOH	White pellets, hydroscopic; M.W. = 40 g/L; Purity, >98%	63 Pure Chemicals/ Japan
Cellulase from *Aspergillus niger*	-	White powder; Activity, 1400 units/g solid; M.W. = 26,000 Daltons	Sigma/ USA
Hemicellulase from *Aspergillus niger*	-	White powder; Activity, 1500 units/g solid;	Sigma/ USA
Cellobiase from *Aspergillus niger*	-	Brown liquid; M.W. = 71,000 - 88,000; Daltons; Activity, ≥250 U/g	Sigma/ USA

Table 3-2 Chemicals used in this study (continuous).

Chemical	Molecular Formula	Properties	Brand/ Country
Sodium acetate · 3 hydrate	$CH_3COONa \cdot 3H_2O$	White crystal; M.W. = 136.08 g/mol; Purity, 98%	Shimakyu/ Japen
Acetic acid	CH_3COOH	Colorless liquid with pungent smell; M.W. = 60.05 g/mol; HPLC grade, >99.8%; Boiling point, 118℃; Acidity (pKa), 4.76	J.T. Backer/ USA
Butyric acid	$CH_3(CH_2)_2COOH$	Colorless liquid with pungent smell; M.W. = 88.11 g/mol; HPLC grade, >99.0%; Boiling point, 163.5℃; Acidity (pKa), 4.82	Alfa Aesar/ USA
Acetone	CH_3COCH_3	Colorless liquid; M.W. = 58.08 g/mol; HPLC grade, >99.5%	Mallinckrodt Backer Chemicals/ USA
n-Butanol	$CH_3(CH_2)_3OH$	Colorless liquid; M.W. = 74.12 g/mol HPLC grade, >99.9%	C-Echo Chemistry/ Taiwan
Ethanol	C_2H_5OH	Colorless liquid; M.W. = 46.07 g/mol Ethanol absolute, >99.8%	Aldrich/ USA
Acetonitrile	CH_3CN	Colorless liquid; M.W. = 41.05 g/mol HPLC grade, >99.9%	J.T. Backer/ USA
Nitrogen gas	N_2	5N, 99.999%	Chiah-Lung/ Taiwan
Hydrogen gas	H_2	5N, 99.999%	Chiah-Lung/ Taiwan
Compress air	$N_2 + O_2$	21% O_2 and 79% N_2	Chiah-Lung/ Taiwan
Helium gas	He	5N, 99.999%	Chiah-Lung/ Taiwan

Table 3-2 Chemicals used in this study (continuous).

Chemical	Molecular Formula	Properties	Brand/ Country
Meat peptone	-	Light yellow powder	Conda/ Spain
Tryptone	-	Light yellow powder	Bio Basic/ Canada
Yeast extract	-	Light yellow powder; Purity, 99%	Scharlau/ Japan
Calcium chloride	$CaCl_2$	White pellets; M.W. = 110.99 g/mol; Purity, >95%	Shimakyu/ Japan
Magnesium sulfate · 7 hydrate	$MgSO_4 \cdot 7H_2O$	White crystalline solid; M.W. = 246.48 g/mol; Purity, 99.8%	Fisher scientific/ UK
Di-potassium hydrogen phosphate	K_2HPO_4	White powder; M.W. = 174.18 g/mol; Purity, 99%	Panreac/ E.U.
Potassium dihydrogen phosphate	KH_2PO_4	White crystal; M.W. = 136.1 g/mol; Purity, >99.5%	Shimakyu/ Japan
Sodium bicarbonate	$NaHCO_3$	White powder; M.W. = 84.01 g/mol; Purity, 99.6-100%	Shimakyu/ Japan
Sodium chloride	NaCl	Colorless/white crystal; M.W. = 58.44 g/mol; Purity, >99.5%	Panreac/ E.U.
Resazurin sodium salt	$C_{12}H_6NO_4Na$	Dark blue powder; M.W. = 251.18 g/mol	Sigma/ USA
Ferrous sulfate · 7 hydrate	$FeSO_4 \cdot 7H_2O$	Green crystal; M.W. = 278.02 g/mol; Purity, >99.5%	Ferak/ Germany
Cysteine-HCl · 1 hydrate	$C_3H_7NO_2S$-HCl · H_2O	White crystal; M.W. = 176.63 g/mol; Purity, >98-100%	Bio Basic/ Canada
Glutathione (Reduced)	$C_{10}H_{17}N_3O_6S$	White powder; M.W. = 307.33 g/mol; Purity, >98%	Bio Basic/ Canada
Glycerol	$C_3H_5(OH)_3$	Color less liquid; M.W. = 92.09 g/mol Purity, 98%	Union Chemical/ Taiwan

Table 3-2 Chemicals used in this study (continuous).

Chemical	Molecular Formula	Properties	Brand/ Country
Ammonium hydrogen carbonate	NH_4HCO_3	White crystal; M.W. = 79.06 g/mol; Purity, 98-100%	Panreac/ E.U.
Ferrous chloride · 4 hydrate	$FeCl_2 \cdot 4H_2O$	Brown solid; M.W. = 198.81 g/mol; Purity, 99-100%	Showa/ Japan
Manganese (II) sulphate · 1-hydrate	$MnSO_4 \cdot H_2O$	Pale pink powder; M.W. = 169.01 g/mol; Purity, 98-100%	Panreac/ E.U.
Sodium molybdate · 2-hydrate	$Na_2MoO_4 \cdot 2H_2O$	White crystal; M.W. = 241.95 g/mol; Purity, 98%	Alfa Aesar/ USA
Sodium sulfide · 9-hydrate	$Na_2S \cdot 9H_2O$	Orange chip; M.W. = 240.18 g/mol; Purity, 90%	63 Pure Chemicals/ Japan
Sodium phosphate dibasic · 12-hydrate	$Na_2HPO_4 \cdot 12H_2O$	Colorless crystal; M.W. = 358.13 g/mol; Purity, 98%	Choneye pure chemicals/ Japan
Sodium dodecyl sulfate	$C_{12}H_{25}NaSO_4$	White powder; M.W. = 288.38 g/mol;	Shimakyu/ Japan
Sodium tetraborate · 10-hydrate	$Na_2B_4O_7 \cdot 10H_2O$	White powder; M.W. = 381.37 g/mol; Purity, 99-100%	Shimakyu/ Japan
EDTA (Ethylenediaminetetraacetic acid)	$C_{10}H_{16}N_2O_8$	White powder; M.W. = 292.23 g/mol; Purity, 99.5%	Choneye pure chemicals/ Japan
2-Ethoxyethanol	$C_4H_{10}O_2$	Colorless liquid; M.W. = 90.12 g/mol; Purity, 99%	Alfa Aesar/ USA
(1-Hexadecyl) trimethylammonium bromide (CTAB)	$C_{19}H_{42}BrN$	White powder; M.W. = 364.46 g/mol; Purity, 98%	Alfa Aesar/ USA

3.2 Rice straw

Rice straw used in the study was supplied by Department of Agriculture at Hsinchu County Government, Jhubei City, Taiwan. The rice straw was cut down and milled to pass through 30 mesh sieves for giving a size around 0.2 to 0.4 mm. Then it was dried at 105℃ by oven to ensure a consistent weight prior to use. This treatment procedure was regarded as physical pretreatment of rice straw in this study. The dry rice straw powder (DRS) went through chemical pretreatment followed by enzymatic saccharification to convert this agriculture residue into fermentable sugars (monosaccharides). The processes of chemical pretreatment and enzymatic saccharification are described in section 3.3 and section 3.4.

3.3 Dilute acid/base pretreatment

The dry rice straw (DRS) with solid contents of 2.4% and 10% were examined in the dilute and base pretreatments. The solid content was defined as the mass of the DRS soaked in a liter of acid or base solution. In the dilute acid pretreatment, 1% sulfuric acid solution was used to pretreat the rice straw. The pretreatment was performed in an autoclave at 121°C for 30 mins and then cooled down to room temperature. The cooled sample was immediately filtered to separate solid and liquid portions. The solid portion was washed with distilled water several times and then oven-dried at 105℃. The identical procedure was carried out for the dilute base pretreatment, whereas 1% sodium hydroxide solution was used in place of 1% sulfuric acid solution. The ratio of the weight loss after pretreatment was determined by using Eq. (a).

$$\text{Loss ratio} = \frac{W_i - W_f}{W_i} \times 100\% \quad \text{..... Eq. (a)}$$

where W_i represents the weight of initial DRS before pretreatment, and W_f is the dry

weight of rice straw residues after pretreatment. Both dilute acid and dilute base pretreated rice straw residues were compositionally analyzed by the method described in section 3.9.1. The experiment conditions and analysis parameters are displayed in Table 3-3.

Table 3-3 The experiment conditions and analysis parameters of chemical pretreatments.

Pretreatment	Solid content [a]	Soaked solution	Analysis and calculation
Dilute acid	2.4% 10%	1% H_2SO_4	1. Composition analysis (see section 3.9.1)
Dilute base	2.4% 10%	1% NaOH	2. Loss ratio (see Eq. a)

[a] Solid content (%) = ((DRS weight (mg) / Soaked solution volume (L)) /10000

3.4 Enzymatic saccharification

A non-pretreated rice straw (NPRS), a pretreated rice straw (PRS), and a mixture of pretreated rice straw and acid hydrolysate (MPRSH) were used as raw materials for the experiment of enzymatic saccharification. The PRS had the solid fraction of 2.4% DRS pretreated by 1% sulfuric acid solution. The PRS was filtered to disregard the acid hydrolysate, and then washed by distilled and deionized (DI) water several times until the pH value reaches more than 4. Thereafter, it was oven-dried at 105℃. The overall scheme of PRS saccharification is shown in Figure 3-2. For the MPRSH, the DRS was subjected to the acid pretreatment with 1% sulfuric acid solution. However, the acid hydrolysate was not removed from the MPRSH. The pH of the MPRSH was adjusted to 5 by 5 N NaOH prior to sacchrification. The overall scheme of MPRSH and NPRS are in Figure 3-3 (a) and (b), respectively.

The enzymatic saccharification experiments were conducted in a series of 500-mL serum bottles under sterile condition. The different conditions of rice straws

mentioned above were placed into the serum bottles. The bottles were filled with 250 mL of acetate buffer solution and 5 g rice straw was added to each bottle to achieve the final solid content of 2%. However, after dilute acid pretreatment and filtration, 5 g DRS was washed and only 2.9 g of PRS were remained. Thus, the solid content in PRS saccharification was 1.2%. Each liter of the acetate buffer solution consisted of 357 mL of 0.1 M acetic acid and 643 mL of 0.1 M sodium acetate. Cellulase, hemicellulase, and cellobiase purchased from Aldrich-Sigma were used for enzymatic saccharification. The characteristics of enzymes are shown in Table 3-4. Different enzyme loadings were evaluated and the loadings are summarized in Table 3-5. The initial pH was controlled at 5.0±0.1 using sodium hydroxide or hydrochloric acid solution under sterile condition. The serum bottles were incubated in a shaker at 170 rpm and 50±1°C for 24-171 h. The samples from enzymatic saccharification were withdrawn every 24 h. All experiments were performed in duplicate.

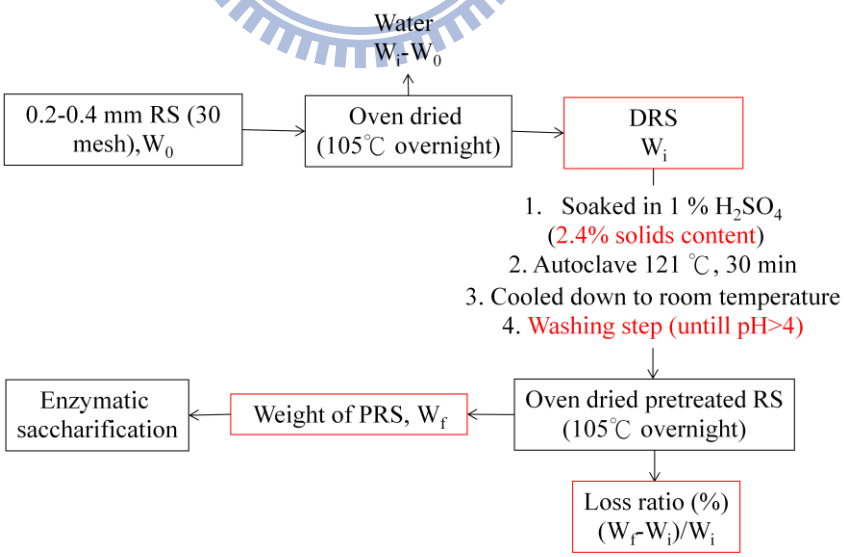


Figure 3-2 The scheme of PRS saccharification.

Table 3-4 The characteristics of cellulase, hemicellulase, and cellobiase.

Enzymes	**Cellulase from *Aspergillus niger***	**Hemicellulase from *Aspergillus niger***	**Cellobiase from *Aspergillus niger***
Synonym	1,4-(1,3:1,4)-β-D-Glucan 4-glucanohydrolase	-	Novozyme 188
Brand (Product number)	Sigma (C1184)	Sigma (H2125)	Sigma (C6105)
Molecular weight (Daltons)	26,000		71,000 - 88,000
Density	-	-	~1.2 g/mL
Unit Definition	One unit will liberate 1 μmole of glucose from cellulose in 1 hr at pH 5.0 at 37℃ (2 hr incubation time).	One unit will produce a relative fluidity change of 1 per 5 minutes using locust bean gum as substrate at pH 4.5 at 40℃.	One unit is defined as 2 μmole of glucose produced per minute at pH 5, 40℃.
Activity (Unit /g enzyme)	1400	1500	≥250
Function	1. Catalyzes the hydrolysis of endo-1,4-β-D-glycosidic linkages in cellulose, lichenin, barley glucan, and the cellooligosaccharides cellotriose to cellohexaose. 2. Cleave intact glycosaminoglycan from a core peptide by hydrolyzing the xylosyl serine linkage. 3. It does not cleave cellobiose or ρ-nitrophenyl-β-D-glucoside.	Usually containing xylanase, mannase and other activities.	The cellobiase hydrolyzes cellobiose to glucose.
Storage	4℃	-20℃	4℃

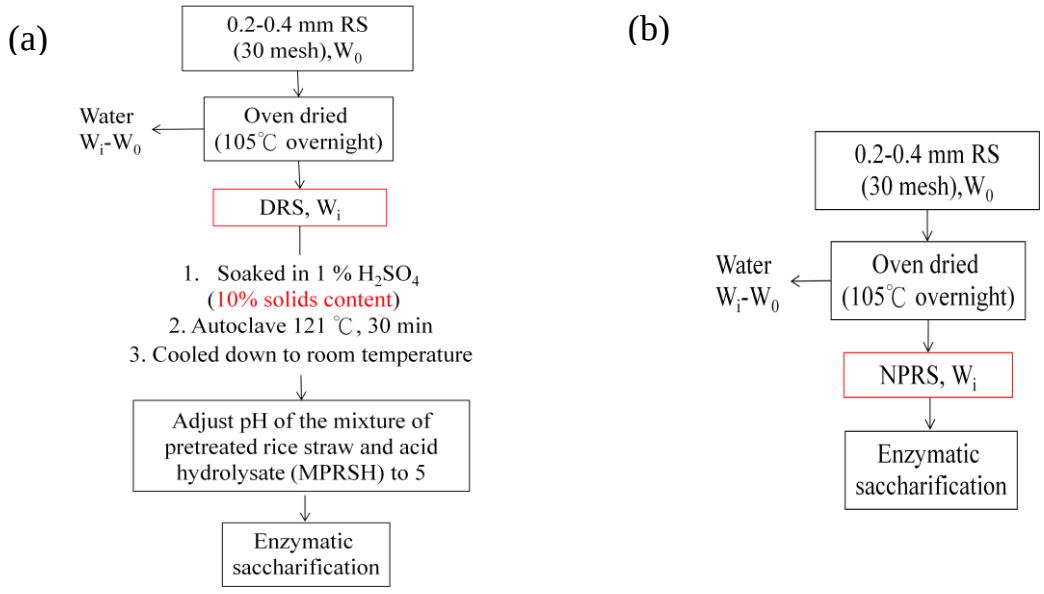


Figure 3-3 The scheme of (a) MPRSH and (b) NPRS saccharification.

Table 3-5 The experimental conditions and parameters of enzymatic saccharification.

	Rice straw	Solid content [a] (%)	pH/ Temp. (℃)	Cellulase (kU/g DRS [b])	Hemicellulase (kU/g DRS)	Cellobiase (kU/g DRS)	Mixture (rpm)
Enzyme loading	PRS [c]	2%	pH 5 / 50℃	0.14 0.28 0.56 1.93	0.14 0.28 0.56 1.93	0.36 0.72 1.44 4.97	170
NPRS-PRS -MPRSH comparison	PRS MPRSH [e] NPRS [f]	1.2% [d] 2% 2%	pH 5 / 50℃	1.93	1.93	4.97	170

[a] Solid content (%): The solid content in acetate buffer (DRS weight (mg) /buffer solution volume (L))/10000.

[b] DRS: Dry rice straw powder.

[c] PRS: DRS was dilute acid pretreated (2.4% solid content), and then discard suspended hydrolysate. Washed solid fraction by DI water several times until pH>4.

[d] Because of the washing step in pretreatment, some rice straw lost. The solid content was lower than 2% in PRS saccharification.

[e] MPRSH : DRS was dilute acid pretreated (10% solid content). Both solid and liquid were directly used in saccharification after pH adjustment by NaOH.

[f] NPRS : DRS without any further treatment were directly used in saccharification.

3.5 Medium preparation

The component of PYG medium was shown in Table 3-6. All PYG media used throughout this study was sealed in anaerobic bottle with open top cap and septum (Figure 3-4). Thereafter, sweep the nitrogen gas across medium face by syringe needles until the PYG medium turned from red into gold color, which indicated anaerobic condition. The color was from resazurin oxygen indicator added in medium. Before inoculation, PYG medium was sterilized at 121℃ for 20 min followed by cool down to room temperature.

Table3-6 The components in one liter of PYG medium.

	Per liter PYG medium
Peptone	5 g
Tryptone	5 g
Yeast extract	10 g
8 g/L $CaCl_2$ stock solution	1 mL
19.2 g/L $MgSO_4 \cdot 7H_2O$ stock solution	1 mL
Mixed salt stock solution (contained 51 g K_2HPO_4, 1 g KH_2PO_4, 10 g $NaHCO_3$, and 2 g NaCl per liter mixed solution)	40 mL
1 mg/mL Resazurin solution	1 mL
0.22 g/L $FeSO_4 \cdot 7H_2O$ stock solution	5 mL
333 g/L Glucose stock solution	30 mL
50 g/L Cysteine-HCl stock solution	10 mL
25 g/L Glutathione stock solution	10 mL

Figure 3-4 Anaerobic bottle with open top cap and septum.

The synthetic NPRS hydralysate was chosen to be medium for ABE fermentation experiments. Assumed 10 g of NPRS was underwent enzymatic saccharification in a series of 500-mL serum bottle filled with 250 mL of acetate buffer solution at enzyme loading of 1.93 kU/g NPRS of cellulase, 1.93 kU/g NPRS of hemicellulase, and 4.97 kU/g NPRS of cellobiase. According to the results of sugar yield in previous experiments, 2.73 g/L arabinose, 28.10 g/L glucose, 10.00 g/L galcatose could be obtained. To simulate ABE fermentation with NPRS hydalysate, 2.73 g/L arabinose, 28.10 g/L glucose, 10.00 g/L galcatose, and additional 6.66 mL/L of nutrient salts solution (Table 3-7) was placed in the anaerobic bottle and was filled with acetate buffer solution (pH 5) to achieve the final working volume of 500 mL. The synthetic NPRS hydrolysate was swept by nitrogen gas through the medium surface for 30 min. Thereafter, the medium for A experimental group need to be sterilized at 121℃ for 20 min followed by cooling down to room temperature before inoculation. On the hand, medium for B experimental group does not sterilized by autoclave. A experimental group was operated under sterile condition while B experimental group was under non-sterile condition.

Table 3-7 The components of nutrient solution in ABE fermentation medium.

Nutrient salts	g/L DI water	Nutrient salts	g/L DI water
NH_4HCO_3	160	$MgSO_4 \cdot 7H_2O$	4
KH_2PO_4	80	$Na_2MoO_4 \cdot 2H_2O$	0.4
NaCl	0.4	$CaCl_2$	0.28
$FeCl_2$	0.278	$MnSO_4 \cdot 1H_2O$	0.37

3.6 Culture development

3.6.1 Laboratory stock *Clostridium saccharoperbutylacetonicum*

Clostridium saccharoperbutylacetonicum N1-4 (ATCC 27021) was purchased from American Type Culture Collection (ATCC 27021), USA. *C. saccharoperbutylacetonicum* N1-4 in freeze-dried powder form was activated in 250 mL PYG medium at 35℃ with 100 rpm agitation for 30 h incubation time inside the anaerobic bag. The activated cells were mixed with glycerol at the ratio of 7:3 (v/v) in microtube, and preserved in -80℃ Freezer as laboratory stock until use. Each microtube contains 1 mL mixed solution of activated cell and glycerol.

3.6.2 Preparation of inoculums for ABE fermentation

2 mL of active cells and glycerol mixed solution (two microtubes) were further subcultured anaerobically in a 500 mL PYG medium under sterile condition, and incubated at 35℃ for 36 h with 100rpm agitation in an incubator shaker, and thereafter used as inoculums for ABE fermentation batch tests. Harvested cells (as inoculums) were used in this study in order to eliminate any residual glucose contained in the PYG subcultured medium. The cells in PYG medium were harvested by centrifugation (4000 rpm for 20 min), and the resulting pellet was collected and inoculated into ABE fermentation medium. To ensure anaerobic condition, the medium surface was swept across by nitrogen gas for 20 min. Then, 1.5 mL of 0.25 M Na_2S was injected into the medium to consume the O_2 residues prior to start ABE fermentation batch experiments.

3.7 Experimental design (central composite design)

The ABE fermentation experiments were designed statistically according to two-factor Central Composite designs (CCD), which enables the construction of second order polynomials relating to one dependent variables i.e. response and two

independent variables. MINITAB® software (Version 15; LEAD Technologies, Inc.) was used to create two-factor CCD and to analyze responses with response surface methodology. In this study, initial cell concentration, X_1 (640-2331 mg/L) and incubation temperature, X_2 (25-45℃) were chosen as two independent variables (factors) in the experimental design. As shown in Figure 3-5, this experimental plan was carried out as a CCD consisting of 11 experiments runs. The total number of runs in CCD depends on the number of independent variables and can be determined by Eq. (b) (Cho and Zoh, 2007; Lu et al., 2008).

$$N = 2^K + 2K + n_c \quad \text{Eq. (b)}$$

where K represents the number of independent variables and n_c is the number of center points. For two variables (K=2), the total number of experiment runs was 11 determined by the expression: 2^2 (factor points) + 2×2 (axial points) + 3 (three replicated central points), which are listed in Table 3-8.

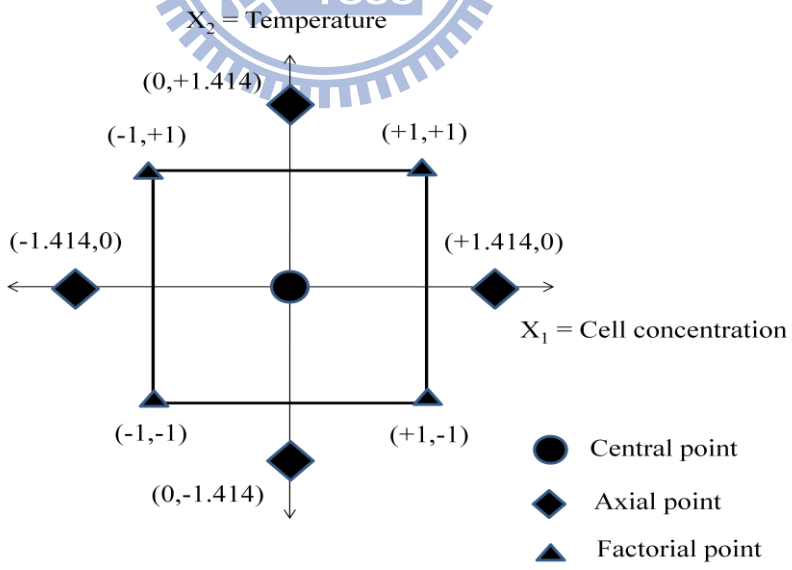


Figure 3-5 Schematic diagram of central composite design (CCD) as a function of X_1 (initial cell concentration), X_2 (incubation temperature) according to the 2^2 factorial design with four axial points and three central points (replication).

3.8 Batch experiments

In this study, ABE fermentation under sterile condition (A group) and non-sterile condition (B group) were performed according to two factorial central composite design as shown in Table 3-8. All ABE fermentation tests were anaerobically conducted in a series of 500 mL anaerobic bottle (Figure 3-4) with 500 mL synthetic NPRS hydrolysate medium. Initial cell concentration and incubation temperature were changed according to the experimental design (Table 3-8). The initial cell concentration was controlled quantitatively by the number of anaerobic bottle centrifuged to harvest inoculums. The numbers of bottle and their corresponding cell concentrations are displayed in Table 3-9. Incubation temperatures were controlled by incubators. The fermentor (anaerobic bottle) was agitated at a constant rate of 100 rpm. The initial pH of ABE fermentation medium was fixed at 5.42±0.03. Samples were taken intermittently and estimated the cell concentration, pH, fermentation products, and sugar concentration as described in section 3.9.

Table 3-8 2^2 factorial central composite design for ABE fermentation experiment.

Run Order	Coded variables		Natural variables	
	X_1	X_2	Cell concentration, X_1 (mg/L)	Temperature, X_2 (℃)
1	0	-1.414	1429±214	25
2	0	+1.414	1429±214	45
3	-1.414	0	640±57	35
4	-1	-1	808±74	28
5	0	0	1429±214	35
6	-1	+1	808±74	42
7	+1	-1	2170±157	28
8	+1	+1	2170±157	42
9	0	0	1429±214	35
10	0	0	1429±214	35
11	+1.414	0	2331+28	35

Table 3-9 The cell concentration harvested from the corresponding number of bottle of subculture PYG.

Numbers of bottle	Average cell conc. (mg MLVSS/L) [a]
0.5	640±57 [b]
0.8	808±74
1.5	1429±214
2.2	2170±157
2.5	2331+28

[a] The data of cell concentration were presented as mg MLVSS per liter of batch medium.

[b] Mean ± standard deviation

3.9 Analytical methods

3.9.1 Composition analysis of rice straw

Analysis of raw material, pretreated DRS, and saccharified DRS residues were including moisture content and composition analysis (celluose, hemicellulose, lignin, and ash). The solid content and the moisture content were measured according to Standard Methods (1998). Composition analysis was based on the methods proposed in 1991 (Van Soest et al., 1991). Van Soest research group used neutral detergent (ND) and acid detergent (AD) to cook 1 g of samples and got neutral detergent fiber (NDF) and acid detergent fiber (ADF), respectively. Subtract the weight of ADF from the weight of NDF is the content of hemicellulose Eq. (c). Then, ADF is continued to treat with 72% H_2SO_4 to get acid detergent lignin (ADL). The weight difference between ADF and ADL is defined as cellulose weight Eq. (d). The residual solids remained after ADL incinerate in 550℃ furnace for 4 h is called ash. The lignin content was calculated as Eq. (e).

Hemicellulose content (%) = (NDF – ADF) / Initial sample weight × 100%.....Eq. (c)

Cellulose content (%) = (ADF – ADL) / Initial sample weight × 100%.....Eq. (d)

Lignin content (%) = (ADL – Ash) / Initial sample weight × 100%.....Eq. (e)

3.9.2 Carbohydrate analysis

The concentration of carbohydrate in hydrolysate solution from pretreatment and enzymatic saccharification, and fermentation broth were analyzed by high pressure liquid chromatography (HPLC) equipped with carbohydrate analysis column (3.9×300 mm, Waters), pump (Hitachi L-2130), and refractive index detector (Waters 410). The temperature was controlled at 35℃. The mobile phase was 80% acetonitrile solution at a flow rate of 1 mL/min. The samples were diluted two fold by acetonitrile followed by filtered through 0.45 μm syringe filter with an injection volume of 20 μL.

3.9.3 Fermentation products analysis

Fermentation products, acetone, butanol, ethanol, acetic acid, and butyric acid, were analyzed by gas chromatograph (Aglient 7890A) equipped with a flame ionization detector (FID) and a 30 m long, 0.53 mm inside diameter capillary column filled with FFAP. Samples were first acidified to pH < 2 by concentrated sulfuric acid. Then, 0.4 μL of sample was injected into GC-FID injector. Helium gas was used as carrier gas. Nitrogen gas was makeup flow gas. Hydrogen gas and air were detector support gases. Gas purify recommendation are 99.999%. Temperatures of injector and detector were both maintained at 250℃. GC oven temperature was initially held at 60℃ for 1 min. Then, temperature was raised with a gradient of 18℃/min and held for 5 min at 230℃. The results of ABE fermentation products concentration can be calculated from the peak by integrator.

3.9.4 Cell concentration analysis

5 mL of fermentation broth was filtered by suction filtration method with glass fiber filter paper (Grade GB-10, Toyo Roshi). The filter paper was washed several times to avoid the interference of sugar residues in fermentation broth. The weight of oven-dried cell on filter paper at 105℃ oven was recorded as W_1. And the weight of residues left after incinerated in 550℃ furnace was W_2. Subtraction of W_2 to W_1 could get the MLVSS weight, which represented cell dry weight in 5 mL of broth. The cell concentration calculated as Eq. (f).

$$\text{Cell concentration } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{(W_2 - W_1)\ (\text{mg})}{\text{V (L)}} \quad \text{Eq. (f)}$$

where V represents the volume of sample, i.e. 0.005 L in this study.

3.10 Data analysis

The loss ratio was the total weight loss of 1 g-based DRS samples in percentage after pretreatment which can be calculated by Eq. (a). The loss ratio of each component (cellulose, hemicellullose, lignin, and ash) presented the weight percentage of corresponding component in total lost weight after pretreatment.

Productivity was calculated as products (solvents in ABE fermentation or sugars in saccharification) produced in g/L divided by time and is expressed as g/L/h or g/L/d. Fermentation time is defined as the time period when a maximum ABE concentration was reached and is expressed in h or d. Sugar yield was calculated as sugar production (g) divided by the additional rice straw (g), while butanol yield was calculated as butanol produced (g/L) divided by the total sugar utilized (g/L).

The modified Gompertz equation, Eq. (g), is a sigmoid function. It has been approved statistically to describe the cumulative hydrogen production in batch tests (Chen et al., 2006) and the cumulative methane production (Chen et al., 2003). This

equation was employed to describe the kinetics of cumulative glucose productions from NPRS PRS and MPRSH, and the cumulative butanol production and the sugar utilization from ABE fermentation.

$$P(t) = P \cdot \exp\left\{-\exp\left[\frac{R \cdot e}{P}(I - t) + 1\right]\right\} \quad \text{.....Eq. (g)}$$

where P(t) is the cumulative glucose/butanol production or utilized sugar concentration at time t; I is time of lag-phase; P is glucose/butanol production potential or sugar utilization potential; R is glucose/butanol production rate or sugar utilization rate; and *e* is exp(1), i.e. 2.71828.

In this study, sugar production curves with respect to time were obtained first from the enzymatic saccharification experiments, and then the first-order kinetics in Eq. (h) was applied to determine the rate constant (k).

$$\log C_t = kt + \log C_i \quad \text{.....Eq. (h)}$$

where C_i represents the initial total sugar concentration (mol/L); C_t is the total sugar concentration (mol/L) at time t; and k is the rate constant of the sugar production (h^{-1}).

MINITAB® software (Version 15; LEAD Technologies, Inc.) was used for the regression analysis of the ABE fermentation experimental data and the response surfaces. Quadratic polynomial equations were developed to predict the responses (butanol productivity, butnaol yield, and butanol production rate), respectively, as function of independent variables and their interaction. The quality of fitness of the polynomial model equation was expressed by the coefficient of regression R^2, and its statistical significance checked by a Fisher F-test. The significance of the regression coefficient was tested by a Student's t-test. The level of significance was given as values of the probability less than 0.05.

Chapter 4 Results and Discussion

4.1 Rice straw composition

The constituent of the rice straw used for enzymatic saccharification in this research was resolved with 38% cellulose, 35% hemicellulose, 7% lignin, and 4% ash. The total of 84% was consistent with the results reported by Abedinifar et al. (2009). However, the rest of 16% was still remained unknown. The possible explanation could be the mass loss during the composition analysis. A comparison with the compositions from other lignocellulosic biomass in previous studies is summarized in Table 4-1. As revealed in the table, cellulose and hemicellulose were the main compositions of the rice straw, whereas lignin was accounted for a minor portion. The lignin content of the rice straw is much less in comparison with wheat straw, corn cob, bagasse, and silvergrass. In plants, lignin surrounds cellulose microfibrils and strengthens the cell wall. The dilute acid or base pretreatment was performed to loose the structure of lignocellulosic biomass for facilitating the hydrolysis (Mosier et al., 2005). Due to the low lignin content, the pretreatment for the rice straw might be disregarded. This could save the energy and the chemical cost. In addition, it should be noticed that cellulose and hemicellulose are the main parts respond to fermentable sugars conversion in lignocellulosic biomass. The rice straw used in our research had 73% of cellulose and hemicellulose, which was high compared with rice straw used in other researches (Abedinifar et al., 2009; Karimi et al., 2006; Teramoto et al., 2009). As a renewable material, the rice straw is more advantageous to other lignocellulosic biomass.

Table 4-1 Common lignocellulosic biomass and their composition.

Raw material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Rice Straw	38	35	7	Our research
	39	27	12	(Karimi et al., 2006)
	24	38	8	(Abedinifar et al., 2009)
	27	30	26	(Teramoto et al., 2009)
Wheat Straw	35-40	20-50	20	(Qureshi et al., 2007)
Corn cob	45	35	15	(Sun and Cheng, 2002)
Bagasse	37	29	19	(Hwang, 2007)
Silvergrass	34	28	19	

Data reported as the percentage of dry weight.

4.2 Different pretreatment methods

Table 4-2 shows the composition of the rice straw under different pretreatments. As apparent from the table, the pretreatment could affect the composition of the rice straw. The dilute acid and base pretreatments with the DRS solid contents of both 2.4% and 10% elevated the content of cellulose to over 50%. In particular, the dilute base pretreatment on 10% rice straw sharply increased the percentage of cellulose to 70%. In contrast to cellulose, the hemicellulose content of 35% in the non-pretreated rice straw were reduced to 7% and 14% in the rice straws of 2.4% and 10%, respectively, while the rice straw was being pretreated by the 1% sulfuric acid solution. The results of the reduction of hemicellulose contents after the dilute acid pretreatment were in consistent with the finding by Abedinifar et al. (2009). It was also found that the hydrolysate solution contained xylose of 3.01 g/L, glucose of 1.95 g/L, and galactose of 1.88 g/L. These monosaccharides are the building blocks of hemicellulose. Hemicellulose is a branched polymer, whereas cellulose is a linear polymer. The structure of hemicellulose is more heterogeneous than cellulose. It

makes hemicellulose easy to destroy. Therefore, the finding elucidates the breakdown of hemicellulose by the dilute acid solution. In other words, dilute acid pretreatment can hydrolyze part of hemicellulose in advance of enzymatic saccharification. Relative to the dilute base pretreatment, however, the analysis illustrates that the content of hemicellulose was not significantly fluctuated by the pretreatment with 1% sodium hydroxide solution.

The data shown in Table 4-2 also indicate that solid content in dilute acid pretreat process mainly affected hemicellulose, and hemicellulose dissolved in 2.4% solid content prtreatment solution more than in 10% solid content solution. The main effect of solid content in dilute base pretreatment was on cellulose. In higher solid content, i.e. 10% solid content, had less loss of cellulose than in 2.4% solid content. Althoght it appeared higher cellulose percentage after dilute base pretreatment, it also caused high loss ratio of rice straw (Table 4-3). It was found that rice straw appeared suspended colloid shape in base pretreatment experiment, which was difficult to saperate from the hydrolysate. Rice straw lost in the process of spaperation. The loss ratio was around 60% for dilute base prtreatment, while it was 40% for dilute acid pretreatment (Table 4-3). Considering the operation difficulties of dilute base pretreatment and the advatages of dilute acid pretreatment, the study chosen dilute acid pretreatment as chemical pretreatment in the following experiments.

Table 4-2 Rice straw composition before and after pretreatment and the loss weight of corresponding component after pretreatment.

	Original DRS	Dilute acid (2.4%)[a]	Dilute acid (10%)[a]	Dilute base (2.4%)[a]	Dilute base (10%)[a]
Cellulose	38±5%	56±1% (-0.08)[b]	50% (-0.09)[b]	59% (-0.21)[b]	70% (-0.10)[b]
Hemicellulose	37±3%	8±1% (-0.36)[c]	14% (-0.30)[c]	31% (-0.28)[c]	28% (-0.33)[c]
Lignin	7±1%	26±0% (0.00)[d]	25% (0.00)[d]	4% (-0.07)[d]	3% (-0.09)[d]
Ash	4±1%	7±1%	7%	0.5%	4%

[a] DRS solid content

[b] Weight loss of cellulose of 1 g-based DRS samples after pretreatment, i.e. W_{pc}-W_{oc}, where W_{pc} is the weight of cellulose in 1-g based pretreated DRS, and W_{oc} is the weight of cellulose in 1-g based original DRS. The unit was grams.

[c] Weight loss of hemicellulose of 1 g-based DRS samples after pretreatment, i.e. W_{ph}-W_{oh}, where W_{ph} is the weight of hemicellulose in 1-g based pretreated DRS, and W_{oh} is the weight of hemicellulose in 1-g based original DRS. The unit was grams.

[d] Weight loss of lignin of 1 g-based DRS samples after pretreatment, i.e. W_{pl}-W_{ol}, where W_{pl} is the weight of lignin in 1-g based pretreated DRS, and W_{ol} is the weight of lignin in 1-g based original DRS. The unit was grams.

Data reported as the percentage of dry weight.

Table 4-3 The loss ratio of rice straw after pretreatment.

	Dilute acid (2.4%)[a]	Dilute acid (10%)[a]	Dilute base (2.4%)[a]	Dilute base (10%)[a]
Cellulose	19%	22%	35%	18%
Hemicellulose	81%	72%	46%	59%
Lignin	-	-	11%	16%
Ash	0%	6%	8%	7%

[a] DRS solid content

Data reported as the percentage of dry weight.

4.3 Enzymatic saccharification

4.3.1 Enzyme loading

The PRS with solid content of 2% was used to conduct the enzymatic saccharification experiments at different enzyme loadings. The results of sugar productivities and yields at different enzyme loadings are listed in Table 4-4. The sugar productivity was determined from the achieved maximum sugar concentrations and the saccharification time. The sugar yield was per gram of sugar produced per gram of PRS. As indicated in the table, both the sugar productivity and yield increase linearly with increasing enzyme loading. The maximum sugar productivity 1.28 mmol/L/h was obtained at the enzyme loading of 1.93 kU cellulase/g DRS, 1.93 kU hemicellulase/g DRS, and 4.97 kU cellobiase/g DRS. This result was compatible to 1.20 mmol/L/h reported by Abedinifar et al. (2009). Meanwhile, this enzyme loading also hydrolyzed the PRS to achieve the maximum sugar yield of 0.94 g sugar/g PRS.

Table 4-4 Sugar productivities and yields of enzyme loading experiment.

Enzyme loading			Incubation [a] time (h)	Sugar productivity (mmol/L/h)	Yield g sugar/g PRS
Cellulase (kU/g DRS)	Hemicellulase (kU/g DRS)	Cellobiase (kU/g DRS)			
0.14	0.14	0.36	165	0.16	0.20
0.28	0.28	0.72	171	0.27	0.27
0.56	0.56	1.44	171	0.41	0.47
1.93	1.93	4.97	168	1.28	0.94

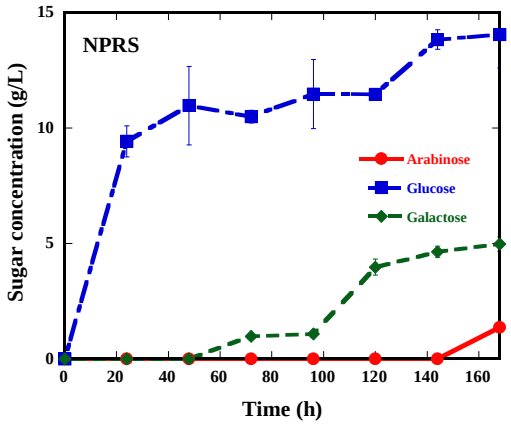
[a] Incubation time: The time reached maximum sugar concentration in saccharification.

4.3.2 Saccharification of NPRS, PRS, and MPRSH

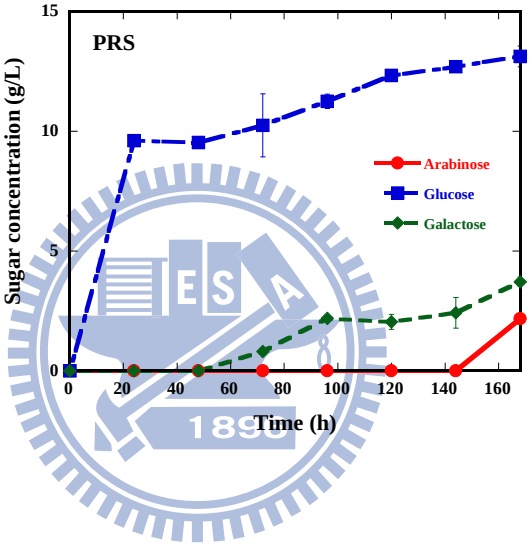
4.3.2.1 Saccharification profiles and performances of NPRS, PRS, and MPRSH

The comparison of saccharification experiments was conducted on 5 g basis of NPRS, PRS, or MPRSH in serum bottle. The saccharification experiments were performed under the enzyme loading of 1.93 kU cellulase/g DRS, 1.93 kU hemicellulase/g DRS, and 4.97 kU cellobiase/g DRS. Figure 4-1 presents the cumulative sugar concentrations from NPRS, PRS and MPRSH during saccharification. Glucose, galactose, xylose, and arabinose were observed during saccharification while glucose was the main final product. As revealed in Figure 4-1 (a), the maximum glucose concentration of 14.05 g/L from NPRS was achieved at the end of saccharification. The glucose concentration increased to 79% of the maximum concentration in 48 h without lags. However, galactose and arabinose were first observed after 48 h and 144 h, respectively. The galactose and the arabinose concentrations at the end of saccharification were 4.99 g/L and 1.37 g/L, respectively. It was apparent that cellulose instantly hydrolyzed to form glucose, resulting in the sharp increase of the glucose concentration, and subsequently galactose and arabinose were gradually produced due to the hydrolysis of hemicellulose. The hydrolysis of hemicellulose was also accompanied with a few productions of glucose. This could explain the slow increase of the glucose concentration after 48 h in the saccharification experiment. The lag phase of galactose, arabinose, and xylose production was possibly caused by the weaker affinity to rice straw powder of hemicellulase than cellulase.

Similar results were also observed from PRS and MPRSH in Figure 4-1 (b) and (c), respectively. The appearing times of galactose and arabinose from PRS were consistent with that from NPRS. However, the galactose and arabinose productions from MPRSH both occurred at 24 h, earlier than that from NPRS. This might be due



(a)



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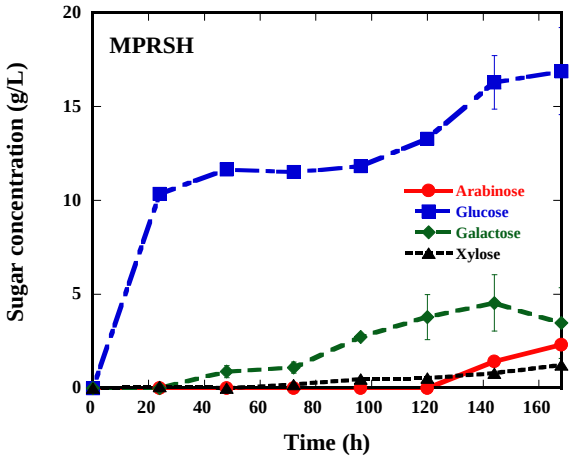
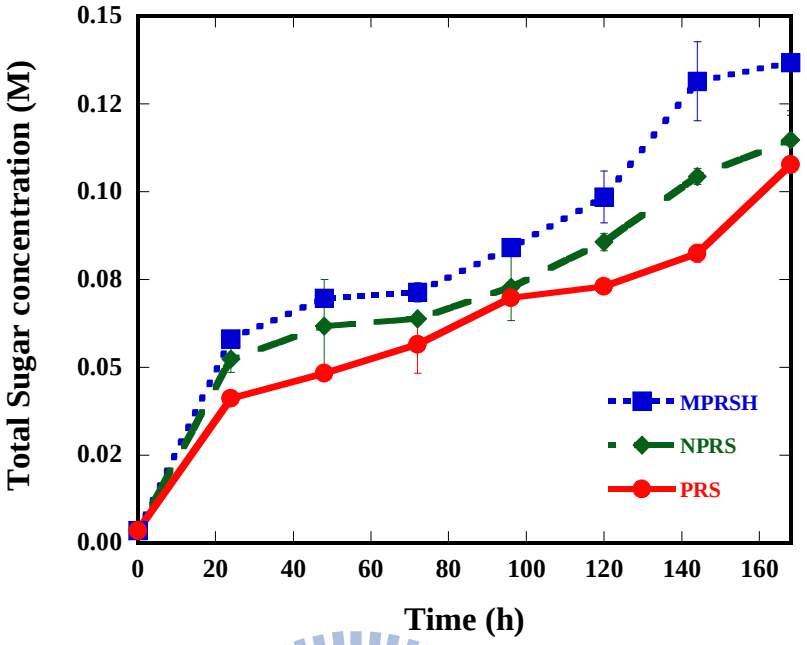


(c)

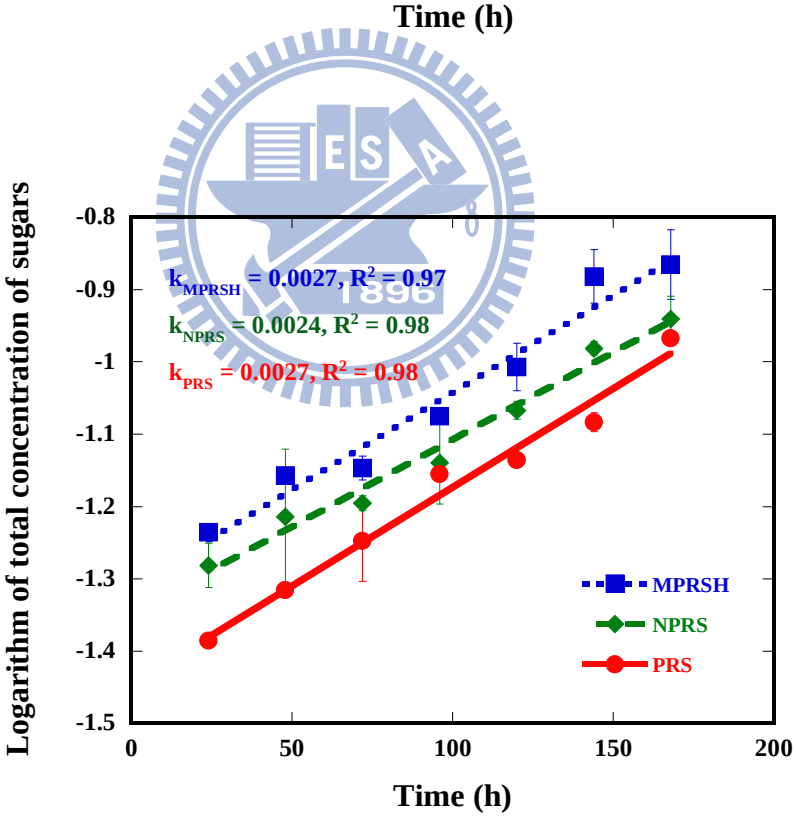
Figure 4-1 The sugar products of rice straw, (a) NPRS, (b) PRS, and (c) MPRSH. Data represent the mean ± standard deviation (SD) of duplicate individual experiments.

to the remained sulfuric acid in MPRSH continued on breaking the rice straw during the saccharification. The more loosen structure of the rice straw facilitates the hydrolysis of hemicellulose. In addition, the xylose concentration of 1.24 g/L was found from MPRSH, whereas it was absence in PRS. The previous experiment indicated that the acid hydrolystate contained some xylose, and this could explain the xylose production from MPRSH. In the study, it was found a 47% of the rice straw loss during the preparation of PRS. The similar finding was also reported in previous study (Cara et al., 2008). The rice straw loss might partly elucidate no xylose production from PRS. Compared to the above monosaccharides, glucose was the main product from either PRS or MPRSH during saccharification. The maximum glucose concentrations were 13.12 and 16.89 g/L for PRS and MPRSH, respectively. The glucose concentrations from both PRS and MPRSH could achieve to 70% of their maximums in 48 h.

Fig. 4-2 (a) shows total sugar concentration produced during incubation time, 0-168 h. The total sugar production is the sum of the produced monosaccharides including pentose and hexose during the course of the saccharification. MPRSH process had the highest total sugar concentration all the time; while PRS process had the lowest total sugar concentration. These results were in accordance with the data of total sugar productivities (NPRS, 0.69 mmol/L/h; MPRSH, 0.93 mmol/L/h; PRS, 0.64 mmol/L/h), which indicated that the enzymatic saccharification efficiency was MPRSH > NPRS > PRS. In the beginning, we expected that SO_4^{2-} anion in MPRSH saccharification may cause the inhibition of enzymes; however, the experimental results shows SO_4^{2-} scarcely affected the efficiency of saccharification in a short incubation time. MPRSH process appeared the highest productivity. In contrast, PRS was removed inhibitor from dilute acid pretreatment by filtration and washing before enzymatic saccharifiction. Thus, not only SO_4^{2-} but also large amounts of



(a)



(b)

Figure 4-2 (a) The total sugar concentration and (b) first order kinetics of MPRSH, NPRS, and PRS processes. Data represent the mean ± standard deviation (SD) of duplicate individual experiments.

hemicellulose and hemicellulose-derived sugars were removed and washed out along with filtration and washing step. Consequently, total sugar concentration of PRS saccharification was lower than MPRSH and even NPRS saccharification.

4.3.2.3 Saccharification kinetics of NPRS, PRS, and MPRSH

The modified Gompertz equation was employed to describe the kinetics of the glucose production in Figure 4-3. The kinetic parameters estimated based on Eq. (g) are listed in Table 4-5. The glucose production was well correlated to the modified Gompertz equation ($R^2 > 0.93$). Predicted glucose yield was calculated from P and added rice straw. Actual glucose yield was calculated from maximum accumulative sugar achieved and added rice straw. As shown in Table 4-5, the glucose production potential and the glucose production rate for NPRS, PRS, and MPRSH were 2.62 g and 3.14 g/d, 2.5 g and 3.81 g/d, and 2.94 g and 3.26 g/d, respectively. There was no lag time during the hydrolysis for the three different conditions of rice straws. The results illustrate the glucose production rates for PRS and MPRSH were higher than that for NPRS. It confirms that the dilute sulfuric acid utilized to soak the rice straw facilitated the biocatalysis to accelerate the saccharification. By contrast, the glucose production potential for NPRS was in between PRS and MPRSH. There was no solid result to conclude that the dilute acid pretreatment profoundly affect the glucose production potential. Similar to the glucose production potential, the glucose yield of 0.52 g glucose/g rice straw for NPRS was compatible to 0.50 and 0.58 g glucose/g rice straw for PRS and MPRSH, respectively. Summarizing the results, it reflects the fact that the rice straw might not need to be chemical pretreated if concerning to save energy and chemical cost.

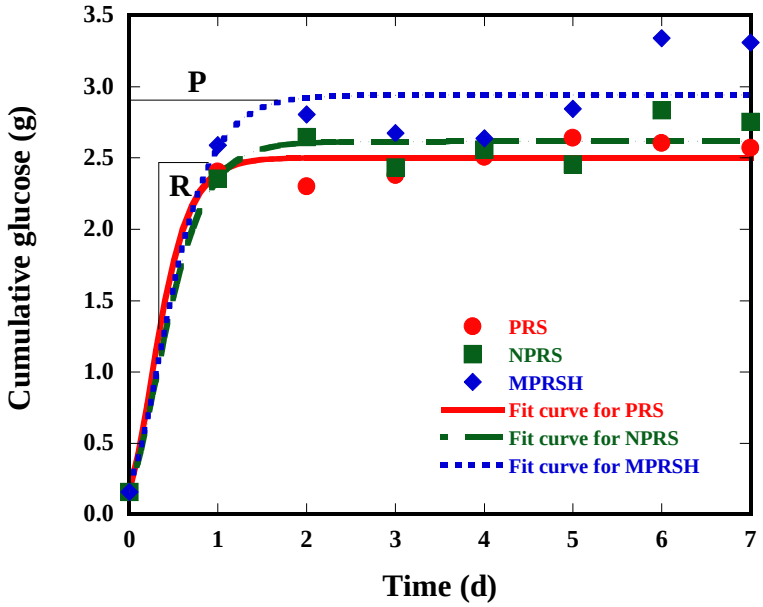


Figure 4-3 Cumulative glucose production of rice straw, NPRS, PRS, and MPRSH saccharification. Markers— experimental data; Nonlinear line—data estimated by Eq. (g).

Table 4-5 Modified Gompertz equation constants of glucose production of NPRS, PRS, and MPRSH saccharification.

	P	R	R^2	Yield	
	(g)	(g/d)		Predicted	Actual
NPRS	2.62	3.14	0.98	0.52	0.56
PRS	2.5	3.81	0.98	0.50	0.52
MPRSH	2.94	3.26	0.93	0.58	0.66

The first-order kinetics of total sugar productions from rice straw is illustrated in Figure 4-2 (b). The total sugar concentrations were well fitted into the first-order kinetics with the correlation coefficients greater than 0.97 in all cases. The rate constants for NPRS, MPRSH, and PRS were 0.0024, 0.0027, and 0.0027 h^{-1}, respectively. The values of k between PRS and MPRSH were no discrepancy. It should be noted that the acid hydrolysate was removed from PRS whereas it was

remained in MPRS. The results of k reflect that the acid hydrolysate would not interfere with the activities of the enzymes. The step of discarding the acid hydrolysate prior to hydrolysis could be neglected, and thus the method of the pretreatment could be simplified. To overlook the three pretreatment conditions, the k value of NPRS was 86% of that of PRS and MPRSH. It suggests the hydrolysis rate of rice straw could be accelerated by the dilute acid pretreatment. However, it should account for the time demand for performing the pretreatment. From the perspective of economic evaluation, the saccharification would be more cost-effective if the dilute acid pretreatment was not employed. Thus, the ABE fermentation studies focused on simulates NPRS hydrolysate fermentation.

4.4 Profiles of ABE fermentation

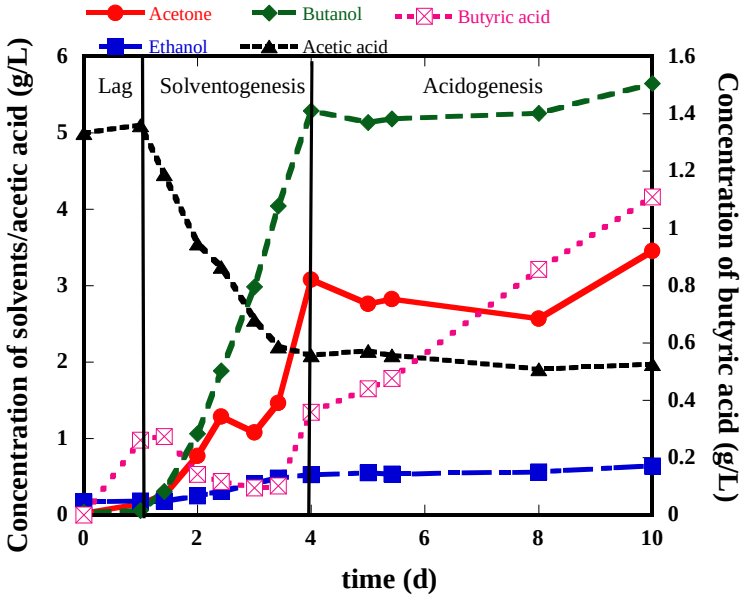
Two experimental groups, A (sterile) and B (non-sterile), of ABE fermentation were conducted under various conditions of initial cell concentration (X_1) and incubation temperature (X_2) designed by CCD at 100 rpm agitation and pH 5.42±0.03 in a series of anaerobic bottle. As revealed in this study, the profiles of ABE fermentation under different conditions could divide into solventogenesis dominant reaction (section 4.4.1) and acidogenesis dominant reaction (section 4.4.2) according to their maximum solvents/acids ratio within the fermentation time. Maximum solvents/acids ratio > 1 indicated that the main products of ABE fermentation within the duration of fermentation were butanol, acetone, and ethanol, while solvents/acids ratio < 1 reflected that organic acids were the major products. The data of maximum solvents/acids ratio of A experimental group and B experimental group are displayed in Table 4-8 and Table 4-10. From the results, the mechanisms of *Clostridium saccharoperbutylacetonicum* N1-4 in Run 3 in A experimental group and Run 3 and 10 in B experimental group were mainly acidogenesis. Other runs except inactive runs

(Run 2, 6, 8 in A group, and Run 2, 4, 6, 8 in B group) were performed solventogenesis in majority. The profiles of metabolic products of all experimental runs could be found at Appendix A, Appendix B and Appendix C. The figures of solvents/acids ratio, pH, total sugar concentration, and cell concentration against fermentation time for all active experimental runs are displayed in Appendix D.

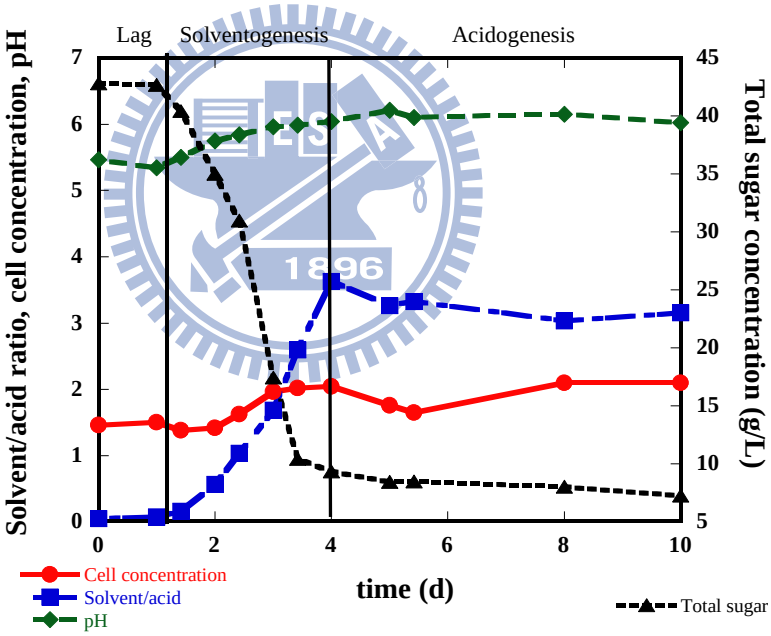
4.4.1 Solventogenesis dominant reaction (maximum solvents/acids ratio > 1)

Figure 4-4 shows the profiles of Run 5 under sterile condition. As revealed, there were three stages of bioreaction, lag stage, solventogenic stage, and acidogenic stage. In lag stage, *Clostridium saccharoperbutylacetonicum* N1-4 rarely consumed sugars and did not grow cell weight. Solvents (butanol, acetone, and ethanol) concentrations were not detected. Acetic acid concentration remained consistent, which indicated no reutilization happened during lag stage. However, butyric acid was produced during the time period of lag stage. It should be noticed that when butyric acid was accumulated to around 0.3 g/L, the solventogenic stage was triggered (Fig. 4-4 (a) and Appendix A). The duration of lag stage was found to be highly relative to incubation temperatures, which will be discussed in section 4.6.

In solventogenic stage, acetic acid and butyric acid were reutilized and converted into final products, butanol, acetone, and ethanol. Under the experimental conditions of Run 5 in group A, the maximum butanol, acetone, and ethanol concentrations of 5.3, 3.1, and 0.5 g/L, respectively, were achieved in the end of fermentation time (4 d). The definition of fermentation time in this study was the time period when a maximum concentration of ABE was achieved, the time of the end of solventogenic stage and the beginning of acidogenic stage on the profiles. The consumption of total sugars was 75% (Table 4-6). From Table 4-8 and 4-10, it was apparent that glucose was an easy



(a)



(b)

Figure 4-4 Profiles of (a) metabolite products and (b) solvents/acids ratio, pH, total sugar concentration, and cell concentration of Run 5 under sterile condition. Initial cell concentration = 1429±214 mg/L, incubation temperature = 35℃.

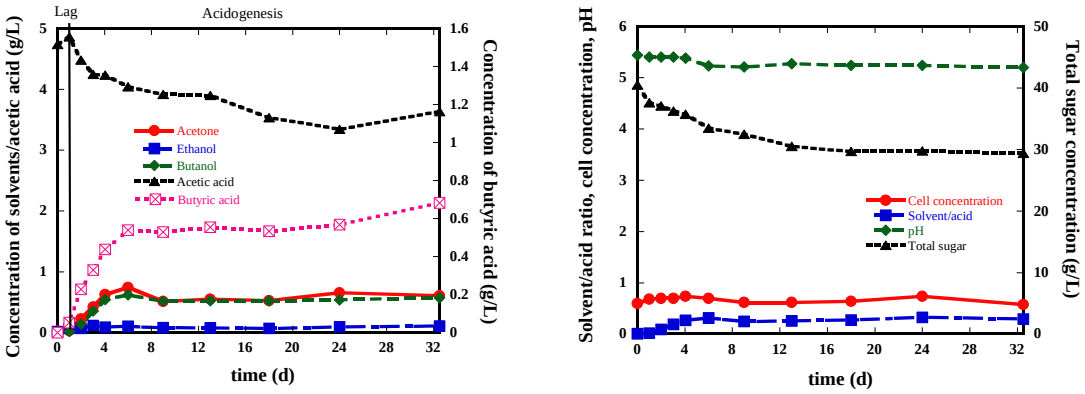
and instant absorbed carbon source for *Clostridium saccharoperbutylacetonicum* N1-4, resulting in the sharp decrease of glucose concentration and subsequently galactose were gradually consumed during fermentation time. However, arabinose was hardly

consumed. As revealed in Figure 4-4, pH was gradually increased with the increasing value of solvents/acids ratio through fermentation time.

In acidogenic stage, solvents production stopped, while butyric acid started to accumulate in concentration. Due to solvents/acids ratio decrease, the pH was gradually dropped. These phenomena were even distinct in other runs such as Run 1 and Run 11 shows in Appendix A and B. It appeared that *Clostridium saccharoperbutylacetonicum* N1-4 was suffered from solvent toxicity, and was shifted back to acidogenesis in this stage. Similar profiles were also observed from other solventogenesis dominant bioreaction runs (Run 1, 4, 5, 7, 9, 10, 11 in A group and Run 1, 5, 7, 9, 11 in B group) which are displayed in Appendix A.

4.4.2 Acidogenesis dominant reaction (maximum solvents/acids ratio < 1)

Figure 4-5 shows the profiles of Run 3 under sterile condition. 0.7 g/L acetone, 0.6 g/L butanol and little ethanol (0.1 mg/L) were detected, which accompanied with a small amount of sugar consumption and acetic acid and butyric acid reutilization. Figure4-5 (b) shows solvents/acids ratio was <1 all the time, indicated organic acids were the main products. Two-stage bioreaction, including lag stage and acidogenic stage, were observed in Run 3. Total sugar utilization was 17% within 32.5 d and pH was slightly dropped from 5.44 to 5.20. Run 3 conducted under non-sterile condition and sterile condition had similar profiles. Only the solvents concentrations were much less which 0.28 g/L of acetone and 0.23 g/L of butanol were detected, and lag stage was longer under non-sterile condition when compared between Run 3 in A and B experimental groups. Other acidogenesis dominant experimental plots are shown in Appendix B.



(a) (b)

Figure 4-5 Profiles of (a) metabolite products and (b) solvents/acids ratio, pH, total sugar concentration, and cell concentration of Run 3 under sterile condition. Initial cell concentration = 640±57 mg/L, incubation temperature = 35℃.

Overall, the main final products were butanol and acetone in solventogenesis dominant reaction, while organic acids were main products in acidogenesis dominant reactions. The profiles of all experimental runs were shown in Appendix A, B and C.

From the data of Table 4-6 and 4-7, it seemed that active ABE fermentation with1000-2000 mg/L initial cell concentration was increased to > 2000 mg/L during fermentation, while with >2000 mg/L or < 1000 mg/L initial cell concentration was slightly grown 0%-17% in concentration. Other characteristics of each ABE fermentation runs were discussed in the following sections. The data of B/A ratio and maximum solvents/acids ratio at the end of fermentation time for all experimental runs are listed in Table 4-8 and 4-10. Data of sugar utilization were in Table 4-9 and 4-11.

Table 4-6 Cell growth for all experimental runs under sterile condition.

Run number	T (℃)	$[Cell]_i$ (mg/L)	t [a] (d)	$[Cell]_t$ [b] (mg/L)	Cell growth [c]
5, 9, 10	35	1429±214	4	2027±12	42±3%
3	35	640±57	6	700	17%
11	35	2331±28	4	2340	0%
7	28	2170±157	7	2080	5%
4	28	808±74	11	880	3.5%
1	25	1429±214	11	2140	84%

The cell growths of Run 2, 6, 8 were 0%.

[a] t represents fermentation time which defined as the duration of maximum ABE concentration was achieved.

[b] The cell concentration at time t.

[c] Cell growth = $([Cell]_t-[Cell]_i) / [Cell]_i$

Table 4-7 Cell growth of all experimental runs under non-sterile condition.

Run number	T (℃)	$[Cell]_i$ (mg/L)	t [a] (d)	$[Cell]_t$ (mg/L)	Cell growth
5 [a]	35	1429±214	5	1740	47%
9 [a]			7	2080	49%
10 [a]			4.25	1980	7%
3		640±57	13.4	700	3%
11		2331±28	4	2240	0%
7	28	2170±157	11	1620	0%
1	25	1429±214	11	2240	70%

The cell growths of Run 2, 4, 6, 8 were 0%.

[a] t represents fermentation time which defined as the duration of maximum ABE concentration was achieved.

[b] The cell concentration at time t.

[c] Cell growth = $([Cell]_t-[Cell]_i) / [Cell]_i$

4.5 Performances of ABE fermentation

4.5.1 ABE fermentation under sterile condition

The ABE fermentation tests in A experimental group were conducted in synthetic 10-g based NPRS hydrolysate under sterile condition, which contained 2.73 g/L arabinose, 28.10 g/L glucose, and 10.00 g/L galcatose and 5 g/L initial concentration of acetic acid. Table 4-8 presents the characteristics of butanol production at various CCD designed incubation temperature and initial cell concentration under sterile condition. Run 5, 9, and 10 were the triplicate central points in CCD design. The central point runs resulted in the production of 5.1±0.3 g/L butanol and 8.1±0.8 g/L ABE in 4 days of fermentation time. During the fermentation, an butanol yield and productivity of 0.16±0.01 and 1.3±0.1 g/L/d were obtained, respectively. Run 3, 11, and central runs were all performed at 35℃ with different initial cell concentrations. Run 3 with relatively low initial cell concentration of 640±57 mg/L caused the lowest butanol and ABE production, while Run 11 with relatively high initial concentration produced the highest butanol and ABE production. Therefore, under the same incubation temperature, ABE production has positive relationship with initial cell concentration increment. The same phenomena can be found when compared between Run 7 and 4, which were conducted at 28℃ with 2170±157 mg/L and 808±74 mg/L initial cell concentrations, respectively.

Run 1 was performed at 25℃ of incubation temperature and 1429±214 mg/L of initial cell concentration. It produced 6.3 g/L of butanol, 9.3 g/L of ABE, and 0.2 of butanol yield, which were higher than central runs (under 35℃ and 1429±214 mg/L cell concentration). The butanol yield was 20% lower when incubation temperature increase from 25℃ to 35℃. The decrease in solvent yield reflected an increased B/A ratio of 25%. This trend was identical with previous finding of E. H. Carnarius, U.S. Patent 2198104, 1940. An increase in the butanol ratio was obtained by decreasing the

temperature of the fermentation from 30℃ to 24℃ after 16 h (Carnarius, 1940). In contrast, the butanol productivity of Run 1 was 56% lower than central runs. It revealed that the metabolic rate of bacteria doubled when temperature elevated 10℃. The high metabolic rate might cause early solvent toxicity to fermentation cell, thus resulted in relative low butanol yield at last, which might another reason to explain higher yield in Run 1 than in central runs.

The results of various sugar utilization and total sugar utilization within fermentation time under sterile condition are listed in Table 4-9. In general, the trend of sugar utilization was consistent with the trend of butanol production. Almost 100% glucose and >70% total sugar were consumed within fermentation time at Run 1, 7, 11, and central runs. Run 3 and 4 had low initial cell concentration which caused low sugar utilization. Run 3 with 640±57 mg/L of initial cell concentration only utilized 22% glucose and 17% total sugar. Run 4 with 808±74 mg/L initial cell concentration had 67% and 52% glucose and total sugar consumption, respectively. *C. saccharoperbutylacetonicum* N1-4 hardly utilized arabinose. Run 2, 6, and 8 had no metabolite production, neither ABE nor organic acids, within 30 d incubation time. The sugars (arabinose, glucose, and galactose) were 0% utilized by cell. The pH was also remained constant. It was apparent that *C. saccharoperbutylacetonicum* N1-4 was dead or inactivated at high incubation temperatures (42℃ and 45℃).

Table 4-8 Characteristics of butanol production of ABE fermentation of component sugars present in non-pretreated rice straw (NPRS) under sterile condition.

Run number	t [a] (d)	Max. butanol conc. (g/L)	Max. ABE conc.(g/L)	Butanol productivity (g/L/d) [b]	Butanol yield (g/g) [c]	B/A ratio [d]	Solvent/ acid ratio [e]
5, 9, 10 [f]	4	5.1±0.3	8.1±0.8	1.3±0.1	0.16±0.01	2.0	3.5±0.3
3	6	0.6	1.5	0.10	0.09	0.9	0.32
11	4	6.3	12.4	1.58	0.20	1.4	5.2
7	7	7.3	13.1	1.04	0.21	1.6	8.6
4	11	4.3	8.1	0.39	0.20	1.3	2.6
1	11	6.3	9.3	0.57	0.20	2.5	7.8
2, 8, 6 [g]	-	-	-	-	-	-	-

[a] t: The fermentation time was defined as the time period when a maximum concentration of ABE was achieved.

[b] Butanol productivities were calculated based on the amount of butanol (g/L) produced within the end fermentation time.

[c] Butanol yield was defined as total grams of butanol produced per total grams of glucose utilized.

[d] Butanol concentration divided by acetone concentration within the end of fermentation time.

[e] Solvent concentration divided by acid concentration at fermentation time.

[f] Three replicated central points. Mean ± SD, n=3.

[g] There was no fermentation happened.

Table 4-9 The percentage of various sugar utilization and total sugar utilization within fermentation time under sterile condition.

Run number	Initial cell conc. (mg/L)	T (℃)	Glucose utilization (%)	Galactose utilization (%)	Arabinose utilization (%)	Total sugar utilization (%)
5, 9, 10 [a]	1429±214	35	98±3	23±9	13±8	75±5
3	640±57	35	22	11	0	17
11	2331+28	35	100	39	6	84
7	2170±157	28	100	56	0	84
4	808±74	28	67	21	0	52
1	1429±214	25	100	35	0	77
2 [b]	1429±214	45	0	0	0	0
8 [b]	2170±157	42	0	0	0	0
6 [b]	808±74	42	0	0	0	0

[a] Three replicated central points. Mean ± SD, n=3.

[b] There was no fermentation happened.

4.5.2 ABE fermentation under non-sterile condition

The CCD designed conditions and medium of ABE fermentation tests in B experimental group were identical with A experimental group except that B experimental group were conducted under non-sterile condition. Table 4-10 shows the characteristics of butanol production at various CCD designed incubation temperatures and initial cell concentrations of B experimental groups. Initial cell concentration might be the key factor that affected the performances, stability and reproducibility of ABE fermentation under non-sterile condition. A trend could be found in Table 4-10:

(1) When initial cell concentrations were ≤ 1500 mg/L, the stability and reproducibility were poor. The variations among central points were relatively large, while the triplicate central points at A experimental group were well reproducibility.

(2) When initial cell concentrations were > 2200 mg/L, the ABE production was stable and the performances were consistent with the results of A experimental group.

(3) When initial cell concentrations were < 800 mg/L, there were no fermentation happened.

In non-sterile fermentation processes, the system was contaminated by other anaerobic bacteria or virus. *C. saccharoperbutylacetonicum* N1-4 and other anaerobic bacteria may be in competitive position. When *C. saccharoperbutylacetonicum* N1-4 was dominant in the fermentation system, the ABE production and performances would not be influenced. On the other hand, the performances of ABE fermentation were poor in reproducibility or low in ABE production when *C. saccharoperbutylacetonicum* N1-4 was inoculated at low cell concentration. The inhibition and competition caused by other anaerobic bacteria or phage infections caused by virus lead *C. saccharoperbutylacetonicum* N1-4 death or inactivation. Summarizing the results, it reflects the fact that ABE fermentation by *C. saccharoperbutylacetonicum* N1-4

conducted under non-sterile condition is feasible when the initial cell concentration is higher than 2200 mg/L. The results of Run 2, 6, and 8 were same as A group, because *C. saccharoperbutylacetonicum* N1-4 was dead or inactivated at high incubation temperatures (42℃ and 45℃).

Table 4-10 Characteristics of ABE production of fermentation of component sugars present in rice straw (NRS) under non-sterile condition.

Run number	t [a] (d)	Max. butanol conc. (g/L)	Max. ABE conc.(g/L)	Butanol productivity (g/L/d) [b]	Butanol yield (g/g) [c]	B/A ratio [d]	Solvent/ acid ratio [e]
5 [f]	5	2.8	4.3	0.55	0.13	2.3	1.1
9 [f]	7	4.8	8.0	0.67	0.17	2.5	2.4
10 [f]	4.25	0.7	1.4	0.17	0.09	1.2	0.32
3	13.4	0.23	0.54	0.016	0.03	0.6	0.11
11	4	6.6	12.5	1.66	0.20	1.28	5.2
7	11	7.9	14.3	0.71	0.22	1.38	7.8
1	11	6.4	9.5	0.58	0.21	2.3	5.3
2, 4, 8, 6 [g]	-	-	-	-	-	-	-

[a] t: The fermentation time was defined as the time period when a maximum concentration of ABE.

[b] Butanol productivities were calculated based on the amount of butanol (g/L) produced within the end fermentation time.

[c] Butane yield was defined as total grams of butanol produced per total grams of glucose utilized.

[d] Butanol concentration divided by acetone concentration within the end of fermentation time.

[e] Solvent concentration divided by acid concentration at fermentation time.

[f] Three replicated central points. Mean ± SD, n=3.

[g] There was no fermentation happened.

Table 4-11 shows the data of sugar utilization in B experimental group within fermentation time. The average total sugar utilization of central runs in B experimental group was 40% lower than A group. And there was 0% of sugar utilization through Run 4 in B group, while there was 52% of sugar utilization in A

group. Other runs' sugar utilization data in B group were compatible with A group. Again, the trend of butanol production was generally consistent with the trend of sugar utilization.

Table 4-11The percentage of various sugar utilization and total sugar utilization within fermentation time under non-sterile condition.

Run number	Initial cell conc. (mg/L)	T (℃)	Glucose utilization (%)	Galactose utilization (%)	Arabinose utilization (%)	Total sugar utilization (%)
5 [a]			70	5	25	49
9 [a]	1429±214	35	88	29	2	66
10 [a]			27	8	0.3	20
3	640±57	35	25	3.6	2.6	18
11	2331+28	35	100	28	19	80
7	2170±157	28	100	68	12	87
4	808±74	28	0	0	0	0
1	1429±214	25	97	26	0	73
2 [b]	1429±214	45	0	0	0	0
8 [b]	2170±157	42	0	0	0	0
6 [b]	808±74	42	0	0	0	0

[a] Three replicated central points. Mean ± SD, n=3.

[b] There was no fermentation happened.

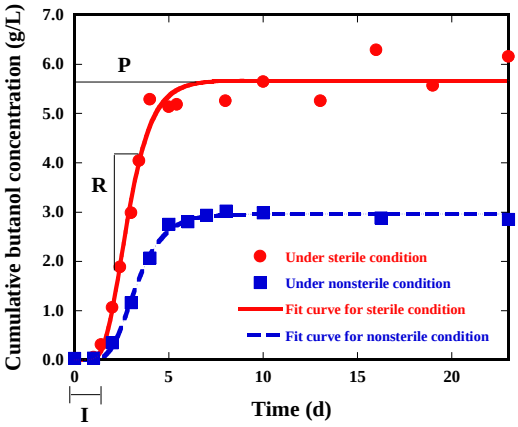


Figure 4-6 Cumulative butanol production curves for ABE fermentation of Run 5 under sterile condition. Markers — experimental data; Nonlinear lines — data estimated by Eq. (g).

4.6 Kinetics of butanol production

The modified Gompertz equation was employed to describe the kinetics of the butanol production. Figure 4-6 shows the predicted values for cumulative butanol production, shown by smooth curves, against experimental values obtained from Run 5 in A experimental group. Similar plots were generated for all active batch experiments in both A and B experimental groups and they are shown in Appendix E. The kinetic parameters estimated based on Eq. (g) are listed in Table 4-12 for A experimental group and Table 4-13 for B experimental group. The ABE production was well correlated to the modified Gompertz equation ($R^2 > 0.97$). Predicted butanol yield was calculated from P and predicted utilized sugar concentration. Predicted sugar utilization was also calculated by modified Gomperz equation, the results are exhibited in Appendix F. Actual butanol yield was calculated from maximum accumulative butanol achieved and utilized sugars. Predicted butanol productivity was P divided by fermentation time, while actual butanol productivity was maximum accumulative butanol concentration divided by fermentation time.

As shown in Table 4-12, the butanol production potential (P) and the butanol production rate (R) for experimental runs in A experimental group were 0.00-7.27 g/L and 0.27-4.82 g/L/d, respectively. From Table 4-13, the P and R for experimental runs in B group were 0.00-7.70 g/L and 0.11-3.21 g/L/d, respectively. The maximum and minimum of butanol production potentials were consistent between A and B group, i.e. under sterile and non-sterile conditions. On the other hand, both the highest and lowest values of production rate in A experimental group under sterile condition were superior to B experimental group under non-sterile condition.

Table 4-12 The parameters of the modified Gompertz equation for butanol production under sterile condition

Run number	Kinetics constants from modified Gomperz equation				Yield (g/g)		Productivity (g/L/d)	
	P (g/L)	R (g/L/d)	I (d)	R^2	Estimated	Actual	Estimated	Actual
5	5.64	2.39	1.61	0.98	0.15	0.16	1.41	1.32
9	5.43	2.92	1.68	0.97	0.15	0.16	1.36	1.26
10	5.55	2.1	1.69	0.98	0.16	0.17	1.39	1.21
3	0.55	0.27	1.54	0.98	0.05	0.09	0.09	0.1
11	6.23	4.82	1.56	0.97	0.18	0.2	1.56	1.58
7	7.27	2.73	2.64	0.99	0.2	0.21	1.04	1.04
4	4.35	1.07	3.43	0.99	0.16	0.2	0.40	0.39
1	6.27	1.47	5.76	0.99	0.2	0.2	0.57	0.57
2	0	0	∞	-	0	0	0	0
8	0	0	∞	-	0	0	0	0
6	0	0	∞	-	0	0	0	0

There were lag time during ABE fermentation for all experimental runs. A experimental group were conducted under sterile condition. The variables were incubation temperatures and initial cell concentrations. As revealed, incubation temperature was the major factor that determine the duration of lag time. Run 3, 5, 9, 10, and 11 were all performed under 35℃, their lag time were about identical regardless of different initial cell concentrations. Run 1 had relative longer lag time, because of its lowest incubation temperature, 25℃. It could be observed that the lag time was 35℃<28℃<25℃. Run 2, 6, and 8 were infinity in lag time, because *C. saccharoperbutylacetonicum* N1-4 was inactive or dead at the incubation temperature of 42℃ or 45℃. On the other side, B experimental group were conducted under non-sterile condition. Besides the variables of incubation temperatures and initial cell concentrations, the contaminations from other microbes also influenced kinetics of ABE fermentation. From Table 4-13, the trend of lag time decreasing with the increasing of incubation temperatures, 35 ℃ <28 ℃ <25 ℃ was still observed.

Nevertheless, the contaminations from other microbes were enlarging the lag time of Run 3 and 4 due to low incubated cell concentration. As stated earlier, high initial cell concentration of *C. saccharoperbutylacetonicum* N1-4 could constrain the effect of contaminations, in contrast, inhibition could occurred at low initial cell concentration under non-sterile condition.

Table 4-13 The parameters of the modified Gompertz equation for butanol production under non-sterile condition.

Run number	Kinetics constants from modified Gomperz equation				Yield (g/g)		Productivity (g/L/d)	
	P (g/L)	R (g/L/d)	I (d)	R^2	Estimated	Actual	Estimated	Actual
5	2.96	1.08	1.88	0.997	0.14	0.13	0.59	0.55
9	4.78	1.16	1.62	0.99	0.15	0.17	0.68	0.67
10	0.69	0.42	1.5	0.97	0.04	0.09	0.16	0.17
3	0.22	0.11	3.08	0.99	0.011	0.03	0.016	0.016
11	6.51	3.21	1.57	0.98	0.18	0.2	1.63	1.66
7	7.7	2.06	2.41	0.99	0.22	0.22	0.7	0.71
4	0	0	∞	-	0	0	0	0
1	6.72	1.43	6.24	0.99	0.22	0.21	0.61	0.58
2	0	0	∞	-	0	0	0	0
8	0	0	∞	-	0	0	0	0
6	0	0	∞	-	0	0	0	0

4.7 Response surface analysis

Full factorial central composite design (CCD) was employed to determine the individual and interactive effects of two independent variables, initial cell concentration (X_1) and incubation temperature (X_2), on butanol production. And response surface methodology (RSM) was used to determine the maximum butanol productivity (Y_1), butanol yield (Y_2), and modified Gomperz predicted butanol production rate (Y_3) in terms of initial cell concentration (X_1) and incubation temperature (X_2) by using the data in Table 4-14. To investigate the optimum

conditions of all experiments conducted under sterile and non-sterile conditions, CCD-RSM analysis were executed for A and B experimental groups.

Table 4-14 Central composite quadratic model and dependent variables for five-level-two-factor response surface analysis.

Run order	Independent variables		Dependent variables (responses)					
	Cell concentration, mg/L (X_1)	Temperature, ℃ (X_2)	Y_1 = Butanol productivity (g/L/d)		Y_2 = Butanol yield (g/g)		Y_3 = Butanol production rate (R) (g/L/d)	
			A	B	A	B	A	B
1	1429±214	25	0.57	0.58	0.20	0.21	1.47	1.43
2	1429±214	45	0.00	0.00	0.00	0.00	0.00	0.00
3	640±57	35	0.1	0.016	0.09	0.03	0.27	0.11
4	808±74	28	0.39	0.00	0.20	0.00	1.07	0.00
5	1429±214	35	1.32	0.55	0.16	0.13	2.39	1.08
6	808±74	42	0.00	0.00	0.00	0.00	0.00	0.00
7	2170±157	28	1.04	0.71	0.21	0.22	2.73	2.06
8	2170±157	42	0.00	0.00	0.00	0.00	0.00	0.00
9	1429±214	35	1.26	0.67	0.16	0.17	2.92	1.16
10	1429±214	35	1.21	0.17	0.17	0.09	2.10	0.32
11	2331+28	35	1.58	1.66	0.20	0.20	4.82	3.21

4.7.1 The second-order model and analysis of variance (ANOVA)

Second-order model was fitted to the uncoded data by least squares. The regression equations are given in Table 4-15. The regression coefficients and the sorted significant parameters are tabulated in Table 4-16 for Y_1, Y_2, and Y_3 where significant parameters are sorted based on t and p-values. The p-values were used as a tool to check the significance level of each coefficient which is necessary to understand the pattern of interactions between the test variables. A p-value < 0.05 is considered to be statistically significant. With a low p-value and high absolute t-value, the corresponding coefficient is highly significant (Hamzaoui et al., 2008).

Table 4-15 Regression equations analysis in uncoded units obtained for butanol productivity (Y_1), butanol yield (Y_2), and modified Gomperz equation predicted butanol production rate (Y_3).

Experimental group	Regression equations
A	$Y_1 = -14 + 3.2\ X_1 + 0.77X_2 - 0.03X_1X_2 - 0.53X_1^2 - 0.01X_2^2$
	$Y_2 = -0.44 + 0.13\ X_1 + 0.04X_2 - 0.0005X_1X_2 - 0.03X_1^2 - 0.0007X_2^2$
	$Y_3 = -26 + 5.1X_1 + 1.5X_2 - 0.08X_1X_2 - 0.27X_1^2 - 0.02X_2^2$
B	$Y_1 = -5.3 + 1.2\ X_1 + 0.28X_2 + 0.04X_1X_2 - 0.18X_1^2 - 0.004X_2^2$
	$Y_2 = -0.99 + 0.59\ X_1 + 0.04X_2 - 0.01X_1X_2 - 0.04X_1^2 - 0.0005X_2^2$
	$Y_3 = -8.5 + 3.3X_1 + 0.41X_2 - 0.10X_1X_2 + 0.47X_1^2 - 0.005X_2^2$

Y_1 is butanol productivity; Y_2 is butanol yield; Y_3 is predicted butanol production rate by modified Gomperz equation; X_1 is initial cell concentration; X_2 is incubation concentration.

As shown in Table 4-16, the responses of Y_1 of A experimental group were significantly affected by constant, linear term of incubation temperature (X_2) and initial cell concentration (X_1), and quadratic term of incubation temperature (X_2^2). Among these statistical significant factors, X_2^2 was the most profound effect on butanol productivity in A experimental group, with a lowest p-value of 0.006 and a highest absolute t-value of 4.5. As for Y_1 in B experimental group, none of the factors were significant in statistics. However, linear term of temperature (X_2) was relatively effective than all the other factors, with lowest p-value and largest t-value. Significant factor for the responses Y_2 in A experimental group was quadratic term of incubation temperature (X_2^2), with p-value of 0.047 and absolute t-value of 2.6, while in B experimental group was linear term of initial cell concentration (X_1), with p-value of 0.028 and absolute t-value of 3.1. Linear term and quadratic term of temperature (X_2 and X_2^2) were the significant factors for butanol production rate (Y_3) in A experimental group where X_2^2 was superior to X_2. None of the terms significantly affected Y_3 in B experimental group. However, comparing the p and t values among

all terms, the term of cross product (X_1X_2) was relatively effective for Y_3 in B group.

Table 4-16 The estimated response surface regression coefficients and their corresponding t and p-values.

Butanol productivity (Y_1)						
Components	A experimental group			B experimental group		
	Coefficient	t-value	p-value	Coefficient	t-value	p-value
Constant	-14	-4.203	0.008	-5.3	-1.143	0.305
X_1	3.2	2.607	0.048	1.2	0.728	0.499
X_2	0.77	4.444	0.007	0.3	1.187	0.288
X_1^2	-0.52	-2.212	0.078	0.18	0.552	0.605
X_2^2	-0.01	-4.546	0.006	-0.004	-1.115	0.315
$X_1 \times X_2$	-0.03	-1.150	0.302	-0.04	-0.909	0.405
Butanol yield (Y_2)						
Components	A experimental group			B experimental group		
	Coefficient	t-value	p-value	Coefficient	t-value	p-value
Constant	-0.44	-1.105	0.319	-0.99	-1.843	0.125
X_1	0.13	0.911	0.404	0.59	3.050	0.028
X_2	0.04	1.963	0.107	0.04	1.614	0.168
X_1^2	-0.03	-1.012	0.358	-0.04	-1.127	0.311
X_2^2	-0.0007	-2.619	0.047	-0.0005	-1.392	0.223
$X_1 \times X_2$	-0.0005	-0.150	0.886	-0.01	-2.454	0.058
The predicted R by the modified Gompertz equation						
Components	A experimental group			B experimental group		
	Coefficient	t-value	p-value	Coefficient	t-value	p-value
Constant	-26	-2.328	0.067	-8.5	-1.119	0.314
X_1	5.1	1.246	0.268	3.3	1.210	0.280
X_2	1.5	2.547	0.051	0.41	1.072	0.333
X_1^2	-0.27	-0.337	0.749	0.47	0.882	0.418
X_2^2	-0.02	-2.600	0.048	-0.005	-0.889	0.415
$X_1 \times X_2$	-0.08	-0.873	0.423	-0.10	-1.625	0.165

X_1 : cell concentration (mg/L)

X_2: Temperature (℃)

t-value was obtained from the Student's t-test, which indicates the significance of the regression coefficients.

p-value was the significant level. ($p<0.05$ is considered to be statistically significant.)

The ANOVA was used as a statistical tool to test the significance and adequacy of the model, i.e. the quality of regressions. The analyses of response surface regression and variance for the quadratic model are shown in Table 4-17. The Fischer variation ratio (F-value) is the mean square due to regression, divided to the mean square due to the residues, which determines the statistic validity of the regression. The F-value greater than 1 implies that the regression adequately explained the data. The p-value tests whether the F-value determined by the model is significant from background; a p-value < 0.05 is considered to be statistically significant (Banerjee et al., 2010). The coefficient of regression (R^2) expressed the degree of model fitness. The more R^2 value approach unity, the better empirical model fits the actual data. Adjusted R^2 is calculated after removing the insignificant terms or factors from a model to compensate and derive a higher R^2 value. However, according to Hierarchy principle, it should contain all of the lower-order terms that compose it (Montgomery, 2001). Therefore, the R^2 was used to express the degree of model fitness rather than R^2_{adj} in this study. From F-value, p-value and R^2 in Table 4-17, it shows the second-order polynomial model was adequate to represent the actual relationship between the responses for butanol productivity (Y_1) and butanol yield (Y_2) in A experimental group and for Y_2 in B experimental group. Although the p-values of Y_3 in A and B experimental groups were 0.067 and 0.060, which was slightly higher than 0.05, the R^2 were considered to be acceptable for bioreactions. On the other hand, the quadratic model was not fit well for Y_1 in B experimental group.

Table 4-17 Analysis of variance for Y_1, Y_2 and Y_3.

	Experimental group	F-value [a]	Probability p-value (>F)	R^2
Y_1	A	8.49	0.017	0.89
	B	2.49	0.171	0.71
Y_2	A	12.85	0.007	0.93
	B	7.67	0.022	0.88
Y_3	A	4.31	0.067	0.81
	B	4.59	0.060	0.82

[a] The F-value is the mean square due to regression, divided to the mean square due to the residues.

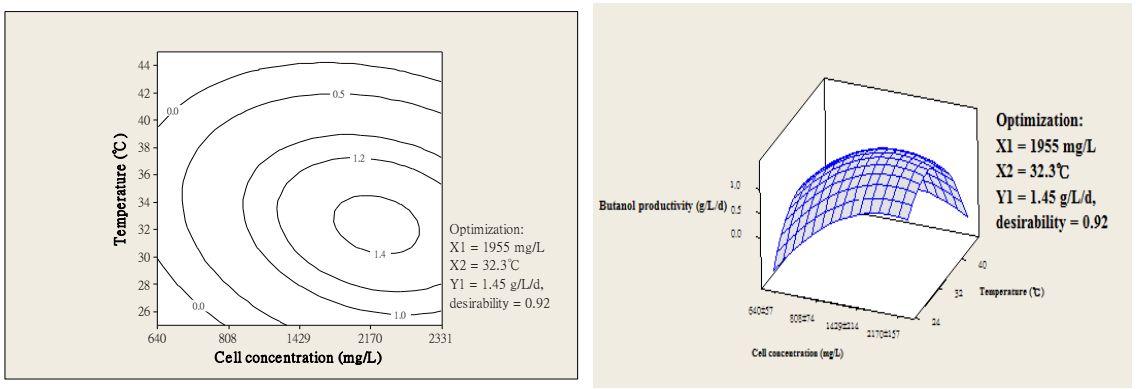
4.7.2 Responses optimization

The three-dimensional response surfaces and two-dimensional contour line plots to estimate butanol productivity, butanol yield, and butanol production rate over independent variables of initial cell concentrations and incubation temperatures under sterile and non-sterile conditions are shown in Figure 4-7 and 4-8, respectively. In Figure 4-7 (a) and (b), under sterile condition, the butanol productivity and yield increased with the increasing initial cell concentration or incubation temperature to their peaks at (X_1, X_2) = (1955 mg/L, 32.5℃) and (2006 mg/L, 26.3℃), respectively, then decreased with further increase in cell concentration or incubation temperature. For Figure 4-7 (c), butanol production rate increased with initial cell concentration or incubation temperature. It should be noticed that actual optimized initial cell concentration may not located in the predetermined range, which inferred a higher production rate could be obtained when initial cell concentration was higher than 2331 mg/L. However, when evaluated butanol production rate within the predetermined ranges of X_1 and X_2, the peak value was obtained at 30.5℃ of incubation temperature and 2331 mg/L of initial cell concentration.

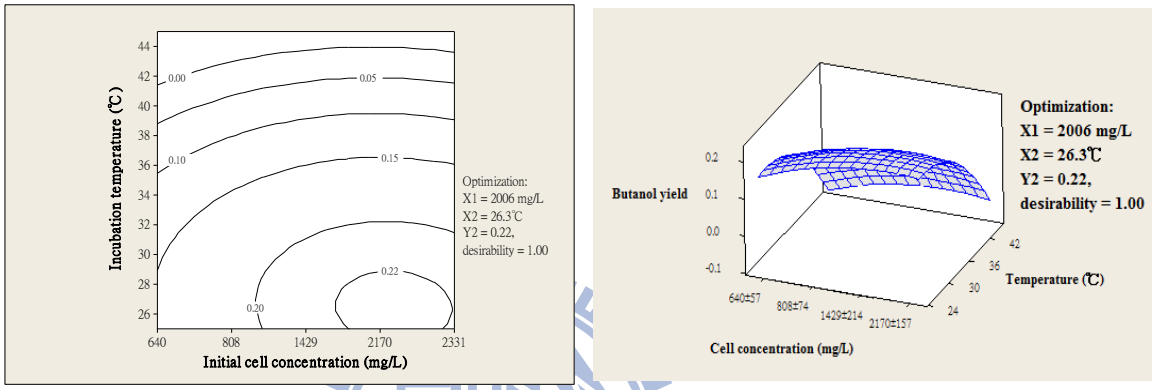
As appears in Figure 4-8, under non-sterile condition, high temperature, and low initial cell concentration (< 800 mg/L) lead butanol productivity, yield, and production

rate to zero level. The peak values of butanol productivity, yield, and production rate were achieved at (X_1, X_2) = (2331 mg/L, 26.4℃), (2331 mg/L, 25.0℃), and (2331 mg/L, 25.0℃) within the predetermined ranges of X_1 and X_2. The predicted maximum values of butanol productivity, yield, and production rate under optimized conditions in CCD were tabulated in Table 4-18 and compared with other previous studies. From the model-predicted values, the butanol productivity of synthetic NPRS hydrolysate fermentation were consistent with the results of fermentation of sludge hydrolysate (Hipolito et al., 2008) and wheat straw hydrolysate (Qureshi et al., 2008b), and were even 10 fold better than the fermentation of barley straw hydrolysate (BSH). Qureshi research group reported the reason for poor BSH fermentation may have been the presence of inhibitory chemicals, acetic acid, furfural, and HMF in BSH substrate (Qureshi et al., 2010a), which were not appears in our NPRSH hydrolysate. The maximum butanol productivity of ABE fermentation by synthetic NPRS hydrolysate were 86% and 65% lower than glucose (Hipolito et al., 2008) and mixed sugars (Ezeji et al., 2007a), respectively. ABE fermentation of synthetic NPRS hydrolysate showed its potential to produce biobutanol by its compatible butanol yield, 0.22-0.32, with the results of glucose and mixed sugars fermentation.

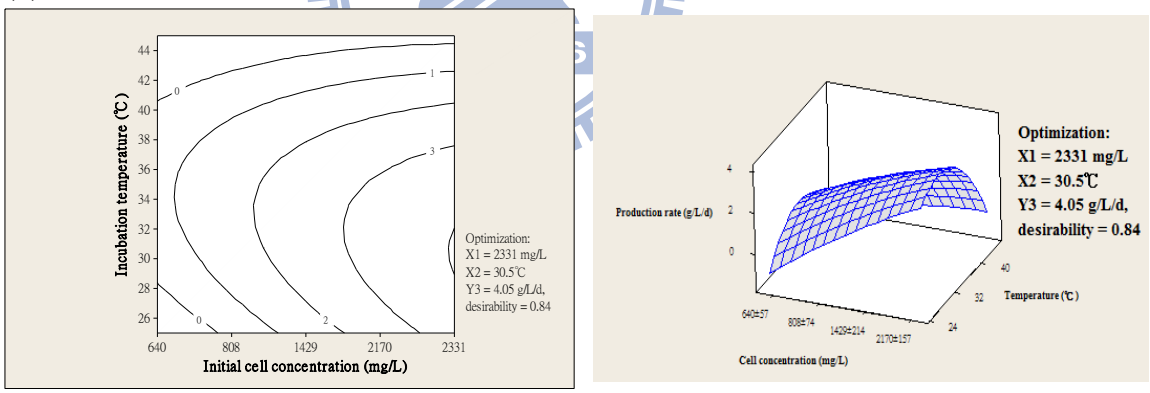
It has been believed that ABE fermentation should conducted under sterile condition to avoid contaminations from other microbes. However, the results in this study elucidated that the maximum values of responses Y_1, Y_2, and Y_3 of A and B experimental groups were at similar level under their corresponding optimized initial cell concentration and incubation temperature. Summarizing the results, it reflects the fact that ABE fermentation of *C. saccharoperbutylacetonicum* Nl-4 by using synthetic NPRS hydrolysate under non-sterile condition was found to be feasible and viable biotechnology to produce biofuels, which reduce cost by recycling agricultural waste and save energy cost and time by skipping the sterilization.



(a)

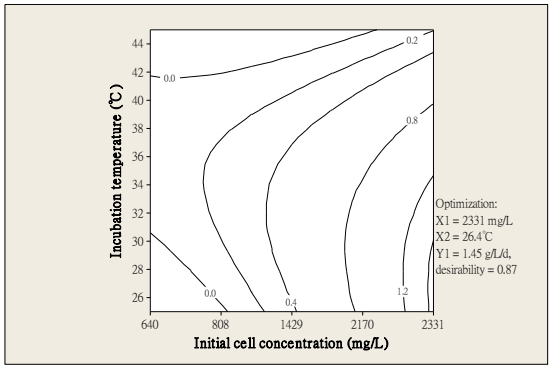


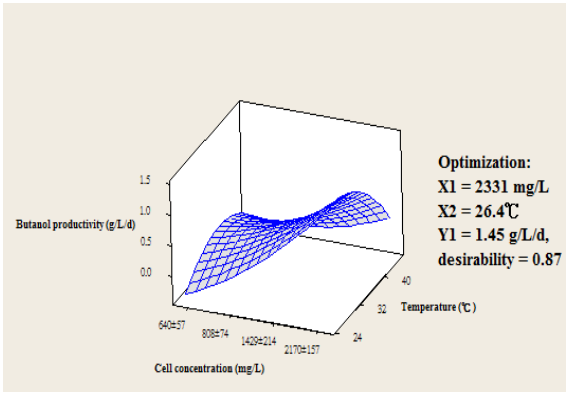
(b)



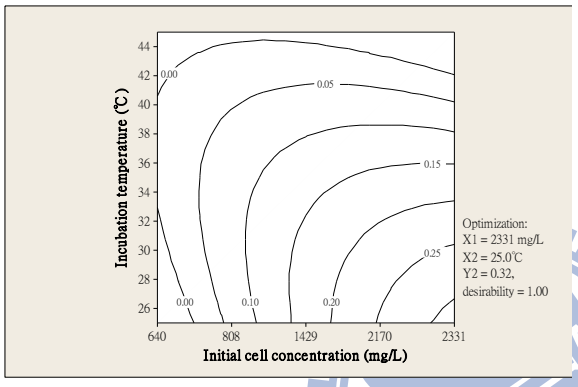
(c)

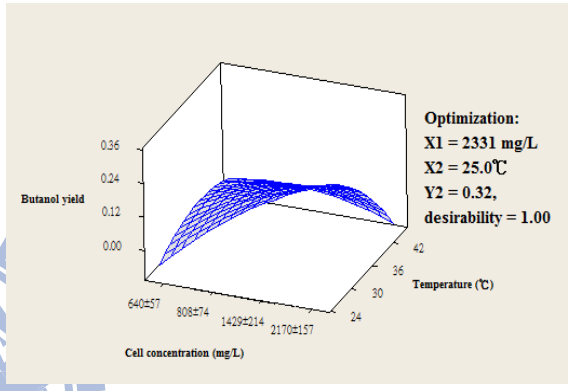
Figure 4-7 Contour plot (right) and response surface plot (left) of (a) butanol productivity (Y_1), (b) butanol yield (Y_2), and (c) butanol production rate (Y_3) in central composite design for A experimental group.



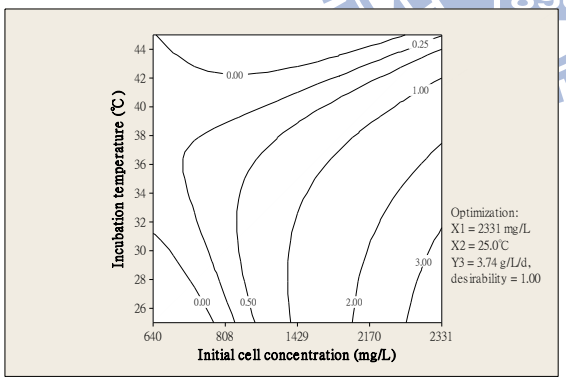


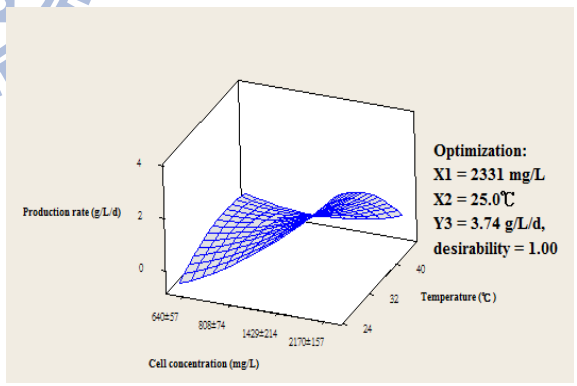
(a)





(b)





(c)

Figure 4-8 Contour plot (right) and response surface plot (left) of (a) butanol productivity (Y_1), (b) butanol yield (Y_2), and (c) butanol production rate (Y_3) in central composite design for B experimental group.

Table 4-18 Predicted maximum values of butanol productivity, yield, and production rate under optimized conditions in CCD in this study and butanol productivity, yield, and production rate in previous studies.

	Substrate	Microorganism	$[S]_i$ [a] (g/L)	T(℃)/ pH_i	$[C]_i$ [b] (g/L)	Butanol conc. (g/L)	Y_1 [c] (g/L/h)	Y_2 [c] (g/g)	Y_3 [c] (g/L/h)	Reference
non-sterile condition	Synthetic NPRS hydrolysate [d]	*C. saccharoperbutylacetonicum* Nl-4	**41**	**26.4/ 5.42**	**2.33**	**6-7**	**0.06**			**This study**
				25.0/5.42	**2.33**			**0.32**		
				25.0/5.42	**2.33**				**0.16**	
Under sterile condition	Synthetic NPRS hydrolysate	*C. saccharoperbutylacetonicum* Nl-4	**41**	**32.3/5.42**	**1.96**		**0.06**			**This study**
				26.3/5.42	**2.01**			**0.22**		
				30.5/5.42	**2.33**				**0.17**	
	Glucose	*C. saccharoperbutylacetonicum* Nl-4	40	30℃/ 5.8	-	10.4	0.43	0.26	-	(Hipolito et al., 2008)
	Sludge hydrolysate		8	30/5.5	-	2.7	0.05	0.34	-	
	Barley straw hydrolyste [e]	*C. beijerinckii* P260	59	35/6.5	-	0.4	0.006	0.03	-	(Qureshi et al., 2010a)
	Wheat straw [f]	*C. beijerinckii* P260	42	35/6.5,	-	8.1	0.08	0.20	-	(Qureshi et al., 2008b)
	Mixed sugar [g] (GXAM=5:4:2 :1)	*C. beijerinckii* BA101	55	35℃ No pH control	-	13.9	0.17	0.30		(Ezeji et al., 2007a)

[a] $[S]_i$: Initial total sugar concentration.

[b] $[C]_i$: Initial cell concentration.

[c] Y_1 = Butanol productivity; Y_2 = Butanol yield; Y_3 = Butanol production rate.

[d] Synthetic NPRS hydrolysate contained 2.73 g/L arabinose, 28.10 g/L glucose, 10.00 g/L galcatose and 5 g/L initial concentration of acetic acid.

[e] Barley straw hydrolysate contained 6 g/L arabinose, 15.6 g/L xylose, 20.2 g/L glucose, and 2.5 g/L galactose. 15.1 g/L glucose was added to the hydrolysate to raise total sugar level to 60 g/L.

[f] Wheat straw hydrolysate which prepared by acid pretreatment and enzyme saccharification contained 2.6 g/L arabinose, 17.1 g/L xylose, 19.1 g/L glucose, 3.1 g/L galactose.

[g] Mix sugars contained glucose, xylose, arabinose, and mannose in the ratio of 5:4:2:1

Chapter 5 Conclusions and Future Prospects

This study evaluated the pretreatment and the saccharification of the rice straw to integrate a most economic approach for the productions of the fermentable sugars. Followed by the optimum operating condition of initial cell concentration and incubation temperature for ABE fermentation of the fermentable sugars by central composite design and response surface methodology under sterile or non-sterile conditions were investigated to seek a most productive and economic viability biotechnology to produce biobutanol.

The composition of the rice straw was determined as 38% cellulose, 35% hemicelluloses, 7% Lignin, and 4% Ash. However, the different pretreatment procedures of the rice straw could vary the composition of the rice straw. Dilute acid pretreatment resulted in the reduction of hemicellulose whereas this was not appeared during the dilute base pretreatment. The removed hemicellulose remained in the acid hydrolysate was hydrolyzed to release xylose, glucose, and galactose. Meanwhile, the reduction of hemicelluose elevated the content of cellulose to more than 50%. This pretreatment was related to the performance of the saccharification of the rice straw. In the saccharification experiments, the total sugar productivity and yield were proportional to the enzyme loadings. The higher enzyme loading was implemented, the higher total sugar productivity and yield were attained. Glucose was the main final product in the saccharification. During the saccharification, there was no lag for the appearance of glucose. However, galactose, xylose, and arabinose were appeared a longer lag than glucose. In fact, the occurrence of cellulose hydrolysis was in advance to that of hemicellulose. The modified Gompertz equation simulated the productions of glucose obtained the glucose production potential and the glucose production rate for NPRS, PRS, and MPRSH were 2.62 g and 3.14 g/d, 2.5 g and 3.81

g/d, and 2.94 g and 3.26 g/d, respectively. The glucose production potential was not profoundly affect by the dilute acid pretreatment. This was in consistent with the glucose yield. The glucose yield of 0.52 g glucose/g rice straw for NPRS was compatible to 0.50 and 0.58 g glucose/g rice straw for PRS and MPRSH, respectively. However, the implementation of the dilute acid pretreatment to the rice straw resulted in the higher production rate compared to the untreated rice straw. It was consistent with the fact of the rate constants of the first-order kinetics. The values of k for NPRS, PRS, and MPRSH were 0.0024 h^{-1}, 0.0027 h^{-1}, and 0.0027 h^{-1}, respectively. There was no discrepancy between PRS and MPRSH. It suggests the activities of hydrolytic enzymes were not inhibited by the byproducts in the acid hydrolysate. However, taking accounts of energy, chemical, and time cost of pretreatment, rice straw grinded without other chemical pretreatment revealed to be the most economical efficiency feedstock to use in saccharification to fermentable sugars. Thus, NPRS hydrolysate was used in the series of ABE fermentation studies.

Under various CCD designed conditions, initial cell concentration (X_1) and incubation temperature (X_2) combinations, ABE fermentation of NPRS hydrolysate could be a solventogenesis or acidogenesis dominant bioreaction, whether operated under sterile or non-sterile condition. (X_1, X_2) = (640±57 mg/L, 35℃) were acidogenesis dominant, while other active runs were solventogenesis, such as (X_1, X_2) = (1429±214 mg/L, 25℃), (1429±214 mg/L, 35℃), (1429±214 mg/L, 45℃), (2170±214 mg/L, 28℃) etc. High incubation temperature, 42℃ and 45℃ lead to the inactivation of ABE fermentation. However, (X_1, X_2) = (808±74 mg/L, 28℃) were inactive under non-sterile condition, due to the contaminations and competition with other microbes.

In general, butanol productivity and yield increased with the increment of initial cell concentration when incubation temperature was remained constant. An increase

in the B/A ratio and butanol yield, and a decrease in butanol productivity was obtained by decreasing the incubation temperature from 35 to 25℃, which was consistent with the results reported by Carnarius (1940). The trend of sugar consumption was consistent with the trend of butanol production. Glucose was an easy and instant carbon source for *C. saccharoperbutylacetonicum* N1-4 to utilize, while arabinose was rarely utilized. The sequence of sugar utilization was glucose, galactose, and then arabinose. Besides initial cell concentration and incubation temperature as variables, contaminated degree was the other important factor that affected butanol production under non-sterile condition. The inhibition and influence cause by contaminations could be restrained by elevate the initial cell concentration to over 2200 mg/L to make *C. saccharoperbutylacetonicum* N1-4 a dominant group in fermentation system.

The modified Gompertz equation predicted the butanol production potential (P), the butanol production rate (R), and delay time (I) for ABE fermentation. The P and R values for experimental runs conducted under sterile condition were 0.00-7.27 g/L and 0.27-4.82 g/L/d, respectively. The duration time of lag time was 35℃<28℃<25℃, which reflected the incubation temperature of ABE fermentation was the main influential factor. As for experimental runs performed under non-sterile condition, P and R were 0.00-7.70 g/L and 0.11-3.21 g/L/d, respectively. Lag time was not only enlarged with decreasing temperature, but also with the deduction of initial cell concentration of *C. saccharoperbutylacetonicum* N1-4.

A deeper and precise investigation of the individual and interactive effect of initial cell concentration (X_1) and incubation temperature (X_2) on butanol productivity (Y_1), yield (Y_2), and modified Gompertz equation predicted butanol production rate (Y_3) and a determination of optimized conditions were achieved by full factorial central composite design and response surface methodology (CCD-RSM). For experimental runs conducted under sterile condition, X_2 and X_2^2 terms were the main factors

determined Y_1, Y_2, and Y_3. The peak value of 0.06 g/L/d of Y_1, 0.22 of Y_2, and 0.17 g/L/d of Y_3 were obtained under the combination conditions of (X_1, X_2) = (1960 mg/L, 32.3℃), (2010 mg/L, 26.3℃), and (2330 mg/L, 30.5℃), respectively; Nevertheless, for experimental runs conducted under non-sterile condition, X_2, X_1, and X_1X_2 terms were the main factors determined Y_1, Y_2, and Y_3, respectively. The peak value of 0.06 g/L/d of Y_1, 0.32 of Y_2, and 0.16 g/L/d of Y_3 were obtained under the combination conditions of (X_1, X_2) = (2330 mg/L, 26.4℃), (2330 mg/L, 25.0℃), and (2330 mg/L, 25.0℃), respectively.

To the final conclusion, ABE fermentation of *C. saccharoperbutylacetonicum* Nl-4 by using synthetic NPRS hydrolysate under non-sterile condition was found to be a feasible and viable biotechnology to produce biofuels, which reduced cost by recycling agricultural waste, and declined the energy cost and time by skipping the sterilization.

Based on this study, it was suggested that ABE fermentation could be conducted under non-sterile condition, when inoculated with high initial cell concentration (2330 mg/L) and low incubation temperature (25℃) of *C. saccharoperbutylacetonicum* Nl-4 at pH 5.42. For future prospects, it recommended that optimum pH value for ABE fermentation of NPRS hydrolysate should be investigated through CCD-RSM model, since many researchers believed pH is a factor required for triggering the onset of solventogenesis. Although, it has been reported that pH of 4.5 is the optimal pH for butanol production using *C. acetobutylicum* (Li et al., 2011). Still, the optimum pH value for solventogenesis appears to vary quite widely depending on the particulate strain and experimental conditions. In general, *Clostridia* could grow and produce solvents under the pH range of mild-acid, while most other bacteria need to grow in the range of neutral pH values. By taking the advantage of acid-resisting natural of *Clostridia*, low pH fermentation system could be built to exclude the contaminations of

other bacteria. By building up the optimum conditions of initial cell concentration, incubation temperature, and pH for non-sterile ABE fermentation, the pilot scale experiment could be conducted eventually.

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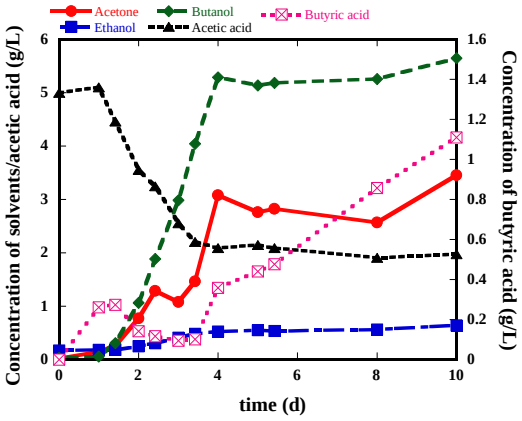
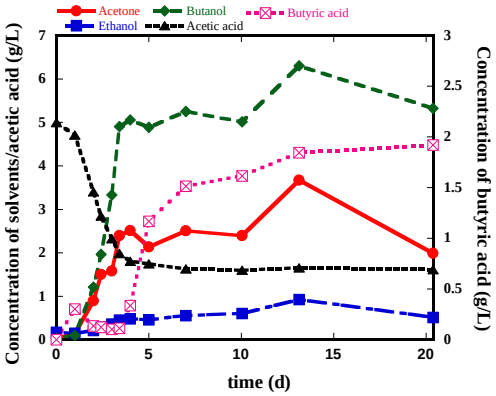
Appendix A Metabolic product (maximum solvents/acids > 1)

(1) Experimental runs under sterile condition



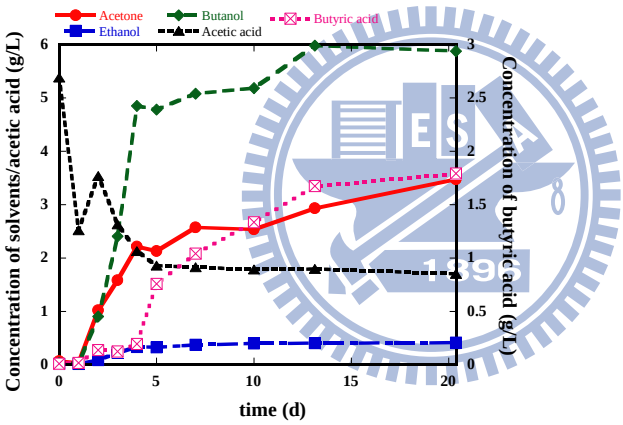
35℃, Cell conc. = 1429±214 mg/L

(Run5)



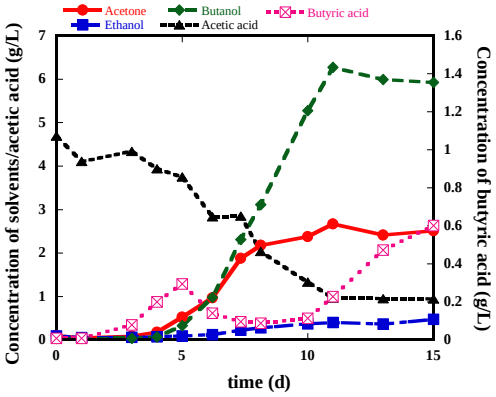
35℃, Cell conc. = 1429±214 mg/L

(Run 9)



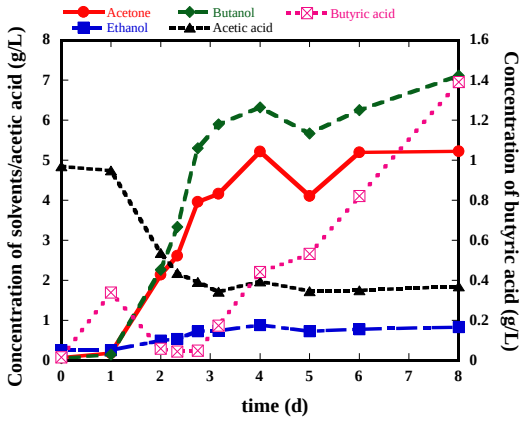
35℃, Cell conc. = 1429±214 mg/L

(Run 10)

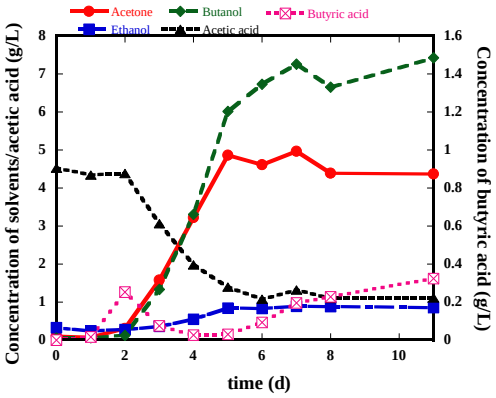


25℃, Cell conc.= 1429±214 mg/L

(Run 1)

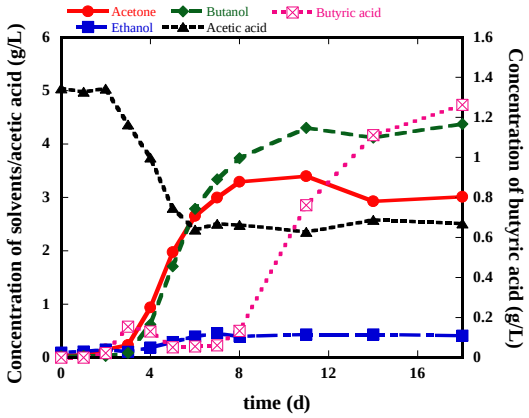


35℃, Cell conc. = 2331+28 mg/L



28℃, Cell conc. = 2170±157 mg/L

(Run 11) (Run 7)

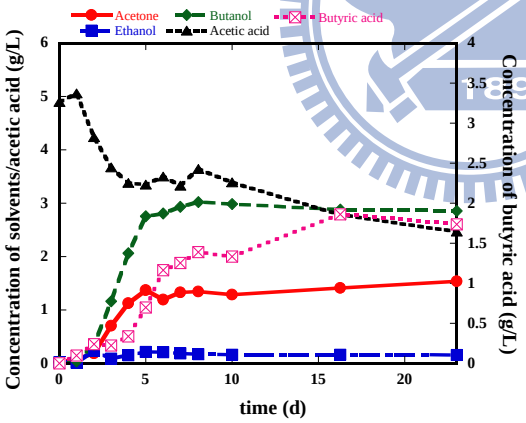


28℃, Cell conc. = 808±74 mg/L

(Run 4)

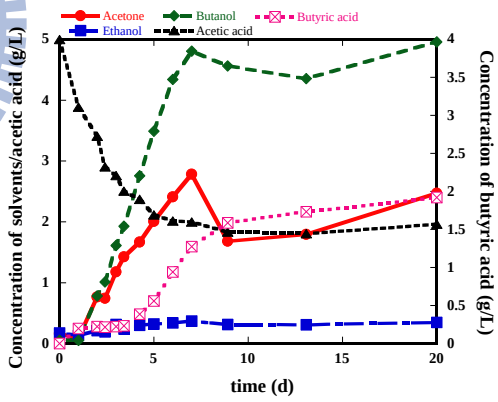
Figure A-1 The profiles of ABE fermentation products with maximum solvents/acids ratio > 1 for all experimental runs in A experimental group.

(2) Experimental runs under non-sterile condition



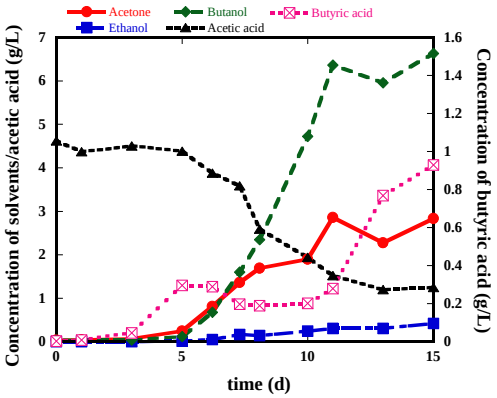
35℃, Cell conc. = 1429±214 mg/L

(Run5)



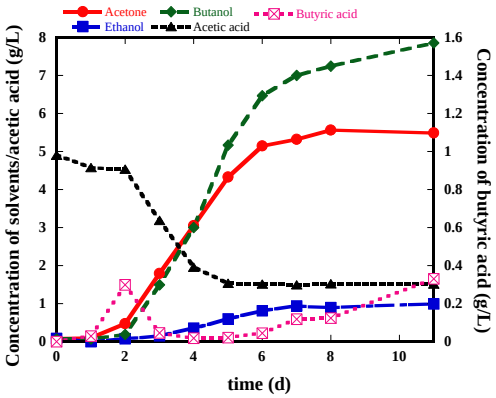
35℃, Cell conc. = 1429±214 mg/L

(Run 9)



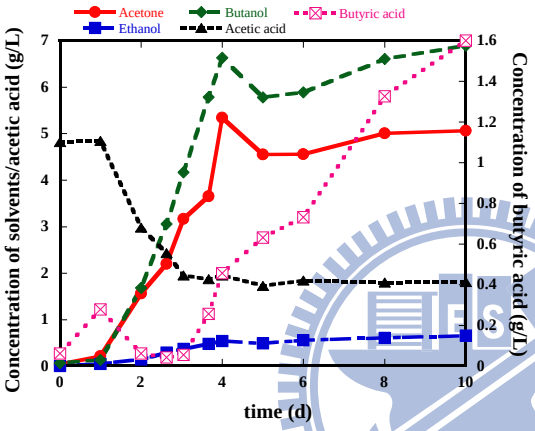
(c) 25℃, Cell conc.= 1429±214 mg/L

(Run 1)



28℃, Cell conc. = 2170±157 mg/L

(Run 7)



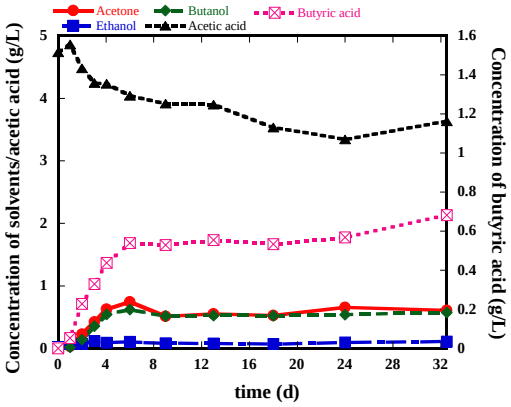
35℃, Cell conc. = 2331+28 mg/L

(Run 11)

Figure A-2 The profiles of ABE fermentation products with maximum solvents/acids ratio >1 for all experimental runs in B experimental group.

Appendix B Metabolic product (maximum solvents/acids ratio < 1)

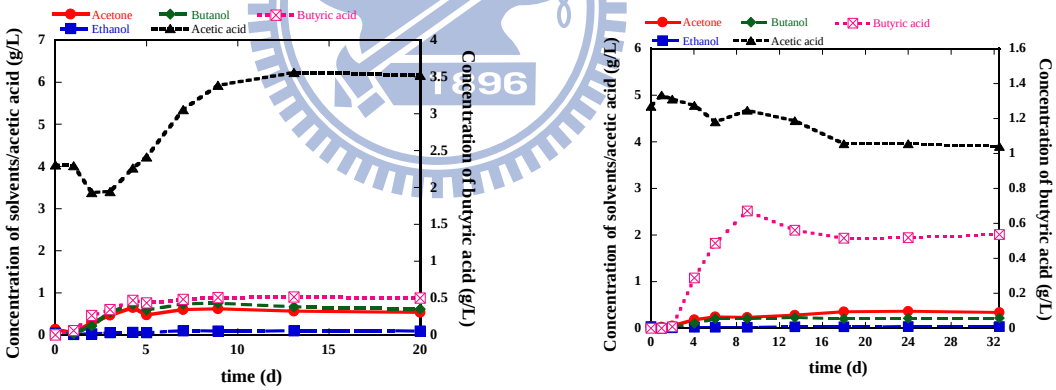
(1) Experimental runs under sterile condition



35℃, Cell conc. = 640±57 mg/L (Run 3)

Figure B-1 The profile of ABE fermentation products with maximum solvents/acids ratio < 1 for all experimental runs in A experimental group.

(2) Experimental runs under non-sterile condition



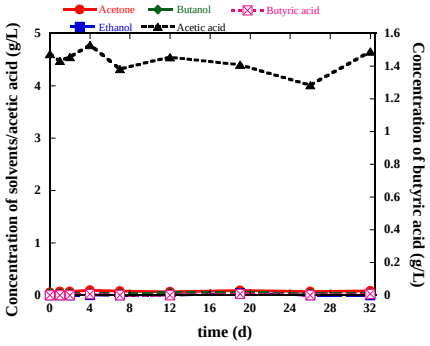
(a) 35℃, Cell conc. =1429±214 mg/L (Run 10)

(b) 35℃, Cell conc. = 640±57 mg/L (Run 3)

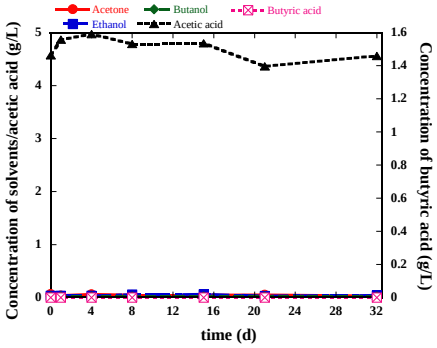
Figure B-2 The profile of ABE fermentation products with maximum solvents/acids ratio < 1 for all experimental runs in B experimental group.

Appendix C Inactive runs with no metabolic products

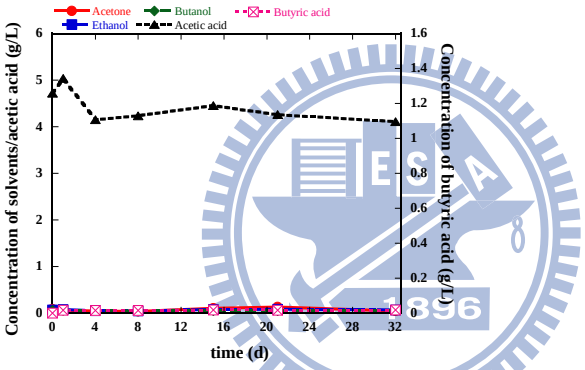
(1) Experimental runs under sterile condition



(a) 45℃, Cell conc. = 1429±214 mg/L (Run 2)



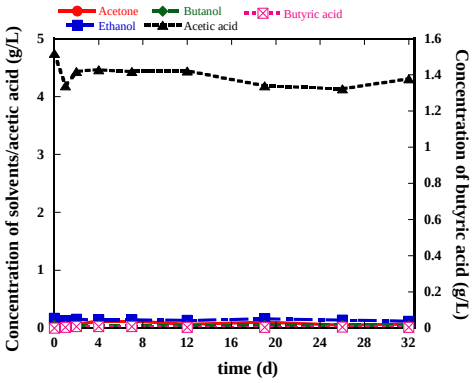
(b) 42℃, Cell conc. = 808±74 mg/L (Run 6)



(c) 42℃, Cell conc. = 2170±157 mg/L (Run 8)

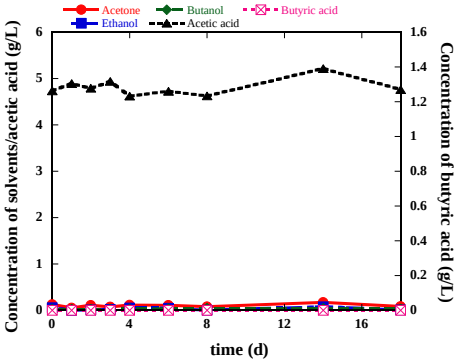
Figure C-1 The profile of metabolic products of inactive runs in A experimental group

(2) Experimental runs under non-sterile condition



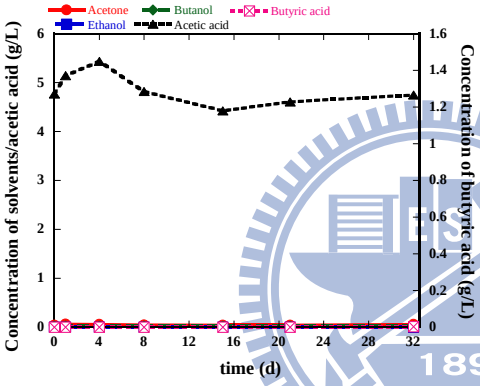
45℃, Cell conc. = 1429±214 mg/L

(Run 2)



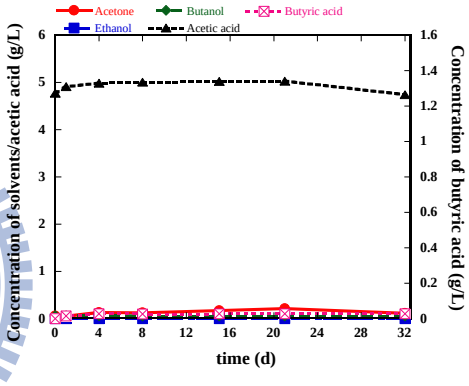
28℃, Cell conc. = 808±74 mg/L

(Run 4)



42℃, Cell conc. = 808±74 mg/L

(Run 6)

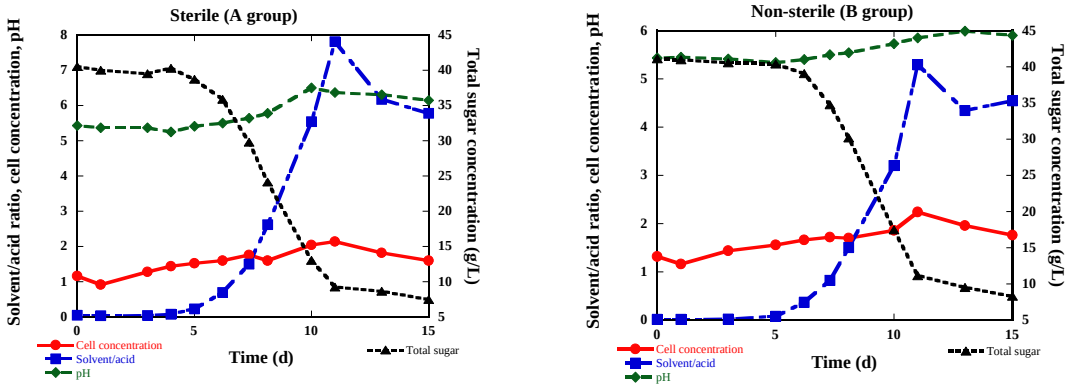


42℃, Cell conc. = 2170±157 mg/L

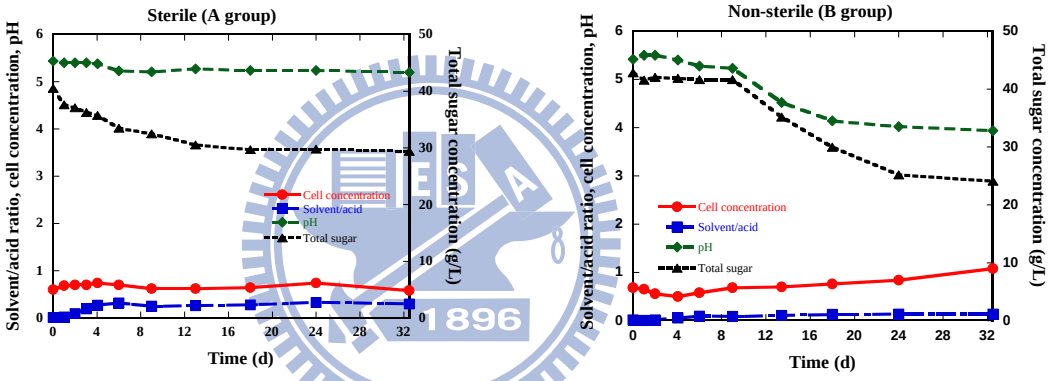
(Run 8)

Figure C-2 The profile of metabolic products of inactive runs in B experimental group.

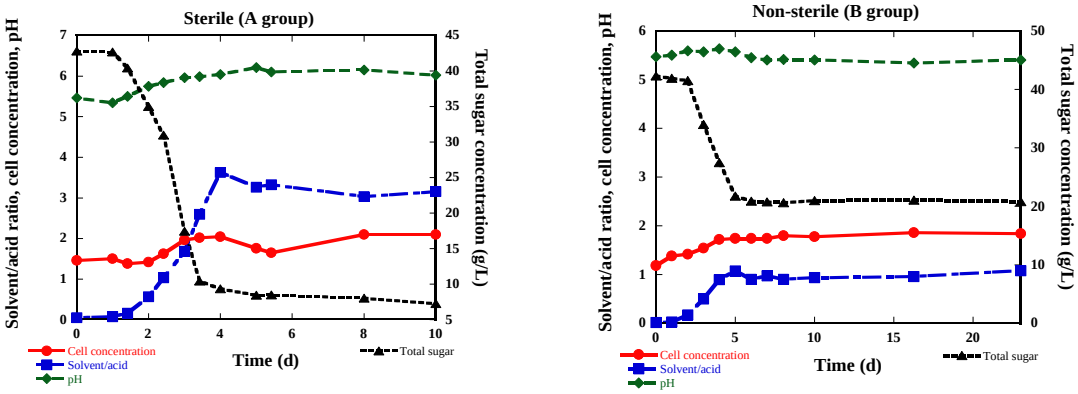
Appendix D Total sugar, pH, and cell concentration, and solvents/acids ratio



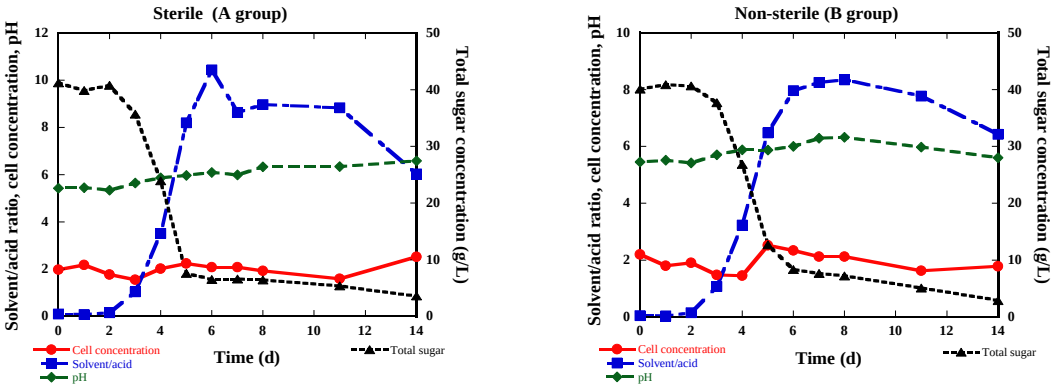
(a) 25℃, Cell conc. = 1429±214 mg/L (Run 1)



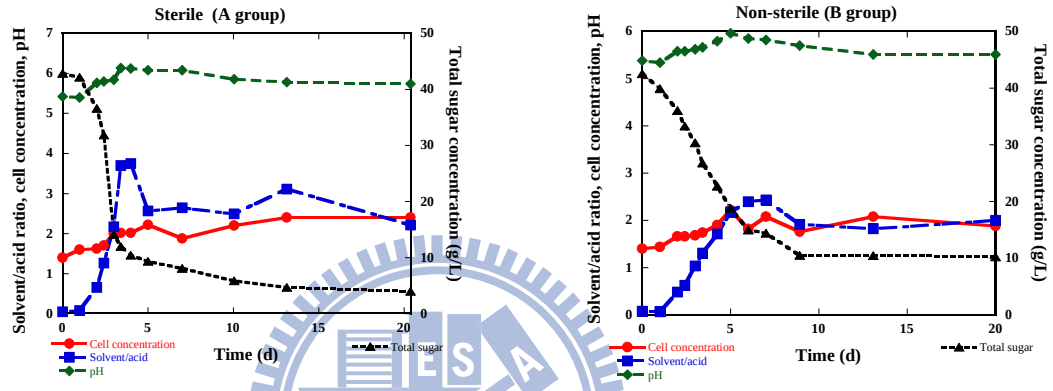
(b) 35℃, Cell conc. = 640±57 mg/L (Run 3)



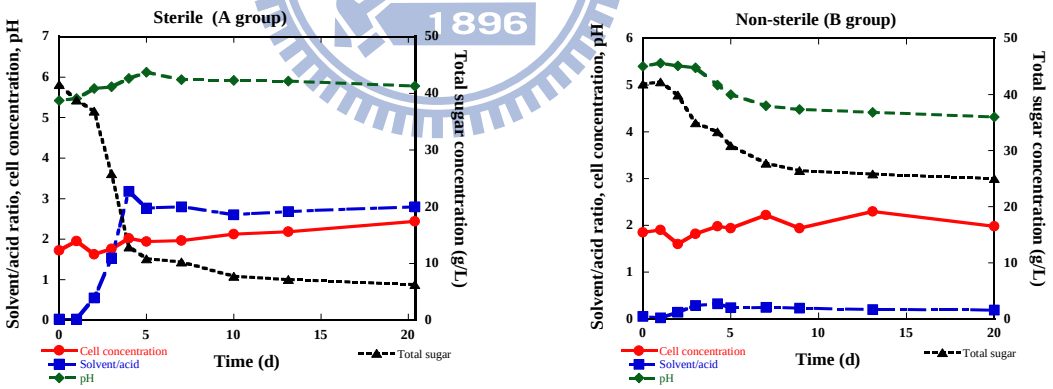
(c) 35℃, Cell conc. = 1429±214 mg/L (Run 5)



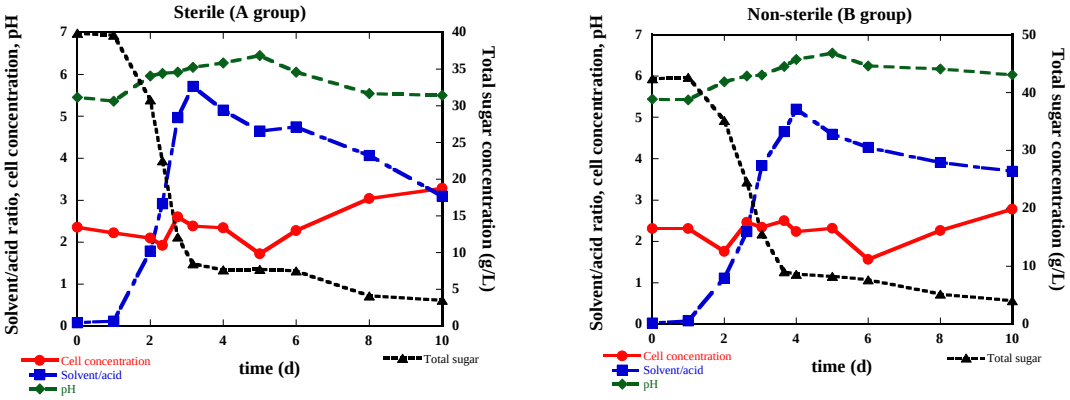
(d) 28℃, Cell conc. = 2170±157 mg/L (Run 7)



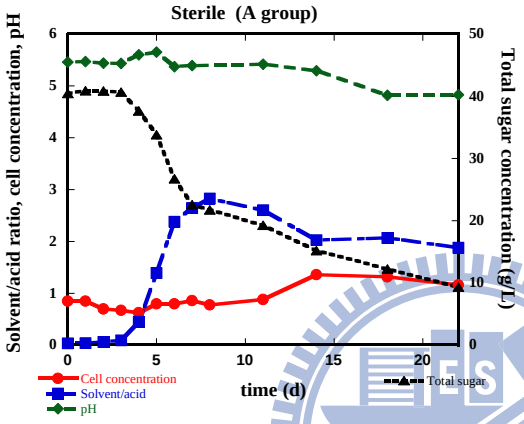
(e) 35℃, Cell conc. = 1429±214 mg/L (Run 9)



(f) 35℃, Cell conc. = 1429±214 mg/L (Run 10)



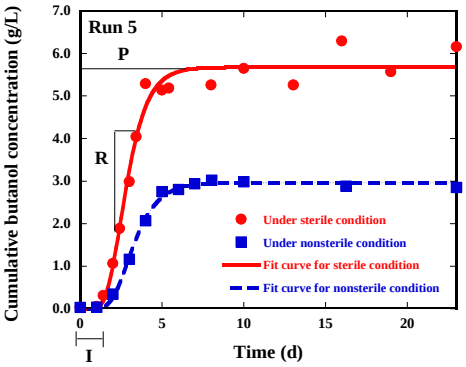
(g) 35℃, Cell conc. = 2331±28 mg/L (Run 11)

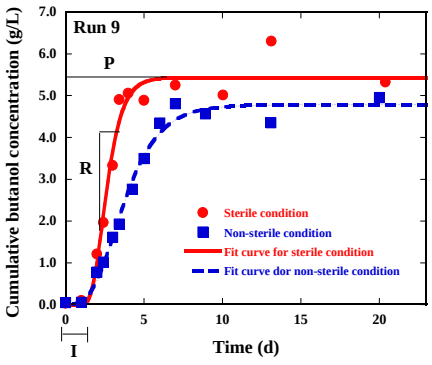


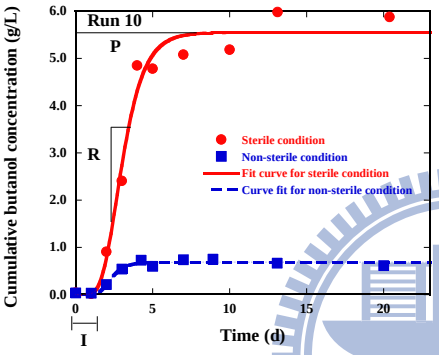
(h) 28℃, Cell conc. = 808±74 mg/L (Run 4)

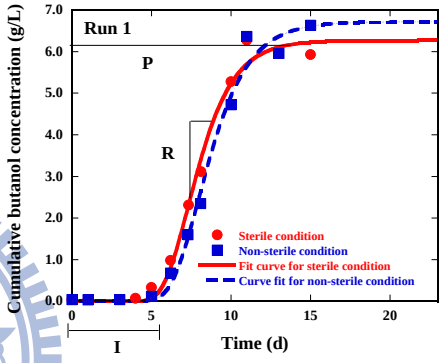
Figure D Relationships of solvents/acids ratio, pH, total sugar concentration, and cell concentration of all experimental runs in A (left) and B (right) experimental groups.

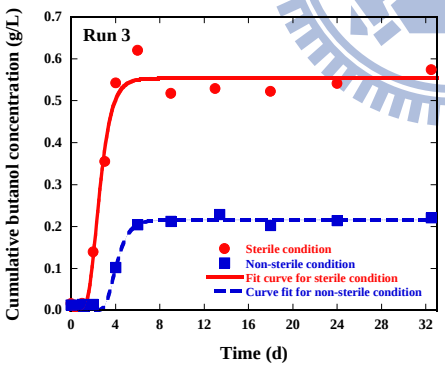
Appendix E Modified Gomperz model for butanol

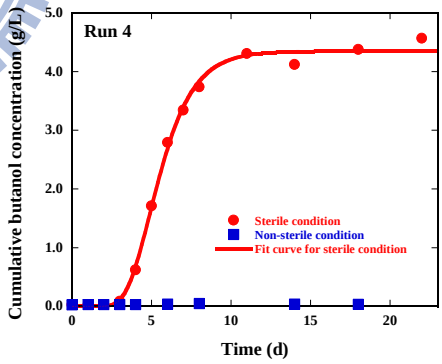


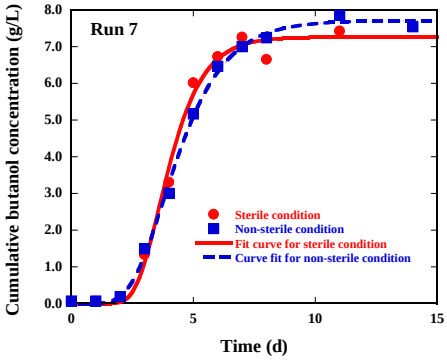












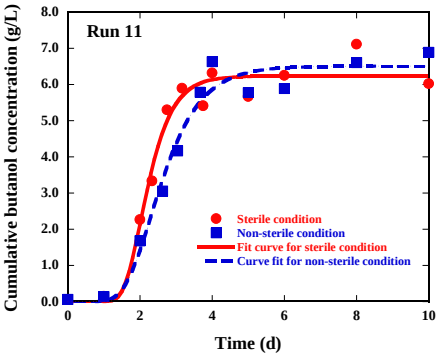
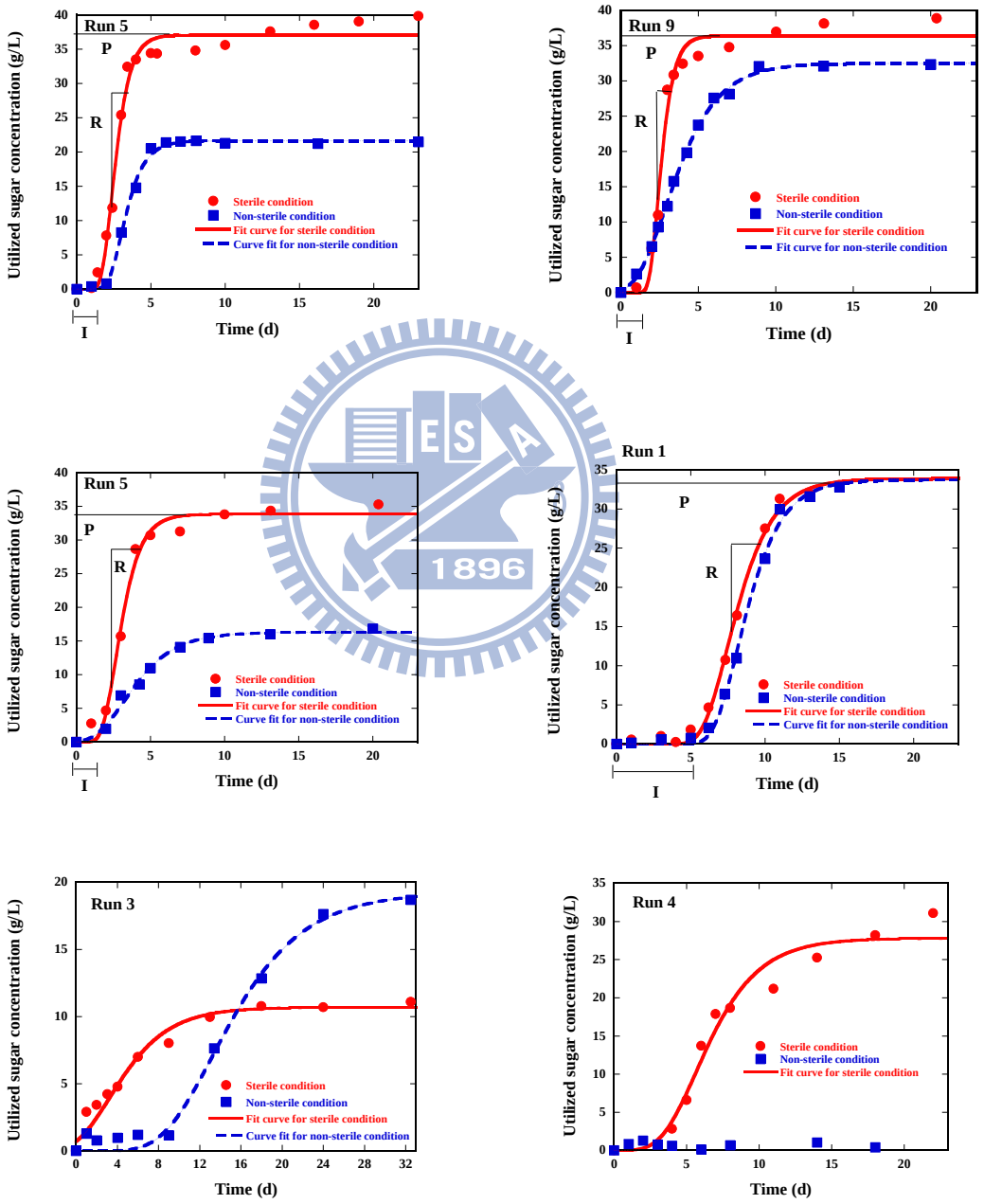


Figure E Cumulative butanol production of all experimental runs in A and B experimental groups. Markers — experimental data; Nonlinear line — data estimated by Eq. (g).

Appendix F Modified Gomperz model for sugar



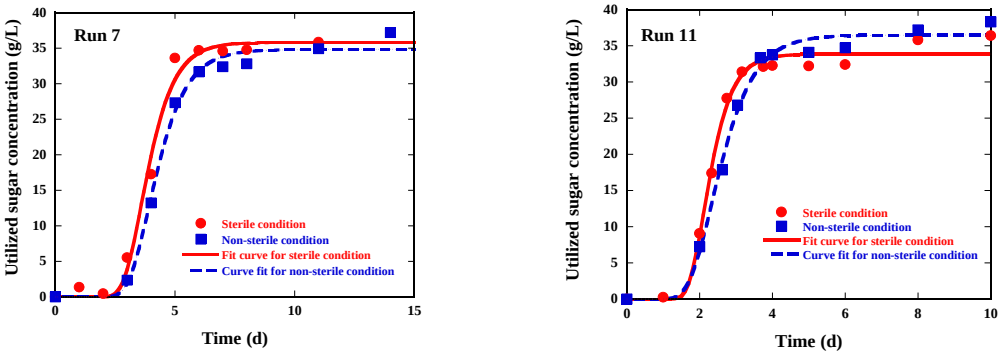


Figure F Utilized sugar concentration of all experimental runs in A and B experimental groups. Markers — experimental data; Nonlinear line — data estimated by Eq. (g).