Optimization of Acetone-Butanol-Ethanol Production from Cassava Starch by a Mixed Culture of *Clostridium* sp. and *Bacillus* sp. in Batch and Fed-batch Fermentation

Tran Thi My Hanh

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Thesis Title	Optimization of Acetone-Butanol-Ethanol Production from Cassava Starch by a Mixed Culture of *Clostridium* sp. and *Bacillus* sp. in Batch and Fed-batch Fermentation
Author	Miss Tran Thi My Hanh
Major Program	Biotechnology

Major Advisor:	**Examining Committee:**
……………………………………	……………………………..Chairperson
(Asst. Prof. Dr.Benjamas Cheirsilp)	(Assoc. Prof. Dr.Poonsuk Prasertsan)
	…………………………………………………
Co-advisors:	(Asst. Prof. Dr.Benjamas Cheirsilp)
……………………………………	…………………………………………………
(Dr.Brian Hodgson)	(Dr.Brian Hodgson)
……………………………………	…………………………………………………
(Dr.Kamontam Umsakul)	(Assoc. Prof. Dr.Penkhae Wanchaitanawong)
	…………………………………………………
	(Dr.Piyarat Boonsawaeng)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biotechnology

………………………………………

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

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ABSTRACT

In this study, a mixed culture of *Clostridium butylicum* TISTR 1032 with a high amylase producing *Bacillus subtilis* WD 161 was attempted to enhance acetone-butanol-ethanol (ABE) production from starch. The possibility for culturing *B. subtilis* WD 161 and *C. butylicum* TISTR 1032 using 20 g/L of soluble starch as a carbon source under culture conditions with and without anaerobic pretreatment was first investigated. The pure culture of *B. subtilis* WD 161 produced 2.6 U/mL and 14 U/mL of amylase activity with and without anaerobic pretreatment, respectively. While the pure culture of *C. butylicum* TISTR 1032 produced amylase activity less than 1.5 U/mL either with or without anaerobic pretreatment and resulted in low ABE production (< 0.94 g/L). The mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 incubated under condition without anaerobic pretreatment was found successfully enhanced amylase activity up to 17 U/mL and the ABE production up to 4.2 g/L or about 4 folds higher than that of the pure culture of *C. butylicum* TISTR 1032.

The enhancements in amylase and ABE production compared to the pure culture of *C. butylicum* TISTR 1032 was also observed when using cassava starch as a carbon source instead of soluble starch. The mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 produced 37 U/mL amylase and 7.4 g/L ABE from 40 g/L of cassava starch which were about 10 and 9 folds higher than those of the pure culture of *C. butylicum* TISTR 1032. The benefits of using this high amylase producing aerobic *Bacillus* in a co-culture with anaerobic *Clostridium* were not only increasing substrate utilization and ABE production but there was also no requirement

to add any costly reducing agent to the medium or flushing with N_2 to ensure anaerobic condition. This thus makes the anaerobic fermentation more economical and cost effective.

The medium optimization for ABE production by the mixed culture without anaerobic pretreatment revealed that cassava starch concentration of 40 g/L, C/N ratio of 4 and the mixed nitrogen sources of 265 mM (33 g/L) yeast extract with 100 mM (4 g/L) ammonium nitrate gave the highest ABE production in terms of final concentration and productivity (9.71 g/L and 0.135 g/L/h, respectively). The use of yeast extract or ammonium nitrate alone had a negative effect on ABE production. Further investigation on the interaction effect of medium components including cassava starch, yeast extract, and ammonium nitrate on the performance of the mixed culture were carried out using response surface methodology (RSM). Among three investigated components, cassava starch concentration contributed a significant effect on amylase and ABE production while yeast extract had less effect. Moderately positive interactions of cassava starch and ammonium nitrate concentrations were observed in amylase activity and consequently ABE production. Based on the response surface plots and economic benefit, the optimum medium components with the minimum requirement of nitrogen sources for ABE production by the mixed culture were 40 g/L of cassava starch, 5 g/L of yeast extract and 8 g/L of ammonium nitrate at which 9.02 g/L of ABE production was obtained.

ABE production by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 was performed in an 1 L anaerobic bioreactor for further process optimization. The study on pH control revealed that the culture maintained at pH 6.0 was optimum for ABE production (10 g/L) compared to pH 6.5, 5.5 and 5.0. On the other hands, pH at 6.5 was favored for acids (butyric and acetic) and amylase production. When the culture was controlled at pH 6.0 and the substrate was fed at the 24 and 36 h in fed-batch culture, the ABE production was increased up to 13.4 g/L. Interestingly, when the substrate was fed every 12 h from 12 to 72 h in semi-continuous culture, the total ABE production reached 15.2 g/L. Moreover, when the cultures were integrated with gas stripping for product recovery, the enhancement of ABE especially butanol production and substrate utilization were observed. Then, the

ABE production was enhanced up to 16.2 g/L and 17.7 g/L, respectively, for fed-batch and semi-continuous cultures.

Cassava pulp waste with and without cellulase pretreatment was used for ABE production by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in fed-batch culture. It was found that, there were considerable amounts of ABE productions from cassava pulp waste either with or without cellulase pretreatment (8.9 and 8.0 g/L, respectively). When semi-continuous fermentation was employed, the ABE production from cassava pulp waste without cellulase pretreatment was increased from 8.0 to 8.7 g/L. These results indicated that mixed culture of aerobic *Bacillus* and anaerobic *Clostridium* may play the key role for developing the industrialized fermentation of ABE from starchy biomass and its waste.

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LIST OF ABBREVIATION AND SYMBOLS

ATP	=	Adenosine Tri-Phosphate
ABE	=	Acetone-Butanol- Ethanol
CO_2	=	Carbon Dioxide
C/N	=	Carbon to Nitrogen Ratio
DSN	=	3, 5-Dinitrosalicylic acid
Fe^{2+}	=	Ferrous II Iron
H_2	=	Hydrogen
H_2O	=	Water
H_2S	=	Hydrogen Sulfide
NADH	=	Nicotinamide Adenine Dinucleotide
NB	=	Nutrient Broth
NaOH	=	Sodium Hydroxide
NH_4Cl	=	Ammonium Chloride
NH_4NO_3	=	Ammonium Nitrate
N_2	=	Nitrogen
RCM	=	Reinforced Clostridia Medium
RSM	=	Response Surface Methodology

CHAPTER 1

INTRODUCTION

The use of energy derived from biological reactions (bio-energy) provides many advantages, perhaps the most important being the reduced dependence on a non renewable fossil fuel source. Also it can provide a good opportunity to convert renewable organic waste materials into energy (Parekh *et al.*, 1999). The most commonly used metabolically derived liquid bio-energy compounds are ethanol and n-butanol. Butanol, along with small amounts of acetone and ethanol, are produced biologically from renewable biomass by *Clostridium* spp. under strictly anaerobic condition. This process is named “the acetone-butanol-ethanol (ABE) fermentation” (Jones and Woods, 1986) and has a high theoretical potential for replacing petro-chemical derived energy. Butanol is more valuable than ethanol as it possesses many favorable physical properties such as a higher energy content, higher boiling point and a reduced need to modify combustion engines made for petroleum compounds. Butanol also has numerous applications in other fields e.g. food, plastics, and other industries (Jesse *et al.,* 2002). This has resulted in a high demand for butanol, but the market for it is still tight due to its high production costs by fermentation reactions. Substrate costs can make up to about 63% of the total cost of butanol production (Dürre, 1998). This is not because of the expense of the substrate itself, but mainly because of the low efficiency of clostridium to convert substrate into butanol (Tashiro *et al.*, 2004). This means that the yield of butanol is often low, and this together with the formation of by-products leads to higher costs for butanol recovery (Dürre*,* 1998; Jesse *et al.*, 2002). In addition, the maintenance of strictly anaerobic conditions for clostridium to grow requires special conditions such as addition of costly reducing agents into the medium and flushing with N_2 gas during the early stages. These are the factors that additionally increase the cost of the fermentation process.

There are several possible ways to reduce the costs of producing butanol by fermentation such as by using a lower cost fermentation substrate, by using

a simple and low cost medium or by optimizing the fermentation conditions to improve the efficiency of converting substrate to butanol. Genetic engineering has been highly effective in producing strains able to utilize substrates more efficiently, so that the need to pretreat substrates is reduced (Dürre, 1998). However, genetic engineering may require that the genetically modified bacteria should be contained to allay fears that there could be problems with safety concerns for human health or their potential effects on the environment (Zaldivar *et al*., 2001). Among the cheap and highly available substrates for ABE production, starch is one of the possible better choices, but starch utilization can be low due to the low activity of the amylases produced by most clostridia. Mutation or genetic engineering can result in a strain with a higher capacity to utilize starch. In addition to the above concerns for negative effects on the environment, some mutated genes do not maintain their activity over a long period of time, and in addition, there is sometimes a need to supply special media and conditions to maintain the transformed or modified genes (Zaldivar *et al.,* 2001). The pre-hydrolysis of starch by either commercial enzymes or by acids with high concentration at high temperature both have negative feedback consequences e.g. processes are costly, produce difficulties in handling procedures, and the treatment of substrate by acids results in a low sugar yield and formation of harmful by-products such as formate, furfurol, melanoids that can seriously inhibit the growth of the butanol producing clostridia (Zverlov *et al.,* 2006). Thus, to increase substrate utilization and butanol yield, there have been several reports of using clostridia and other organisms to first facilitate hydrolyses of the substrates such as by a fungus/clostridial mixture. The fungus first hydrolyses the starch by producing amylase, and then butanol production was achieved separately by adding another clostridium species (Soni *et al.,* 1982; Fond *et al.*, 1983; Yu *et al.*, 1985). However, all these mixed culture experiments were carried out under strictly anaerobic conditions and the cultures were randomly established. There has been one report of butanol production by the simultaneous co-culture of a *Clostridium* with a *Bacillus* that had been randomly isolated from the same soil sample as the *Clostridium* but the role of the *Bacillus* in the co-culture was not clearly mentioned (Stevens *et al.*, 1988) and there was no suggestion on how to set up this mixed culture for enhancement of the target product(s).

The utilization of starch for ABE production by a *Clostridium spp* includes two processes, starch hydrolysis by amylolytic enzymes to produce glucose for the cells growth together with acid (acetic and butyric) production (acidogenesis) and the conversion of these acids into ABE products (solventogenesis). These two processes are influenced by a number of factors e.g. the amylolytic activities and ABE producing capacity of *Clostridium* both being influenced by the medium composition (starch concentration, nitrogen source, and C/N ratio). A high sugar concentration (160 g/L) was found to be toxic to clostridial cells (Jones and Woods, 1986); but with a low sugar concentration organic acid reassimilation is terminated due to an insufficient amount of energy-rich metabolites e.g. ATP or NADH (Shinto *et al,.*, 2007). In the presence of excessive amounts nitrogen (corresponding to a low C/N ratio); carbon utilization is completed more rapidly and cells grow better. In contrast, when the nitrogen supply becomes limiting carbon utilization is less effective. However, the relationship between the C/N ratio and ABE production is quite complicated. It was found that better growth and ABE production are normally observed at a lower C/N ratio. At higher C/N ratios (> 7.27), cell growth and ABE production were decreased (Lai and Traxler, 1994). On the other hand, the absolute concentrations of carbon and nitrogen were found to have more effect than the C/N ratio in the research of Madihah *et al*. (2001).

The conventional method for optimizing a process is to change one variable at a time while keeping the others at constant levels (Liu and Tzeng, 1998). Thus, the interactions between many variables are not considered. This limitation can be overcome by using response surface methodology (RSM) where the combined effects of all variables are determined through mathematical and statistical inference from experimental design to the analysis of results (Silva and Roberto, 2001). There have been several research programs that have used conventional method to optimize ABE production from starch using pure cultures of *Clostridium* (Linden *et al*., 1985; McNeil and Kristiansen, 1986; Lai and Traxler, 1994; Madihah *et al*., 2001). Only in the research of Badr and Hamdy (1992), was RSM used to optimize ABE production from sweet potato using the pure culture of *C. acetobutylicum* P262.

In addition, the onset of ABE production is normally associated with a decrease in the pH of the medium, and this is related to the accumulation of undissociated acids (acetic and butyric). At a pH 6.0 only 6% of the total amount of

butyric acid is in the undissociated form, whereas at pH of 4.5, 66% occurs in the undissociated form (Haggstrom, 1985). Solventogenesis is triggered when the undissociated butyric acid reaches a critical concentration (1.5-1.9 g/L) that initiates solvent production only after the pH of the mash had decreased to around 4.5 to 5.0. During solventogenesis butyric and acetic acids are utilized and ABE are produced resulting in a pH increase of the broth. It has been reported that organic acids are produced more rapidly at a higher pH whereas solvent production is enhanced at lower pH (Jone and Woods, 1986). As, butyric and acetic acids are growth associated products, and *Clostridium* normally grows better at a high pH value; a low pH value is not suitable for Clostridial growth therefore acids production, but at low pH value acids exist in the form of undissociated forms which would stimulate ABE. Thus, the effect of controlling the pH on growth, amylase, acids and ABE production should be investigated.

In addition to the problems of low substrate utilization and production of by- products; product and substrate inhibition are also among the factors that cause limitations for butanol production. When butanol in the culture reaches 13 g/L or substrate is provided at more than 160 g/L, conditions will become toxic to the cells and the fermentation process ceases, but when the carbon is limited (e.g. glucose is below 7 g/L), only acids are formed (Jone and Woods, 1986). It has been suggested that using high substrate concentration would increase productivity, and shorten the fermentation time. However, in the case of using starch as the substrate, high starch concentrations cause a higher viscosity that hinders amylase excretion and ABE production (Madihah *et al.,* 2001). To maintain a sufficient amount of substrate in the culture to allow for optimum cell growth, amylase and ABE production over the fermentation process, and to reduce the product inhibition problems, fed-batch integrated with gas stripping to remove the product from the culture has been employed. Gas stripping was selected due to a number of advantages over the other techniques e.g. simpler to handle, and more economically beneficial (Ezeji *et al.,* 2004). There have been a few reports on investigating the efficiency of a fed batch fermentation (Tashiro *et al.,* 2004; Zains *et al.*, 2007). This differs from the batch system in that the substrate concentration within the reactor can be maintained at a lower level and introducing fresh medium can prevent product inhibition caused by product accumulation.

In this study, screening for a suitable medium and culture conditions for cell growth and ABE production of a pure culture of *Clostridium butylicum* TISTR 1032 various medium types and conditions with and without anaerobic pretreatment were tested. A high amylase producing *Bacillus subtilis* WD 161 was used to co-culture with *C. butylicum*. Firstly, cell growth and amylase production of *B. subtilis* was investigated under aerobic and anaerobic conditions. Then, the mixed culture of *C. butylicum* and *B. subtilis* were evaluated for enhancement of ABE production from cassava starch. The medium components for ABE production by the mixed culture were optimized by using conventional methods. Additionally, the effect of each medium component as well as their interaction on amylase and ABE production by the mixed culture was determined by using RSM. The mixed culture was set up in an anaerobic bioreactor. Optimizing the process including pH control, fed-batch mode, and product recovery by gas stripping techniques were employed for further enhancement of ABE production.

Objectives of the study

1. To study the growth and ABE production of a pure culture of *Clostridium butylicum* TISTR 1032 under conditions with and without anaerobic pretreatment using various media.
2. To enhance ABE production using a mixed culture of *C. butylicum* TISTR 1032 with the amylase producing *Bacillus subtilis* WD 161.
3. To optimize the medium components for ABE production from cassava starch by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161.
4. To determine the effect of each medium component as well as their interactions on amylase and ABE production by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 using Response Surface Methodology (RSM).
5. To optimize the process for ABE production by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 from cassava starch and its pulp waste.

CHAPTER 2

LITERATURE REVIEWS

1. Overview on butanol

Butanol is a four carbon alcohol classified in the same family with methanol (1-carbon), ethanol (2-carbon) and propanol (3-carbon). Butanol is used primarily as an industrial solvent e.g. in producing synthetic rubber, lacquer, paint, rayon, detergents, and brake fluids and as solvents for fat, waxes, and resins (http://www.butanol.com). Besides sharing the common advantages of biological fuels with ethanol butanol provides significant additional environmental benefits: forming no green house gases, no sulfur oxide (SOx) or nitrogen oxide (NOx) when burned; reducing the dependence on fossil fuels, and opening of new markets for agricultural and diary food wastes. Butanol has more advantageous over ethanol due to its more favorable physical properties such as low vapor pressure, low solubility with water, and complete solubility with diesel fuel. Butanol has a higher energy content than ethanol and almost the same level as gasoline: 110,000 Btu's per gallon for butanol vs. 84,000 Btu per gallon for ethanol, while gasoline produces about 115,000 Btu's per gallon. Butanol is six times less "evaporative" than ethanol and 13.5 times less evaporative than gasoline, making it safer to use in high temperature areas. Butanol can be shipped through existing fuel pipelines whereas ethanol must be transported via rail, barge or truck. Butanol can be used as a complete replacement for gasoline e.g. 100%, or any other percentage. Ethanol can only be used as an additive to gasoline up to about 85% and then only after significant modifications to the gasoline engines (http://www.butanol.com). The world market for butanol is about 350 million gallons per year. In the US butanol currently sells for about US $ 3.70 per gallon in bulk (barge) and US $ 6.80 in 55 gallon drums (http://www.butanol.com). Butanol can be produced by the anaerobic fermentation process of the strictly anaerobic bacterium clostridia in which butanol is produced along with acetone and a small amount of ethanol. This process is named as the ABE fermentation. An alternative way to produce butanol is by chemical synthesis from fossil-oil-derived ethylene, propylene, and triethyl-aluminum

or carbon monoxide and hydrogen (Zverlov *et al.*, 2006). However, the production of butanol by chemical synthesis has a number of disadvantages e.g. dependence on materials from fossil oil, forming green house gases: carbon dioxide, and hydrofluorocarbons (HFCs) (http:www.butanol.com).

2. History of butanol production from biological source

The formation of butanol in a bacterium was first reported by Pasteur in 1861. At the beginning of the 20th century, when the prices for natural rubber drammatically increased due to the high demand this led to efforts to produce synthetic substitutes. Chaim Weizmann, a chemist, realized that the key to the success of a synthetic rubber manufacturing process was the production of butanol or isoamyl alcohol by fermentation. Between 1919 and 1914, he successfully screened several productive strains, one of them, later named as *Clostridium acetobutylicum* produced the highest amounts of acetone and butanol from various starchy substrates (Jones and Woods, 1986). Weizmann is the person who first ran the production plant for butanol from starches. During the World War I, due to the need for acetone for the production of munitions, the first large scale industrial plants were set up in Canada and USA, by that time butanol was considered an unimportant by-product and kept in storage tanks. However, when new methods of automobile production were introduced in the USA, there were more cars produced and these needed to be painted and it was found that butanol was the ideal solvent for lacquer paints. Thus, more new butanol plants were built, but due to the constraint of substrate price, most of the industrial plants changed the substrate to molasses and screened for new clostridial strains, and one of the best isolated strains was *C. sacchaributylicum* (Keis *et al.*, 2001). In the 1950s, butanol production in Western countries was very productive, but because of the persistent problems with bacteriophage infections, and the unavailability of molasses (as the sugar processing was improved and molasses was also used as feed addictive for pig breeding), the production declined. Moreover, cheaper butanol was being produced from chemicals and fuels so ABE fermentation ceased in Western industrialized countries during the 1960s. Even after the oil crisis in the 1970s, the potential utilization of renewable resources e.g. cane molasses, corn, soft wood and by-products e.g. whey

from the dairy industries to produce biological fuels were raised, and much research was carried out to investigate the production of ABE from various different sources of biomass (Jones and Woods, 1986). However only a few plants survived into the 1980s, including the plant in Germiston, South Africa. Now, only China still runs biological ABE, and about 50% of acetone requirements are still met by fermentation processes (Dürre, 1998; Zverlov *et al.*, 2006).

The acetone-butanol-ethanol (ABE) fermentation industry in China was started in the early 1950s in Shanghai and expanded rapidly thereafter. At its peak, there were about 30 plants all over the country and the total annual production of solvents reached 170,000 tons. But due to the same factors that existed in other areas in the world, at the end of the 20th century due to the rapid increase of petrochemicals, the production of butanol also decreased and by the end of the 1990's all fermentation plants were closed . In general the strategies of the plants in China were to operate continuous fermentation processes, to maintain the maximal growth and acid production phase, with adoption of multiple stages in the solvent phase to allow gradual adaptation to increasing solvent, and the incorporation of stillage to offer enough nutrients to delay cell degeneration (Chiao and Sun, 2007; Ni and Sun, 2009).

3. General characteristics of *Clostridium*

A member of the genus *Clostridium* is characterized as: a Gram positive, anaerobic, rod shaped bacterium forming heat-stable endospores. About 120-160 species have been described since the establishment of the genus in 1880. Between 70 to 80% of these species are saprophytic bacteria that are harmless to animals. Nevertheless, up to 25 species represent minor pathogens and 13 species can be regarded as major pathogens. Solvents producing *Clostridium* are harmless saprophytes and have been investigated from various sources e.g. broad bean roots, market potatoes, broken maize grains (Calam, 1980) and soil from different depths in Colombia (Montoya *et al.*, 2000). Among these, the best strains for butanol production in the research of Calam (1980) came from bean roots and potatoes. Result in the research of Montoya *et al*. (2000) showed the best chance to find good solvent producing *Clostridium* was soil from cultures of tubers, chrysanthemum and grass.

So far, a large number of solventogenic clostridia have been described, but of them only about 40 solventogenic strains have been deposited in public strain collection (Zverlov *et al.*, 2006). It has generally been accepted that the industrial clostridial strains are classified as *Clostridium acetobutylicum* as they are all phenotypic similar. But, the research of Woods (1995) based on DNA hybridization and 16 S ribosomal RNA gene- sequencing studies concluded that the solventogenic strains could be differentiated into at least two different groups named as amylolytic and saccharolytic clostridia (Woods, 1995). Lately, the clostridial solventogenic strains have been classified into four genetically distinct groups including *C. acetobutylicum* (Weizman strain and the type strain ATCC 824 are in this group), *C. beijerinkii*, and two other groups of unnamed species. Differences were also found in butanol producing capacity (ranging from 10 to 24 g/L) and solvent yield (between 6.8 and 33.2%) of these four species (Zverlov *et al.*, 2006).

4. Butanol formation

4.1 Solvent production pathway of *Clostridium*

ABE are produced during the later stages of a batch fermentation of clostridial culture under the appropriate conditions. There are two distinct phases in the ABE formation pathway acidogenesis followed by solventogenesis. Typically, during acidogenesis, cells grow exponentially with the formation of acetic and butyric acids and ATP. The formation of these acids causes the pH of culture broth to decrease. When the culture enters the stationary phase, the metabolism of the cells is shifted to solvent production (solventogenic phase), acetone, ethanol and butanol are produced. The solventogenesis is initiated by the accumulation of undissociated fatty acids. During the solventogenic phase, the organic acids are reutilized and converted into the reduced end products butanol and acetone that result in an increase in pH of the broth (Jones and Woods, 1986). It is reported that organic acids (butyric and acetic) production is enhanced at higher pH, while solvents are mainly produced at a lower pH. The organic acids in broth have been proved to trigger the metabolic switch from the acidogenic stage to solventogenesis since the addition of organic acids into the fermentation

medium can stimulate solvents production (Jones and Woods, 1986; Tashiro *et al.*, 2004). However, the exact mechanism of this is still not fully understood.

The solvent producing *Clostridium spp* ferment glucose, sucrose, and starch via the Embden-Meyerhof pathway (EMP) (Figure 1). Moreover, they also utilize glycerol, other hexoses, pentoses, and oligosaccharides e.g. cellobiose, lactose, raffinose, mannose, xylose and arabinose (Woods *et al.*, 1995). The pentoses are metabolized through the pentose phosphate pathway via pentose-5-phosphate resulting in the formation of fructose 6- phosphate and glyceraldehydes-3 phosphate that join the glycolytic pathway.

In the primary metabolism of *C. acetobutylicium*, hexoses and pentoses are converted to pyruvate, ATP and NADH are formed. Subsequently, pyruvate is oxidatively decarboxylated to acetyl-CoA by a pyruvate-ferredoxin oxidoreductase. Some of the reducing equivalents generated in this step are converted to hydrogen by an iron-only hydrogenase. Acetyl-CoA is the branch-point intermediate, leading to the production of organic acids (acetate and butyrate) and solvents (acetone, butanol and ethanol). Acety-CoA is converted to acetate by phosphotransacetylase and acetate kinase. In the central pathway, thiolase catalyses the condensation of two acety-CoA molecules to form one acetoacetyl-CoA molecule, the precursor of the four carbon solvents. This reaction plays an important role in determining the ratio between the two-carbon (acetate, ethanol) and the three- carbon (acetone) and four-carbon products- (butyrate and butanol). The activity of thiolase is regulated *in vivo* by the coenzyme-A to acetyl-CoA ratio. In three consecutive steps, acetoacetyl-CoA is further reduced to butyryl- CoA by butyryl-CoA hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase. Butyryl-CoA is converted to butyrate by phosphotransbutyrylase and butyrate kinase. Acetone formation from acetoacetyl-CoA involves a CoA transferase and an acetoacetate decarboxylase. Ethanol and butanol are produced from acetyl-CoA and butyryl-CoA, respectively, in two reductive steps catalysed by aldehyde and alcohol dehydrogenases.

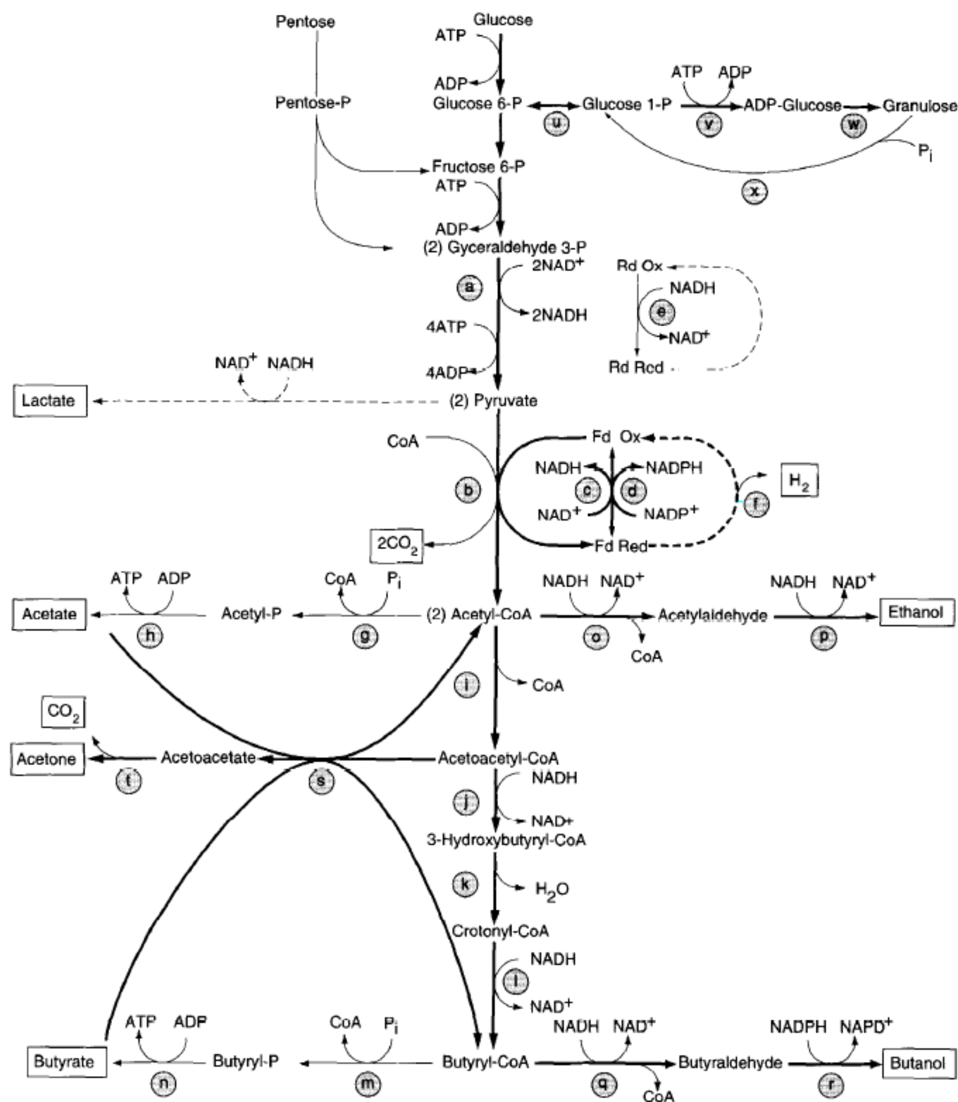

Figure 1. Biochemical pathways in *Clostridium acetobutylicum.*

Reactions that predominate during the solventogenic phase of the fermentation are shown by thick arrows. (a) glyceraldehyde-3-phosphate dehydrogenase; (b) pyruvate-ferredoxin oxidoreductase;(c) NADH-ferredoxin oxidoreductase; (d) NADPH-ferredoxin oxidoreductase; (e) NADH-rubredoxin oxidoreductase; (f) hydrogenase; (g) phosphate acetyltransferase (phosphotransacetylase); (h) acetate kinase; g) thiolase (acetylCoA acetyltransferase); (i) 3 hydroxybutyryi CoA dehydrogenase; (k) crotonase; (I) butyryl-CoA dehydrogenase; (m) phosphate butyltransferase (phosphotransbutyrylase); (n) butyrate kinase; (m) acetyldehyde dehydrogenase; (p) ethanol dehydrogenase; (q) butyraldehyde dehydrogenase; (r) butanol dehydrogenase; (s) acetoacetyl-CoA:acetate/butyrate:CoA transferase; (t) acetoacetate decarboxylase; (II) phosphoglucomutase; (v) ADP-glucose pyrophosphorylase; (w) granulose (glycogen) synthase; (x) granulose phosphorylase. Abbreviations: CoA, coenzyme A; Pi, inorganic phosphate; Rd Ox, rubredoxin oxidase; Rd Red, rubredoxin reductase; Fd Ox, ferredoxin oxidase; Fd Red, ferredoxin reductase. Dotted lines indicate pathways that are not operational under these conditions; numbers in brackets represent the

Source: Woods (1995)

The solventogenetic genes of *C. acetobutylicum* are located on a large plasmid thus it is unstable and easily causes degeneration of the solventogenesis during

long fermentations which is characterized by acid accumulation without a switch to solventogenesis (Kashket and Cao, 2007). Moreover, sporulation genes of this species are also located on the plasmid. So, degeneration is commonly found in this species group. In *Clostridium beijerinckii* and possibly also other butanologenic strains the solventogenic genes are located on the chromosome (Wilkinson *et al.*, 1995). The solventogenic degeneration observed with these strains may have other causes and/or be less dominant.

4.2 Factors affecting solvent production

4.2.1 pH and butyric acid

pH has been considered to be a key factor in determining the productivity of ABE fermentation. Along with some other factors e.g. undissociated butyrate concentration, it is most likely the trigger that shifts from acidogenesis to solventogenesis. A number of reports have confirmed that cultures maintained at high pH produce mainly acids, whereas in cultures maintained at a low pH solvent production is usually predominant (Jones and Woods, 1986; Kim *et al.,* 1984; Stevens *et al*., 1988). However, the pH range over which solvent formation may occur appears to vary quite widely depending on the particular strain and the culture conditions used.

It is found that the pH range in which solvents production occurred was around 4.5 to 5.0. However, this also varies with particular strains and culture conditions used. Bahl *et al.* (1982) found that when *C. acetobutylicum* was grown in continuous culture under glucose limitation at neutral pH and varying dilution rates the only fermentation products formed were acetate, butyrate, carbon dioxide and molecular hydrogen. Acetone and butanol were only formed when the pH was decreased below 5.0 (optimum pH was 4.3). The addition of butyric acid (20 to 80 mM) to the medium with a pH of 4.3 resulted in a switch from acidogenesis to solventogenesis (Bahl *et al.*, 1982).

Kim *et al*. (1984) reported that in a pH controlled batch fermentations, no solvents were produced at pH 5.8 whereas good solvent levels were obtained at a pH of 4.5 (Kim *et al.*, 1984). The optimum pH range for solvent production is much higher for *C. acetobutylicum* P262 and related strains (P265, P270) (Robson *et al.*, 1982). In laboratory scale experiments, these strains provided good levels of solvent production

within the pH range of 5.0 to 6.5, and when it was decreased to a pH below 4.5, during the early part of the fermentation, growth and metabolism of the cells were inhibited and no solvents were produced. In the industrial fermentation the initial pH of the fermentation medium was about pH 6.0, and during the initial phase of the fermentation the pH decreased to about 5.2 at the breakpoint, after which it increased, reaching about 5.8 at the end of the fermentation.

An Egyptian isolate of *C. acetobutylicum* was reported to produce more solvents when the cultures were maintained at a pH of 6.4 (Fouad *et al.*, 1982). The production of solvents by a strain of *C. beijerinckii* (VPI 13436) maintained at a pH of 6.8 was reported by George and Chen (1983). Other research work on *C. thermosaccharolyticum* showed that the butanol/butyrate ratio was higher during the growth at neutral pH (7.0) than at acidic pH (4.5) and the production of butanol could be further stimulated by the addition of butyrate (Jones and Woods, 1986).

In an experiment of Marchal *et al.* (1985) the pH of the unbuffered broth was initially decreased by self-acidification to a value of 5. Then, the pH was brought back to 6.5-6.7 stepwise and then freed of control. This resulted in a solvent concentration of 23-24 g/L (Marchal *et al.*, 1985). The switch from acidogenesis to solventogenesis is normally associated with a fall in the pH of the medium to about 5 linked to the accumulation of acid end products e.g. butyric, acetic acids. At pH 6.0 only 6% of the total amount of butyric acid is in the undissociated form, whereas at pH 4.5, 66% occurs in the undissociated form (Jones and Woods, 1986). Butyric, especially in its undissociated form has been found to play an important role in triggering solvent formation. The presence of a critical concentration of undissociated butyric acid (1.5-1.9 g/L) was also reported to be required for the triggering of solvent formation (Jones and Woods, 1986). Gottschal and Morris (1981) reported that the addition of acetate and butyrate (10 mM each) to batch culture of *C. acetobutylicum* maintained at pH 5.0 resulted in a rapid induction of solventogenesis, and this was accompanied by a decrease in the specific growth rate and the rate of hydrogen production. The addition of butyric acid (20 to 80 mM) to the medium with a pH of 4.3 resulted in a switch of acidogenesis to solventogenesis (Bahl *et al.*, 1982). However, the excretion of butyric acid ceased when the total butyric acid concentration reached 8.7 g/L (Soni *et al.*, 1982) or the undissociated butyric acid reached 1.7 to 1.9 g/L (Monot *et al.*, 1984).

Besides butyric acids, acetic acid has also been found to have certain effects on the production of butanol. The addition of acetate or propionate to an uncontrolled-pH batch culture of *C. acetobutylicum* does not affect the initiation of solventogenesis but does enhance the final solvent concentration. When 30 mM acetate was added at 14 h the final concentration of butanol and acetone were 37 mM and 28 mM, respectively, higher than those in the control (18 mM, and 4.6 mM, respectively) (Hüsemann and Eleftherios, 1990). Chen and Hans (1999) found that the addition of sodium acetate to MPS medium increased and stabilized solvent production of *C. beijerinckii*. When growing *C. beijerinckii* BA 101, a mutant derived from *C. beijerinckii* NCIMB 8052, in MP2 medium containing 60 mM sodium actate and 8 % glucose the concentration reached 32.6 g/L total solvents and of this 20.9 g/L was butanol. This is the highest solvent and butanol concentration produced by that strain when grown in batch culture (Chen and Hans, 1999; Zverlov *et al*., 2007). However, Tashiro *et al.* (2004) found that addition of acetate to the culture of *C. acetobutylicum* did not enhance specific butanol production rate significantly, but specific acetone production rate was enhanced from 0.02 g/g/h to 0.088 g/g/h.

The effect of pH (between 5.0 and 6.3) on butyric acid fermentation of xylose by *Clostridium tyrobutyricum* was studied. At pH 6.3, the fermentation gave a high butyrate production of 57.9 g/g/h with a yield of 0.38–0.59 g/g/h xylose and a reactor productivity up to 3.19 g/g/h. However, at low pHs (<5.7), the fermentation produced more acetate and lactate as the main products, with only a small amount of butyric acid (Zhu and Yang, 2004).

It is obvious that the pH and organic acid like undissociated butyric acid are the key factors for formation of solvents, but the mechanism of how they work still remains unclear. However it has been confirmed that the influence of pH could be correlated with the control by undissociated butyric acid (Jones and Woods, 1986).

4.2.2 Temperature

Some research has been carried out to determine the effect of temperature on solvent production. McCutchan and Hickey (1954) found that solvents productivity by three different solvent-producing strains remained fairly constant at around 31% at 30 and 33°C, but decreased to 23-25% at 37°C. Similar results were obtained in the study with *C. acetobutylicum* NCIB 8052, in which solvent yields were

found to decrease from 29% at 25°C to 24% at 40°C, although the fermentation time decreased as the temperature was increased, but reduction was found for only acetone production, while the yield of butanol was unaffected. This is in contrast to earlier findings in which an increase in the butanol ratio was obtained by decreasing the temperature of the fermentation from 30 to 24°C after 16 h. This result was found in the research of Carnarius on the U.S. Patent 2,198,104, 1940 (Jones and Woods, 1986) and other experiments with *C. acetobutylicum* fermentation in which the temperature ranged from 25 to 40°C it was found that the solvent productivity reached maximum when the fermentation temperature was 35°C (McNeil and Kristiansen, 1985).

Low temperature was found to be the cause of retrogradation when using starch solution as feed for ABE production. The continuous reactor fed with cornstarch solution (feed temperature 19°C) produced approximately 6.0 g /L total ABE. Increasing the feed storage temperature to 37 °C improved ABE production to 7.2 g/ L suggesting that retrogradation was occurring more rapidly at 19 °C. The use of soluble starch, which is less prone to retrogradation, resulted in the production of 9.9 g/ L ABE at 37 °C feed storage temperature, as compared to 7.2 g/ L ABE when cornstarch was used (Ezeji *et al*., 2004).

4.2.3 Butanol concentration

During the solvent-producing phase, cell metabolism usually continues until the concentration of the total ABE reaches inhibitory levels of around 20 g/ L (or butanol concentration of 13 g/L), after which further cell metabolism ceases (Jones and Woods, 1986; Ezeji *et al*., 2004). This causes a major limitation in the industrial scale production. Of the solvents produced, butanol is the most toxic, and it is the only one produced in inhibitory concentrations during the fermentation. Solvent production ceases when the concentration of butanol reaches about 13 g/L in the industrial fermentation process (Jones and Woods, 1986). Butanol at the level of 13 g/L was completely inhibitory to the growth of *C. saccharoperbutylacetonicum* (Soni *et al.*, 1982). The concentrations of acetone and ethanol, on the other hand, do not appear to reach inhibitory levels during the fermentation. The addition of acetone and ethanol reduced growth by approximately 50% at a concentration of around 40 g/L, and total growth inhibition occurred at a concentration of about 70 g of acetone and 50 to 60 g of ethanol per liter (Costa and Moreira, 1983). Butanol (and other aliphatic alcohols with

more than four carbon atoms) caused an increase in the ratio of saturated to unsaturated fatty acids of the cell membrane. This resulted in an increase in membrane fluidity that led to the destabilization of the membrane due to the disruption of the phospholipid components and disruption of membrane-linked functions. However, the sequence and relationship of these events are not known (Jones and Woods, 1986; Ezeji *et al*., 2004).

4.2.4 Roles of C and N

Only acids were formed when carbon limited growth. In batch culture, when glucose was below 7 g/L or in fed batch culture with a feed rate less then 4 g/L per day no solvent was produced. It is generally accepted that under carbon limited conditions, the amount of acid end products formed was not sufficient to induce the switch from acidogenesis to solventogenesis or organic acid reassimilation is terminated due to an insufficient amount of energy-rich metabolites e.g. ATP or NADH (Shinto *et al*., 2007). A sufficient amount of sugar (glucose higher than 7 g/L) is determined to be essential for maintaining of solvent production, with the presence of a large amount of sugar. However, a high sugar concentration (160 g/L) has been shown to be toxic to clostridial cells (Jones and Woods, 1986; Ezeji *et al*., 2005b).

There have been a few reports on the optimum starch concentration for direct fermentation of starches into ABE this aspect by pure culture of *Clostridium*, but there has not been any research using mixed culture for direct fermentation of ABE from starch. It was reported that corn starch at a concentration of 5-6.5% (w/v) was found suitable for solvent production up to 3.8% (w/w) based on sugar consumed (McNeil and Kristiansen, 1986). Maize and potato starch at a concentration of 5% (w/v) have also been used for solvent fermentation to give the yield ranging from 1.6 to 2.6% (w/v) (Linden *et al*., 1985). Sago starch at the concentration of 50 g/L was found optimum for ABE production (18.82 g/L) by the pure culture of *C. acetobutylicum* (Madihah *et al.*, 2001).

The C/N ratio in the medium has been determined to be one of the important factors that affect the growth and solvent production of *Clostridium*. Lai and Traxler (1994) found that cells grew well and more solvents were produced at a lower C/N ratio e.g. 0.2. When the ratio is higher than 0.2, the cell growth and sugar utilization was reduced and no solvents were produced due to the limitation of nitrogen even when sugar was abundant (Long *et al.*, 1984; Lai *et al.*, 1994). Roose *et al.* (1985)

also found that, at a very low C/N ratio (1.25) with a pH of 4.5, acids were predominantly produced. However, when pH was controlled at 3.7, solvent production was enhanced at a low C/N ratio. Recently, Madihah *et al.* (2001) reported that when *C. acetobutylicum* was grown in gelatinized sago starch to produce ABE, the individual concentrations of nitrogen and carbon influenced solvent production to a greater extent than did the C/N ratio. In fermentations using a fixed concentration of starch of 50 g/L, total solvent concentration decreased from 26.98 (g/L) to 2.63 (g/L) along with an increasing C/N ratio from 3.6 to 42.8. In contrast, for a fermentation using a fixed concentration of nitrogen (5 g of yeast extract and 2 g of NH_4NO_3) total solvent increased with increasing C/N ratio up to a value of 20 and then decreased slightly above this value (Madihah *et al.*, 2001).

5. Overview on mixed culture for ABE production

Clostridia and *Bacilli* are commonly associated in nature e.g. water and sludge. Some research work that has investigated the feasibility of growing bacilli in anaerobic conditions found that anaerobic growth of strains of *B. anthracis*, *B. cereus*, and *B. subtilis* occurred in both synthetic and nonsynthetic glucose-containing media. No spores were formed by either organism under anaerobic conditions, but there was production of lactic, succinic, formic, and acetic acids; acetylmethylcarbinol, 2, 3-butylene glycol and glycerol as fermentation products of glucose (King and Stein, 1950; Puziss and Rittenberg, 1957). These confirmed that oxygen limited *Bacillus* would still survive.

Several types of mixed culture have been used. The earliest co-cultures used to try to increase butanol production were with fungi either separately or together with a fungus that hydrolyzed cellulose and hemicelluloses. Mixed-culture filtrates from the cellulolytic fungi *Trichoderma reesei* and *Aspergillus wentii* were used to obtain fermentable sugars from bagasse and rice straw. After treatment to remove undesired impurities, *C. saccharoperbutylacetonicum* produced 16 g/L from the hydrolysate. Fermentation of alkali-pretreated wheat straw, using *C. acetobutylicum* in a fermentation medium supplemented with a cellulase preparation from *T. reesei*,

produced solvent concentrations of 17.3 g/L and solvent yields of 18.3% with respect to pretreated wheat straw. These results were obtained after 36 h (Soni *et al.*, 1982).

C. thermocellum cocultured with *C. acetobutylicum* showed an efficient utilization of all hydrolysis products derived from the lignocellulose substrates but the majority of the fermentation products were acids, and little or no solvent was detected just as happened in the case of Fond *et al.* (1983). The results did not change even with the addition of glucose into the culture (Yu *et al.*, 1985). Berstrom and Foutch (1983a) designed a series of mixed cultures and found that using the co-culture of *C. butylicum* and *C. pasteurianum* they obtained consistently higher product concentrations when compared with the mono cultures. In their system, the butyric acids produced by an acid producing species (*C. butylicum*) was then converted into butanol by the second species (*C. pasteurianum*). But the total butanol concentration yield from their research was lower when compared to the single-culture fermentation reported in the literature. Other clostridial species have been co-cultured with other organisms such as *C. thermolacticum* with *Methanothermobacter thermoautotrophicus* and *Moorella thermoautotrophica* in an acetate fermentation from lactose (Collet *et al.*, 2003) or *C. butyricum* and *Enterobacter aerogenes* for hydrogen production from sweet potato starch (Yokoi *et al.*, 2001). In both these cases, the target products obtained from mixed cultures were higher than those of the monoculture. However, references on butanol production by mixed cultures of a clostridia and an aerobe are very poor. We are aware of only one research report on the production of butanol from mixed cultures of *C. beijerinkii* and *Bacillus cereus* under a controlled pH of 5.5. This research obtained higher butanol concentrations (2.00 g/L) than that of the monoculture of *C. beijerinkii* (0.8 g/L) (Stevens *et al.*, 1988). Recently, Chang *et al.* (2008) employed mixed culture of aerobic *Bacillus* and anaerobic *Clostridium* for bio-fuels and bio-hydrogen production. The mixed culture enhanced Hydrogen production from 40 mL/L of the pure culture of *Clostridium* to 90 mL/L (Chang *et al.,* 2008)

6. Overview on the production of butanol from starch

6.1 Characteristics of starch

Starch is the major reserve carbohydrate in higher plants that exists in the form of water soluble granules and includes two types of polymers: amylose (normally 20-30%) and amylopectin (normally 70-80%) (Madihah *et al.*, 2001). Amylose is made up of glucose units linked by an α -1, 4-glycosidic bond. The carbon 1 of a glucose unit is linked to carbon 4 of the other glucose unit. The majority of linear chains are interlinked at branch points by α -l, 6 glycosidic bonds to form a lightly branched structure but compared to amylopectin the degree of branching in amylose is very much less. In amylopectin, there are the same α -1, 4 glycosidic linkages between two glucose units but linear chains contain about 20-25 α -1, 4 linked glucose residues interlinked by α -l, 6 glycosidic linkages to form a multibranched structure (Manners, 1989). The major impurities in commercial starch are fats, proteins and ash. Fats and proteins are present at levels of up to 1 and 0.5% dry basis respectively. The small quantities of minerals present are usually less than 0.2%.

The association of polymers by glycosidic linkage within the granules produces crystals that prevent the starch from being diluted in water. To provide a suitable substrate for rapid enzymatic hydrolysis it is necessary to disrupt the starch granules. This can be done by heating the starch in the presence of excess water at 55-90°C when starch is gelatinized. Gelatinized starch gels are thermo-dynamically unstable structures, and on cooling re-association of the starch molecules through H-bonding involving both amylose and amylopectin occurs, with a corresponding increase in viscosity, a phenomenon termed retrogradation. The rate of retrogradation depends on a number of variables including the structure of the amylose and amylopectin, the ratio of amylose to amylopectin, temperature, concentration of starch, botanical source of starch, and the presence and concentration of other ingredients (Kim *et al.*, 1997; Jacobson *et al.*, 1997). Retrogradation has been shown to significantly lower the enzymatic susceptibility of gelatinized starch to hydrolysis and this is a considerable problem especially in the production of butanol by fed-batch fermentation process when the fed medium is stored for a period of time before being used.

The utilization of starch for solvent production involves a two-step process: breaking down internal α-1,4-glycosidic linkages of starch by α-amylase to oligosaccharides and hydrolysis of oligosaccharides by de-branching enzymes such as glucoamylase or amyloglucosidases that cut α- 1,4- and α-1,6-glycosidic linkages to release glucose from the none reducing ends of starch, and the subsequent fermentation of the glucose to solvents.

Cassava (*Manihot esculenta*), also called manioc, tapioca or yuca, is one of the most important food crops in the humid tropics, being particularly suited to conditions of low soil nutrient availability and able to survive drought. In Thailand Cassava starch is an important export commodity of Thailand, about $2x10^6$ tonnes are exported annually, with cassava being mainly processed to meal and flour (Kosugi *et al.*, 2009). Cassava contains 17-21% amylose. Due to the high market demand for cassava products, the Thai cassava starch industry is well established and has developed from small to large-scale with improved processing technology. At present, a production capacity of one factory is, on average, 200 tonnes starch per day (Sriroth *et al.*, 2000).

6.2 Amylolytic enzymes from *Bacillus* and *Clostridium*

In order to optimize the fermentation of the mixed culture, it is necessary to understand the characteristics of the amylolytic enzymes from each organism in the mixed culture. Because the excretions and activities of these enzyme will determine the level of substrate formation which directly affects the product formation. Amylases have been derived from different sources e.g. fungi, plants, bacteria but for commercial applications α-amylase is mainly derived from the genus *Bacillus*: *B. licheniformis*, *B. stearothermophilus*, *B. amyloliquefaciens* (Priest, 1977; Konsula and Liakopoulou-Kyriakide, 2004). The characteristics of amylases from different hosts are different, in general amylases from bacilli are unstable at low pH, quite stable at high temperature, and perform best on potato starch e.g. an amylase isolated from a moderately thermophilic *B. subtilis* strain displayed maximal activity at 135°C and a pH 6.5 (Priest, 1977). Potato starch hydrolysis resulted in a higher yield of reducing sugars in comparison to the other starches including rice, corn and oat (the amylase activity ranged from 30- 50 U/mL) (Konsula and Liakopoulou-Kyriakide*,* 2004)**.** However, α-

amylase from *B. acidocaldarius* is stable at an acidic pH but very sensitive to thermal inactivation (Priest, 1977).

From *Clostridia*, several amylases are also found, such as an α-amylase from *C. acetobutylicum* ATCC 824**,** with an optimal pH of 5.6 and temperature of 45°C, was stable at an acidic pH but very sensitive to thermal inactivation (Swamy and Seenayya, 1996). Shih and Labbe (1995) found from *C. perfringens* NCTC 8679 produced an α-amylase that showed a maximal activity (0.40 U/mL) at a pH of 6.5 and 30°C in the absence of calcium. Swamy and Seenayya (1996) found another α-amylase produced by *Clostridium thermosulfurogenes* SV9 with optimal temperature and pH values for its formation of 60°C and 7.0, respectively. Maximum amounts of α-amylase (0.60 U/mL) were produced at the end of the exponential growth phase (12 h).

Glucoamylases have been found in some *Clostridium* including *Clostridium thermohydrosuffuricum, C. acetobutylicum and C. thermosaccharolyticum.* Notably, the glucoamylases produced by these bacteria, are thermostable. These enzymes allowed the hydrolysis of starch without significant *a*-amylase activity (Specka and Mayer, 1993). There are only a few references on glucoamylase from bacilli and it is possible that such an enzyme may be completely absent from bacilli (Priest, 1977; Rowe and Agyrios, 2004).

6.3 Butanol production from starch

Initially, maize mash served as the main fermentation substrate for industrial production of butanol and provided a very positive amount of product (the original Weizmann strain, *C. acetobutylicum*, produced a yield of 28-30% solvents from 3.8% starch) but later due to the shortage of substrate, by the beginning of the 1930s most fermentation processes were switched to molasses (Ennis and Maddox, 1985). The molasses based fermentations lasted until the beginning of the 1960s when the petroleum industry started to be dominant. Since then there have been few works on using starches as the substrate for butanol production (Grobben *et al.*, 1993; Gutierrez *et al.*, 1998, Nimcevic *et al.*, 1998, and Madihah *et al.*, 2001).

With the revival of interest in producing butanol from biological sources and with the huge availability of cheap starches, several research workers have attempted to employ different types of starch as the fermentation substrate for the

production of butanol. Starches recently investigated include potato, sago, corn, and tapioca (Grobben *et al.*, 1993; Gutierrez *et al.*, 1998, Nimcevic *et al.*, 1998, and Madihah *et al.*, 2001; Ezeji *et al.*, 2004). Sago starch seems to be a more productive source than potato and tapioca starch due to its higher amylose content, 27-18% compared to 17-21%. The highest total solvent production (18.82 g/L) was obtained using a sago starch concentration of 50 g/L (Madihah *et al.*, 2001). These results have confirmed that starch could serve as a promising source of substrate for butanol production.

7. Overview on fermentation modes and product recovery by gas stripping

7.1 Fermentation mode

Butanol can be produced by either a batch fermentation mode, fed batch, immobilized cells or continuous fermentation. Of these the batch fermentation mode has been widely applied in industrial butanol production. Batch fermentation does not require much equipment and can provide higher final concentrations of butanol than a continuous fermentation process. However, it does suffer from a number of drawbacks.

As butanol is highly toxic to biological systems at quite low concentration (13 g/L), thus, the level of solvents obtainable in the final fermentation broth could reach only the maximum concentration of 2% maximum this is equal to 20 g/L of total solvents (13 g/L of butanol). At higher concentrations cell growth is inhibited. This causes profound effects on the economics of product recovery and also limits the sugar concentration in the fermentation medium resulting in the requirement for large process volumes and reactor sizes and as the result, the ratio of products is sometimes not desirable. In addition, the fermentation process is quite complex and needs to be run under sterile conditions. Contamination, particularly due to phage infections, causes problems and evaporative loss of solvents is also encountered (Jones and Woods, 1986).

A fermentation process operated in a continuous mode may provide some advantages over a batch process e.g. only one batch of inoculum culture would be needed over the course of the acetone-butanol-ethanol (ABE) production period, the volume of the fermentor can be reduced without a reduction in productivity, and the

time necessary for cleaning and sterilization of the equipment would be drastically reduced. In addition, the continuous flow culture system is an important research tool for the determination of parameters responsible for changes in the physiology and activity of solvent-producing clostridia (Qureshi *et al.*, 2000a).

Fed-batch systems with continuous feeding of concentrated substrate solution can be used, coupled with simultaneous product removal. This differs from the conventional system in that the sugar concentration within the reactor can always be maintained at low levels. However, the product formation in this system does still not proceed effectively, possibly due to the accumulation of mineral salts in the fermentation medium (Maddox *et al.*, 1994).

Immobilized cell systems able to maintain high cell concentrations, generally have improved reaction rates, and are stable at high dilution rates with little cell washout and simplicity of operation. Other advantages are that the process can be relatively simple, and support structures can often be reused (Jones and Woods, 1986). However, the residence time of the fermentation medium inside the reactor will be low; starch hydrolysis may not be carried out efficiently and within the reactor separate hydrolysis may be necessary and reactor blockage may occur due to excessive cell growth (Ezeji *et al.*, 2005).

Ezeji *et al.* (2005) compared the productivity of different fermentation modes including batch fermentation, fed-batch with integrated evaporative recovery cell immobilization and continuous fermentation. They found that cell immobilization and continuous fermentation can improve the economics of butanol fermentation. Because these provided higher productivity (15.8 g/L/h) compared to 0.39 g/L/h in batch fermentation and then the size of the reactor could be reduced by a factor of 40 (Ezeji *et al.*, 2005). Batch fermentation is the most cost intensive, followed by fed-batch fermentation and immobilized-cell continuous fermentation. The productivities are in order of 0.39, 0.98 and 15.8 g/L/h. At the productivity of 15.8 g/L/h with the dilution rate 2.0/h, solvent concentration would be 7.9 g/L as compared to 26.5 g/L in a batch reactor with low membrane flux that would require a larger membrane area.

7.2 Study on fed-batch fermentation

Fed-batch fermentation is the technique that industry employs to produce chemicals and biochemicals. In fed-batch mode, the reactor is started with a small volume of fermentation medium and when the sugar is utilized a new amount of concentrated sugar will be added. This fermentation is especially beneficial in the production of butanol as butanol accumulation is toxic to the culture. However, the removal of product is necessarily carried out simultaneously.

To the best of our knowledge, the research of Ezeji *et al.* (2004) would be the most intensive research on the operation of a fed batch mode for ABE production by *C. beijerinckii* BA101. Starch was used as the feed with a dilution rate of 0.02 h^{-1}, the operated temperature was 37°C and starch solution/feed volume (3 L) was replaced every 72 h. This system provided the best ABE concentrations with a 30 g/L starch solution. The effect of feed storage temperature was investigated and the results showed that at the feed storage temperature of 19°C produced approximately 6.0 g/L of total ABE, but when the feed storage temperature was increased to 37°C ABE production of 7.2 g/L was obtained. At the lower temperature, retrogradation of starch occurred, and this caused a reduction in hydrolysis of the starch.

Several research workers have used the fed batch fermentation process for butanol production using substrates other than starch with positive results. Qureshi *et al.* (2000b) used a glucose-based P2 medium as the feed medium for the fermentation of *C. beijerinckii* BA101 (mutant strain) and *C. beijerinckii* 8052 (wild type) and found that the solvent productivity increased from 0.35 in a batch reactor to 0.98 g/L/h in fed-batch reactor. In another research paper of Qureshi *et al.* (2000 a), a fed-batch mode along with the removal of butanol by using a pervaporation membrane the solvent yield was higher (0.34–0.37 g/L/h) than with the batch reactor (0.29–0.30 g/L/h).

Tashiro *et al.* (2004) employed a pH-stat fed-batch culture and fed butyric acid and glucose to *C. saccharoperbutylacetonicum* N1-4. They found that feeding only butyric acid alone did not support butyric acid utilization and butanol production, but by feeding a mixture of butyric acid and glucose, butyric acid was utilized and butanol was produced. The maximum butanol production was 16 g/L and the residual glucose concentration in the fermentation broth was very low at a butyric acid/glucose ratio of 1.4. Moreover, yields of butanol in relation to cell mass and

glucose utilization were 54% and 72% respectively, much higher in pH-stat fed-batch culture with butyric acid than that of a conventional batch culture (24 % and 32 %, respectively)

7.3 Overview on gas stripping techniques

A number of butanol removal techniques including membrane-based systems, such as pervaporation (Groot *et al.*, 1984; Qureshi and Maddox, 1990; Qureshi *et al.*, 2000a; Qureshi and Blaschek, 2001), reverse osmosis, adsorption (Ennis *et al.*, 1987), liquid–liquid extraction and gas stripping (Groot *et al.*, 1984) have been examined. The application of some of these recovery techniques can allow the use of concentrated sugar solutions in the fermentation medium thereby reducing the volumes of the process streams (Maddox *et al.*, 1995; Qureshi *et al.*, 2001). In such systems, up to 100% utilization of the available sugar has been demonstrated. Among these techniques gas stripping has a number of advantages e.g. simpler to handle, causes no harm to cells, and most of all it is more economically beneficial (Ezeji *et al*., 2004; Groot *et al*., 1992). In a batch reactor *C. beijerinckii* BA101 utilized 45.4 g /L glucose and produced 17.7 g/L ABE while in the integrated process it utilized 161.7 g/L glucose and produced total ABE of 75.9 g/L (Ezeji *et al*., 2004).

Gas stripping allows for selectively removing volatiles from the fermentation medium and uses no membranes or expensive chemicals. Gas (CO_2 and H_2) is sparged into the bioreactor through a sparger that will form bubbles. Bubbles formed or broken in the bioreactor will result in vibrations that remove volatiles from the reaction mixture. The volatiles can then be condensed and separated from the condensate. Bubbles size has profound impact on the transfer and mixing hydordynamics in a gas-liquid agitated vessel. Small gas bubble sizes provide maximal mass transfer but large gas bubble size provides maximal recirculation and mixing in the bioreactor.

It has been found that gas recycle rates of 80 cm^3/s and a K_sa of 0.058/h are sufficient for keeping the butanol concentration below toxic levels in a 2-L bioreactor (1-L reaction volume) during the course of the ABE fermentation. It was also demonstrated that bubble sizes < 0.5 and 0.5–5.0 mm had no effect on the stripping rate of butanol, smaller bubbles size (<0.5 mm) led to larger amounts of foam in the reactor,

which required the addition of high levels of antifoam thus affecting ABE production negatively. The ABE productivities of the bioreactor using an impeller (larger size bubble delivery system) or sparger (smaller size bubble delivery system) based gas delivery systems were 0.47 and 0.25 g/L/h, respectively. The presence of acetone, and ethanol (using a model solution) had no affect on the butanol removal rate. It is recommended that a gas bubble size in the range of 0.5–5 mm in diameter (produced by the impeller) be used for gas stripping to provide good mass transfer and avoid problems associated with excessive foaming.

8. Anaerobic metabolisms of *Bacillus*

It is reported that *Bacillus anthracis* and *Bacillus cereus* could grow anaerobically in both synthetic and nonsynthetic glucose containing medium. Under anaerobic condition, *Bacillus* produced lactic, succinic, formic, and acetic acids, acetylmethylcarbinol, 2, 3-butylene glycol and glycerol as fermentation products of glucose (data shown in Table 1) (Puziss and Rittenberg, 1957). Several research reports have determined anerobic growth and identified end-products of *B. subtilis*. The results prove that *B. subtilis* can grow in the absence of oxygen using nitrate as terminal electron acceptor (Nakano *et al*., 1997; Ramos *et al*., 2000)**.** When the cells were grown in the minimal medium with glycerol in the absence of nitrate, no anaerobic growth was observed after the optical density at the wave length of 600 nm (OD_{600}) doubled and the cells began to lyse. The cells grew well when the glycerol medium was supplemented with nitrate but not when it was supplemented with fumarate (Clements *et al*., 2002).

B. subtilis grows anaerobically by fermentation either when both glucose and pyruvate were provided or when glucose and mixtures of amino acids were present. However, it was found that the fermentation process was stimulated in the presence of pyruvate. In the presence of glucose, *B. subtilis* cells were unable to grow well under anaerobic condition. The same fermentation products including lactate, acetate and 2,3-butanediol were identified in the presence of glucose and pyruvate was observed in much lower amounts. Since pyruvate was produced by fermentation of glucose, the stimulatory effect of pyruvate was not easy to explain (Nakano *et al*., 1997; Ramos *et al*., 2000).

Table 1. Anaerobic fermentation products in mg per 100 ml of medium, quantitative assays on 72-h-old cultures

Product Assayed	*Bacillus subtilis*	*Bacillus anthracis* strains	
		Weybridge[a]	Vollume[a]
2,3-Butylene glycol	12.2	17.1	8.3
Acetylmethylcarbinol	3.6	1.8	1.7
Glycerol	1.6	1.4	2.8
Lactic acid	6.1	3.1	8.0
Succinic acid	6.3	5.6	9.5
Formic acid	3.1	6.5	5.0
Acetic acid	7.8	8.8	10.7
Butyric acid	Nil	Nil	Nil

[a]Name of bacillus strain.

Source: Puziss and Rittenberg (1957).

Ramos *et al*. (2000) studied the anaerobic fermentation of *B. subtilis* strains in defined minimal media using 50 mM glucose and 50 mM pyruvate as carbon sources. They proposed the pathways for anaerobic fermentation and related catabolism in *B. subtilis* as shown in Figure 3. Total product concentration (56.4 mM) included lactate (23.3 mM), acetate (16.4 mM), 2,3-butanediol (16.7 mM) and the concentration of cells was 8.0 (g [wet wt]/L). A few studies have determined the formation and activities of amylases of *Bacillus* under anaerobic condition. However, the results were different from each research. Thus, it has not been possible to answer the question if the lack of oxygen has any effect on the amylase enzymes. Nomura *et al*. (1957) whose results implied that α-amylase formation by stationary-phase cells of *B. subtilis* does not require oxidative phosphorylation, in contrast, Coleman and Elliot, (1961) found the opposite results.

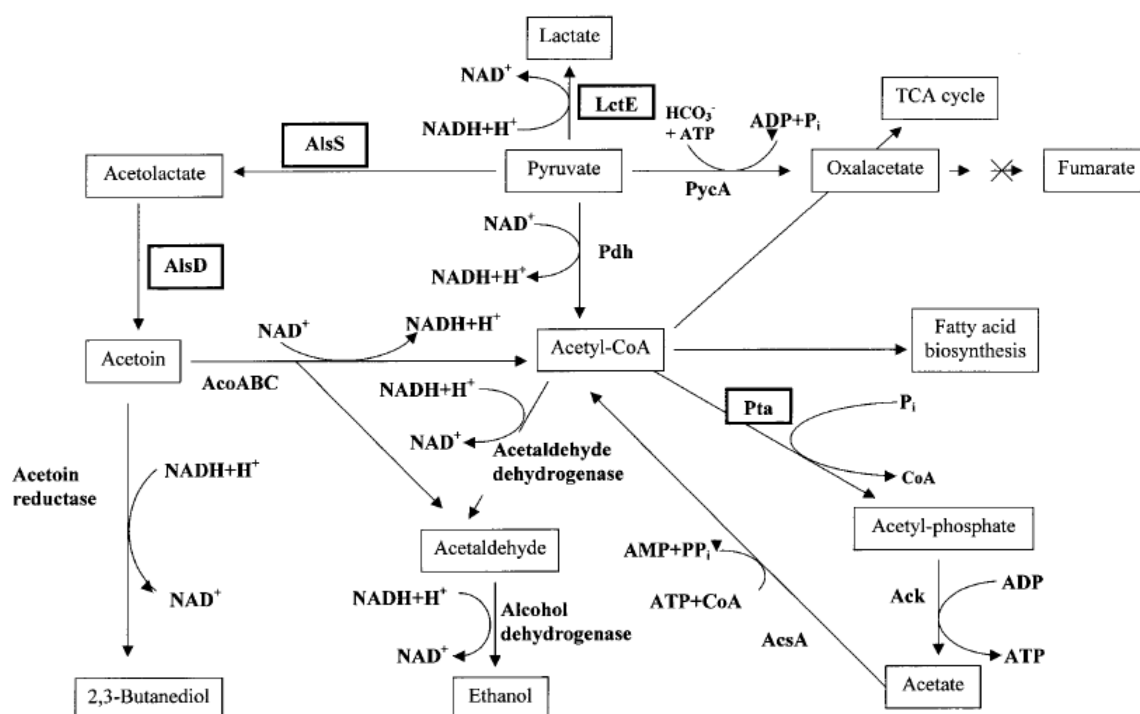

Figure 2. Proposed pathways for anaerobic fermentation and related catabolism in *B. subtilis*.

Note: Enzymes with known coding genes are as follows: LctE, lactate dehydrogenase; AlsS, acetolactate synthase; AlsD, acetolactate decarboxylase; Pta, phosphotransacetylase; Ack, acetate kinase; AcoABC, acetoin dehydrogenase; Pdh, pyruvate dehydrogenase; PycA, pyruvate carboxylase; AcsA, acetyl-CoA synthetase. TCA, tricarboxylic acid

Source: Ramos *et al*. (2000)

9. Cassava starch waste

Annual cassava production in Asia is about 48 million tonnes, mainly in Thailand (18), Indonesia (15), India (6), China (4) and Vietnam (2). In Thailand, the total annual cassava production (about 18 million tonnes) is converted to 4 million tonnes of chips/pellets, and about 1.6-1.8 million tonnes of starch (Sriroth *et al*., 2000). Like the other starch type production process, there are two types of waste derived from cassava starch production which includes solid and liquid form (waste water and effluent). Solid waste from cassava starch processing is divided into three categories:

peelings from the initial processing, fibrous by-products from crushing and sieving (pulp waste) and starch residues after starch settling. An indication of the proportion of solid waste produced during cassava processing is shown in Figure 4. In starch processing, pulp waste is the main problem, especially for the bigger factories, that produce massive quantities (each year the 51 starch processors in Thailand will generate about 1 million tonnes of pulp waste). Table 2 shows the composition of cassava pulp produced after starch extraction at a starch factory in Thailand. Significant amounts of starch (60.6%) and non-starch polysaccharide (29% as fiber) were detected in the pulp. Analysis of the non-starch polysaccharides indicated that glucans, such as cellulose, were the major polysaccharide. The analyzed compounds accounted for 94.7% of the total dry pulp weight (Kosugi *et al*., 2009).

Table 2. Composition of cassava pulp

Components	g/100 Dry pulp
Starch	60.6
Reducing sugars (glucose)	4.7
Nitrogen	0.4
Non-starch polysaccharides	
Glucan	19.1
Xylan	4.2
Arabinan	1.4
Galactan	0.5
Mannan	0.7
Others	0.9
Klason lignin	2.2
Total	94.7

Source: Kosugi *et al*. (2009)

Dealing with this waste is difficult, as it is not easily dried, due to its high moisture and starch contents (Sriroth *et al.*, 2000). In Thailand, most fiber waste is sun dried and mixed with ground chips to be used as animal fed. As starch production

increases in the future this may not take care of the problem. Finding an alternative ways to use pulp waste is in high demand.

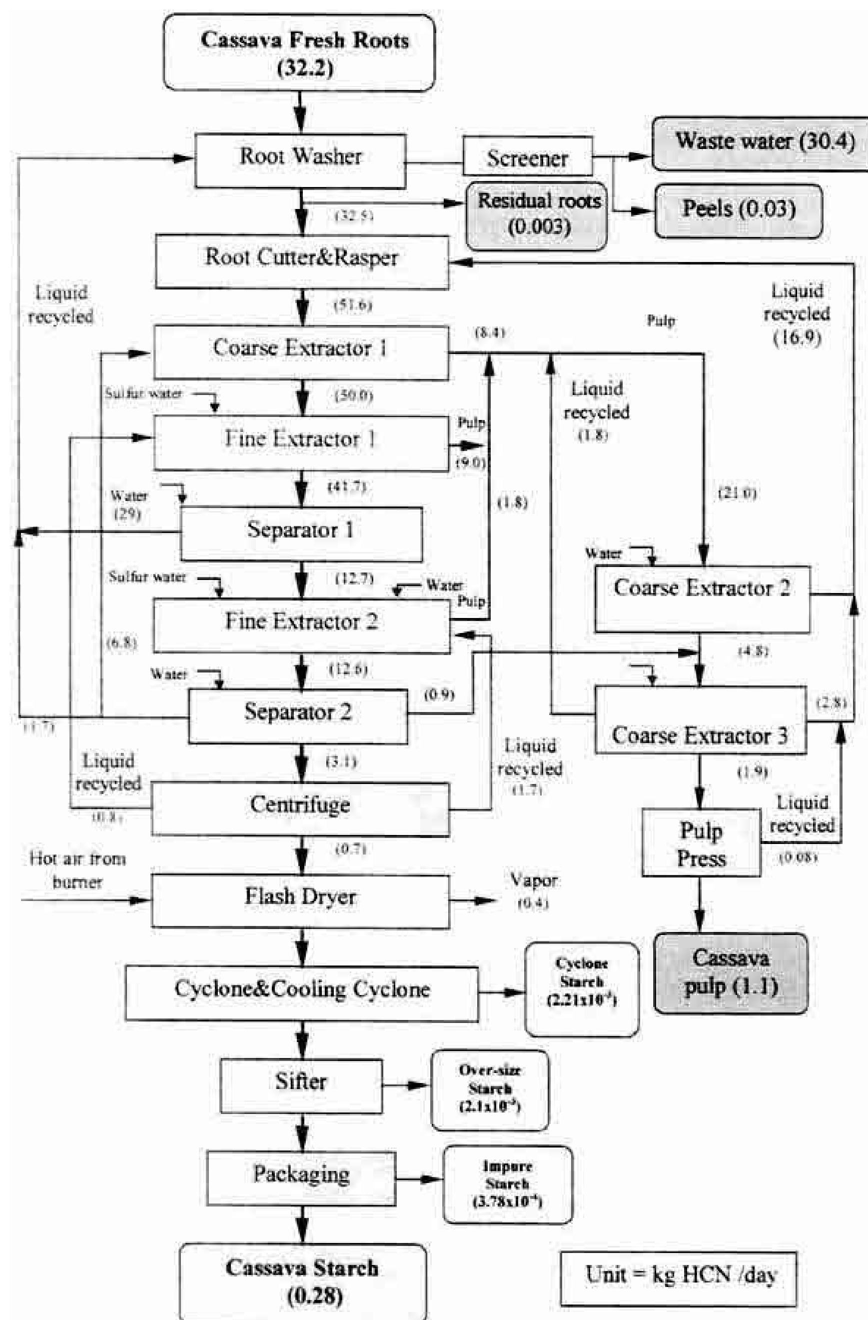

Figure 3. Flow chart for large-scale production of cassava starch.

Source: Sriroth *et al*. (2000)

CHAPTER 3

MATERIALS AND METHODS

Materials

1. Microorganisms

Clostridium butylicum TISTR 1032 was purchased from the Culture Collection of the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. The stock culture was maintained in the form of a spore suspension in 25% glycerol and frozen at -20°C.

Bacillus subtilis WD 161 was a generous gift from Associate Professor Dr. Poonsuk Prasertsan, (Environmental Biotechnology Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University). The stock culture was maintained at 4°C on a nutrient agar slant and subcultured monthly.

2. Cassava starch and cassava starch pulp waste

Cassava starch was purchased from local markets in Hat-Yai, Songkhla Province, Thailand.

Cassava pulp waste was obtained from Srima Inter Product Co., Ltd, located in Cha-Choeng Sao Province, Thailand.

3. Media employed for primary studies

To investigate the economic feasibility of the medium type on ABE production, AB medium, B medium, TPS medium and modified Reinforced Clostridia Medium (RCM) were employed; 20 g/L soluble starch was used as the sole carbon source in all media. The original Reinforced Clostridia Medium (RCM, Oxoid) me
(1 L RCM medium contains: 10 g meat extract; 5 g peptone; 3 g yeast extract; 5 g

glucose; 1 g soluble starch; 5 g sodium chloride; 3 g sodium acetate; 0.5 g L-cysteine). The components of each medium are listed in Table 3.

Table 3. Components of starch based AB medium, B medium, TPS medium and RCM medium

Component (g/L)	Medium			
	AB[b]	B[c]	TPS[b]	Modified RCM[a]
Tryptone	10	-	5	-
Peptone	-	-	5	5.0
Yeast extract	2	3	10	3.0
Meat extract	-	-	-	10
Soluble starch	20	20	20	20
KH_2PO_4	-	0.5	1	-
K_2HPO_4	-	-	1	-
NaCl	-	0.02	-	5.0
CH_3COONa	-	-	-	3.0
NH_4Cl	-	1.5	-	-
$MgSO_4 \cdot H_2O$	-	0.3	-	-
$MnSO_4$	-	0.02	-	-
$FeSO_4$	0.2	0.02	-	-
Na_2SO_3	0.05	-	-	-
$Na_2S_2O_3$	0.05	-	-	-

[a]modified RCM with 20 g/L of soluble starch and no L- cysteine.

Source: [b]Bard *et al*. (2001); [c] Chauvatcharin *et al*. (1997).

Initial pH of all media was adjusted to 6.5. To create anaerobic culture, 0.5 g L-cysteine was added into each medium.

Methods

1. Inoculum preparation

A stock culture of *C. butylicum* TISTR 1032 was maintained as a spore suspension in glycerol 25% at – 20°C. Spores (0.5 mL) was heat shocked at 75 ^{o}C for 3.5 minutes and on ice for 1 minute (Quireshi *et al.*, 2001). The heat shocked spores then was anaerobically pre-cultured in RCM medium. It was then incubated under static condition at 37°C for 18-24 h when the log phase was reached (OD 660 of 2.0). *B. subtilis* WD 161 was aerobically pre-cultured in a nutrient broth (NB) medium (HiMedia) under shaking condition at 200 rpm and 37°C for 18 h when the log phase was reached (OD 660 of 2.0) (Yokoi *et al.*, 2001; Chang *et al.*, 2008).

2. Fermentation

B medium was employed throughout the study (Table 3) (Chauvatcharin *et al.*, 1997). Where noted, NH_4Cl was replaced with the same weight amount of NH_4NO_3. Under aerobic condition, *B. subtilis* WD 161 was grown in 100 mL B medium in a 250 mL flask and shaken on a rotary shaker at 200 rpm. The culture conditions with and without anaerobic pretreatment by addition of reducing agent (L-cysteine) and flushing with N_2 gas over the medium were established in 120 mL butyl rubber seal-serum bottles. Starch based media were liquefied by boiling (dissolved oxygen in the medium was supposed to be removed by this step), then as soon as the process was complete, 90 ml of the medium was quickly dispersed into the 120 ml serum bottle, and then the serum cap was tightly sealed. The conditions without anaerobic pretreatment were stirred at 120 rpm during the fermentation process. The working volume of all cultures was 100 mL, and the fermentation process was carried out at 37°C. The mixed culture was prepared by dispersing a 5% inoculum of each organism (*C. butylicum* TISTR 1032 and *B. subtilis* WD 161) grown as previously described. All experiments were performed at least in duplicate.

3. Cassava starch pulp waste pretreatment

Cassava starch pulp was suspended in water at the starch concentration of 40 g/L (equal to 66.0 g/L cassava pulp waste) and autoclaved at 121°C for 60 min. The pH of the slurry was then adjusted to 5.0 with sodium hydroxide, and 3 M sodium acetate buffer at pH 5.0 was added at a final concentration of 50 mM. Cellulase (LAB-SCAN) was used in the ratio of 3 U/ g dry pulp, the cellulose hydrolysis was conducted at 50° C for 72 h, and pH of the slurry was readjusted to 6.0 before fermentation (Kosugi *et al*., 2008).

4. Optimization of medium components using RSM

The effect of three variables including cassava starch concentration (x_1), yeast extract concentration (x_2) and ammonium nitrate concentration (x_3) with three levels (low: -1; medium: 0; and high: +1) for each variable on ABE (Y_1), butanol (Y_2) and amylase production (Y_3) were investigated. A Box-Behnken design was employed for study of the interactions between these three variables. Response surface plots for the models were obtained by using the Statistica for Window version 5.0 by plotting as a function of two variables, while keeping other variables at a constant value (Box and Behnken, 1960).

5. Process optimization

The mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 was performed up in a 1 L anaerobic bioreactor that had a pH probe and the temperature was controlled at 37°C. The medium was stirred at 120 rpm using a magnetic bar. To study the effect of pH on ABE production, the initial pH was first adjusted and controlled at 5.0, 5.5, 6.0 and 6.5 through the fermentation for each treatment using 3M sodium hydroxide. For fed-batch fermentations, the culture was operated in batch mode for 24 h with a 700 mL working volume (1 L medium contains: 40 g/L cassava starch; 5 g/L yeast extract; 8 g/L ammonium nitrate; 0.5 g KH_2PO_4; 0.3 g $MgSO_4 \cdot 7H_2O$; 0.02 g $MnSO_4 \cdot 7H_2O$; 0.02 g $FeSO_4 \cdot 7H_2O$; 0.02 g NaCl). 100 mL of 80 g/L cassava starch (the

maximum starch concentration which could be dissolved in water) with optimum C/N ratio was fed at the 24 and 36 h time after initiation of the culture. For the semi-continuous fermentations, the culture was operated in batch mode for 12 h with a 900 mL working volume (1 L medium contains: 40 g/L cassava starch; 5 g/L yeast extract; 8 g/L ammonium nitrate; 0.5 g KH_2PO_4; 0.3 g $MgSO_4 \cdot 7H_2O$; 0.02 g $MnSO_4 \cdot 7H_2O$; 0.02 g $FeSO_4 \cdot 7H_2O$; 0.02 g NaCl). Then, 200 mL of medium containing 80 g/L cassava starch was added every 12 hours from the 12 h to 72 h. Each time, before adding the new medium, 200 mL culture broth was taken out, and the pH of culture was controlled at 6.0. The cassava starch used for the fed-batch experiments was contained in Duran-bottle and placed at 37°C after being autoclaved to reduce the possibility of retrogradation. For the fed- batch fermentation with gas stripping, gas stripping was initiated from about 36 h by recycling oxygen free N_2 gas through the system to create gas bubbles in the culture using a twin-head peristaltic pump. The ABE vapors were cooled (to 5°C) in a condenser which had been previously fluxed with oxygen free N_2. The stripped ABE was collected into the solvent collector (125 mL flask). To maintain a constant liquid level inside the reactor, O_2-free distilled water was regularly added to the reactor (as some water was lost due to gas stripping). Samples were withdrawn at intervals for amylase, organic acids and ABE analysis (Ezeji *et al*., 2004).

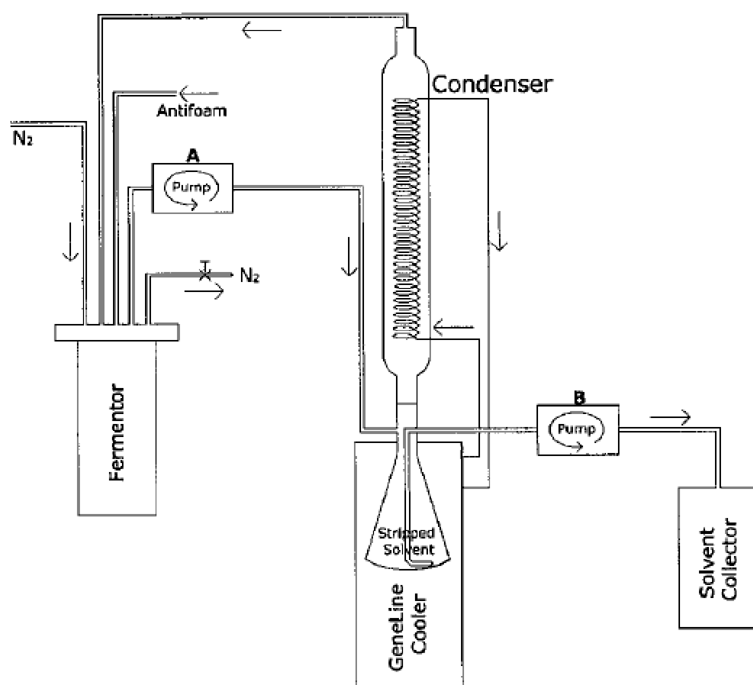

Figure 4. A schematic diagram of butanol production and *in situ* recovery by gas stripping. Pump A: gas recycle pump; pump B: condensed solvent removal pump.

Source: Ezeji *et al*. (2004)

6. Analytical methods

Cell growth was determined by measurement of the optical density at 660 nm (OD_{660}) by a spectrophotometer. Where noted, Colony Forming Unit (CFU) of *Clostridium* and *Bacillus* were determined by sample serial dilution followed by spread plate technique for *Bacillus* and pour plate technique for *Clostridium*. Clostridial plates were placed into an anaerobic jar. All the samples were incubated at 37°C for 5-7 days. For sampling, during the fermentation period (72 h), a 3.0 ml sample was taken every 12 h using a syringe and centrifuged at 8000 rpm, 4 ^{o}C for 25 min. The supernatant was used to analyze for ABE, organic acids, residual reducing sugar concentrations and amylase activity. ABE and organic acids were measured by gas chromatography (Hewlett Packard) using a glass column (HP- INNOWax Polyethylene Glycol) and a

flame ionization detector with helium as the carrier gas. The temperature of the detector and injector were maintained at 270°C and 230°C, respectively (Gapes *et al.*, 1996). The reducing sugar amounts were estimated by the dinitrosalicylic acid (DNS) method of Miller (1959) using a glucose standard calibration curve.

Amylase activity was determined by the method of Okolo *et al.* (1995). The reaction mixture consisted of 1.25 mL of 1 % soluble starch, 0.5 mL of 0.2 M acetate buffer (pH 5.0), and a 0.25 mL sample. After 10 min of incubation at 50°C, the reaction was stopped by boiling at 100°C for 10 min. The control was carried out in the same manner using a sample previously inactivated by boiling for 15 min. The liberated reducing sugars were estimated by the DNS method as previously mentioned. One unit (U) of amylase is defined as the amount of enzyme that releases one μmole of glucose equivalent per min under the assay conditions. The C/N ratio was calculated based on a molar basis. One gram of starch/L was assumed to be converted into 1.1 gram of glucose/L. The formula then used for the conversion of the mass concentration of starch (g/L) to mM concentration of carbon was [starch (g/L) × 36.7 = mM carbon]. The total nitrogen content in the yeast extract used in this study was 11.1% (data was provided by the LAB-SCAN company). The conversion of yeast extract mass concentration (g/L) to mM concentration of nitrogen was [yeast extract (g/L) × 7.93 = mM nitrogen] (Madihah *et al.*, 2001).

CHAPTER 4

RESULTS AND DISCUSSION

1. Growth and ABE production by pure cultures of *C. butylicum* TISTR 1032 incubated under various conditions

As mentioned previously, the main obstacle that prevents bio-butanol from being marketable is the high cost of the product. Substrate cost is a major factor that has a major impact on the economics of butanol production (Dürre, 1998). To improve the economics of butanol production, searching for a low-cost medium is essential. Previous research work on the ABE production process mostly had the same purpose of reducing the cost of the butanol product; however there has been little attempt to use a low-cost medium for ABE fermentation. As documented earlier, addition of vitamins, amino acids, and reducing agents were often required in their research (Badr *et al*., 2001; Madihah *et al*., 2001; Ezeji *et al*., 2004; Hipolito *et al*., 2008). These components are remarkably costly, so addition of them will definitely increase the overall cost of the product. In this study, several medium types (AB medium, B medium, TPS medium and modified RCM) were employed for investigations of ABE production by pure cultures of *C. butylicum* in order to select one that will be the most economic for further studies.

On the other hands, *Clostridia* are considered to be obligate anaerobes; for example, oxygen is harmful or lethal to these bacteria. However, it was known that some of them have shown some tolerance to oxygen and they can survive limited exposure to air (Hillmann *et al*., 2008). It was thought that the culture without anaerobic pretreatment (with addition of L-cysteine and flushing N_2) would be beneficial to improve the cost effectiveness of ABE production since then there was no need to add any costly reducing agents or flush with oxygen free N_2 gas. The possibility of culturing this *C. butylicum* TISTR 1032 under condition without anaerobic pretreatment was tested. *C. butylicum* TISTR 1032 was cultured under condition with and without anaerobic pretreatment.

1.1 Effect of anaerobic pretreatment on pure culture of *C. butylicum* TISTR 1032

C. butylicum TISTR 1032 was cultured in conditions with and without anaerobic pretreatment by addition of a reducing agent (L-cysteine) and flushing with N_2. The results are shown in Figures 5 and 6. It is interesting to note that even though *Clostridia* are well-known as strictly anaerobic organisms, the clostridial strain in our research grew reasonably well in all the investigated media where no nitrogen sparging or addition of reducing agent was used (without anaerobic pretreatment). However, the presence of small amounts of oxygen in the culture in all media tested did have some negative impact on the growth profiles of this strain as the lag phase under this condition was longer than that under condition with anaerobic pretreatment. As the lag phase was longer then the time for the culture to obtain its maximum growth under the condition without anaerobic pretreatment was also longer and the maximum growth was lower than when cultures were incubated under conditions with anaerobic pretreatment. Under anaerobic pretreated conditions, every culture reached the maximum OD_{660} values after just 48 h, but for those under conditions without anaerobic pretreatment the maximum OD_{660} values were obtained in the range of 48-72 h. A longer lag phase phenomenon with the clostridium culture under conditions without anaerobic pretreatment was also found in the research of Hipolito *et al*. (2007). This is understandable since *Clostridi*um *spp* are strictly anaerobic; the presence of oxygen in the culture does not provide the ideal conditions for their growth. However in either this research or the research of Hipolito, the amount of oxygen in the culture was probably not high enough to have a serious effect on the growth of this *Clostridium spp*.

In our research, the starch based media were liquefied by boiling (dissolved oxygen in the medium was supposed to be removed by this step), then as soon as the liquefaction was complete, 90 mL of the medium was quickly dispersed into the 120 mL serum bottle, and then the serum cap was tightly sealed. Therefore, there would not be much oxygen either in the form of dissolved or un-dissolved form in the medium.

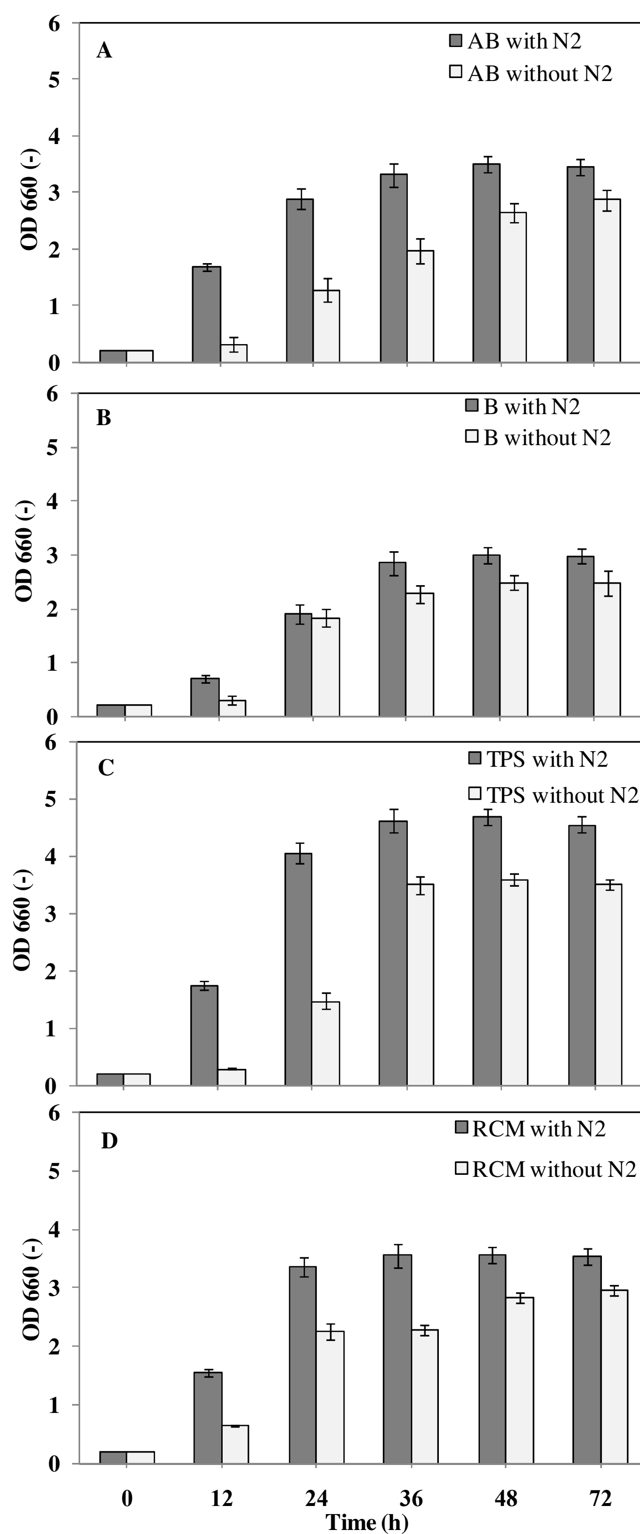

Figure 5. Growth profiles of pure culture of *C. butylicum* TISTR 1032 in various media: AB medium (A), B medium (B), TPS medium (C), RCM medium (D) with either anaerobic pretreatment (with addition of L-cysteine and N_2 flushing) or without anaerobic pretreatment (without N_2 flushing).

Besides that, in the culture without anaerobic pretreatment, as the medium was being stirred during the fermentation process by employing a magnetic bar and stirrer at the speed of 160 rpm cells became well dispersed throughout the medium that positively supported cell growth and prevented them from sedimenting whereas, the anaerobic cultures did not have this advantage. This somehow narrowed the differences in the growth of *Clostridium* under conditions with and without anaerobic pretreatment.

In terms of ABE production, all media with anaerobic pretreatment provided higher ABE production compared to that without anaerobic pretreatment. This again would be explained by the effect of oxygen in the condition without anaerobic pretreatment (Figure 6). The presence of oxygen may influence ABE production of the culture without anaerobic pretreatment in two ways. One was as mentioned above in that oxygen causes a longer lag phase and less growth resulting in lower amounts of the ABE precursors: acetic and butyric acids. In another way, oxygen is supposed to have a negative effect on the functions of some enzymes in the ABE pathway and prevents them from working properly to produce precursors for ABE or converting precursors into ABE (Hipolito *et al.*, 2007). The effect of the types of media on the growth and ABE production of *C. butylicum* TISTR will be discussed in the next section.

1.2 Effect of the medium composition on pure cultures *C. butylicum* TISTR 1032

In terms of growth, *C. butylicum* TISTR 1032 grew best in TPS medium, followed by modified RCM, AB and finally B medium. Some factors that we anticipate might have affected the growth of the *Clostridium* are sources and amount of organic nitrogen, buffer capacity, and the presence and the amount of mineral salt in the medium. In a poorly bufferred medium (AB medium), when high amount of acids are produced and this rapidly reduced pH of the medium and caused toxicity to cells. A medium with a high buffering capacity (modified RCM) could achieve a higher concentration of acids over a longer period of time without a rapid decrease of pH and this would probably increase the growth and carbohydrate utilization (Ezeji *et al.*, 2005b; Hüsemann and Eleftherios, 1990). The amount of nitrogen source affects the utilization of carbohydrate. Normally, the presence of higher amount of nitrogen makes the utilization of carbon source more complete, and better growth would be obtained.

Mineral salts are well-known as co-factors for many enzymes. The presence and amount of them affect the activities of enzymes (Ezeji *et al.*, 2005b). TPS medium is the medium that contains three nitrogen types with higher total amounts compared to the other media (tryptone 5 g, peptone 5 g and yeast extract 10 g). That the best growth was obtained from this medium is possibly reasonable. These three organic nitrogen sources not only provide nitrogen, but also various amino acids, vitamins, minerals and growth factors that promote good growth of the bacteria.

Modified RCM medium contains smaller amount of organic nitrogen (peptone: 5 g and yeast extract: 3 g) compared to TPS medium, but it is the only medium containing CH_3COONa. This is present as both a buffer and a solvent enhancing factor (Chen and Blaschek, 1999). That is probably the reason why modified RCM medium showed better growth than AB medium that has a higher amount of an organic nitrogen source (tryptone 10 g and yeast extract 2 g) but no CH_3COONa or mineral salts. B medium is poor in organic nitrogen (B medium comprises only 3 g of yeast extract), but it contains various minerals that are required as cofactors for enzymes in the ABE pathway thus the B medium still allowed for normal growth, even though the content of nitrogen was less than the other media (Table 3).

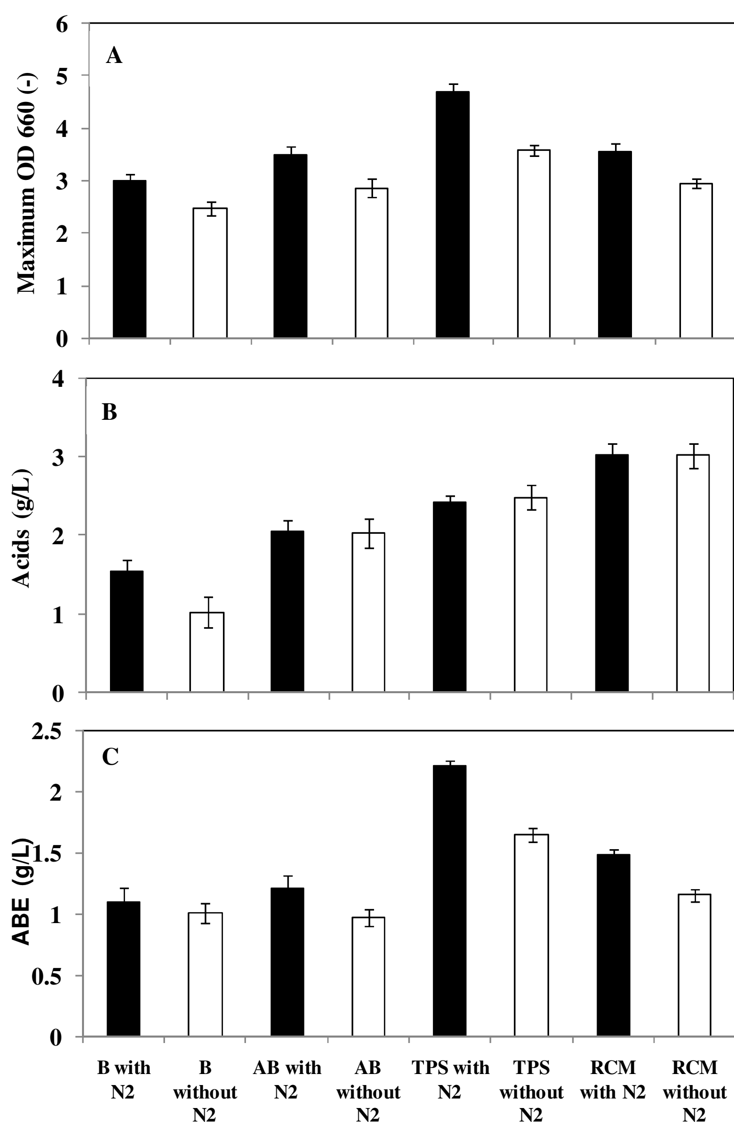

Figure 6. Maximum OD 660 (A), butyric acid concentration (B) and ABE production (C) by a pure culture of *C. butylicum* TISTR 1032 in various media either with anaerobic pretreatment with addition of L-cysteine and N_2 flushing) or without anaerobic pretreatment (without N_2 flushing), at 72 h.

Acetone-ethanol-butanol (ABE) and acid production by *C. butylicum* TISTR 1032 in different media under conditions with and without anaerobic pretreatment are shown in Figure 6. In terms of total ABE formation, either under conditions with or without anaerobic pretreatment, the most productive medium was TPS medium (2.20 g/L and 1.60 g/L under conditions with and without anaerobic pretreatment, respectively), followed by modified RCM medium (1.49 g/L and 1.15

g/L), AB (1.20 g/L and 0.97 g/L) and B medium (1.10 g/L and 1.01 g/L). In terms of butanol production, the same order was observed: TPS medium (1.84 g/L and 1.58 g/L under anaerobic condition with and without N_2, respectively), modified RCM medium (1.14 g/L and 0.91 g/L), AB medium (1.00 g/L and 0.80 g/L) and B medium (0.93 g/L and 0.790 g/L) (Figure 6). Although, ABE are not growth associated products, their precursors i.e. acetic and butyric acids are. In general, factors affecting growth such as the nitrogen content, mineral salts, and buffer would also affect ABE production, as better growth would result in a higher amount of acid concentrations then subsequently higher amounts of ABE. Besides the factors governing the growth of *Clostridia* mentioned above, CH_3COONa and $FeSO_4$ are determined to be very important components that cause significant effects on ABE formation. It was found, (Hüsemann and Eleftherios, 1990; Chen and Blaschek, 1999) that additions of sodium acetate to the fermentation medium increased and stabilized solvent production. The conversion of pyruvate to acetyl-CoA in the solventogenesis reactions of clostridia involves a ferredoxin oxidoreductase iron-sulfur protein that accepts and donates electrons at a very low potential, thus the maintenance of a low redox potential is important for the ABE production. Therefore, the supplementation of some mineral salts in the medium is necessary (Ezeji *et al*., 2005b). Parekh *et al.* (1999) reported that the addition of $FeSO_4{\cdot}H_2O$ to 6% glucose and 1.6% solids corn steep water medium resulted in a 26% increase in butanol concentration, and the butanol and acetone ratio was higher than that of the culture without ferrous ion addition. In general, ferrous iron has a dramatic effect on the butanol concentration and butanol/acetone ratio, therefore ABE production. Thus, even though the B medium is much poorer in nitrogen source compared to the other media, it does contain various types of mineral salts thus the amount of ABE obtained from the B medium was comparable to the AB and modified RCM medium. Considering the yield of ABE, B medium contains only 3 g yeast extract, but produced 0.93 g/ L butanol in the total 1.10 g/L ABE under conditions with anaerobic pretreatment. While the TPS medium produced higher amounts, 1.84 g/ L butanol in the total 2.20 g/L ABE, the medium contained yeast extract 5 g/L, tryptone 5 g/L and meat extract 10 g/L. The nitrogen content of the TPS medium is about 6.5 fold higher than that of the B medium, but butanol and the total ABE produced from the TPS medium are only two fold higher than those from the B medium. As the same amount of soluble

starch (20 g/L) was used for both media, and because the price of the media are mainly decided by the price of organic nitrogen, the amounts of ABE over the amount of nitrogen were compared. It was found that the B medium is definitely more economic than the other investigated media as it gave the highest yield of ABE production per unit of nitrogen. It should be noted here that all these four media in this research provided lower amount of ABE compared to most of the reported data elsewhere (6-10 fold lower). This may be due to the lower concentration of starch used in this study. Therefore further optimization of the starch concentration is needed.

2. Enhancement of ABE production from cassava starch by a mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

Bacilli are aerobic microorganisms, but some species are able to grow under anaerobic condition especially in the presence of nitrate that can act as the terminal electron acceptor to facilitate growth and metabolic activities of *Bacillus* in the absence of oxygen (Coleman and Elliott 1962; Nakano and Hullet 1997; Clements *et al*., 2002). In this study, the growth and amylase production of a pure culture of *B. subtilis* WD 161 under conditions with and without anaerobic pretreatment by addition of a reducing agent and flushing with N_2 were compared to those under aerobic conditions with the presence of either ammonium nitrate (NH_4NO_3) or ammonium chloride (NH_4Cl) as the inorganic nitrogen source. Then, a preliminary study of co-culturing *B. subtilis* with *C. butylicum* TISTR 1032 and its effect on ABE production from soluble starch was performed under conditions with and without anaerobic pretreatment.

2.1 Effect of O_2 on growth and amylase production of *B. subtilis* WD 161

To investigate the possibility of using *B. subtilis* WD 161 for co-culturing with *C. butylicum*, it was cultured under aerobic and condition with and without anaerobic pretreatment and the culture medium was assayed for amylase activity and growth (OD_{660}). Previous reports on the anaerobic growth of *Bacillus* have shown that some *Bacillus* species grow in the presence of NH_4NO_3. NO^-_3 replaced oxygen as an electron acceptor and the NO^-_3 is reduced to N_2 gas, a process known as

denitrification (Coleman and Elliott, 1962; Nakano and Hullet, 1997; Clements *et al.*, 2002). Thus, the initial inorganic nitrogen source, NH_4Cl, of the soluble starch based B medium was replaced by the same weight amount of NH_4NO_3 (1.5 g/L) and the growth and amylase production of *B. subtilis* in the two media were compared (Figures 7 and 8). *B. subtilis* grew well under aerobic condition with NH_4Cl as inorganic nitrogen source and produced 20 U amylase/mL after 24 h then increased up to 26 U/mL after 72 h. With NH_4NO_3 as inorganic nitrogen source growth was slightly better and amylase production increased from 14 U/mL at 24 h and after growth ceased up to 45 U/mL at 48 h. The phenomenon that the amylase production is not always associated with bacillus growth was also found by Nomura *et al.* (1957) and this is quite distinghished from the other bacilli of which amylase production is growth associated product (Coleman and Elliot, 1961; Konsula and Liakopoulou-Kyriakide, 2004).

Under conditions without anaerobic pretreatment, some growth did occur over the first 12 h but then ceased and the increased growth with NH_4NO_3 compared to that with NH_4Cl was only small (Figure 7b). However, the amylase activity with NH_4NO_3 as inorganic nitrogen source, increased from 5 U/mL at 12 h up to 14 U/mL at 36 h after growth ceased, while the amylase levels of the NH_4Cl culture did not increase beyond 5 U/mL (Figure 8b). There was no growth under conditions with anaerobic pretreatment and only 2.6 U/mL of amylase was produced within the first 12 h with NH_4Cl and this did not increase further whereas with NH_4NO_3, amylase increased to 4.8 U/mL (Figure 8c). It could be assumed that without anaerobic pretreatment, some oxygen did gain access to the anaerobic medium and allowed some growth of *B. subtilis* and amylase production in the same way as when it was grown aerobically but this was prevented with anaerobic pretreatment when no oxygen was present.

There has been very little published work about amylase production by *Bacillus* under oxygen limited conditions. Coleman and Elliott (1962) determined the effects of anaerobiosis on amylase formation by *B. subtilis*. Their results showed that amylase formation was reduced 93% (from 64 to 4.6 U/mL) when *B. subtilis* was cultured under anaerobic instead of aerobic conditions. The decrease of amylase formation under oxygen limitation was thought to be due to the loss of the respiratory energy required for enzyme production. This would indicate that although *B. subtilis* WD 161 used in our experiments could not produce enough energy to grow

anaerobically with nitrate as terminal electron acceptor, the presence of nitrate in some way did facilitate the production of amylase after growth ceased.

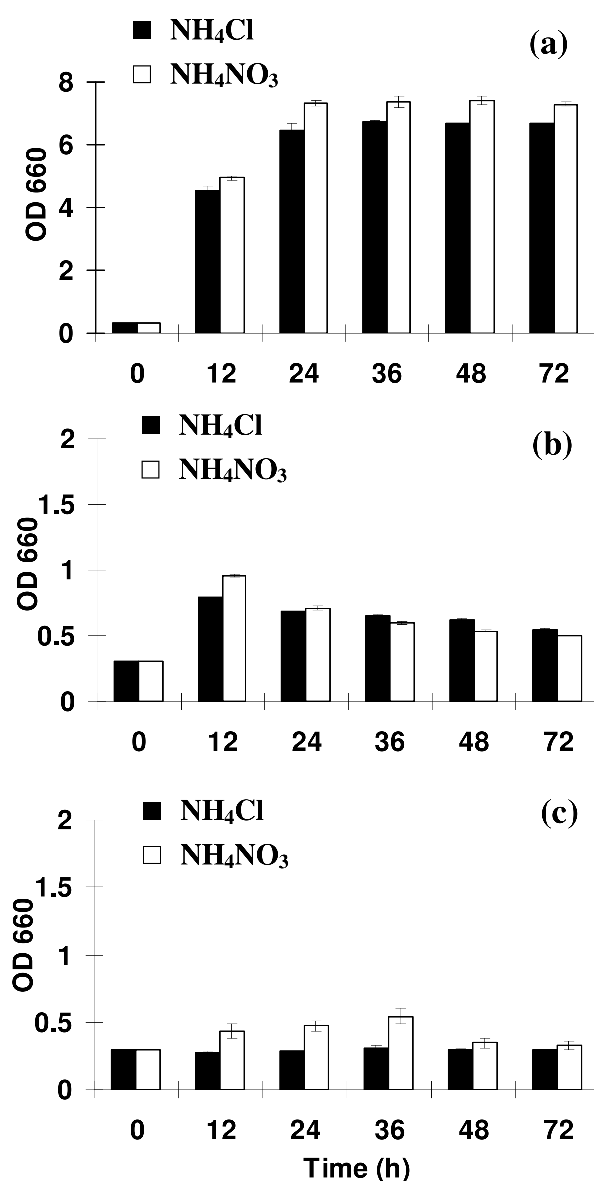

Figure 7. Growth of *B. subtilis* WD 161 with different inorganic nitrogen sources under aerobic conditions (a), conditions without anaerobic pretreatment (b) and conditions with anaerobic pretreatment (c).

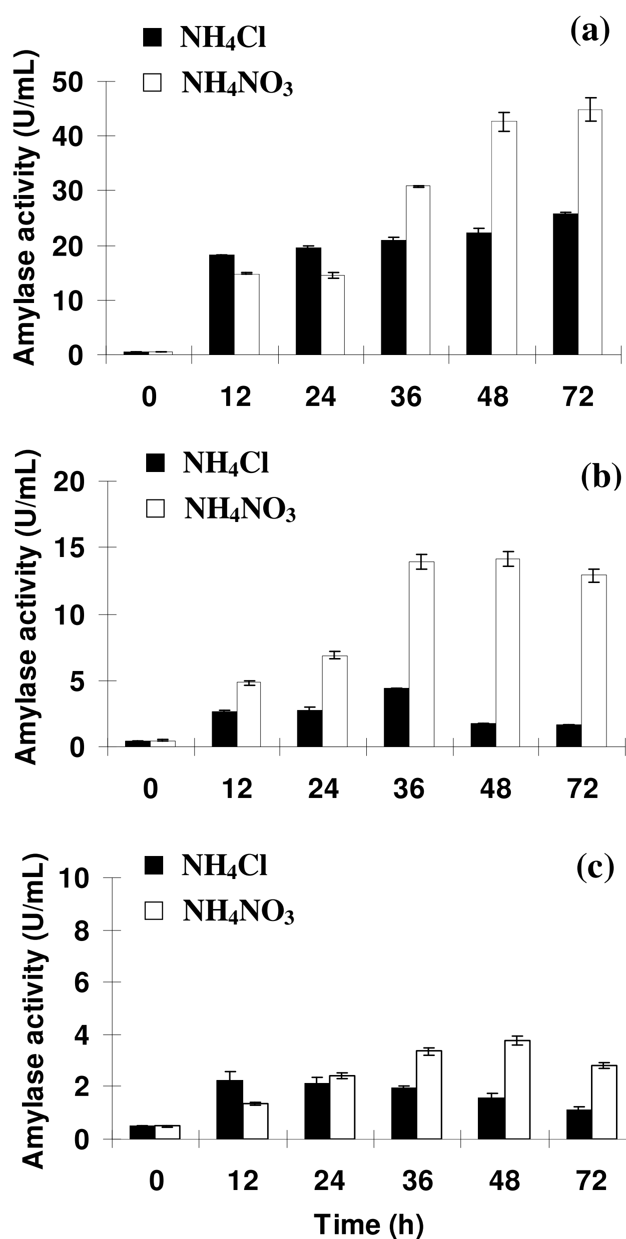

Figure 8. Amylase profiles of *B. subtilis* WD 161 with different inorganic nitrogen sources under aerobic conditions (a), conditions without anaerobic pretreatment (b) and conditions with anaerobic pretreatment (c).

Of most interest for our work was the finding that *B. subtilis* could produce amylase under conditions without anaerobic pretreatment and its activity was relatively stable over a period of 72 h (Figure 8b). It was thus possible that a culture of *B. subtilis* under conditions without anaerobic pretreatment would produce amylase and thus provide the clostridium with a ready supply of reducing sugar to carry out the ABE

fermentation. A further study was then carried out to investigate whether the clostridium would survive under conditions without anaerobic pretreatment in the presence of *B. subtilis*.

2.2 ABE production of *C. butylicum* TISTR 1032 under conditions with and without anaerobic pretreatment

As mentioned previously, *Clostridia* are considered to be obligate anaerobes, e.g. oxygen is harmful or lethal to these bacteria. Nevertheless, it is known that some of them can survive limited exposure to air (Hillmann *et al.,* 2008).This information indicates a possibility for co-culturing a clostridium with an aerobic organism for enhancement of ABE without paying much effort to maintain strictly anaerobic condition, as the aerobic organism will quickly consume oxygen and create the anaerobic conditions for clostridium (Yokoi *et al.,* 1998). The aim of this experiment was to again check the oxygen tolerating capacity of clostridium to investigate the possibility of culturing *C. butylicum* under conditions without anaerobic pretreatment, before setting up a co-culture with *B. subtilis*. ABE and acid (acetic and butyric) production by a pure culture of *C. butylicum* in a soluble starch based B medium with NH_4NO_3 as the inorganic nitrogen source under conditions without and with anaerobic pretreatment were compared (Figure 9). With anaerobic pretreatment, a slightly higher amount of ABE (0.94 g/L) was produced compared to that without pretreatment (0.78 g/L). The same results were observed in the case of using NH_4Cl as the inorganic nitrogen source (data not shown). This was most likely due to the presence of a small amounts of oxygen. This effect of anaerobic pretreatment was similar to that found by Hipolito *et al*. (2008) and also see Figs 5 and 6. Although, the *C. butylicum* in this research produced comparatively low amount of ABE, it did show a capacity to tolerate low amounts of oxygen.

Since the difference in the ABE production of *C. butylicum* with or without anaerobic pretreatment was small and *B. subtilis* cultured under condition without anaerobic pretreatment could survive and produce amylase at up to 14 U/mL and its activity was stable for 72 h of cultivation, these results indicated the possibility of studying the effects of amylase activity produced by *B. subtilis* on the ability of *C. butylicum* to produce ABE from starch. Preliminary work has indicated that *C.*

butylicum does not produce very active amylases. Therefore, we decided to use these two organisms to investigate the hypothesis of product enhancement using a syntrophic co-culture system.

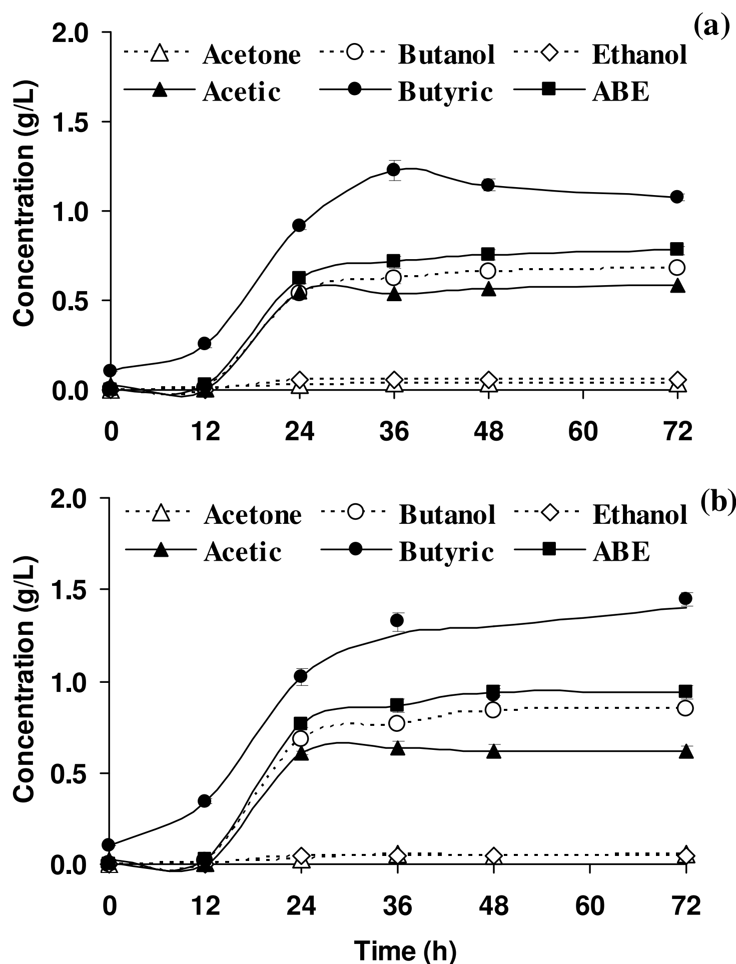

Figure 9. ABE production by a pure culture of *C. butylicum* TISTR incubated with 20 g/L soluble starch medium using NH_4NO_3 as an inorganic nitrogen source under conditions without (a) and with anaerobic pretreatment (b).

2.3 A mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

A mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 was established under conditions with and without anaerobic pretreatment. The growth, amylase activity, ABE, acids (acetic and butyric) is shown in Figures 10-12. Compared to the pure cultures of *C. butylicum* either with or without anaerobic pretreatment and even the mixed culture with anaerobic pretreatment, the mixed culture without

anaerobic pretreatment (Figure 10a) had a much faster rate of increase of OD_{660} up to a final value of 5.0 compared to about 2.5 by the mixed culture with anaerobic pretreatment (Figure 10b). This difference in OD is most likely due to the fact that in the mixed culture without anaerobic pretreatment some oxygen from the empty space of the serum bottle and from the aerobically pre-cultured inoculum of *B. subtilis* allowed some growth and amylase production that had an enhancing effect on cell growth and metabolism of the *C. butylicum*.

From Figures 11 and 12, it is quite clear that the mixed culture without anaerobic pretreatment produced much higher amounts of ABE (4.2 g/L), than that of any other cultures especially the pure culture of *C. butylicum* grown with anaerobic pretreatment (0.94 g/L). The mixed culture with anaerobic pretreatment produced higher amount of butyric and acetic acids during the first 12 h (Figure 11b) compared to those of the mixed culture without anaerobic pretreatment (Figure 11a), but neither culture, at this early time, produced much butanol. After 12 h when the butyric acid concentrations increased up to 2 g/L, butanol production occurred in both cultures but more quickly in the mixed culture without anaerobic pretreatment and in this culture the amount of butanol increased until 72 h. whereas in the mixed culture with anaerobic pretreatment, the amount of butanol during 24-72 h was almost unchanged. Of most interest was that the main effect of *B. subtilis* in the mixed culture without anaerobic pretreatment was an increase of butanol production. The amount of butanol was 3.9 g/L, whereas the amount of acetone and ethanol were 0.1 and 0.2 g/L, respectively. The ratio of these products is interesting as it is reported that pure culture of clostridium normally produces ABE with the ratio of 2:3:1 (Jones and Wood 1986). Since butanol was the dominant product in the mixed culture, product recovery would be much easier to handle. This also appears to be another advantage of the mixed culture.

The amylase levels in the pure culture of *C. butylicum* without and with anaerobic pretreatment did not exceed 1.5 U/mL and 1.8 U/mL, respectively (Figure 12). In the mixed culture with anaerobic pretreatment, the amylase level was slightly higher than that of the pure culture but did not exceed 2.5 U/mL (Figure 12a) whereas with no anaerobic pretreatment, the amylase level reached about 13.5 U/mL after 12 h and increased further to 17 U/mL after 24 h and thereafter remained fairly constant (Figure 12 a). This confirms that the production of amylase by *B. subtilis* did increase

with a small amount of oxygen. This is consistent with the evidence obtained with the results from the pure *B. subtilis* culture grown without anaerobic pretreatment (Figure 8). The production of ABE in the mixed culture without anaerobic pretreatment was about 4 fold greater than that of the other cultures (Figure 11a). It seems as though in the mixed culture without anaerobic pretreatment the extracellular bacillus amylase rapidly converted starch to glucose and this available sugar soon stimulated the metabolism of the *C. butylicum* to grow and eventually have enhanced ABE production.

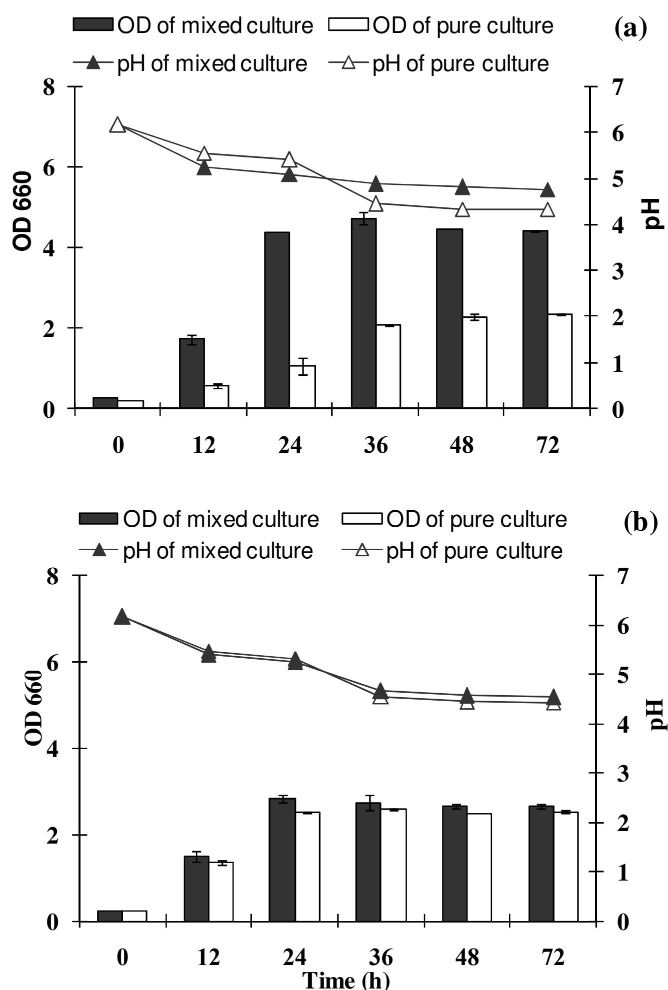

Figure 10. Growth and pH profiles of a pure culture of C. *butylicum* TISTR 1032 incubated with 20 g/L soluble starch medium under conditions without (a) and with anaerobic pretreatment (b).

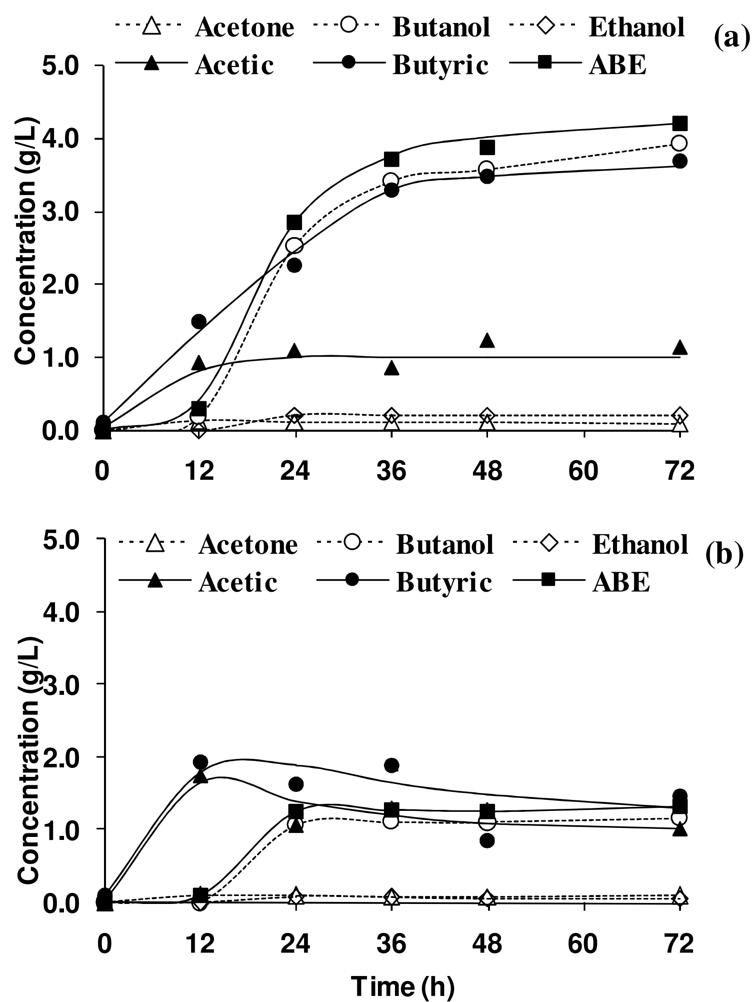

Figure 11. Acids and ABE production by a mixed culture of C. *butylicum* TISTR 10321 and *B. subtilis* WD 16 (a) and a pure culture of *C. butylicum* TISTR 1032 (b) incubated with 20 g/L soluble starch medium under conditions without anaerobic pretreatment.

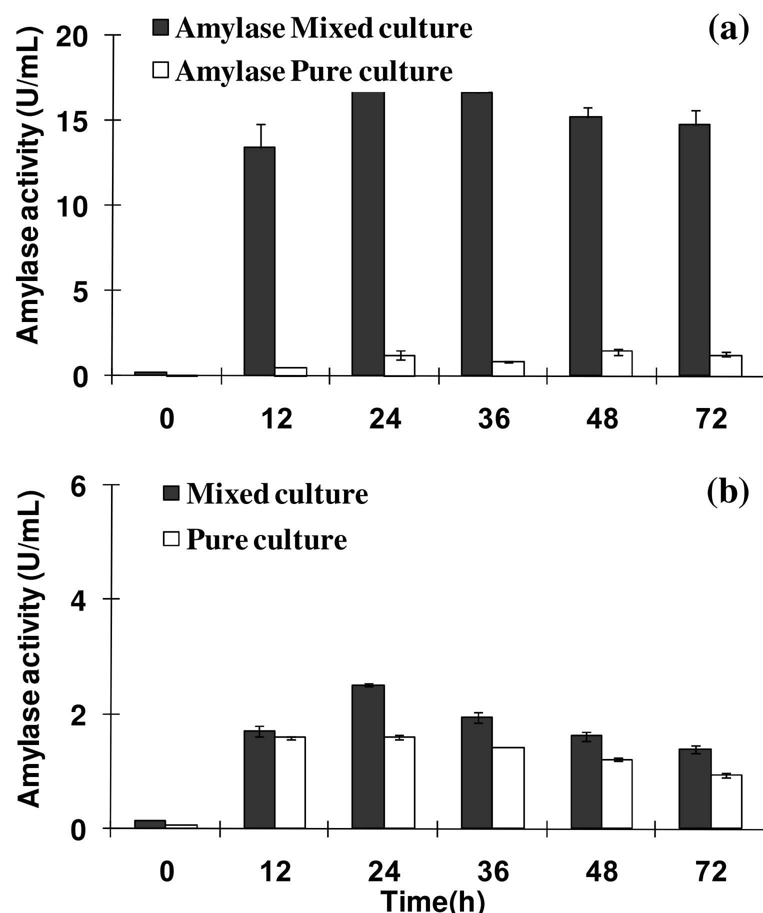

Figure 12. Amylase production by a pure culture of C. *butylicum* TISTR 1032 incubated with 20 g/L soluble starch medium under conditions without (a) and with anaerobic pretreatment (b).

2.4 Application of a mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 for ABE production from cassava starch

The production of amylase and the enhancement of ABE by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 when using 40 g/L of cassava starch as carbon source was investigated under conditions without anaerobic pretreatment. The results are shown in Figure 13. Enhancement of amylase, butyric acid, and ABE productions by the mixed culture of *Clostridium* and *Bacillus* compared to those of the pure culture of *Clostridium* were observed. In the mixed culture of *Clostridium* and *Bacillus*, the amylase activity (36.7 U/mL) and ABE production (7.4 g/L) was increased 10 fold and 6.5 fold, respectively, over those of the pure culture of *Clostridium*. This result also indicated that the amylase activity was the key factor in

ABE production from starch. Hence, as increased amylase activity was produced in the mixed culture of *Clostridium* and *Bacillus* in this study, a rapid starch hydrolysis to sugar would ensue. Consequently, the availability of sugar in the culture was high and encouraged the *Clostridium* to grow rapidly and produce large amounts of acids and ABE products in a short period of time. It was noted that the fermentation time where the optimum ABE was obtained by the mixed culture of our research was shorter (48 h) compared to that of the other research (72 h) using pure culture of *Clostridium* at the same starch concentration of 40 g/L (Madihah *et al.*, 2001). This is another advantage of using a mixed culture for ABE production, since the fermentation time is shorter, the cost of ABE products are accordingly expected to be reduced. In accordance with Gapes (2000), if the batch fermentation can be maintained for 40-60 h, the acetone-butanol fermentation should be industrially viable. Therefore, in terms of the economical benefits, the ABE production by the mixed culture in this research is most encouraging.

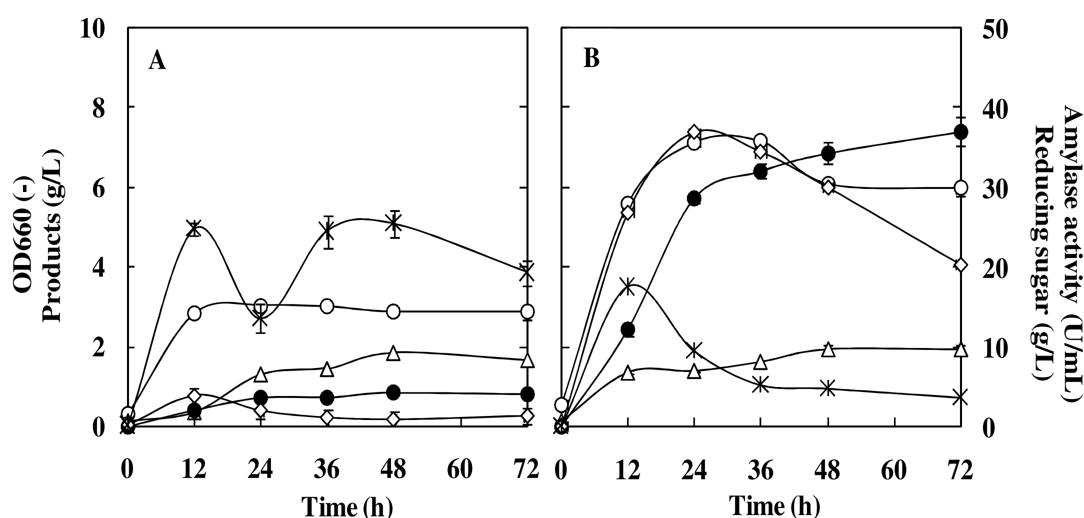

Figure 13. Growth and metabolic activity of pure culture of *C. butylicum* TISTR (A) 1032 and a co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 16 (B) incubated with 40 g/L cassava starch medium under condition without anaerobic pretreatment. Symbols: (○) OD_{660}; (◊) amylase activity; (*) reducing sugar; (Δ) butyric and acetic acid; (●) ABE. Values are means and standard deviations of duplicates.

3. Optimizing the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 for ABE production from cassava starch

The utilization of starch for ABE production by a *Clostridium* includes two processes, starch hydrolysis by amylolytic enzymes to produce glucose for the cells growth and production of acids (acidogenesis by acetic and butyric) and the conversion of these acids into ABE products (solventogenesis). These two processes are influenced by a number of factors e.g. the amylolytic activities and ABE producing capacity of *Clostridium* and especially the medium composition (starch concentration, nitrogen source, and C/N ratio). A high sugar concentration (160 g/L) has been shown to be toxic to clostridial cells (Jones and Woods, 1986); but with a low sugar concentration organic acid reassimilation is terminated due to an insufficient amount of energy-rich metabolites e.g. ATP or NADH (Shinto *et al*., 2007). With the presence of an excessive amount of nitrogen (corresponding to a low C/N ratio); carbon utilization is carried out more rapidly and completely, cells then can grow better. In contrast, the lack of nitrogen makes carbon utilization less effective. However, the relationship between the C/N ratio and ABE production is thought to be quite complicated; it has been found that better growth and ABE production was observed at a lower C/N ratio. At a higher C/N ratio (> 7.27), cell growth and ABE production were decreased (Lai and Traxler, 1994). On the other hand, the absolute concentrations of carbon and nitrogen were found to have more effect than the C/N ratio in the research of Madihah *et al*. (2000).

In our preliminary study, a mixed culture of *C. butylicum* TISTR 1032 with *B. subtilis* WD 161 for ABE production from soluble starch was successfully established under anaerobic condition without the addition of L-Cysteine or N_2 flushing. When *B. subtilis* WD 161 was co-cultured with *C. butylicum* TISTR 1032 the amylase activity was increased and ABE production of *C. butylicum* TISTR 1032 was enhanced up to 4.2 g/L compared to 0.94 g/L by the pure culture (Figure 11). This present work was designed to optimize ABE production by the mixed culture of these two organisms using cassava starch as a carbon source as this is highly available and of low cost in Thailand. Besides the important roles of starch concentration and C/N ratio on ABE production as mentioned above, the investigation of the ratio of yeast extract and ammonium nitrate was expected to obtain useful information for the strategy to reduce

medium costs. Even thought yeast extract will provide various amino acids, vitamins, minerals and factors that promote the growth of microorganisms, it was previously shown that the *Bacillus subtilis* grown in the presence of ammonium nitrate produced amylase under anaerobic conditions that directly influenced starch utilization and ABE production in the *Clostridium* mixed culture (Coleman and Elliott, 1961; Nakano *et al*., 1997; Clements *et al*., 2002).

3.1 Effect of cassava starch concentration

The ability of amylase and enhancement of ABE by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 when using cassava starch as carbon source was evaluated. The mixed culture was cultured with various cassava starch concentrations (from 20 g/L to 50 g/L) under condition without anaerobic pretreatment. The yeast extract and NH_4NO_3 were used as organic and inorganic nitrogen sources, respectively, with the ratio of 39.755/50.0 mM/mM (5.0/2.0 g/g). The results are shown in Figures 14-17 and Table 4. The activity of the amylase and presence of reducing sugar increased with increasing starch concentration (from 30.5 to 45.8 U/mL, and from 1.7 to 10.1 g/L, respectively). The highest amylase activity and reducing sugar level were obtained at 50 g/L starch concentration (45.8 U/mL and 10.1 g/L, respectively) (Figure 17). This would be because high starch concentration could induce the production of amylase and resulted in a high rate of hydrolysis of starch to sugar. It is well-known that higher amylase activity results in higher available sugar from starch for production of acids and ABE by the *Clostridium*. An excess of sugar is determined to be essential for the triggering and maintainance of ABE production (Long *et al*., 1984). With support from the high amylase activity in the mixed culture, the ABE production from cassava starch increased from 4.0 to 7.4 g/L when the starch concentration was increased from 20 to 40 g/L but a decrease in ABE production to 6.5 g/L was observed at 50 g/L starch concentration. The metabolism of *Clostridium* for ABE production involves first the acidogenic phase followed by a shift to the solventogenic phase. In the mixed culture, the most effective conversion of acids to ABE was observed at 40 g/L starch concentration. The high ABE production (7.4 g/L) correlated with a low accumulation of acids (1.93 g/L) at 40 g/L starch concentration (Figure 16).

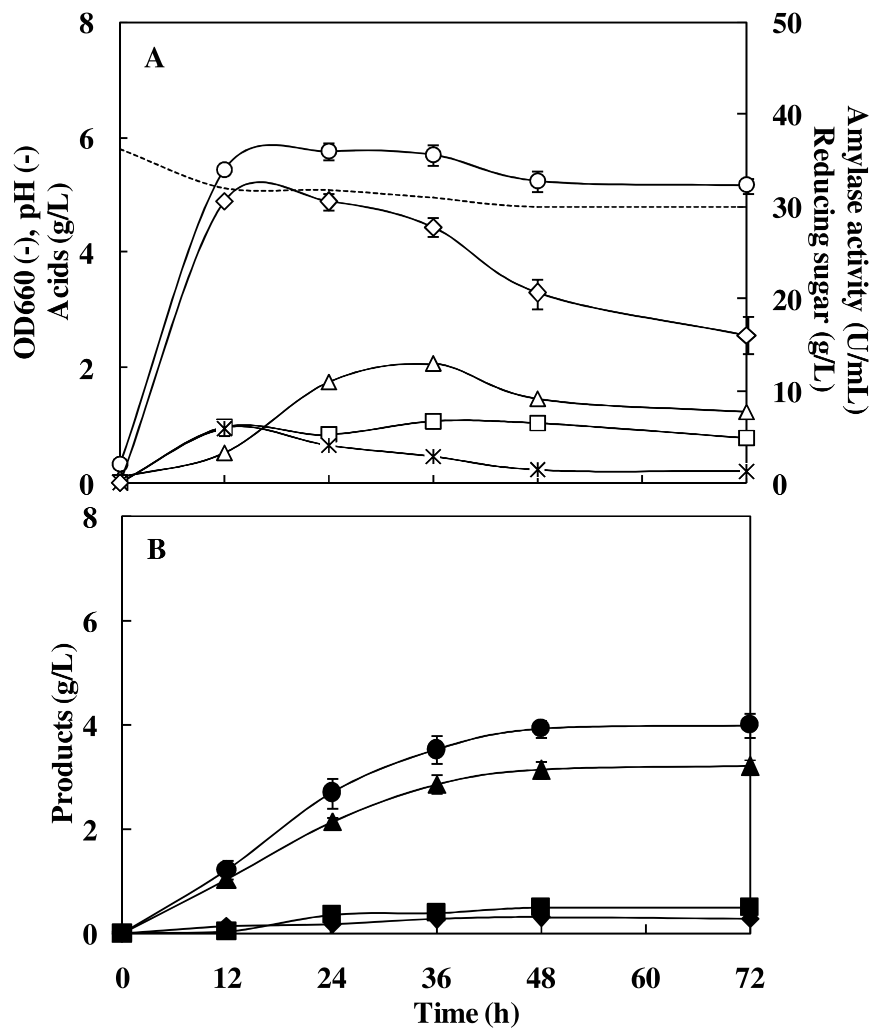

Figure 14. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at 20 g/L cassava starch. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (······) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicates.

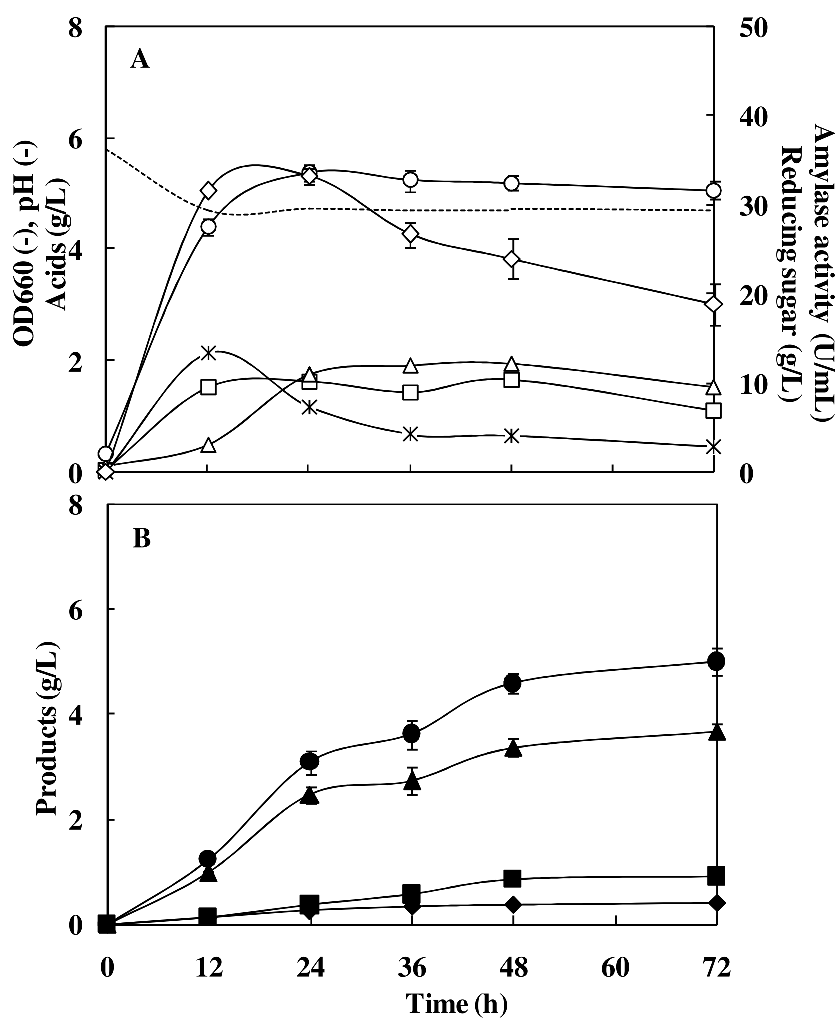

Figure 15. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at 30 g/L cassava starch. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (······) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicates.

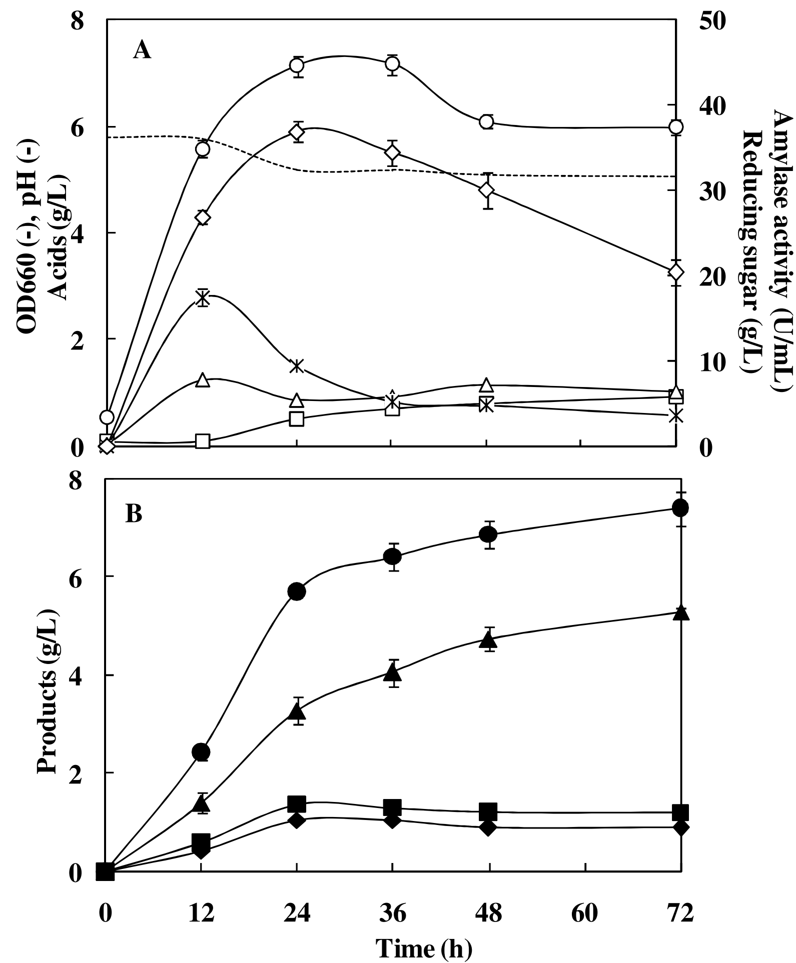

Figure 16. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at 40 g/L cassava starch. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (······) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicates.

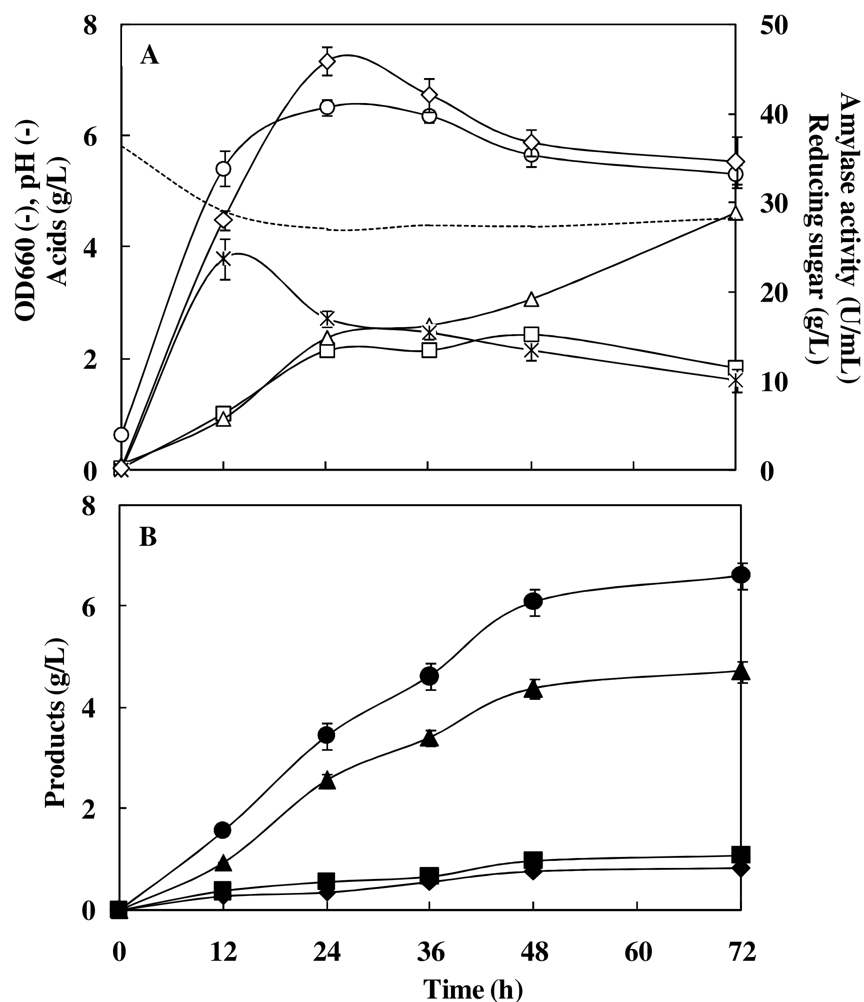

Figure 17. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 without anaerobic pretreatment at 50 g/L cassava starch. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (------) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicates.

Table 4. The performance of ABE production from cassava starch by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 with various culture conditions

Culture condition	Amylase activity[b] (U/mL)	ABE concentration[c] (g/L)	ABE productivity (g/L/h)	ABE yield (g/g)	Butanol Ratio[e] (-)
Starch conc. (g/L)					
20	30.5	4.01	0.082	0.200	0.80
30	33.2	4.99	0.104	0.166	0.74
40	36.9 (3.8)[d]	7.40 (1.1)[d]	0.103(0.01)[d]	0.18(0.02)[d]	0.72(0.78)[d]
50	45.8	6.59	0.092	0.131	0.72
C/N					
32	18.9	4.22	0.090	0.105	0.65
16	36.9	7.40	0.103	0.185	0.72
8	49.1	8.04	0.112	0.201	0.65
4	53.9	8.89	0.123	0.222	0.66
YE[a]/NH_4NO_3					
115/250	73.7	8.45	0.112	0.211	0.65
165/200	53.9	8.89	0.123	0.222	0.65
265/100	49.3	9.71	0.135	0.242	0.69
365/0	17.4	3.45	0.048	0.086	0.82
0/365	5.4	2.51	0.035	0.062	0.69

[a]Yeast extract/ NH_4NO_3 ratio (mM/mM).

[b]indicates the maximum values attained during the fermentation (at 24 - 36 h).

[c]indicates the final concentration at 72 h.

[d]values in parentheses are the results of the pure culture of *C. butylicum* TISTR 1032.

[e]indicates the ratio of butanol to total ABE at 72 h.

Values are means of at least duplicate experiments.

Although, a high concentration of reducing sugar at 50 g/L starch concentration enhanced acid production during the acidogenic phase, the conversion of acids to ABE during solventogenic phase was less effective and reflected in a low

consumption rate of the reducing sugar (Figure 17). This result indicated that the ABE production was not stimulated by the higher amount of acids. One possibility might be because the nitrogen source was used up during the high production rate of acids in the acidogenic phase and it was apparently insufficient for cells to further convert acids to ABE in the solventogenic phase. It was also reported that the high production rate and accumulation of an excessive amount of acids in the culture could be toxic to the cells leading to less sugar consumption and cell growth, hence reducing the total ABE production (Madihah *et al*., 2001; Ezeji *et al*., 2004). In the study of Madihah *et al.* (2001), they found that the ABE production appeared to depend on the amount of the undissociated form of the acids rather than the total amounts of acids.

Table 4 also shows that the amylase activity increased with the starch concentration. The ABE production in terms of the final concentration increased with increasing starch concentration and reached a maximum value at 40 g/L starch concentration. The highest ABE productivity at 30 g/L starch concentration was not much different from that at 40 g/L. It is interesting to note that the butanol ratio (the ratio of butanol to total ABE product) in this study was higher (from 0.71-0.8) compared to the commonly reported ratio (0.5-0.6). This means that butanol was the dominant component of the three fermentation products, acetone, butanol, and ethanol. Increasing the total ABE production without seriously interfering with the butanol ratio is a valuable property, because when butanol is dominant, the recovery of it from the culture will be easier to handle.

3.2 Effect of C/N ratio

It has been reported that the C/N ratio plays an important role in carbon utilization; at a low C/N ratio, carbon utilization is carried out more rapidly and completely, and cells can then grow better. In contrast, a high C/N ratio makes carbon utilization less effective. Generally, a low C/N ratio is required to achieve high levels of ABE production (Lai and Traxler, 1994). In order to investigate the effects of the C/N ratio on ABE productions by the mixed culture, the C/N ratio was varied from 32, 16, 8 and 4. The results are shown in Figures 18-20 and Table 4.

The amylase activity increased when the C/N ratio decreased from 32 to 4, as a result of the increase in nitrogen sources. It is well-known that enzyme

production strongly depends on the amounts of the nitrogen source. At the high C/N ratio of 32, low amylase activity consequently resulted in lower sugar consumption rate. Low ABE production at this high C/N ratio was clearly due to the deficiency of the nitrogen source that limited both the amylase activity and the ABE production. The increase in amylase activity with decreasing C/N ratio enhanced the sugar consumption rate and ABE production. The maximum ABE production was obtained at a C/N ratio of 4. The increased amount of nitrogen accelerated the carbon utilization (Figures 18-20). Although high amounts of ABE were obtained at this C/N ratio, high amount of acids also accumulated. The cessation of the conversion of acids to ABE might be due to the depletion of sugar resulting in an insufficient amount of energy-rich metabolites such as ATP or NADH. This result was consistent with the result of Shinto *et al.* (2007). Therefore, the right balance of carbon and nitrogen sources is necessary to further the conversion of acids to ABE. Another factor that affects the conversion of acids to ABE would be the amount of inorganic nitrogen. It has been reported that the anions from an inorganic nitrogen source such as NH_4NO_3 could be metabolized by *Clostridium* with a positive effect on the accumulated acids being converted to ABE (Welsh and Valiky, 1987). Although the highest butanol ratio was obtained at a C/N ratio of 16, the final concentration (7.4 g/L) and productivity of ABE (0.103) at this C/N ratio were lower than those (8.89 g/L and 0.123, respectively) from a C/N ratio of 4 (Table 4). Generally, the result of this research was coincided with that of Lai and Traxler (1994) which reported that butanol production was favored at low C/N ratio, and reduced at a C/N ratio above 7.23. However, Madihah *et al.* (2001) reported that the individual concentrations of nitrogen and carbon influenced ABE production by *C. acetobutylicum* to a greater extent than did the C/N ratio. As for the fermentation using a fixed concentration of starch (50 g/L), the ABE concentration decreased (26.9 to 2.63 g/L) along with the increasing of C/N ratio (3.6 to 42.8). In contrast, for a fermentation using a fixed concentration of nitrogen, ABE increased with increasing C/N ratio up to value of 20 and decreased slightly above this value (Madihah *et al.*, 2001).

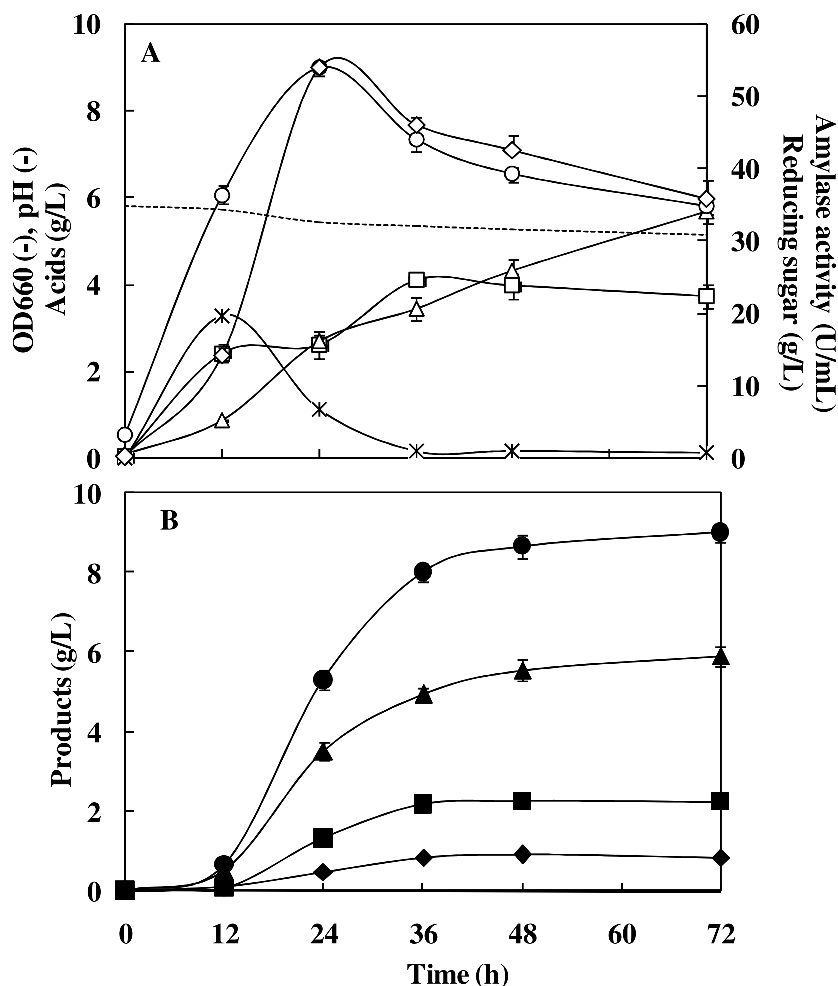

Figure 18. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a C/N ratio of 4. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (·····) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

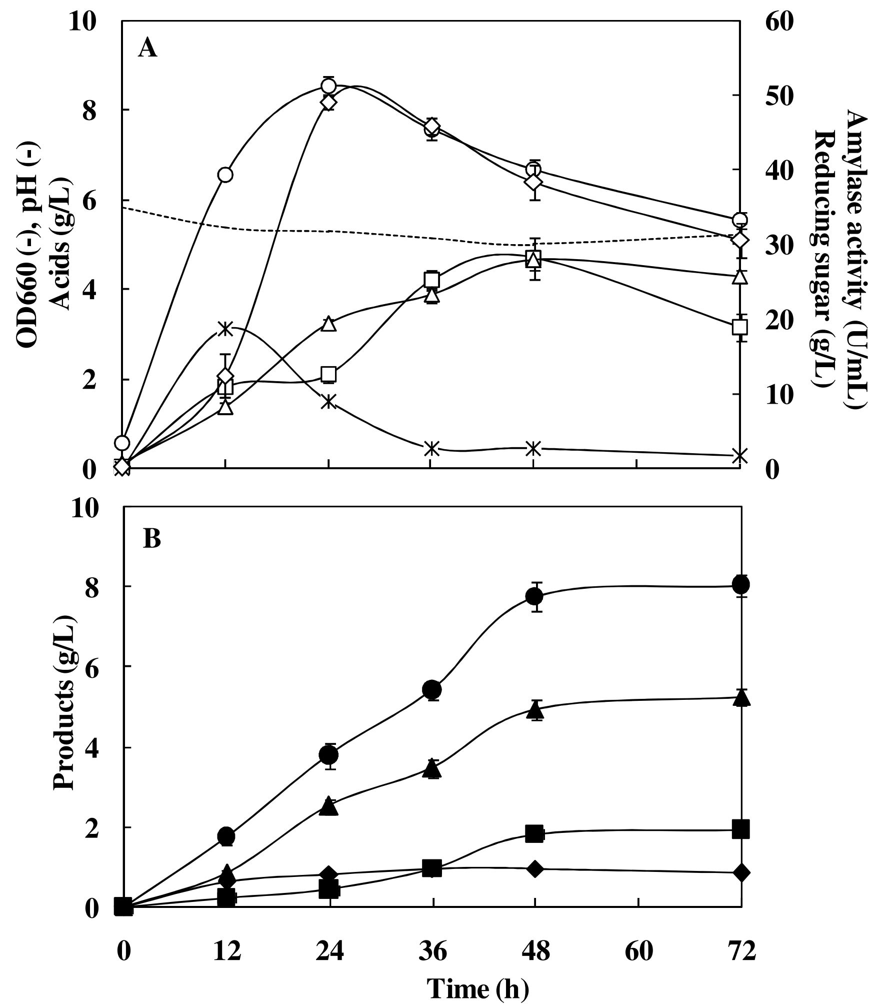

Figure 19. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a C/N ratio of 8. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (······) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

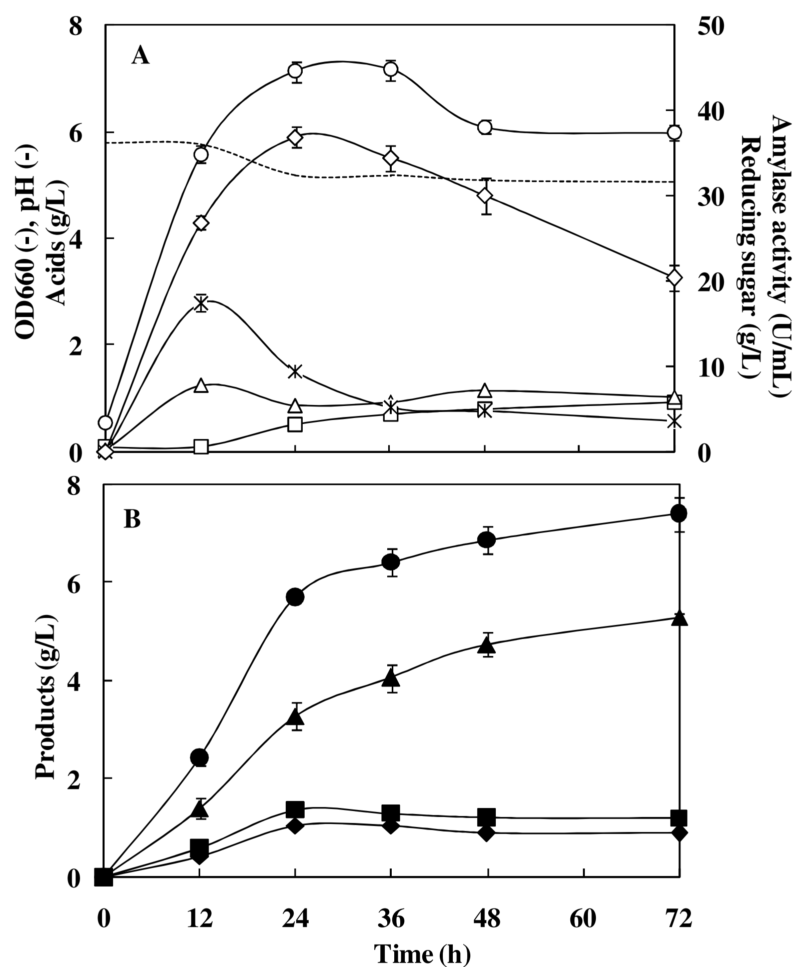

Figure 20. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a C/N ratio of 16. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (┈) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

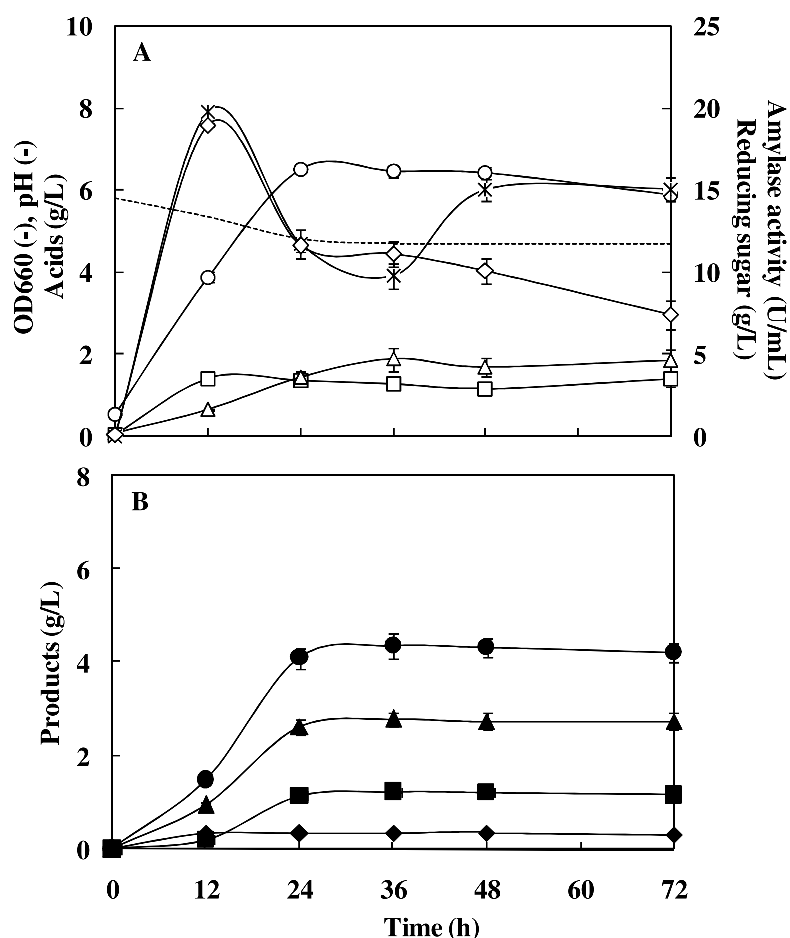

Figure 21. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a C/N ratio of 32. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (·····) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

3.3 Effect of organic and inorganic nitrogen sources

Yeast extract is well-known for providing various amino acids, vitamins, minerals and factors that promote the growth of microorganisms. Previously the presence of NH_4NO_3 was found to support growth and amylase production by *Bacillus*. In this study yeast extract and NH_4NO_3 were used as organic and inorganic nitrogen sources, respectively. A further investigation on the ratio of yeast extract to NH_4NO_3 was expected to determine the best ratio for optimum ABE production and also useful information that could reduce the costs since NH_4NO_3 is much less expensive than yeast

extract. The ratio of yeast extract to NH_4NO_3 was varied at 0/365, 115/250, 165/200, 265/100 and 365/0 mM/mM. The results are shown in Figures 21-24 and Table 4.

An increase in NH_4NO_3 enhanced amylase activity and sugar consumption. The use of yeast extract alone resulted in a low amylase activity and low sugar consumption and, consequently, low ABE production (Figures 22). The use of NH_4NO_3 only (365 Mm) was also found to have a negative effect on both amylase and ABE production (Figure 25). The final concentration and productivity of ABE increased with increasing yeast extract to 265 mM with the combination of 100 mM NH_4NO_3 (Table 4). This result was acceptable since yeast extract contains various amino acids, vitamins, minerals and growth factors that promote the growth of bacteria. On the other hand, NH_4NO_3 was previously found to enhance the growth and amylase production of *Bacillus*. This has direct effects on starch hydrolysis and the availability of sugar for growth and ABE production by *Clostridium*. Thus, a mixture of organic and inorganic nitrogen sources was required to maximize ABE production by the mixed culture of *Clostridium* and *Bacillus*. Although the 115/250 ratio of yeast extract/ NH_4NO_3 resulted in the highest amylase activity and production rate of acids, the ABE production was lower compared to the 265/100 ratio. These results could be explained by the lower yeast extract content reducing the ability of the microbial activity to further convert acids to ABE. The results of this present study coincide with those of other researchers that reported that using a mixture of yeast extract and NH_4NO_3 for the pure culture of *C. acetobutylicum* resulted in a lower accumulation of undissociated acids and increased the ABE production (18.78 g/L) about 4.5-fold higher over that using yeast extract alone (3.75 g/L) (Madihah *et al*., 2001). This result again demonstrated that the presence of NH_4NO_3 enhanced amylase production but also assisted with ABE production by the *Clostridium*. As the content of yeast extract increased so did the ratio of butanol to the other fermentation products increase. However although this ratio was highest (0.85) when using yeast extract alone the yields were then unacceptably low. The most suitable ratio of yeast extract/NH_4NO_3 in terms of the final concentration (9.71 g/L) and productivity of ABE (0.135) (Table 4) was 265 mM /100 mM, respectively.

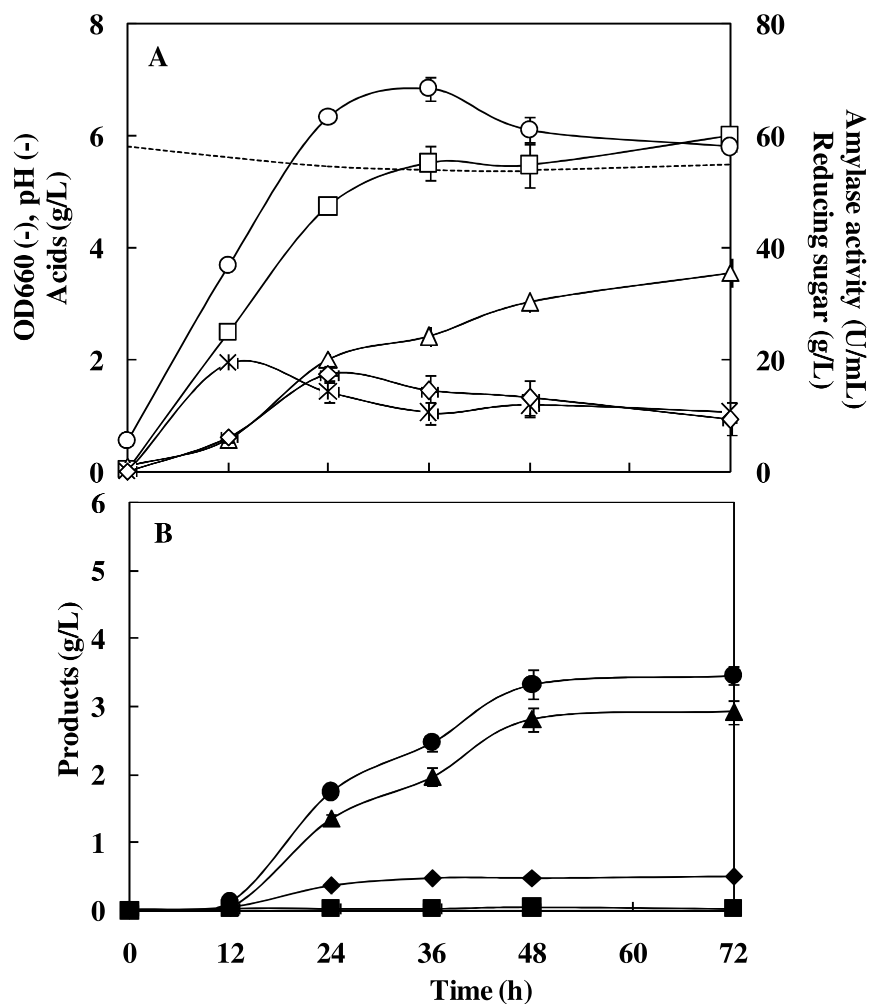

Figure 22. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a yeast extract/NH_4NO_3 ratio of 365/0. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

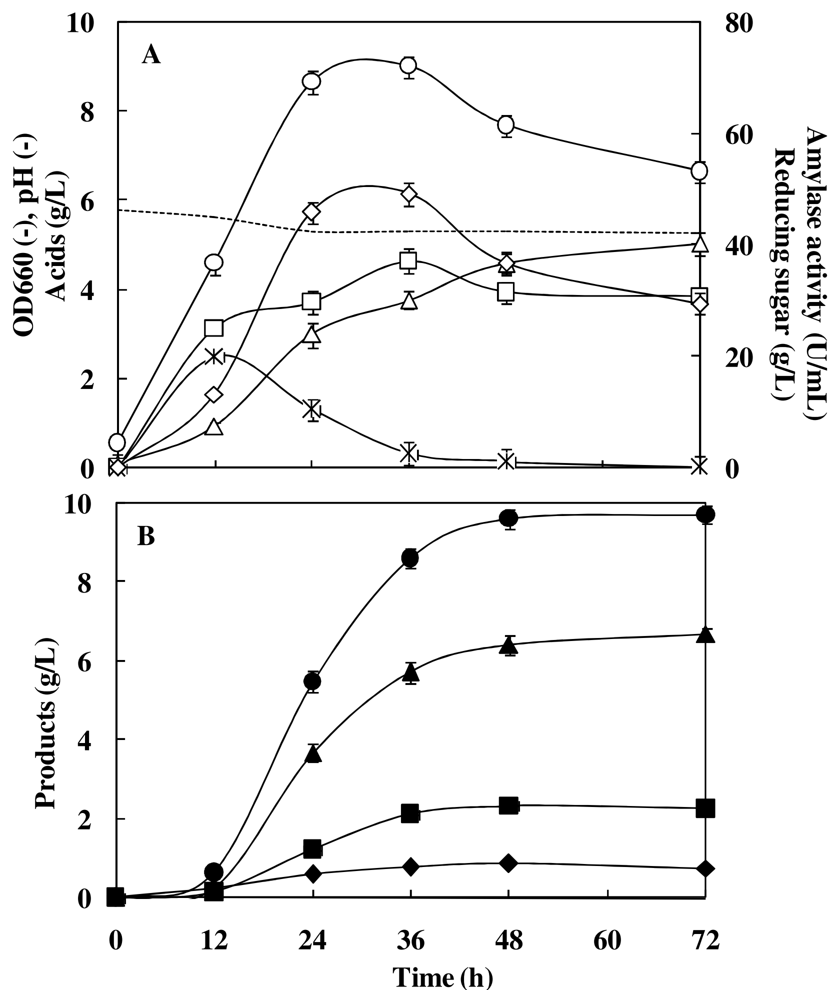

Figure 23. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a yeast extract/NH_4NO_3 ratio of 265/100. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (····) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

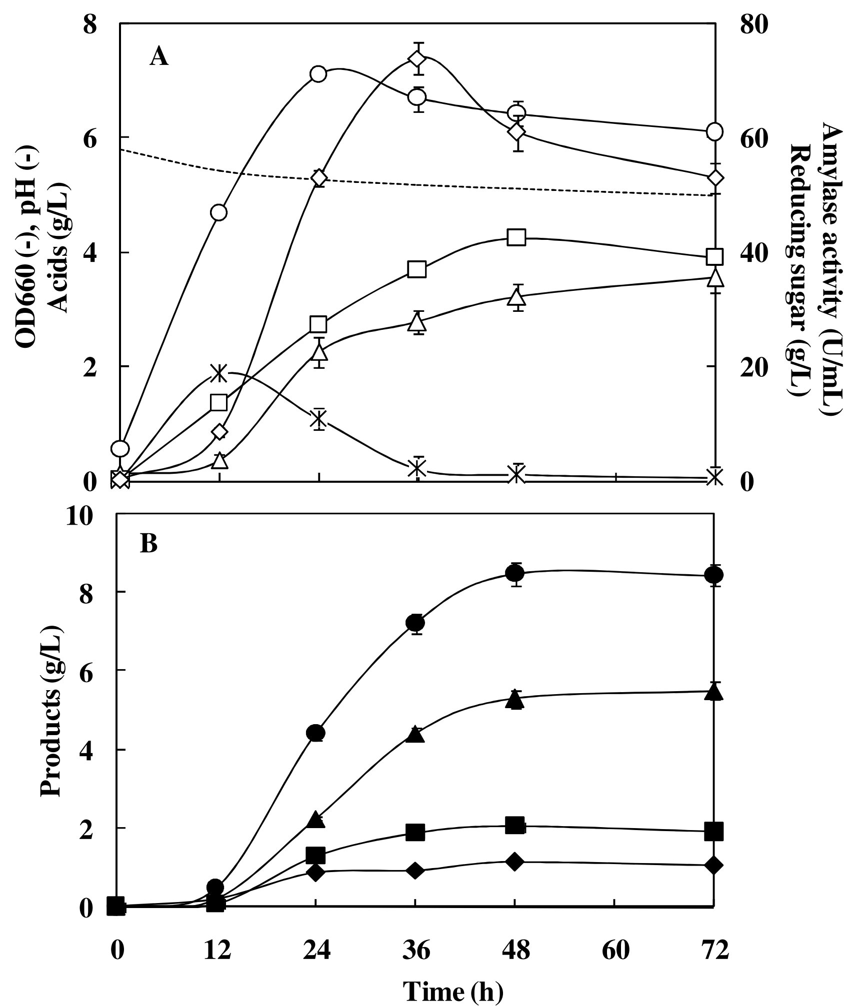

Figure 24. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a yeast extract/NH_4NO_3 ratio of 115/250. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

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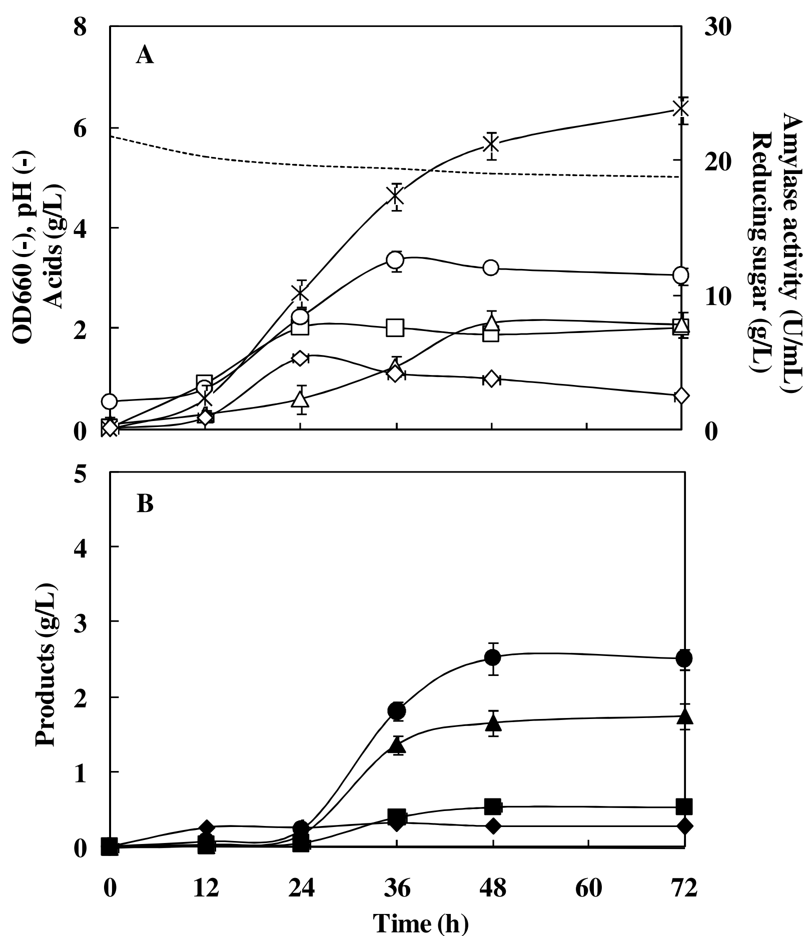

Figure 25. Growth and metabolic activity of mixed cultures of *C. butylicum* TISTR1032 and *B. subtilis* WD 161 without anaerobic pretreatment at a yeast extract/NH_4NO_3 ratio of 0/365. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (-----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. Values are means and standard deviations of duplicate experiments.

4. Optimizing a syntrophic co-culture of TISTR 1032 and *B. subtilis* WD 161 for ABE production from cassava starch using RSM

The utilization of starch for ABE production by *Clostridium* actually includes two processes, starch hydrolysis by amylolytic enzymes to produce glucose for cells growth and producing acids (acidogenesis, acetic and butyric) production and the conversion of these acids into ABE products (solventogenesis). Medium components such as the substrate or starch concentration, nitrogen source and content, have been reported to have profound effects on these two processes (Linden *et al*., 1985; McNeil and Kristiansen, 1986; Lai and Traxler, 1994; Madihah *et al*., 2001). Thus, optimizing the medium components to create the most favorable condition for substrate hydrolysis, acids and ABE production is extremely important.

The conventional method optimizes a process by changing one variable at a time and keeping the others at constant levels (Liu and Tzeng, 1998). Thus, any interactions between variables are not considered. This limitation can be overcome by using response surface methodology (RSM) where the combined effects of all variables are determined through mathematical and statistical inferences from experimental design to analysis of results (Silva and Roberto, 2001). The aim of this research was to determine the effect of each medium component as well as their interaction on amylase and ABE production from cassava starch by a syntrophic co-culture of *Clostridium butylicum* TISTR 1032 with amylase producing *Bacillus subtilis* WD 161 using RSM. It is known that starch concentration is important for the production of amylase and ABE. In addition to starch concentration, the combination of organic nitrogen source and inorganic nitrogen source is also important for enhancement of amylase and ABE production. In this study, yeast extract and ammonium nitrate were used as organic and inorganic nitrogen source, respectively. Yeast extract can provide various amino acids, vitamins, minerals and growth factors that promote growth of microorganisms. While, ammonium nitrate has been previously found to support growth and amylase production by *Bacillus* under anaerobic condition (Coleman and Elliott, 1961; Nakano *et al*., 1997; Clements *et al*., 2002). Thus, the effects of these three factors on ABE, butanol and amylase production were investigated using RSM.

4.1 Response surface methodology for optimizing co-culture of C. *butylicum* TISTR 1032 and *B. subtilis* WD 161

Experimental design

The effect of three variables including cassava starch concentration (x_1), yeast extract concentration (x_2) and ammonium nitrate concentration (x_3) with three levels (low: -1; medium: 0; and high: +1) for each variable were investigated. The Box-Behnken design was employed to study the interaction between these three variables; the complete design consisting of 15 trials in total each containing three replicates at the central point for estimating the purely experimental uncertainty variance (Table 5) (Box and Behnken, 1960). Responses under observation included total acetone-butanol-ethanol concentration (ABE) (Y_1), butanol concentration (butanol) (Y_2) and amylase activity (amylase) (Y_3). The experimental values were compared with the predicted values from the models**.** In addition, the butanol ratio was also calculated by Y_2/Y_1.

Statistical analysis

The response surface analysis was based on multiple linear regressions taking into account the main, quadratic and interactive effects, according to the following equation**:**

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x^2_i + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response; x_i and x_j are input variables that influence the response variable Y; β_o is the offset term; β_i is the i th linear coefficient*;* β_{ii} is the i th quadratic coefficient and β_{ij} is the ij th interaction coefficient.

Table 5. Experimental data for the three-factor with three level response surface analysis

Trial	Independent variables			Dependent variables			
	Cassava starch (g/L)	Yeast extract (g/L)	Ammonium nitrate (g/L)	ABE (g/L)	Butanol (g/L)	B ratio (-)	Amylase (U/mL)
	x_1	x_2	x_3	Y_1	Y_2	Y_2/Y_1	Y_3
1	1(60.0)	1(20.0)	0(7.0)	4.37	2.50	0.57	46.0
2	1(60.0)	-1(5.0)	0(7.0)	5.49	2.17	0.51	39.0
3	-1(20.0)	1(20.0)	0(7.0)	3.15	2.89	0.91	20.1
4	-1(20.0)	-1(5.0)	0(7.0)	3.20	2.50	0.78	25.6
5	1(60.0)	0(12.5)	1(12.0)	3.50	2.00	0.64	33.4
6	1(60.0)	0(12.5)	-1(2.0)	2.80	1.80	0.57	27.0
7	-1(20.0)	0(12.5)	1(12.0)	2.70	1.75	0.65	26.4
8	-1(20.0)	0(12.5)	-1(2.0)	1.92	1.81	0.94	15.0
9	0(40.0)	1(20.0)	1(12.0)	9.37	6.12	0.71	75.0
10	0(40.0)	1(20.0)	-1(2.0)	8.10	5.60	0.69	64.6
11	0(40.0)	-1(5.0)	1(12.0)	8.90	5.80	0.72	67.2
12	0(40.0)	-1(5.0)	-1(2.0)	6.70	5.00	0.75	36.0
13	0(40.0)	0(12.5)	0(7.0)	9.00	6.00	0.71	69.0
14	0(40.0)	0(12.5)	0(7.0)	9.05	5.90	0.69	67.3
15	0(40.0)	0(12.5)	0(7.0)	9.13	6.00	0.70	66.7

Note: values in parentheses are the un-coded independent variables.

x_1: cassava starch concentration; x_2: yeast extract concentration; x_3: ammonium nitrate concentration. Y_1: ABE; Y_2: butanol; B ratio: the ratio of butanol to total ABE; Y_3: amylase.

Responses surface plots for these three models were carried out by using the Statistica for Window version 5.0 by plotting as a function of two variables, while keeping other variables at the constant value. The combined effect of three variables; cassava starch concentration (x_1), yeast extract concentration (x_2) and ammonium nitrate concentration (x_3) on ABE, butanol and amylase production was examined by performing the 15 trials designed by the Box-Behnken. The experimental design and respective experimental results are given in Table 5. The regression coefficients (β) and analysis of variances are shown in Table 6. The polynomial equations for ABE (Y_1), butanol (Y_2) and amylase production (Y_3) are listed as follows:

$$Y_1 = -6.6998 + 0.6820x_1 - 0.0404x_2 + 0.2652x_3 - 0.0078x_1^2 + 0.0011x_2^2 - 0.0086x_3^2 + 0.0018\, x_1x_2 - 0.0011x_1x_3 - 0.0020\, x_2x_3$$

$$Y_2 = -4.2271 + 0.4091x_1 + 0.0614x_2 + 0.2360x_3 - 0.0049x_1^2 - 0.0027\, x_2^2 - 0.0137\, x_3^2 + 0.0006\, x_1x_2 + 0.0003\, x_1x_3 + 0.0008\, x_2x_3$$

$$Y_3 = -89.0804 + 6.0833x_1 - 0.7859x_2 + 5.3586x_3 - 0.0784\, x_1^2 + 0.0247x_2^2 - 0.1536x_3^2 + 0.0541\, x_1 x_2 - 0.0254\, x_1x_3 - 0.1524\, x_2 x_3$$

Generally, the adequacy of a model is determined through R^2 (multiple correlation coefficient), CV (coefficient of variation) and P value. R^2 values closer to 1 denote better correlation between the experimental and predicted values. As shown in Table 5 these three models above are adequate since their R^2 values were found to be close to 1: 0.99, 0.97, and 0.98, respectively, for models of ABE, butanol and amylase indicating that 99 %, 97% and 98% of the variability in the response could be explained by the model of ABE, butanol and amylase, respectively. The CV value indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV (> 20). In the present case, acceptable CV values were observed for the model of ABE, butanol and amylase (6.7, 10.1 and 10.3, respectively) denoting that the experiments performed are reliable. The P values of these three models were ≤ 0.05 to indicate the significance of the coefficients.

Table 6. Regression of coefficients and analysis of variance of the second order polynomial for response variables

Coefficient	ABE (g/L) Y_1	Butanol (g/L) Y_2	Amylase (U/mL) Y_3
β_o	- 6.6998*	- 4.2271*	- 89.0804*
Linear			
x_1	0.6820*	0.4091*	6.0833*
x_2	- 0.0404	0.0614	- 0.7859
x_3	0.2652	0.2300	5.3586*
Interaction			
x_1x_2	0.0018	0.0006	0.0541*
x_1x_3	- 0.0011	0.0003	- 0.0254*
x_2x_3	- 0.0020	0.0008	- 0.1524
Quadratic			
x_1^2	- 0.0078*	- 0.0049*	- 0.0784*
x_2^2	0.0011	- 0.0027	0.0247
x_3^2	- 0.0086	- 0.0137	- 0.1536
Variability			
R^2 of model	0.99	0. 97	0.98
F value of model	63.17	4.19	29.95
$P > F$	0.016	0.002	0.032
CV of model	6.7	10.1	10.3

x_1, x_2, x_3 are cassava starch, yeast extract and ammonium nitrate concentrations, respectively.* means significant at 5% level.

In terms of determination of the interaction between the variables, *P* values can provide understanding of the pattern of the mutual interactions between the variables, as well as the effect of each variable on the investigated responses. Further statistical analysis showed that only cassava starch concentration (x_1) had a significant effect on all the responses ($P<0.05$). In the case of amylase production, besides cassava starch concentration, ammonium nitrate (x_3) also had a significant effect ($P<0.05$). The quadratic effect of cassava starch concentration (x_1^2) also had a significant effect for the three investigated responses. Among those significant affecting terms, cassava starch concentration (x_1) had the largest effect which is referred to as the highest value of the coefficient (for ABE, butanol, and amylase production were 0.6500, 0.4091 and 6.0833, respectively). A high value of coefficient for ammonium nitrate (x_3) was also observed for amylase production (5.3587). In addition, the interactive terms of x_1x_2 and x_1x_3 were found to be significant for amylase production (Table 6). In the research of Bard and Hamdy (1992) where RSM was employed to investigate the interactive effect of a number of medium components for ABE production by *C. acetobutylicum* P262, the obtained statistical analyses indicated that the concentration of starch and calcium carbonate significantly affected yields and productivities, while phosphate and nitrogen did not.

4.2 Optimal condition for ABE production

The effect of starch concentration on ABE, butanol and amylase were studied using a response surface plot (Figures 26-28). When the concentration of starch was increased higher than the optimal level, a reduction in ABE, butanol and amylase were observed. This might be due to the high viscosity of the culture and it may hinder mass transfer for enzyme hydrolysis and microbial reactions (Bard *et al*., 2007; Madihah *et al*., 2001). The production of ABE from starch includes two steps, starch hydrolysis by amylolytic enzymes to produce glucose for cells growth and acids production during acidogenesis phase and the conversion of these acids into ABE products during solventogenesis phase. Therefore, the amylolytic enzymes were determined to be a key factor in ABE production (Lin and Blaschek, 1983). When the amylase activity in the culture was high, starch hydrolysis would be more complete. The availability of sugars for cell growth, acids and ABE production would also be high. It

was reported that maize, potato, sago and tapioca starch at a concentration of 50 g/L was optimum for solvent production by a pure culture of *C. acetobutylicum* (Linden *et al*., 1985; Madihah *et al*., 2001). At lower concentrations of starch less sugar is available for cells due to the lack of carbon, but higher starch concentrations causes a high viscosity of medium that hinders amylolytic enzymes excretion and proper function. Moreover, it was also found that a higher starch concentration caused a higher accumulation of organic acids that would cause toxicity to cells (Madihah *et al*., 2001).

The interactive effects and optimal levels of cassava starch, yeast extract, and ammonium nitrate concentration were determined by plotting the response surface curves. Based on the statistical analysis, among the three investigated variables, yeast extract had the least effect on all responses (Table 6). Thus, response surface curves of two variables, cassava starch and ammonium nitrate concentrations were plotted by fixing the yeast extract concentration at three values in the selected range (5.0, 12.5 and 20 g/L). The shapes of the response surface curves showed a positive interaction between the two variables on the production of ABE, butanol and amylase. These products were found to increase with a simultaneous increase of both variables. In the ABE fermentation, acetone, butanol, and ethanol are normally produced in the ratios of 2:3:1 (Jones and Woods, 1986). Increasing the ABE concentration without any reduction in the proportion of butanol is the target for most optimizing work on the ABE fermentation process. When butanol is present as the major product in the culture, the recovery process is much easier to handle. Thus, in this research in addition to the total ABE production, the butanol concentration was also taken into account for the selection of the optimal conditions that provided both high ABE and butanol concentrations, namely a high butanol ratio.

Figure 28 shows that the amylase activity increased with increasing yeast extract concentration. Although, the highest amylase activity (55 U/mL) was achieved at 20 g/L of yeast extract, a comparable level of amylase activity (46 U/mL) was also obtained at a 5 g/L yeast extract concentration by increasing the amount of ammonium nitrate to 14 g/L. This result indicates that at the 5 g/L yeast extract concentration the synergic effect of cassava starch and ammonium nitrate were more obvious than those at 12.5 and 20 g/L yeast extract concentration. This also means that at high yeast extract concentration, the amylase production mostly depends on the concentration of cassava

starch. The ABE and butanol production was also increased in line with the increasing ammonium nitrate concentration. This is probably due to the effect of it on the growth and amylase production of the *Bacillus* in the mixed culture. However, it is noted here that even though the amylase concentration increased along with the increase in ammonium nitrate, a concentration of ammonium nitrate higher than 8 g/L no enhanced effect was observed on either ABE or butanol concentrations. This is possibly due to a requirement to limit the amount of amylase for the optimum ABE or butanol production. It was found that the presence of ammonium nitrate supported the growth of *B. subtilis* under anaerobic condition. It has also been reported that, NO^{-}_3 could replace oxygen as an electron acceptor in the absence of oxygen (Coleman and Elliott, 1961; Nakano *et al*., 1997; Clements *et al*., 2002).

In addition to the information on the interaction between the studied variables, results that come from this RSM study would also provide necessary information to determine the optimal process for producing product and for economic purposes. The finding that the ABE and butanol production were mainly influenced by the concentration of cassava starch rather than yeast extract or ammonium nitrate was important for a strategy to maximize ABE and butanol production with minimum cost. As the adjustment of medium composition could be focused only on cassava starch. Then, the concentration of ammonium nitrate would be adjusted in the range suitable for the achievement of a sufficient amylase concentration. Additionally, RSM provided not only an optimal point, but also an optimal area from which the suitable point from the economic view point could be selected. Figures 26 and 27 show that the maximum ABE and butanol concentrations obtained in the selected range of yeast extract concentration (5.0, 12.5 and 20 g/L) were not significantly different. This result indicated that the maximum ABE and butanol could be achieved by varying only two variables, cassava starch and ammonium nitrate concentration and fixing yeast extract concentration at minimum level (5 g/L).

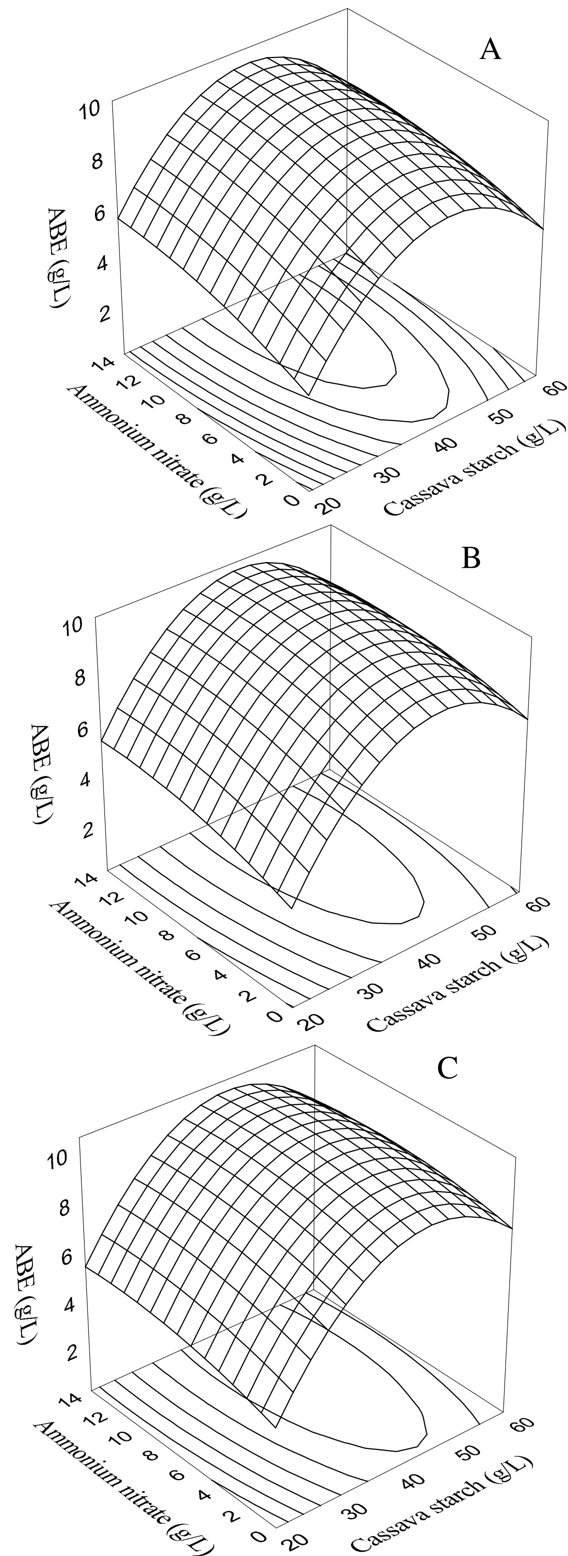

Figure 26. Response surface plots representing the interaction between cassava starch and ammonium nitrate concentrations on ABE production at a given yeast extract concentration: 5 g/L (A), 12.5 g/L (B) and 20 g/L (C).

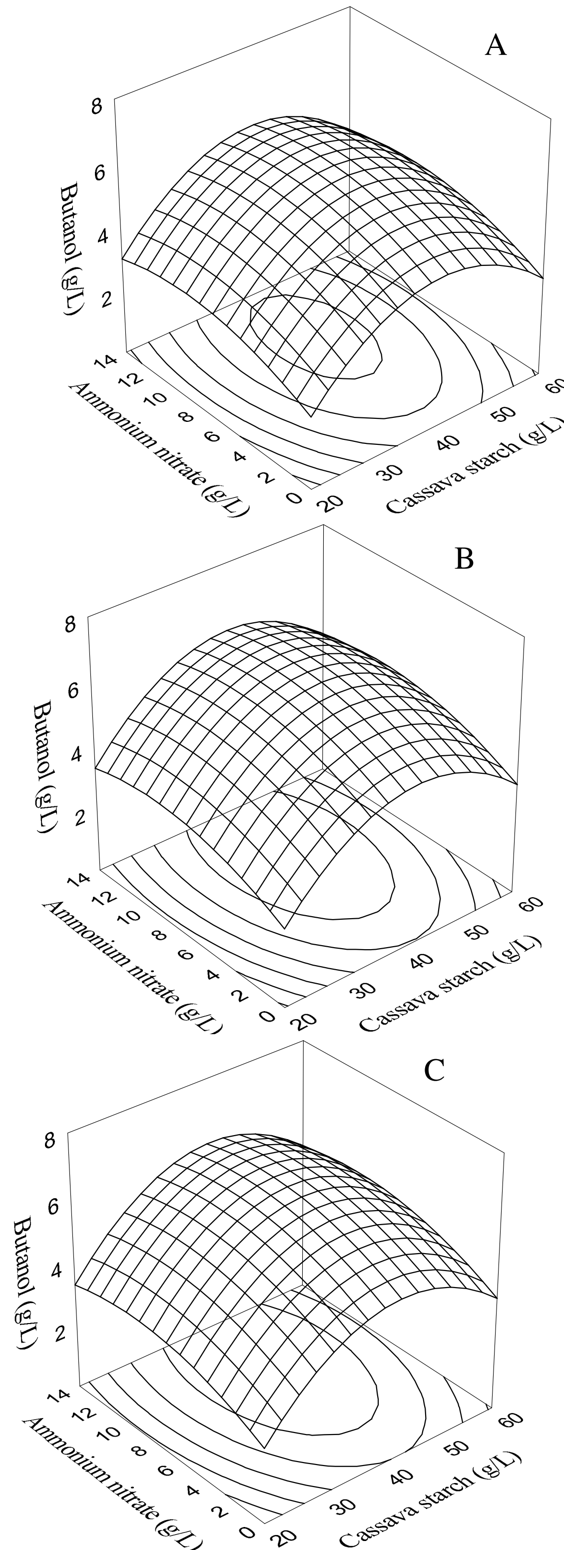

Figure 27. Response surface plots representing the interaction between cassava starch and ammonium nitrate concentrations on butanol production at a given yeast extract concentration: 5 g/L (A), 12.5 g/L (B) and 20 g/L (C).

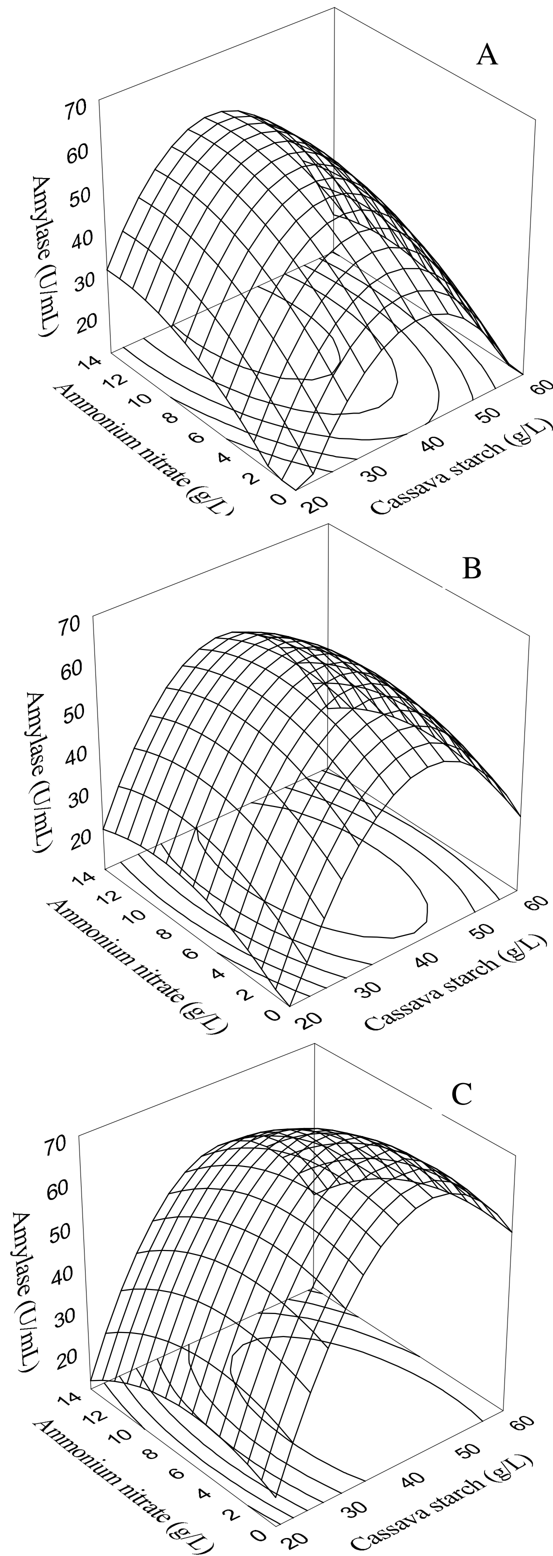

Figure 28. Response surface plots representing the interaction between cassava starch and ammonium nitrate concentrations on amylase production at a given yeast extract concentration: 5 g/L (A), 12.5 g/L (B) and 20 g/L (C).

To optimize both ABE and butanol production using RSM, superimposing performance in the optimal area for ABE and butanol production using the Lotus Freelance Graphics at 5 g/L yeast extract concentration was carried and the optimal points for both ABE and butanol production were in the center of an overlapping area as shown in Figure 29. The superimposed graph revealed that the conditions for the optimum productions of ABE and butanol were over a large range. The central point of this area with 40 g/L cassava starch, 5 g/L yeast extract, and 8 g/L ammonium nitrate was therefore selected for maximizing both ABE and butanol production.

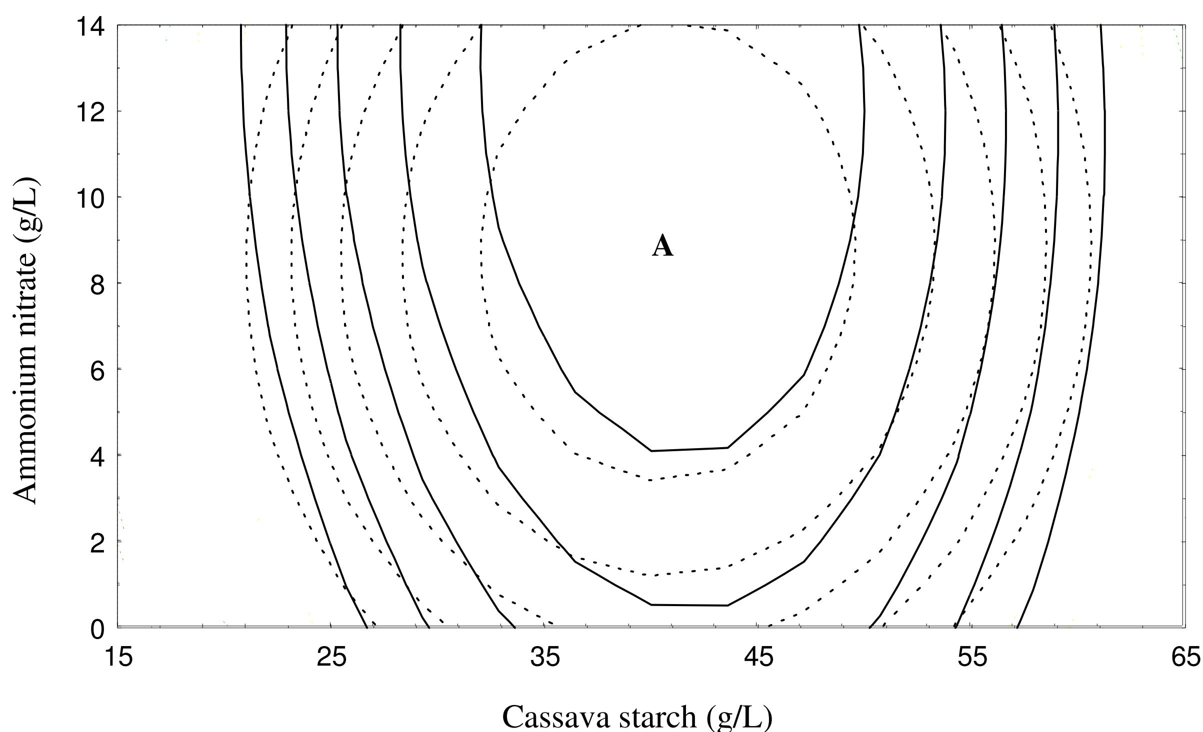

Figure 29. A superimposed graph of the optimal areas for ABE and butanol production. ABE production (solid line), butanol production (dashed line). The center of the overlapping area is the optimum point for ABE and butanol productions (solid line). The central point A contains 40 g/L cassava starch, 5 g/L yeast extract, and 8 g/L ammonium nitrate.

The predicted conditions were tested. The conditions and responses obtained from the predicted conditions are shown in the Table 7. The low value of CV indicated a close correlation between the experimental and predicted values. The optimum condition for ABE, butanol and amylase production by the mixed culture of *C. butylicum* TISTR 1032 and *Bacillus subtilis* WD 161 was cassava starch concentration (40 g/L), yeast extract concentration (5 g/L) and ammonium nitrate concentration (8.0 g/L) at which 9.02, 5.60 g/L ABE and butanol, respectively and 56.70 U/mL amylase were obtained.

Table 7. Predicted and observed values for the independent variables

Response	Predicted value	Observed values ± SD	CV
ABE (g/L)	9.43	9.02 ± 0.17	1.92
Butanol (g/L)	5.80	5.60 ± 0.13	2.37
Amylase (U/mL)	55.0	56.7 ± 6.70	13.4

Note. The central point with 40 g/L cassava starch, 5 g/L yeast extract, and 8 g/L ammonium nitrate were replaced into the polynomial equations for predicted values of ABE (Y_1), butanol (Y_2) and amylase (Y_3). The observed values are the values obtained from results of experiment carried out in this condition.

Both the two optimum conditions contained the same amount of cassava starch (40 g/L). However, the optimum condition obtained from the result of conventional method was different from that of the RSM by having higher amount of yeast extract and smaller amount of ammonium nitrate. The ABE production from the culture employing maximum condition obtained by the conventional method was higher than that of the RSM, although amylase production from the RSM condition was higher. The higher amount of ABE production would be explained by the presence of high amount of yeast extract, and the higher amylase activity in the culture employing RSM optimum condition would be explained by higher amount of ammonium nitrate. Yeast extract can provide various amino acids, vitamins, minerals and growth factors that promote growth of microorganisms. While, ammonium nitrate has been previously

found to support growth and amylase production by *Bacillus* under anaerobic condition (Coleman and Elliott, 1961; Nakano *et al*., 1997; Clements *et al*., 2002).

Table 8. Comparison in amylase, ABE and butanol production at optimum condition obtained by conventional method and RSM

Method	Optimum medium component s (g/L)			Product	
	Cassava starch	Ammonium nitrate	Yeast extract	ABE (g/L)	Amylase (U/mL)
RSM	40	8	5	9.02	56.7
Conventional	40	2	32	9.71	49.3

The synergic interaction between cassava starch and ammonium nitrate found by the RSM method allowed the achievements of maximum production without requiring a high amount of yeast extract concentration. This is an economic benefit of using RSM method for optimization.

5. Process optimization for ABE production by a mixed culture system of *C. butylicum* and *B. subtilis* using cassava starch and its pulp waste

pH plays an important role in the solventogensis process. Solventogenesis is said to be triggered when undissociated butyric acid reaches a critical concentration. High pH values favoured acid production and lower pH values could stimulate ABE production (Jone and Wood, 1986). In addition, amylase which was the key factor in ABE production from starch by *Clostridium* (Madihah *et al*., 2001), was reported to be considerably influenced by the pH of the culture. An increase of pH from 4.4 to 5.2 led to a remarkably higher production of amylase (Soni *et al*., 1992) and an amylase was also found to exhibit maximal activity at pH 6.5 and 30°C without the presence of calcium (Shih and Labbe, 1995).

Carbon concentration plays an important role in ABE production. When the carbon concentration is high (glucose concentration is above 160 g/L) that could inhibit cell growth, amylase, acids, and ABE production, but when the carbon is limited

(e.g. glucose is below 7 g/L), only acids are formed (Jone and Woods, 1986). It has been suggested that using a high substrate concentration would increase productivity, and shorten fermentation time. However, in the case of the substrate being starch, high starch concentration causes higher viscosity that hinders amylase excretion and ABE production (Madihah *et al*., 2001). To maintain an appropriate amount of substrate in the culture for cell growth, amylase and ABE production over the fermentation process, feeding substrate is required.

Product inhibition is among the major factors causing limitations for the butanol market. In the case of a pure culture of *Clostridium*, normally when butanol in the culture reaches 13 g/L or ABE concentration is more than 20 g/L, the conditions will be toxic to cells and the fermentation process will cease. Thus, removal of butanol from the culture will be advantageous. For the case of a mixed culture, there has not been any report on the critical amount of ABE that causes toxicity for the cells. Among the available techniques for solvent recovery, gas stripping seems to be more beneficial due to a number of advantages over the other techniques e.g. easy to handle, and more economically beneficial (Ezeji *et al*., 2004). Also nutrients and reaction intermediates are in this case not removed from the reaction mixture.

Though, cassava starch is readily available at low cost in Thailand in particular and in Asia in general, in the long term the use of a cassava crop for ABE production may not be practical, because this crop is also an important source of food, animal feed, and material for production of other products e.g. enzymes, antibiotic and ethanol. Using cassava waste from manufacturers that use cassava for other processes as substrates for ABE production is a promising alternative (Kosugi *et al*., 2008). In this context, cassava pulp waste has also been evaluated as feedstock for butanol production due to its high availability from the large number of cassava starch factories in Thailand and Asia (approximately one million ton generated from cassava starch factories per year). Cassava pulp waste was obtained from Srima Inter Product Co., Ltd, located in Cha-Choeng Sao province, Thailand. The waste normally contains about 60.6% cassava starch, and about 29% non-starch polysaccharides of which 20% is cellulose (Kosugi *et al*., 2008). Besides starch, cellulose in cassava pulp waste can also be a carbon source for butanol production if provided with appropriate enzymes. Therefore cassava pulp waste was employed in two ways that included direct use, and pulp waste pretreated

hydrothermally and enzymatically before use for ABE fermentation. In this study, an ABE production process from cassava starch by mixed culture of an oxygen tolerating *Clostridium* (*C. butylicum* TISTR 1032) and a amylase producing *Bacillus* (*B. subtilis* WD 161) was optimized by employing pH control, fed-batch, and product recovery by gas stripping. The optimum system was then applied for ABE production from cassava pulp waste.

5. 1 A mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 for ABE production

The mixed culture of *B. subtilis* WD 161 with *C. butylicum* TISTR 1032 were established under conditions without anaerobic pretreatment compared to the pure cultures of *C. butylicum* TISTR 1032 using optimum conditions obtained from the RSM study. The fermentation was carried out in a 100 mL serum bottle with 90 mL working volume, at 37°C. Optimized medium components (40 g/L cassava starch, 5 g/L yeast extract, and 8 g/L ammonium nitrate, pH 6.5) obtained from the previous RSM study was employed. The medium was stirred at 120 rpm using a magnetic bar. The time course of OD_{660}, amylase activity, reducing sugars, acids and ABE production are shown in Figure 30.

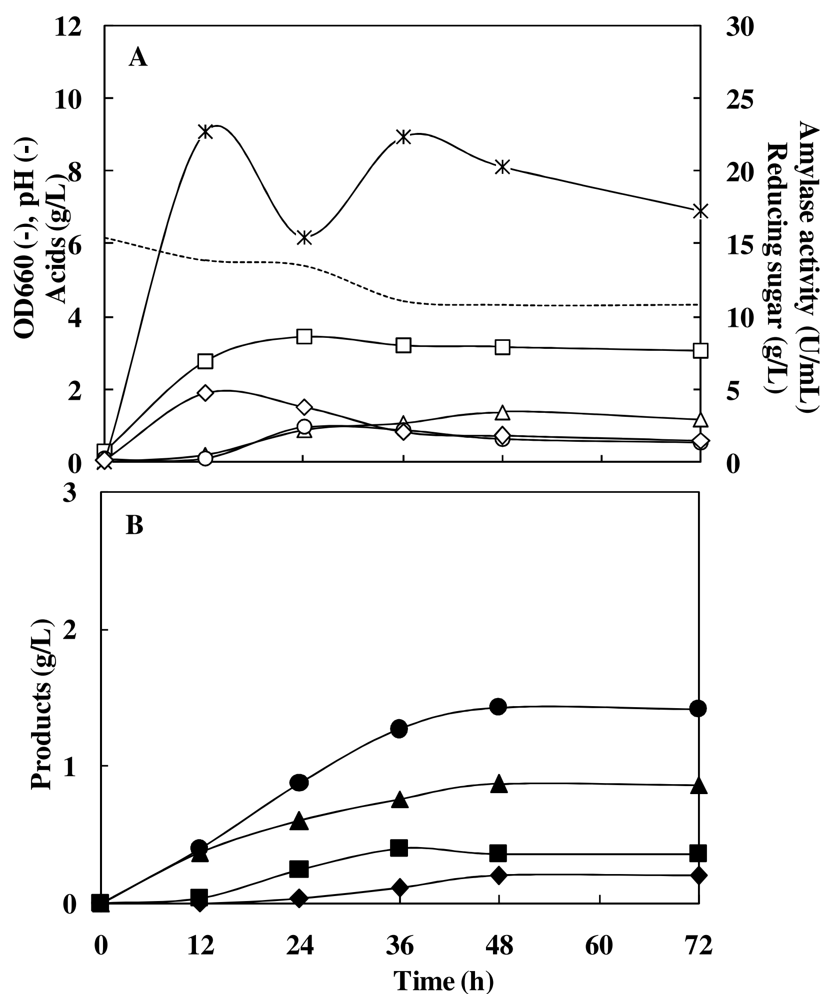

Figure 30. Growth and metabolic activity of pure culture of *C. butylicum* TISTR 1032 **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (-----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

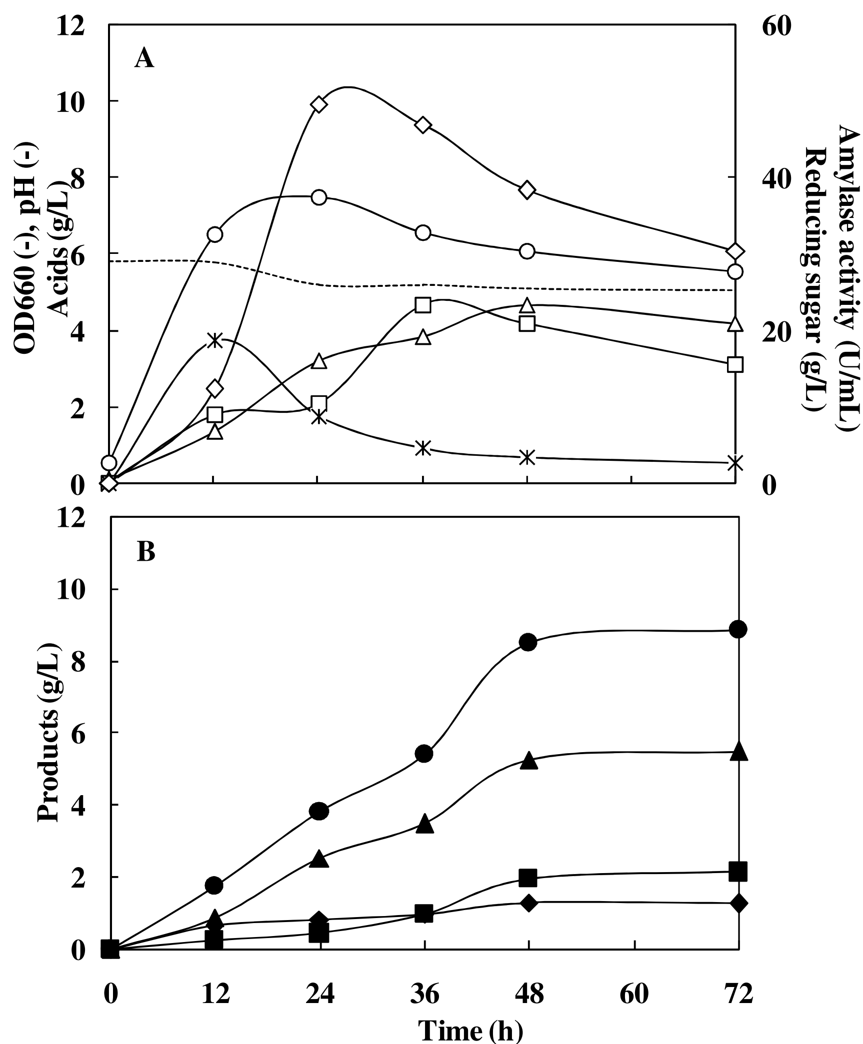

Figure 31. Growth and metabolic activity of co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (------) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

The mixed culture of *Clostridium* and *Bacillus* performed better growth and amylase production than the pure culture of *Clostridium*. The maximum amylase production was 50 U/mL or about 10 fold that of the pure culture (Figure 31). The substrate utilization in the mixed culture was found to be more complete than in the pure culture. The residual reducing sugars were 17.2 g/L and 2.77 g/L, respectively for the pure culture of *Clostridium* and mixed culture of *Clostridium* and *Bacillus*. This is probably due to the difference in amylase production. The low amylase production in the pure culture of *Clostridium* might not be sufficient to convert cassava starch into ready to use sugars. But, the high amylase activity in the mixed culture might rapidly convert starch to available sugar that soon stimulated the metabolism of *Clostridium* to grow and enhanced ABE production. Consequently, the mixed culture produced much higher amounts of ABE (9.02 g/L) or about 6.3 fold higher than that of the pure culture. The results showed that in the mixed culture without anaerobic pretreatment *Bacillus* plays an important role on the enhancement of *Clostridium* by providing high amylase production in the culture to create more available substrate for *Clostridium*. The butanol ratio of the mixed culture was 0.62 which is higher than the common ABE ratio from a pure culture of *Clostridium* reported in literature (2:3:1) (Parekh *et al*., 1999). Since butanol was the dominant product in the mixed culture, product recovery would be much easier to handle. This is also another advantage of the mixed culture. In the next study, the mixed culture was scaled up and optimized for enhancement in ABE production.

5.2 ABE production by using mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in 1 L anaerobic bioreactor

In this study, mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 was scaled up in a 1 L anaerobic bioreactor which was connected with a pH probe and temperature was controlled at 37°C. The optimum medium obtained from RSM study was employed (1 L medium contains: 40 g/L cassava starch; 5 g/L yeast extract; 8 g/L ammonium nitrate; 0.5 g KH_2PO_4; 0.3 g $MgSO_4{\cdot}7H_2O$; 0.02 g $MnSO_4{\cdot}7H_2O$; 0.02 g $FeSO_4{\cdot}7H_2O$; 0.02 g NaCl); the medium was stirred at 120 rpm using a magnetic bar. The obtained results are shown in Figure 32. Compared to the small scale (100 mL serum bottle) of the mixed culture, OD, amylase, butanol and ABE

productions at this scale (6.7, 46 U/mL, 5.0 g/L and 7.9 g/L, respectively) were slightly smaller than those of the small scale (the amylase, butanol and ABE production at small scale was 7.5, 50.0 U/mL, 5.47 g/L and 8.85 g/L, respectively. The butanol ratio was not significantly different at 0.615 for the small scale and 0.62 for the large scale. Additionally, the fermentation time at the large scale was also approximately the same as with the small scale (72h). Notably, the amount of reducing sugars in the large scale (5.0 g/L) was remarkably higher than that at the small scale (2.8 g/L). The reduction of substrate utilization and products concentration when scaling up is probably due to less effective cells dispersion throughout the culture during the fermentation process and this may hinder metabolic activities of the cells (Ezeji *et al*., 2005 b; Hipolito *et al*., 2007).

Generally, the performance of the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in the anaerobic bioreactor was consistent with that in the serum bottle. This indicates that the anaerobic bioreactor is usable for the larger scale study. However, either at the small or large scale, the accumulation of acids, especially butyric, in the culture was considerably high (4.2 and 4.7 g/L for small scale and large scale, respectively). This information indicated that the conversion of butyric into butanol was less effective and also most of the energy might be used for acids formation rather than butanol production. Producing a further conversion of these acids to ABE products was needed pH control, fed-batch, and product recovery were modified for enhancement of ABE production by the mixed culture.

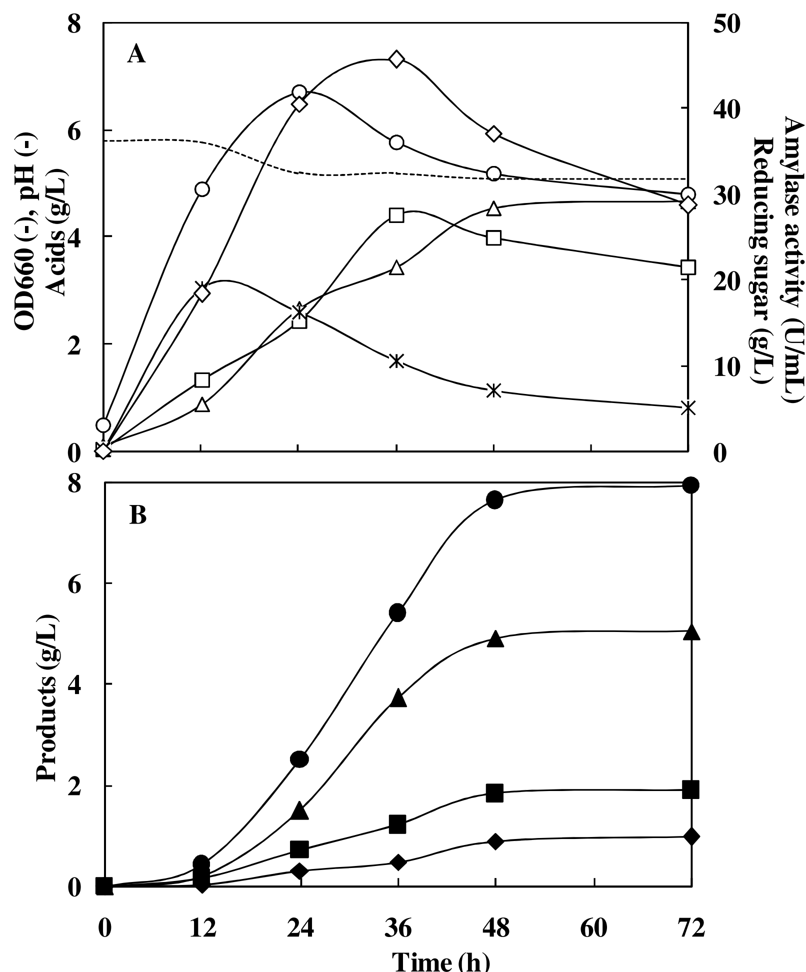

Figure 32. Growth and metabolic activity of co-cultures of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in anaerobic bioreactor. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (·····) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

5.3 Effect of pH on ABE production by co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

In this study on the effect of pH on ABE production, the initial pH was first adjusted and controlled at 5.0, 5.5, 6.0 and 6.5 throughout the fermentation using 2.5 M sodium hydroxide. Fermentation was carried out in the 1 L anaerobic bioreactor which was connected with a pH probe and temperature was controlled at 37°C. The medium was stirred at 120 rpm using a magnetic bar. The results are shown in Figure

31. The pH control at 6.5 was found to favor growth (at early time), amylase and acid production. At the end of the fermentation, there was about 7.5 g/L butyric accumulated in the culture at pH 6.5. At pH 6.5 butyric acid exists mostly in its disassociated form so the conversion of butyric acid into butanol is not favored. These results were consistent with the results of Monot *et al*. (1983). Thus, butyric acid accumulated in the culture and caused toxicity to the cells. Consequently, sugars utilization was less complete and the reducing sugars remained higher at pH 6.5 than that at pH 6.0. On the other hand, pH 6.0 favored ABE production (10.2 g/L). At pH 6.0, there was good growth that resulted in production of sufficient amounts of butyric and acetic acids. At this pH, the undissociated form of butyric and acetic acids could stimulate the conversion of them to the ABE products. Therefore, the final ABE product at pH 6.0 was much higher than for the other cultures (Figures 33-36). A lower final concentration of butyric acid at pH 6.0 (2.5 g/L) compared to the culture without pH control (4.7 g/L) was also observed. However, there still remained a quite high amount of butyric in the culture (2.5 g/L). Substrate utilization was also more complete at pH 6.0, with the residual reducing sugars of this culture being about 2.0 g/L (Figures 33-36).

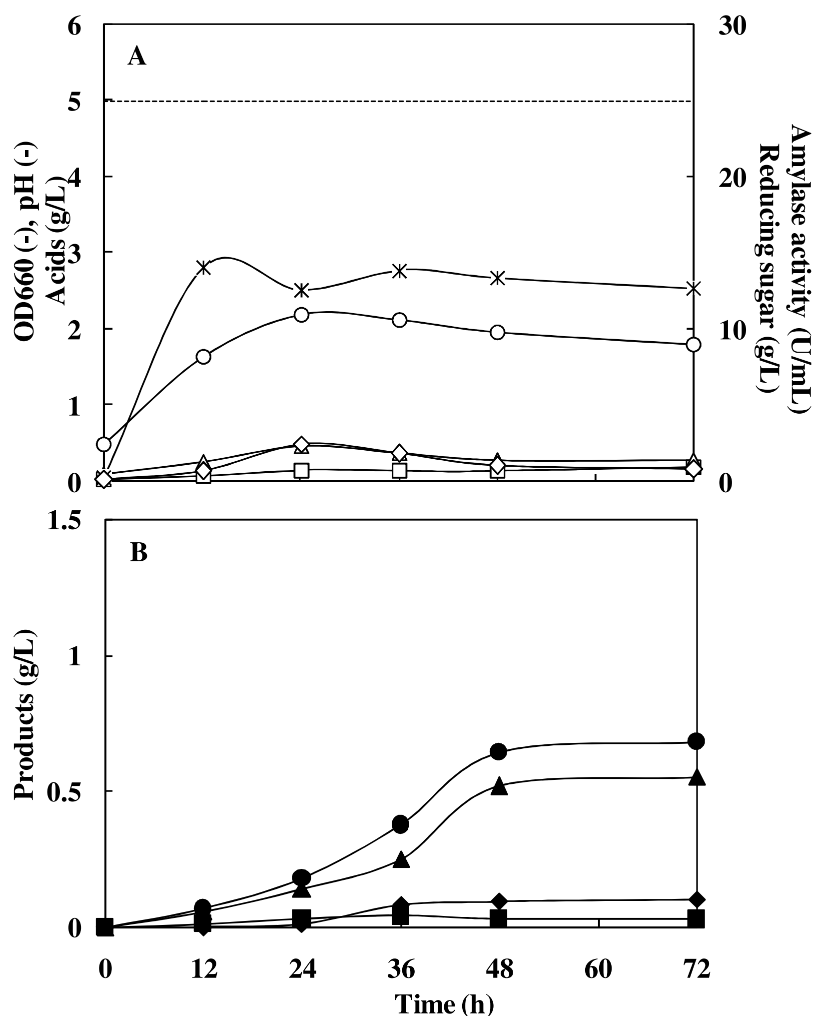

Figure 33. Effect of pH on growth and metabolic activity of a co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in an anaerobic bioreactor at pH 5.0. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (⋯⋯) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

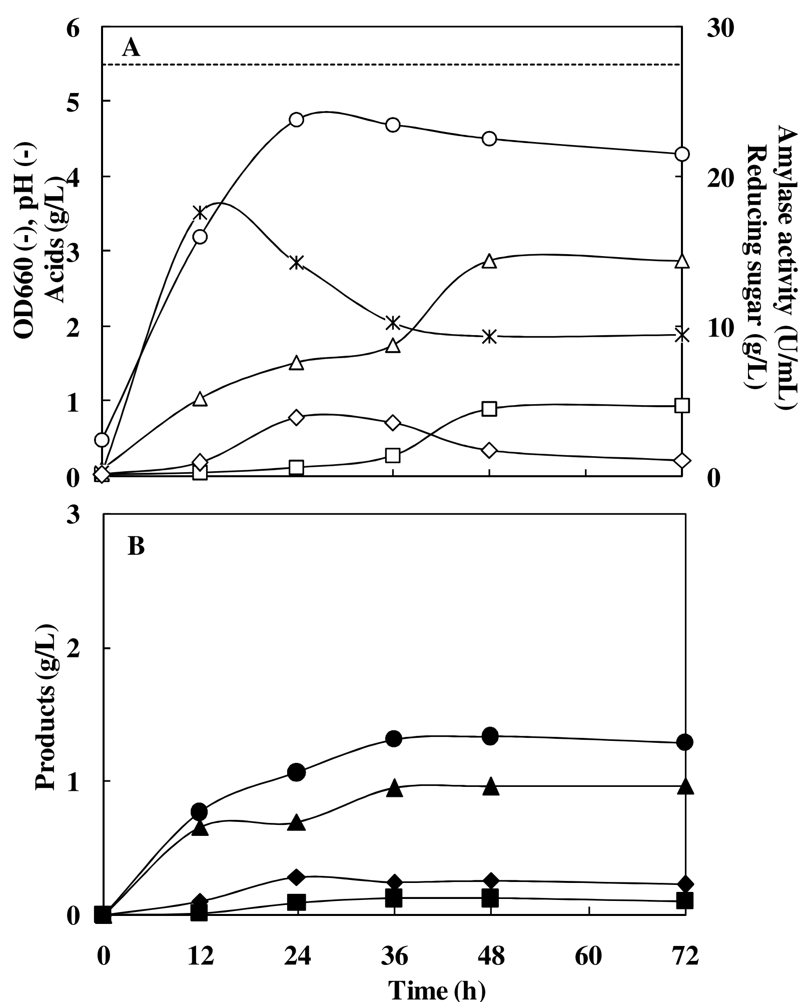

Figure 34. Effect of pH on growth and metabolic activity of a co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in an anaerobic bioreactor at pH 5.5. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (------) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

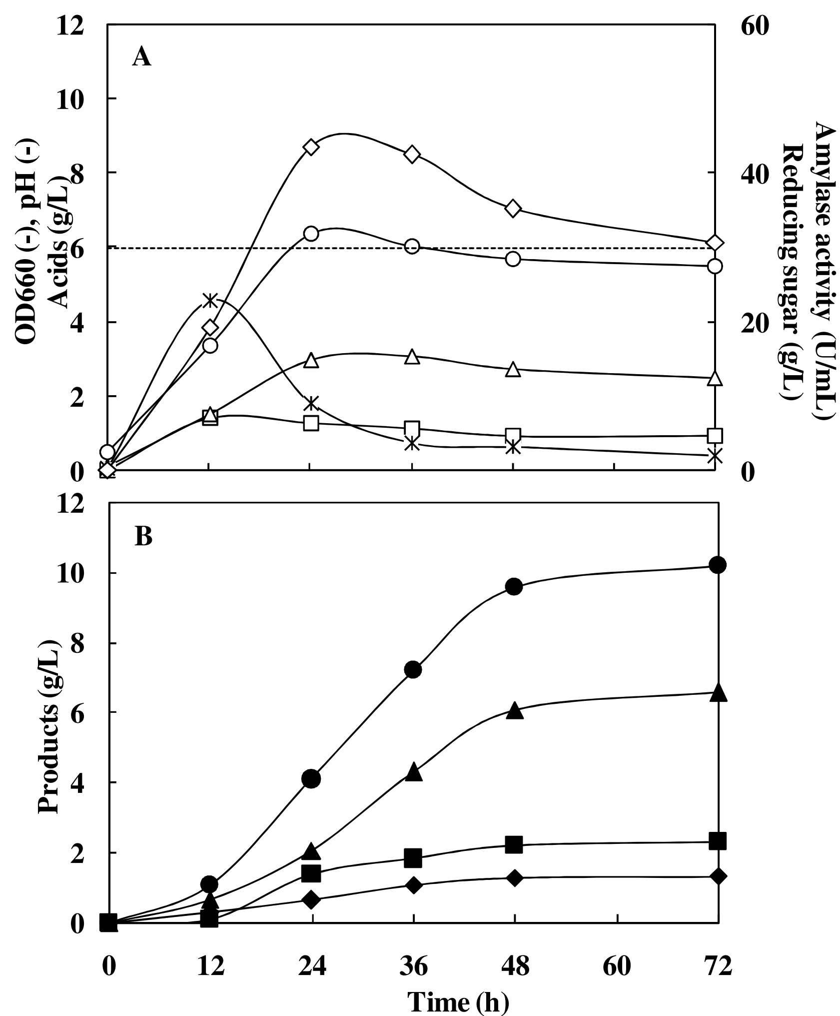

Figure 35. Effect of pH on growth and metabolic activity of a co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in an anaerobic bioreactor at pH 6.0. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (------) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

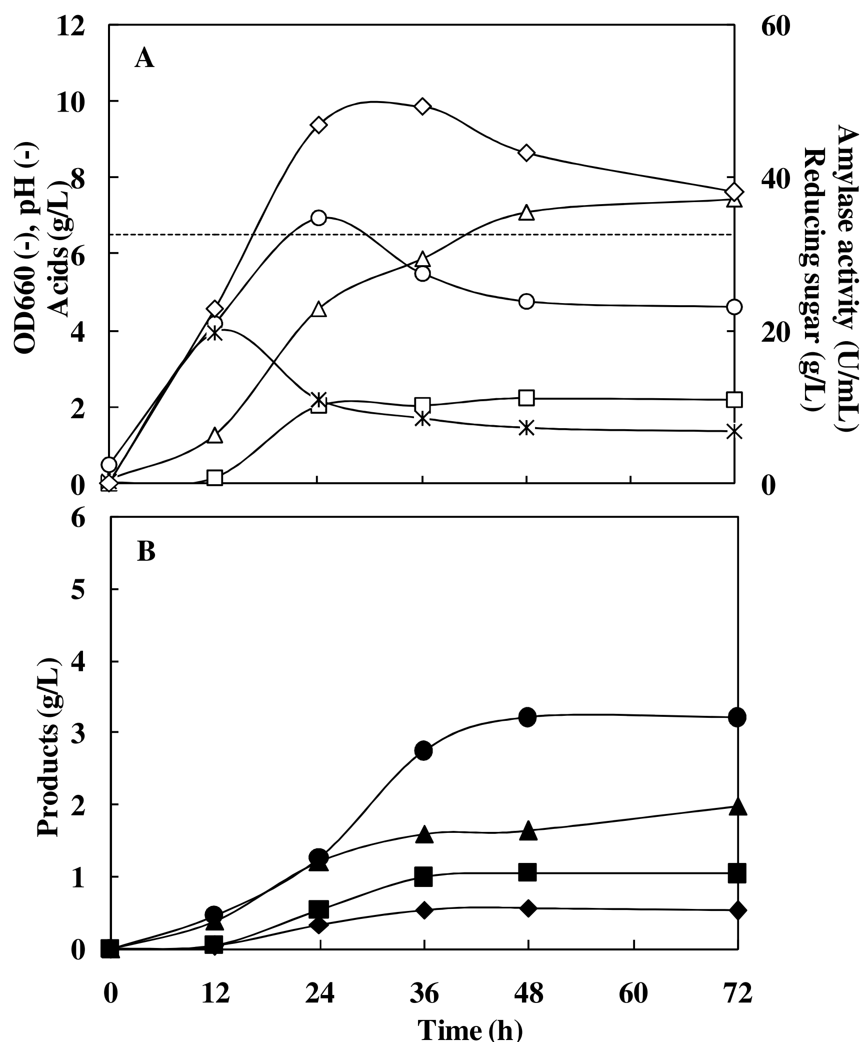

Figure 36. Effect of pH on growth and metabolic activity of a co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in an anaerobic bioreactor at pH 6.5. **A**. (○) OD_{660}; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (-----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

Remarkably, in the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 the pH control did not show significant effects on the B ratio (B ratio at pH 6.0 was 0.66). Among the three products including acetone, butanol, and ethanol; butanol was dominant regardless of whether the culture had the pH controlled or not. This is also one of the advantages, since controlling pH could increase the total ABE without changing the ratios of product and this would make the product recovery easier to handle. The productivity (g/L/h) and the yield of butanol/starch (g/g) of the mixed culture at pH 6.0 were about 0.14 and 0.25, respectively which are comparable with those of other researches 0.16 g/L/h and 0.2 g/g, respectively reported by Madihah *et al.* (2001) and 0.28 g/g reported by Ezeji *et al.* (2004). It is even higher than that reported in the research of Badr *et al.* (2001) (0.054 g/L/h and 0.104 g/g, respectively) and Hipolito *et al.* (2008) (0.09 g/L/h). All further experiments in the bioreactor were therefore carried out with pH control at 6.0.

5.4 Fed-batch of mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

5.4.1 Fed-batch without integrated with gas stripping

The previous study on controlling the pH found that when the pH of the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 was controlled at 6.0, the residual reducing sugars was very small (2.0 g/L). This fact indicated that perhaps an addition of new substrate might be necessary for further ABE production. In this study, the mixed culture with an initial working volume of 700 mL was first operated in the batch mode for 24 h. Then, 100 mL of medium with 80 g/L of cassava starch (the maximum starch concentration which could be dissolved in water) with optimum C/N ratio was added at 24 and 36 h where reducing sugars concentration decreased or started to be decreased. The pH of culture was controlled at 6.0 throughout the fermentation process. The profiles of growth, amylase, reducing sugars, acids and ABE production are shown in Figure 37.

The OD value and amylase production in the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 employing the fed-batch mode were higher than those of the mixed culture in the batch fermentation. In the batch mode the reducing sugars rapidly reduced after the first 12 h, but in the fed-batch fermentation the

reducing sugars increased after the addition of new substrate then gradually reduced after 36 h. At the end of the fermentation process, the amount of residual reducing sugar in the fed-batch culture was 3.0 g/L that is slightly higher compared to the batch culture (2.0 g/L) (Figure 37). The addition of cassava starch provided a new carbon source and hence more energy for cell growth and maintenance as well as for conversion of acetic and butyric acids into ABE product. The ABE production in the fed-batch culture (13.4 g/L) was higher than that of the batch culture (10.0 g/L) (Figures 35 and 37). The productivity (g/L/h) and the yield of butanol/starch (g/g) of the mixed culture at pH 6.0 were about 0.14 and 0.25 and those of the fed batch culture without stripping were 0.19 and 0.24, respectively. In the batch culture, ABE was rapidly produced until 48 h, but there was only a 0.4 g/L increase during 48- 72 h. This was probably due to the depletion of substrate. In the fed- batch culture ABE was produced faster and in greater amounts during 48-72 h (Figure 37) compared to that in batch mode. Accumulation of butyric acid in the fed-batch culture was less than that of the batch culture. This is probably explained by there being more available substrate in fed-batch culture for further conversion of acids into ABE. There was no difference in the butanol ratio in the fed-batch or batch culture, butanol was found to be dominant in both batch and fed-batch cultures and higher compared to the typical product ratios in the literature. Remarkably, the fermentation times required to obtain the optimum ABE were the same in both cultures (72 h). Compared to other research work where a single culture of *Clostridium* was used for ABE production from starch or other substrates, the fermentation time in this study was much shorter (Ezeji *et al*., 2005a; Qureshi *et al*., 2007). This property is important for reduction of the cost of butanol. Since the fermentation time was short, it would require less energy to maintain fermentation process. The shorter fermentation time found in this research might be due to the fast starch hydrolysis resulting in higher available sugars for cell growth, acids and ABE production. In the research of Kosugi *et al*. (2009) the slow production of ethanol from cassava starch waste by *Saccharomyces cerevisiae* K7 was found to be due to the time required for saccharification of starch by the glucoamylase.

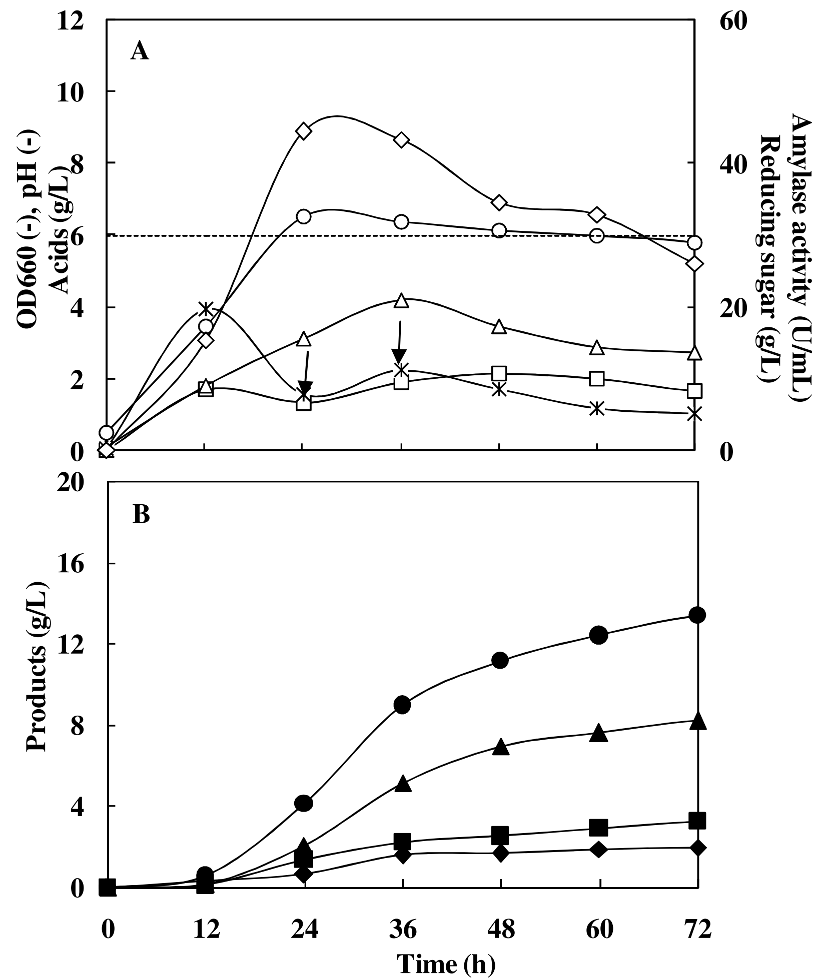

Figure 37. Growth and metabolic activity of a co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in an anaerobic bioreactor in fed-batch mode; pH controlled at 6.0. **A**. (○) OD 660; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (-----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively. The arrows indicate the times when the substrate was fed.

So far, there has been no report on ABE production from starch by mixed culture of *Clostridium* with a high amylase producing *Bacillus*, especially in fed-batch culture. In the research of Ezeji *et al*. (2004), corn and soluble starch were used to feed the culture of *C. beijerinckii*. It was found that the fed-batch culture process did improve ABE production.

The enhancement of ABE production and the reduced accumulation of acids using a fed-batch culture technique indicated that the addition of carbon source would facilitate the acid conversion process. This is different from the batch culture method when the presence of an initial high amount of starch in the culture time stimulated amylase excretion. Therefore, starch hydrolysis happened rapidly and resulted in a large amount of available sugars in the culture. Cells might use this large amount of sugars for growth and acid production and might not have sufficient energy for further conversion of acids into ABE. On the other hand, the accumulation of a large amount of acid in the culture would cause toxicity for the cells. In fed-batch culture, the extra addition of cassava starch provided more energy and this made further conversion of acids into ABE products possible.

5.4.2 Fed-batch integrated with gas stripping for ABE recovery

Product inhibition is among the major factors causing limitations for the butanol market. With a pure culture of *Clostridium*, normally when butanol in the culture reaches 13 g/L or ABE concentration is more than 20 g/L, cells will die from the toxicity and the fermentation process will cease. Thus, removal of butanol from the culture will reduce this possibility. In the fed-batch mixed culture, acids still accumulated in the medium. It was possible that ABE yields could be increased by product recovery using gas stripping. Among the available techniques for solvent recovery, gas stripping seems to be of more benefit due to a number of advantages over the other techniques e.g. easy to handle, and more economically beneficial (Ezeji *et al*., 2004). It is better because nutrients and reaction intermediates are not removed from the reaction mixture. Before applying a gas stripping method, a model solution containing acetone, butanol and ethanol was tested to check its ability. The result revealed that when the system was applied to the model solution contained 5, 10 and 2 g/L of acetone, butanol and ethanol, respectively; about 35% of butanol was recovered after 36 h and about 20% acetone and ethanol thus should significantly reduce the ABE

concentrations in the fermentation medium. In order to examine the effect of gas-stripping on ABE production with the fed-batch mixed culture, the mixed culture was operated in fed-batch mode for 36 h at which time butanol and ABE concentrations should be 5.0 and 9.1 g/L, respectively. At this stage, ABE recovery by gas-stripping was started by sparging oxygen free nitrogen into the culture and the fermentation was continued for another 36 h. Condensate was collected every 12 h for ABE and acids determinations (Ezeji *et al*., 2005a). The obtained results are shown in Figure 38.

Acetic, butyric, acetone and ethanol could not be detected in the condensate. However the result did indicate that gas stripping might be a useful tool to selectively recover butanol from the medium. The finding that no acetic and butyric acids could be detected in the condensate has been reported by several research workers (Ezeji *et al*., 2003; Ezeji *et al*., 2005a). It is not known why gas stripping did not strip out acetone and ethanol as it did in the case of using the model solution. However the concentrations of acetone and ethanol in the culture were very low less than 3 g/L. The presence of cells might also reduce the stripping ability for these compounds.

There was not much difference between growth and amylase production of the culture with and without gas stripping (Figures 37 and 38). However, there was an improvement in terms of substrate utilization after gas striping. The amount of residual reducing sugars (2.0 g/L) was also reduced compared to that of the culture without gas stripping (3.0 g/L) (Figure 32). It is probably due to the removal of butanol by gas stripping. The lower amount of butanol in the medium may allow the cells to consume more sugars and produce acids, consequently the conversion to ABE was higher.

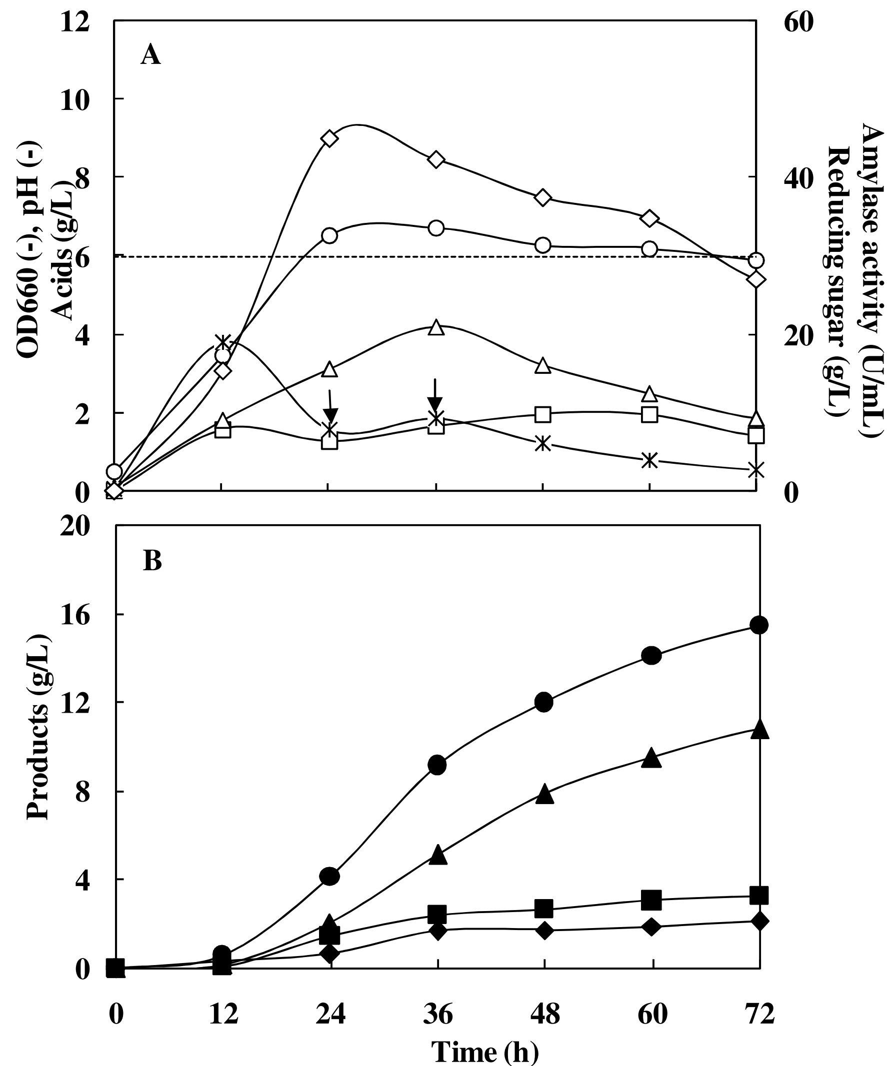

Figure 38. Growth and metabolic activity of a co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in an anaerobic bioreactor in fed-batch integrated with gas stripping, pH controlled at 6.0. **A**. (○) OD 660; (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (·····) pH. **B**. (●) ABE; (♦) butanol; (■) acetone; (▲) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively. The arrows indicate the times when the substrate was fed.

At the end of the fermentation process, the medium contained 6.8 g/L butanol, 1.4 g/L acetic and 1.5 g/L butyric acid. The condensate was recovered three times (every 12 h during the period of 36-72 h) with the volumes of 47 and 39 and 36 mL per time and containing butanol of 27.6, 23.0 and 20.0 g/L, respectively. The total ABE in the culture is shown in Figure 38. The removal of butanol from culture by sparging N_2 over the medium from 36 h produced a positive effect on the utilization of substrate and ABE production compared to the system without the gas stripping. The total ABE production in the culture with gas stripping (16.2 g/L) was considerably higher than that of the culture without gas stripping (13.4 g/L).

The positive effect of gas stripping on substrate utilization, acid assimilation and ABE production has been previously reported by several research workers (Ezeji *et al*., 2003; Ezeji *et al*., 2005a). Compared to the others the improvement in ABE production by gas stripping in our research was limited. The slight improvement in total ABE production in our research might be due to the amount of butanol concentration which was not yet critical for cell growth and ABE production. However, the positive effect of this system on butanol production and starch utilization revealed a potential positive use in cultures with a higher butanol concentration.

5.5 Semi-continuous fermentation of mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

5.5.1 Semi-continuous without integrated with gas stripping

The previous study (section 5.4, Figures 37 and 38) on fed batch studies found that the addition of fresh substrate at 24 and 36 h enhanced final ABE production with or without gas stripping. However, the reducing sugar prolife revealed that at 24 h, the amount of reducing sugars in the cultures was small (7.8 g/L) and this continued to fall until the end of the experiment after a small increase following the 36 h addition of substrate (Figures 37 and 38). This indicated that further additions of new substrate during the fermentation process might further improve substrate availability. In this study, the mixed culture with an initial working volume of 900 mL was first operated in the batch mode for 12 h. Then, 200 mL of medium containing 80 g/L cassava starch with the optimum C/N ratio was added every 12 h from the 12 h to 72 h. Each time, before adding the new medium, 200 mL of culture broth was removed, and the pH of

the culture was controlled at 6.0. The profiles of growth, amylase, reducing sugars, acids and ABE production are shown in Figures 39. Generally, there were only small differences in the maximum amylase activity of the semi-continuous substrate fed mode (49 U/mL) compared to that of the fed-batch mode (44. 4 U/mL). However, the amylase was more stable in the semi-continuous feed mode. Maximum amylase production occurred in the fed batch mode at the 24 h, but with the semi-continuous feed mode the maximum was at 36 h. A reduction in amylase production was observed in both modes of feeding, but that of the semi-continuous feed mode was slower (Figures 38 and 39). The higher amylase production and its slower rate of reduction might be caused by the presence of higher amounts of starch present during the fermentation process in the semi-continuous feed mode and this stimulated the production of amylase. Madihah *et al*. (2001) showed that, an increase in starch concentration led to an increase of amylase production.

The reducing sugars in the semi-continuous feed mode were also higher than those in the fed-batch mode; at the end of the fermentation process, the residual reducing sugars in the semi-continuous feed mode was 8.5 g/L whereas with the fed-batch mode it was 4.5 g/L. Production of ABE and butyric acid in the semi-continuous feed mode was 15.8 and 3.7 g/L, respectively while in the fed-batch mode they were 2.5 and 13.5 g/L, respectively. This result might be explained by the higher amount of sugar present in the semi-continuous feed mode and this could enhance growth and production of acids leading to an increase of ABE production. The maximum ABE production in the semi-continuous feed mode was obtained at 96 h compare to 84 h for the fed-batch mode.

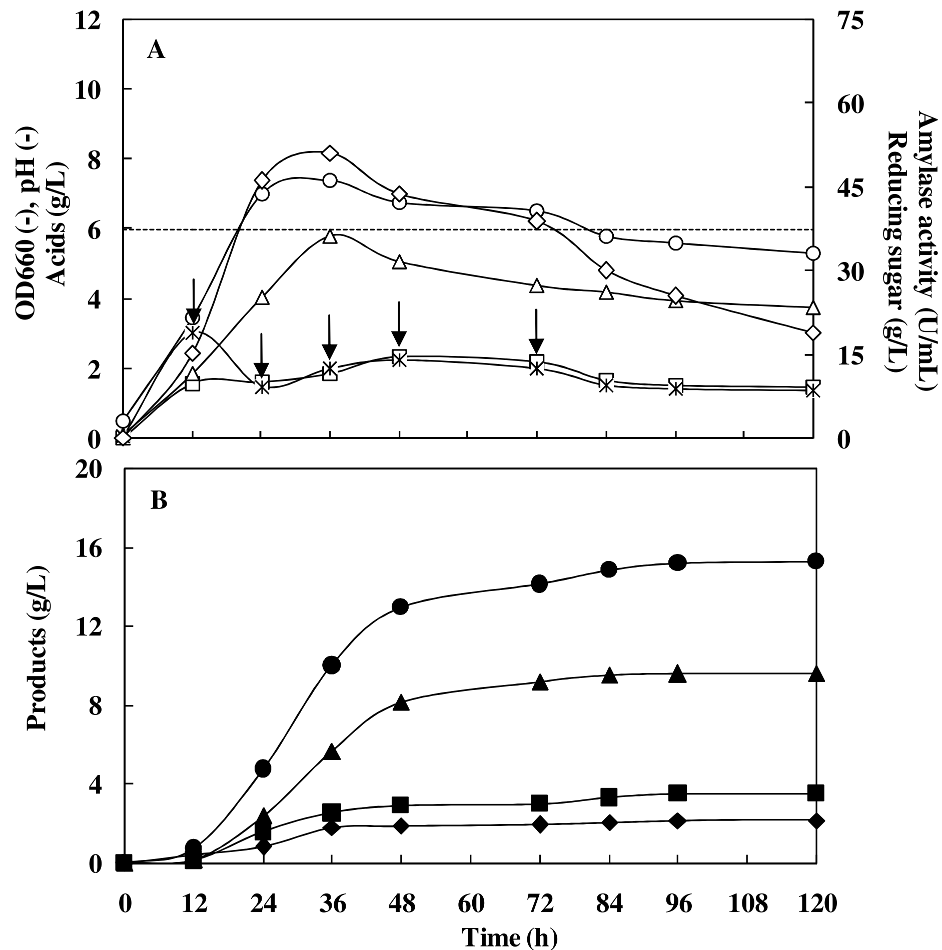

Figure 39. Growth and metabolic activity of co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in the anaerobic bioreactor using a semi-continuous mode with cassava starch, pH controlled at 6.0. **A**. (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (-----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively. The arrows indicate the times when the substrate was fed.

5.5.2 Semi-continuous fermentation integrated with gas stripping

Previously it was found that gas stripping had very little effect on growth and amylase production with the fed-batch mode of fermentation (Figure 40). However, there was an improvement in terms of substrate utilization after gas striping. The amount of residual reducing sugars (7.0 g/L) was also reduced compared to that of the culture without gas stripping (8.5 g/L) (Figure 39). This is probably due to the removal of butanol by gas stripping. The lower amount of butanol in the medium may allow the cells to consume more sugars and produce more acids, and consequently their conversion to ABE was higher.

At the end of the fermentation process, the medium contained 7.4 g/L butanol, 1.33 g/L acetic and 3.39 g/L butyric acid. The condensate was recovered three times (at the 48, 72 and 84 h) with volumes of 50 and 85 and 37 mL and contained butanol of 30.0, 25.2 and 23.0 g/L, respectively. There was a trace of acetone and ethanol in the condensate; however the amounts in the broth culture were probably too small to allow for their recovery (Ezeji and Blaschek, 2004). The total ABE in the culture combined with the amounts in the condensates is shown in Figure 40. The removal of butanol from the culture by sparging N_2 over the medium from 36 h produced a positive effect on the utilization of substrate and ABE production compared to the system without the gas stripping. The ABE concentration in the culture integrated with gas stripping was 17.75 g/L which was higher than that of the culture without gas stripping (15.2 g/L). Calculation of the amount of butanol that was present in each of the 200 mL samples removed at 12, 24, 36, 48 and 72 h and adding this to the butanol present in the culture broth plus that in the condensates showed that the total ABE production by the semi-continuous substrate feed culture with gas stripping (25.8 g/L) was higher than that of the culture without gas stripping (23.1 g/L) (Table 9).

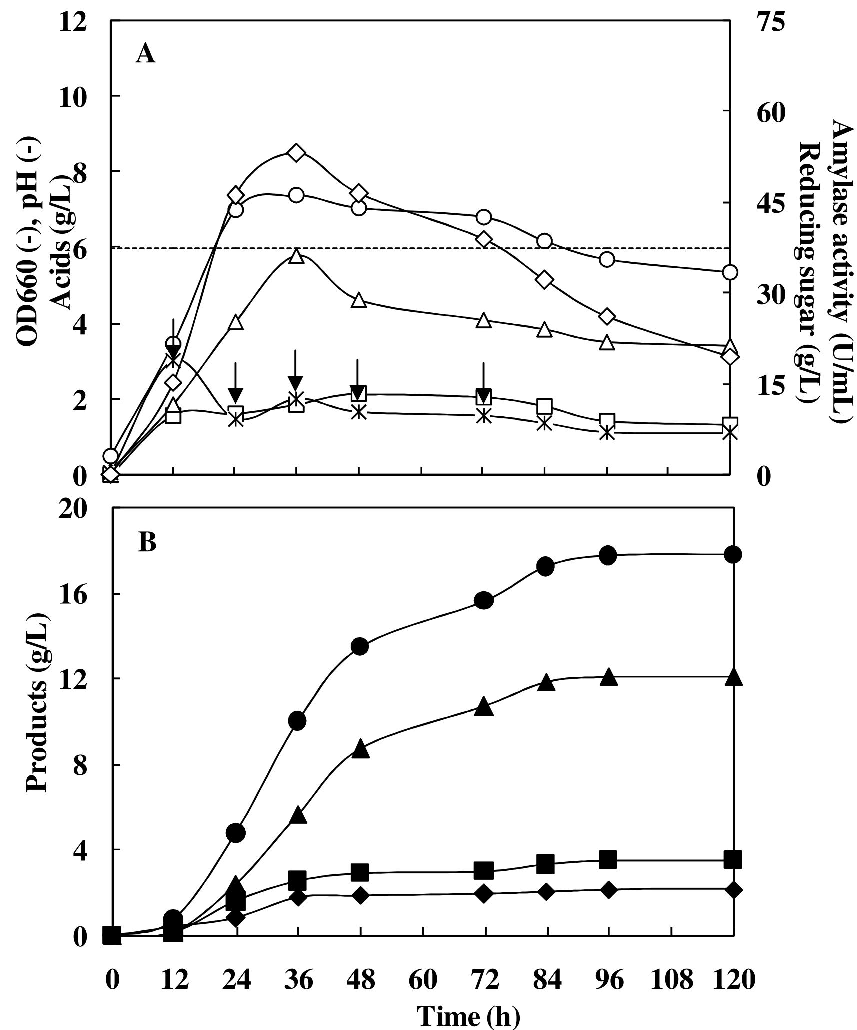

Figure 40. Growth and metabolic activity of co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in the anaerobic bioreactor using a semi-continuous mode with cassava starch, pH controlled at 6.0. **A**. (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (-----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively. The arrows indicate the times when the substrate was fed.

It is noted that after 72 h of culture, further production of ABE was limited in the cultures with or without gas stripping. It is possible that after 72 h the numbers of viable cells of *Clostridium* and *Bacillus* were significantly reduced. In order to check this, viable counts were determined during the fermentation (Figure 41). It is of interest that at the start both cultures were present at about the same level however the Bacillus increased by 2 logs and reached its maximum level over the first 12h then gradually decreased by about 1 log after 72 h. It is not known if the increase in the bacillus occurred very soon after inoculation when a low amount of oxygen might have been present. In contrast the Clostridium culture increased rapidly by 6 logs and reached its maximum level over the first 24 h. This was followed by a logarithmic rate of decrease until the end of the experiment by again 6 logs. The reasons for decreasing of viable cells were probably due to the accumulation of toxic compounds (Madihah *et al*., 2001)

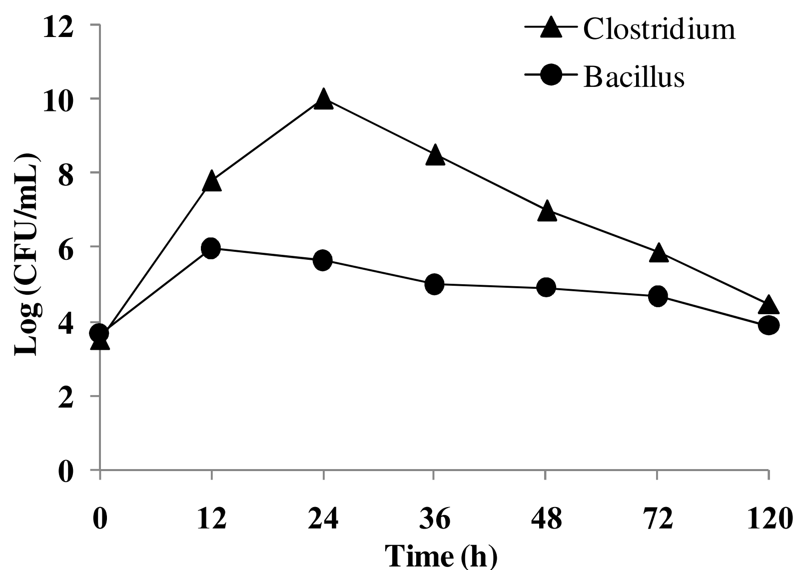

Figure 41. Growth (Log CFU/mL) of *Clostridium* and *Bacillus* in the co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in the anaerobic bioreactor using a semi-continuous mode integrated with gas stripping, pH controlled at 6.0. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively.

Table 9. Effect of fermentation mode on metabolic activity of co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

Parameter	Batch mode	Fed-batch mode	Semi-continuous
Amylase activity (U/mL)[a]	43.45	44.4 (45.0)	51.0 (53.0)
ABE concentration (g/L)[b]	10.18	13.4 (16.2)	15.2 (17.5)
Total ABE (g)[c]	10.18	13.4 (16.2)	23.1(25.8)
Butanol ratio (-)[b]	0.644	0.61 (0.66)	0.63 (0.67)
Residual sugar (g/L)[b]	2.05	5.06 (2.67)	8.50 (7.03)
ABE productivity (g/L/h)[d]	0.141	0.186 (0.195)	0.219 (0.245)
ABE yield (g/g)[b]	0.25	0.24 (0.28)	0.22 (0.24)

[a]The maximum activity of amylase.
[b]Data were obtained at 84 h.
[c]The total ABE including the amount removed from the fermentor.
[d]Data were obtained at 72 h.
Data in parenthesis obtained from cultures integrated with gas stripping.

The semi-continuous substrate feed mode provided a larger enhancement in ABE production either in the case with or without integration with gas stripping, and also provided the higher productivity and product yield over the batch and fed-batch cultures (Table 9). These could be explained by the higher availability of substrate in the semi-continuous cultures which made the acid production and further conversion of acids into ABE production more effective.

5.6 ABE production from cassava starch pulp waste by mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

5.6.1 ABE production from cassava starch pulp waste using fed-batch fermentation

Cassava pulp contains about 60.6% cassava starch, and about 29% of non-starch polysaccharide of which 20% is cellulose. Thus, besides starch, cellulose could also be a carbon source for butanol production. Therefore cassava pulp waste was employed in two ways including direct use, and after being hydrothermally and enzymatically pretreated before use in the ABE fermentation. A fed batch mode was also employed; new substrate being fed after 24 h and 36 h. Cell concentration could not

be measured by OD_{660} due to the opaque nature of these substrate solutions. The results on amylase, residual reducing sugar concentration, acids and ABE production are shown in Figures 42 and 43. Amylase production in the mixed culture using cassava starch waste pulp waste with pretreatment was similar to that found in the mixed culture using cassava starch pulp waste without pretreatment, but there was higher amount of residual reducing sugars (13.0 g/L) in the culture with pretreatment. This higher amount of reducing sugar probably arises from the hydrolysis of cellulose. ABE production in the culture with pretreatment (8.9 g/L) was higher than that of the culture without pretreatment (8.0 g/L). However, there was not much difference in the butanol ratio which was 0.64 and 0.66 for the culture with and without pretreatment, respectively. Cellulose hydrolysis would result in more available sugars for growth and ABE production in the culture with pretreatment. Compared to the fed-batch mixed culture using cassava starch, the amylase, and ABE productions in the culture using cassava starch pulp waste were smaller either with or without pretreatment. The maximum amylase and ABE productions in starch culture was 42 U/mL, and 14.5 g/L, respectively but those of the culture using cassava starch pulp waste were 36 U/mL and 8.9 g/L, respectively. The utilization of reducing sugars was less complete in the culture using cassava pulp waste. The smaller amounts of the products in the culture using cassava starch pulp waste was probably due to the presence of impurities in the waste which might cause toxicity to the cells, or hinder some metabolic processes (Kosugi *et al*., 2008).

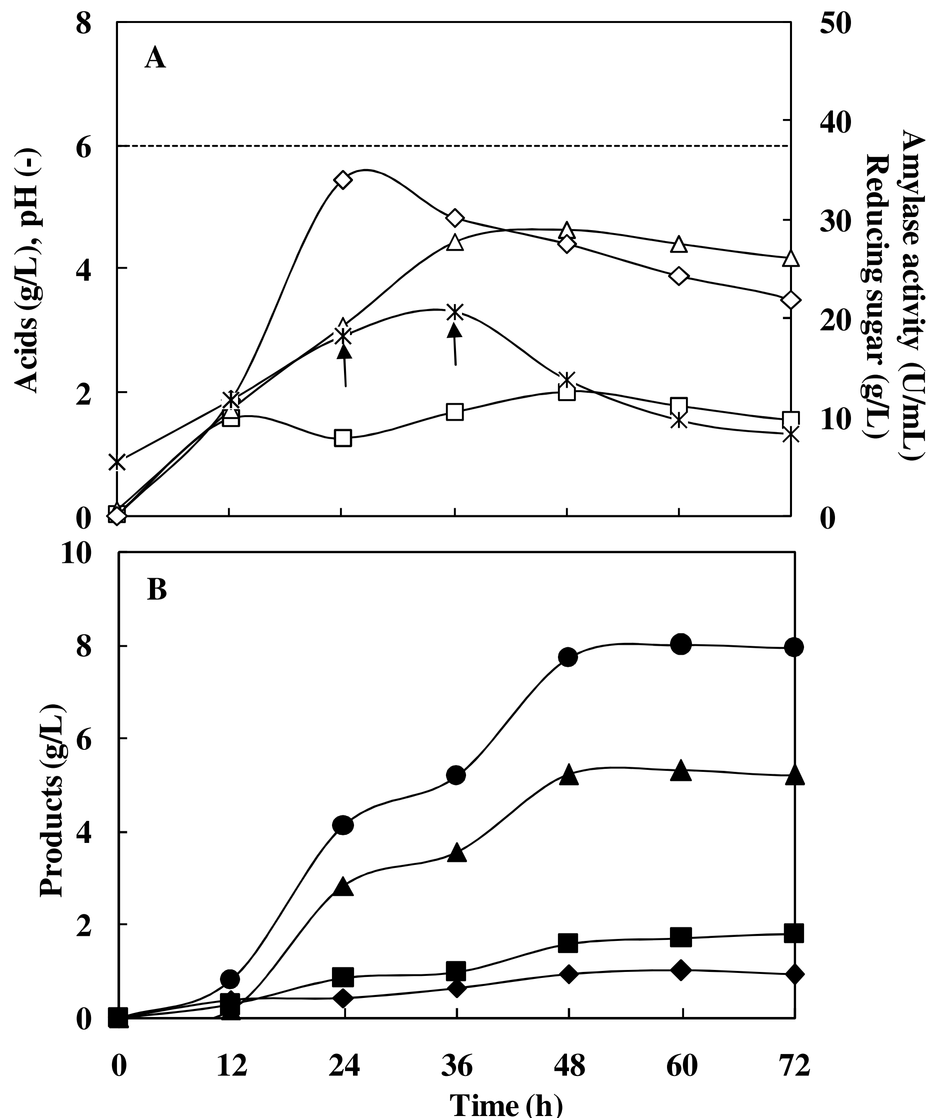

Figure 42. Growth and metabolic activity of co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in the anaerobic bioreactor using a fed-batch mode with cassava starch pulp waste without pretreatment, pH controlled at 6.0. **A**. (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively. The arrows indicate the times when the substrate was fed.

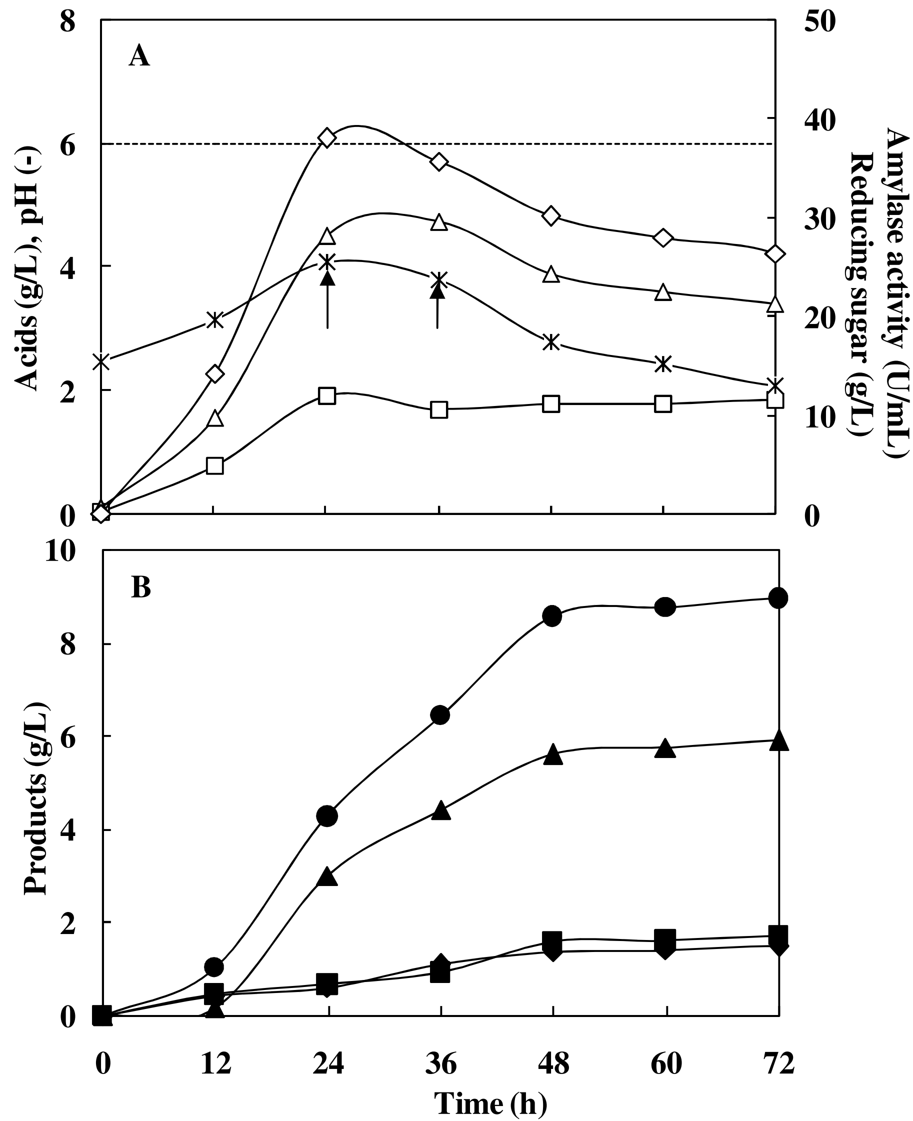

Figure 43. Growth and metabolic activity of co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in the anaerobic bioreactor using a fed-batch mode with cassava starch pulp waste with pretreatment, pH controlled at 6.0. **A**. (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (----) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 40 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively. The arrow indicates the time when the substrate was fed.

5.6.2 ABE production from cassava pulp using semi-continuous fermentation

Now that we have established the best conditions for converting cassava starch into ABE including; substrate levels of 40 g/L cassava starch semi continuous substrate feed, continuous control of pH at 6, gas stripping after the culture had grown for 36 h. It was necessary to determine what could be achieved by replacing pure cassava starch, a versatile product used in many manufacturing processes, with untreated cassava pulp waste, a much cheaper product with few applications. Preliminary experiments using non treated or treated cassava pulp waste has already been described (Section 5.6.1). In this study, we have used untreated cassava pulp waste and the conditions as described in the previous section (5.6.1) except for the gas stripping as previous study found that the butanol produced from pulp was lower than critical concentration (Section 5.6.1). The profiles of growth, amylase, reducing sugars, acids and ABE production are shown in Figures 44. Amylase production in the semi-continuous feed culture (37.0 U/mL) was slightly higher than that of the fed batch culture (34 U/mL), but the residual reducing sugar level in the semi-continuous feed mode (12.38 g/L) was considerably higher than that in the fed batch mode(8.2 g/L). The semi-continuous feed mode enhanced ABE production from 8.0 g/L fed-batch fermentation to 8.78 g/L. However, the final butyric acid concentration in the semi-continuous feed mode (5.26 g/L) was also higher than that of the fed-batch mode (4.1 g/L). These results could be explained by the presence of a larger amount of starch in the semi-continuous culture and this supported more growth and acid production, and consequently ABE production. However, the accumulation of higher amount of acids in the semi-continuous feed mode probably hindered increased reducing sugar consumption. As the result, the residual reducing sugars in this culture remained high. It might also include the possibility that the presence of impurities in wastes caused some negative effects on further substrate utilization (Kosugi *et al*., 2009).

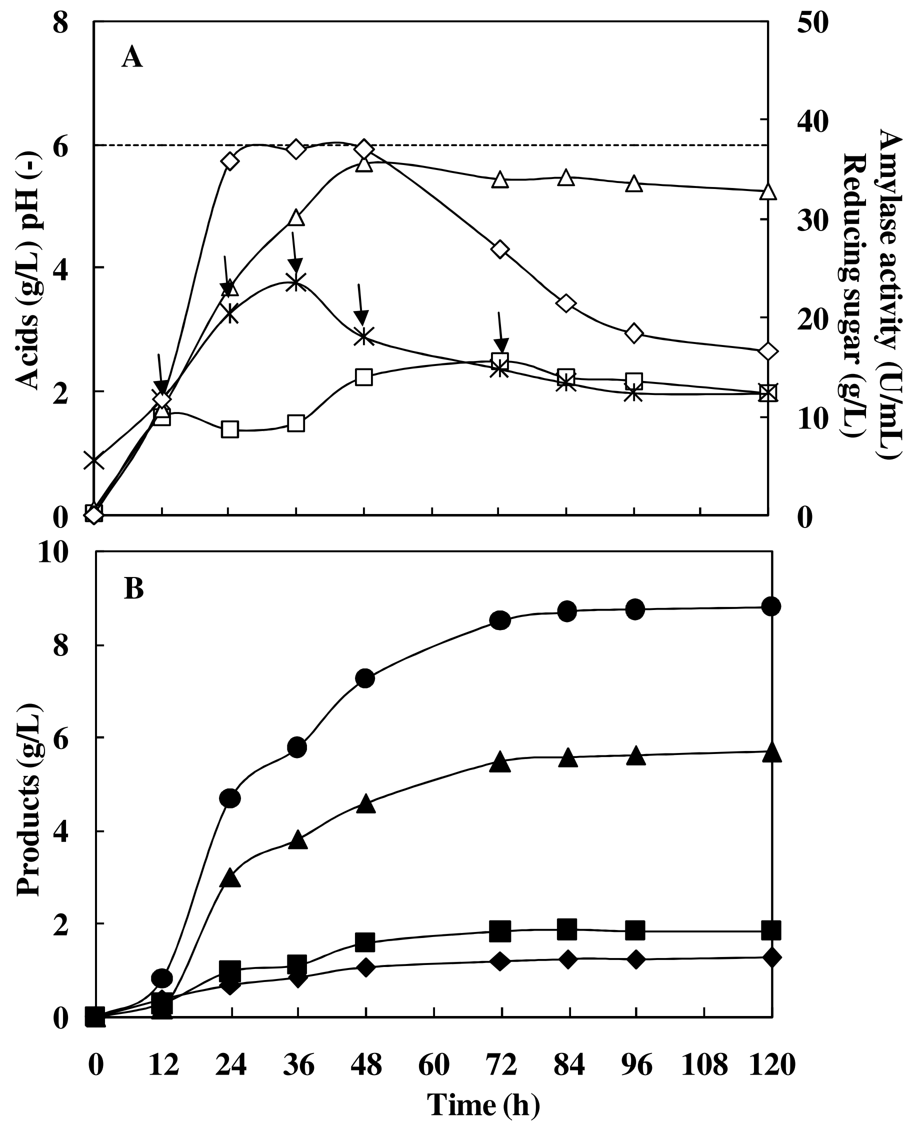

Figure 44. Growth and metabolic activity of co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in the anaerobic bioreactor using a semi-continuous mode with cassava starch pulp waste with pretreatment, pH controlled at 6.0. **A**. (◊) amylase activity; (□) acetic acid; (Δ) butyric acid; (*) reducing sugar; (·····) pH. **B**. (●) ABE; (▲) butanol; (■) acetone; (♦) ethanol. 66 (g/L) cassava starch, 5 (g/L) yeast extract, and 8 (g/L) ammonium nitrate was used as carbon, organic nitrogen, and inorganic nitrogen source, respectively. The arrows indicate the times when the substrate was fed.

Table 10. Effect of fermentation mode on ABE production from cassava starch pulp waste by mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 in 1 L anaerobic bioreactor

Parameter	Batch mode	Fed-batch mode	Semi-continuous
Amylase activity (U/mL) [a]	34.0	35.7	37.0
ABE concentration (g/L)[b]	8.00	8.90	8.70
Total ABE production (g)[c]	8.00	8.90	13.9
Butanol ratio (-)[b]	0.62	0.66	0.64
Residual sugar (g/L)[b]	8.20	12.5	12.3
ABE productivity (g/L/h)[d]	0.111	0.123	0.120
ABE yield (g/g)	0.200	0.158	0.138

[a]The maximum activity of amylase
[b]Data were obtained at 84 h
[c]The total ABE including the amount removed from the fermentor
[d]Data were obtained at 72 h

The semi-continuous substrate feed mode provided a larger amount of total ABE production (13.9 g) compared to the batch (8.0 g) and fed batch mode (8.9 g). In terms of productivity, the productivity of the semi-continuous feed mode (0.12 g/L/h) was almost the same as with the fed-batch mode (0.123 g/L/h) and slightly higher than that of the batch mode (0.11 g/L/h). However, the product yield of the batch mode (0.2 g/g) was higher than that of the fed-batch and semi-continuous mode (0.16 and 0.138 g/g) (Table 10). The lower product yield could be explained by the lower efficiency of substrate utilization in this culture due to the presence of toxic compounds at higher concentrations in the pulp waste (Kosugi *et al*., 2009).

So far, there has been no reported research using cassava starch pulp waste as the substrate for ABE production. Kosugi *et al*. (2008) employed hydrothermally and enzymatically pretreated cassava pulp waste for ethanol production by *Saccharomycetes cerevisiae*. The impurities in the waste and the by-products generated from the pretreatment processes were also found to cause a reduction in ethanol production (Kosugi *et al*., 2008). Notably, in terms of the fermentation time, which in our research (84 h) was much shorter compared to that of their research (7

days). This again proved that the use of the mixed culture could also effectively reduce the fermentation time with different substrates (either starch or starch waste).

Studies on ABE production by pure cultures of *Clostridium* from other wastes rather than cassava starch waste were previously conducted by several research groups. It was reported that the direct use of wastes generally produced low amounts of ABE. Addition of the other compounds (e.g. glucose, vitamins) is required. Compared to other research work where wastes were directly used for ABE production (Table 11), the ABE production in our research is somewhat higher. However, when compared to the other research where wastes were supplemented with the other components e.g. corn steep water supplemented with glucose (Pareckh *et al*., 1998), sludge supplemented with sago starch (Hipolito *et al.*, 2008), wheat straw hydrolysate supplemented with glucose (Qureshi *et al*., 2007), the ABE production in this research is less.

Accordance to Gapes (2000), substrate and production costs are the most important factors to make the ABE fermentation process economic. From this point of view, the direct use of cassava starch waste as the substrate without addition of other compounds for ABE production by the mixed culture in our research has a great potential for making ABE industrially viable.

Table 11. List of ABE production from natural wastes by pure cultures of *Clostridium*

Wastes	Bacterial species	ABE (g/L)	Reference
Sludge hydrolysate	*C. saccharoperbutylacetonicum*	6.4	Hipolito *et al*., 2008
Excess sludge	*C. saccharoperbutylacetonicum*	0	Kobayashi *et al*., 2005
Excess sludge-glucose		9.3[a]	
Domestic organic waste	*C. acetobutylicum*	1.5	Claassen *et al*., 2000
	C. beijerinckii B-592	0.9	
	Clostridium LMD	1.9	
Palm oil mill effluent	*C. aurantibutyricum*	7.2	Somrutai *et al*., 1996
Cassava starch waste	*Clostridium* and *Bacillus*	8.0[b]	This study
		8.9[c]	
		13.9[d]	

[a]only butanol production was measured.

[b]total ABE in fed-batch culture without substrate pretreatment.

[c]total ABE in fed-batch culture with substrate pretreatment.

[d]total ABE production in semi-continuous without substrate pretreatment; the values of [b, c, d] were obtained at 84 h.

CHAPTER 5

CONCLUSIONS

This study has shown that the use of high amylase producing *B. subtilis* WD 161 for co-culturing with *C. butylicum* TISTR 1032 could enhance ABE production from starch without anaerobic pretreatment. The mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 increased amylase activity 10 fold and enhanced ABE production 4 and 6.5 fold from soluble starch and cassava starch, respectively, compared to those of the pure culture of *Clostridium* itself. The benefits of using this high amylase producing aerobic *Bacillus* in a co-culture with anaerobic *Clostridium* were not only increasing substrate utilization and ABE production but there was also no requirement to add any costly reducing agent to the medium or flushing with N_2 to ensure anaerobic condition. This thus makes the anaerobic fermentation more economical and cost effective.

The medium optimization for ABE production by the mixed culture showed that the optimum cassava starch concentration was 40 g/L. A low C/N ratio of 4 enhanced amylase activity and starch utilization and, consequently, the production of ABE. The use of higher amounts of yeast extract or ammonium nitrate alone had a negative effect on ABE production. The optimum ratio of yeast extract to ammonium nitrate was found to be 265/100 mM/mM. Response surface methodology (RSM) revealed, that among three investigated variables including cassava starch, yeast extract, and ammonium nitrate concentrations, only cassava starch concentration significantly influenced butanol production, total ABE and amylase production. In the case of amylase production, beside cassava starch concentration, ammonium nitrate also had a significant effect. A moderately positive interaction of the cassava starch and ammonium nitrate concentration on the productions of butanol, ABE, and especially amylase were also observed. The economically profitable optimum condition for ABE production was selected from response surface regression as follows: 40 g/L cassava

starch concentration; 5 g/L yeast extract and 8 g/L ammonium nitrate at which ABE production was 9.02 g/L.

ABE production by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 was scaled up by using an anaerobic bioreactor. The studies on the effect of a controlled pH on growth, amylase and ABE production by the mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 revealed that a pH control of 6.0 favored ABE production, but a pH controlled at 6.5 was favored for acids production. An enhancement in ABE production was observed when the culture with pH 6.0 was operated in fed-batch and semi-continuous modes together with product recovery by gas stripping.

When the optimum process was applied for ABE production from cassava pulp waste by mixed culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 using fed-batch fermentation, there were considerable amounts of ABE production in the cultures either with or without enzymatic pretreatment of cassava pulp waste (8.0 and 8.9 g/L, respectively). Semi-continuous fermentation enhanced ABE production to 13.9 g/L without substrate pretreatment. However, to increase the ABE production from cassava pulp waste more work is required to optimize the conditions. This study proved that the mixed culture of aerobic *Bacillus* and anaerobic *Clostridium* may contribute greatly to developing industrialized ABE production.

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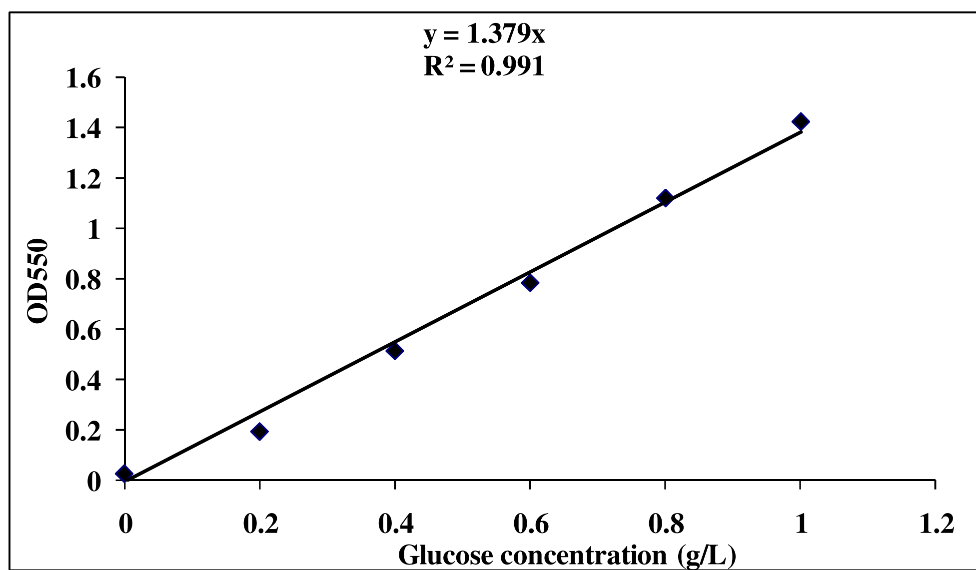
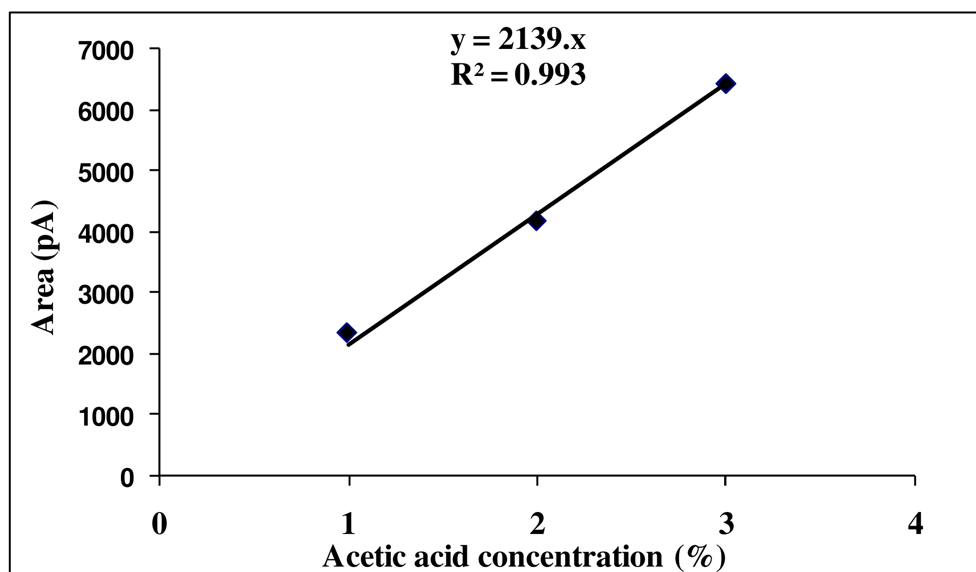
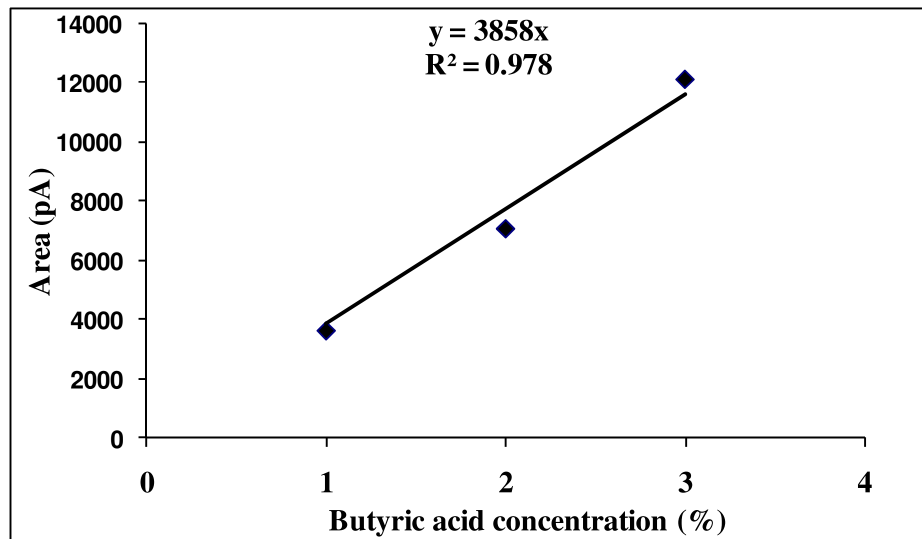
APPENDIX

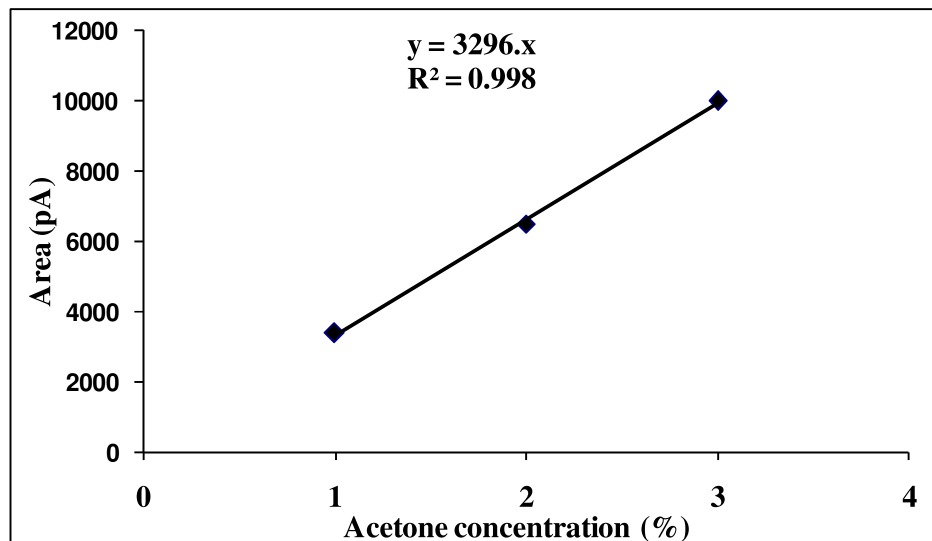
1. Glucose standard curve for DNS method

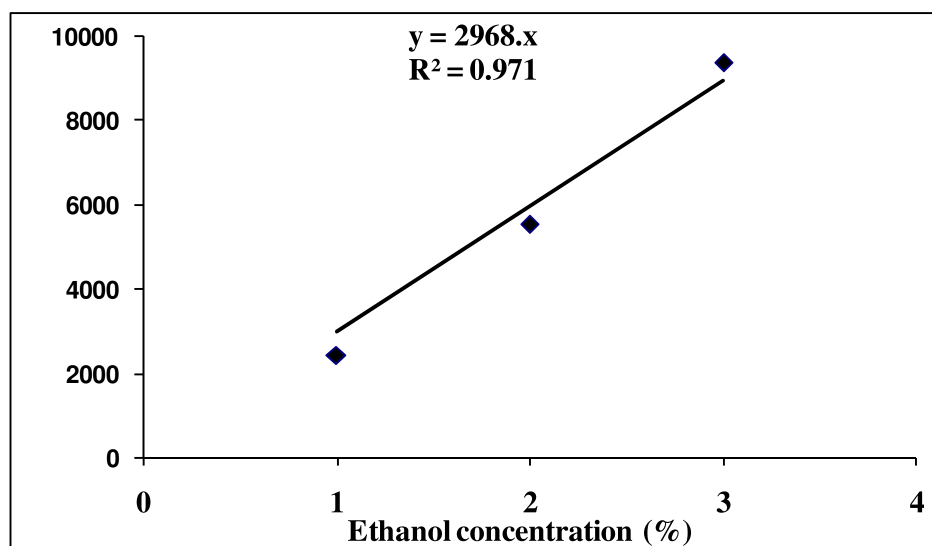


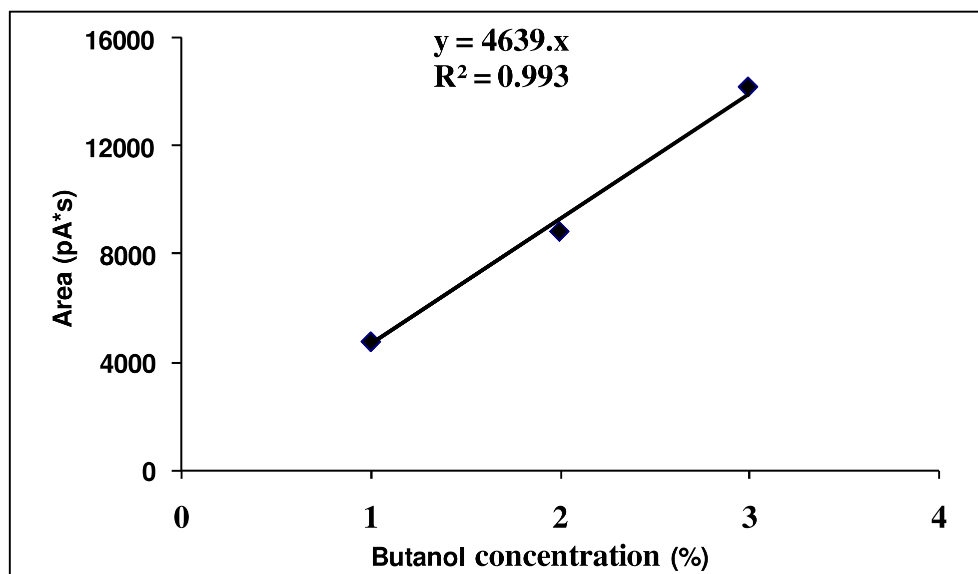
2. Acids and ABE standard curve




y = 3858x
R² = 0.978
Area (pA)
14000
12000
10000
8000
6000
4000
2000
0
0
1
2
3
4
Butyric acid concentration (%)


y = 3296.x
R² = 0.998
Area (pA)
12000
10000
8000
6000
4000
2000
0
0
1
2
3
4
Acetone concentration (%)


y = 2968.x
R² = 0.971
10000
8000
6000
4000
2000
0
0
1
2
3
4
Ethanol concentration (%)



y = 4639.x
R² = 0.993
Area (pA*s)
16000
12000
8000
4000
0
0
1
2
3
4
Butanol concentration (%)

VITAE

Name Miss Tran Thi My Hanh

Student ID 5011030013

Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc. in Agro-Biology	Hanoi University of Education	2003
M.Sc. in Tropical Program	Kasetsart University	2006

Scholarship Awards during Enrolment

Songklanakrind scholarship

List of Publication and Proceedings

Publications

Tran, T.M.H., Cheirsilp, B., Hodgson, B. and Umsakul, K. 2009. Potential use of *Bacillus subtilis* in a co-culture with *Clostridium butylicum* for acetone-butanol-ethanol production from cassava starch. Biochemical. Eng. J. (Accepted)

Tran, T.M.H., Cheirsilp, B., Hodgson, B. and Umsakul, K. 2009. Optimizing a syntrophic co-culture of *Clostridium butylicum* and *Bacillus subtilis* for acetone-butanol-ethanol production from cassava starch using response surface methodology. Process Biochem. (Submitted)

Tran, T.M.H., Cheirsilp, B., Hodgson, B. and Umsakul, K. 2009. Process optimization for acetone-butanol-ethanol production by a co-culture system of *Clostridium butylicum* and *Bacillus subtilis* using cassava starch and its pulp waste. (Preparing)

Presentations

Tran, T.M.H., Cheirsilp, B., Hodgson, B. and Umsakul, K. 2008. Enhancement of Acetone-Butanol-Ethanol Production from Starch by A Mixed Culture of *Clostridium butylicum* and *Bacillus subtilis*. International Conference of the Thai Society for Biotechnology, 14-17 October, 2008, Maha Sarakham, Thailand.