ACETONE-BUTANOL-ETHANOL (ABE) PRODUCTION FROM CASSAVA AND GLYCEROL BY *CLOSTRIDIUM ACETOBUTYLICUM*

Apichai Sawisit

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การผลิตอะซิโตน-บิวทานอล-เอทานอล (เอบีอี) จากมันสำปะหลัง และกลีเซอรอลด้วยเชื้อ *Clostridium acetobutylicum*

นายอภิชัย สาวิสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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วัตถุประสงค์หลักของงานวิจัยในครั้งนี้ เพื่อศึกษาความเป็นไปได้ในการใช้มันสำปะหลังและกลีเซอรอลเป็นแหล่งคาร์บอน ร่วมกับยีสต์ใช้แล้วในอุตสาหกรรมการผลิตเบียร์เป็นแหล่งไนโตรเจน เพื่อผลิตตัวทำละลาย อะซิโตน บิวทานอลและเอทานอลด้วยกระบวนการหมักแบบกะ โดยใช้เชื้อ *Clostridium acetobutylicum* TISTR 1462 ทั้งนี้การทดลองที่ใช้มันสำปะหลังเป็นแหล่งคาร์บอนนั้น ได้ศึกษาผลของการควบคุมค่า pH ที่แตกต่างกันในช่วง pH 4.5-6.5 ศึกษาผลของความเข้มข้นของแป้งมันสำปะหลังในช่วง 20-80 g/L และศึกษาผลของการใช้แหล่งคาร์บอนและไนโตรเจนที่แตกต่างกันต่อการผลิตตัวทำละลาย จากผลการทดลองพบว่า *Cl. acetobutylicum* TISTR 1462 สามารถใช้แป้งมันสำปะหลังในการผลิตตัวทำละลายได้อย่างมีประสิทธิภาพ และเทียบเท่ากับการใช้กลูโคส เป็นแหล่งคาร์บอน โดยการทดลองแบบกะที่ไม่มีการควบคุม pH สามารถผลิตตัวทำละลายทั้งหมด 14.33 g/L ซึ่งให้ผลใกล้เคียงกับการผลิตโดยใช้กลูโคสที่ผลิตตัวทำละลายทั้งหมด 15.39 g/L นอกจากนี้ ยังพบว่าการใช้เอนไซม์ย่อยแป้งมันสำปะหลังก่อนนำไปหมัก ซึ่งทำให้ได้มอลโตสและกลูโคสเกิดขึ้นนั้น ไม่ได้มีผลช่วยให้การผลิตตัวทำละลายเพิ่มขึ้นเมื่อเทียบกับการหมักที่ไม่ได้ย่อยด้วยเอนไซม์ ขณะที่การย่อยด้วยกรดก่อนนำไปหมัก พบว่าให้ผลผลิตน้อยกว่าการย่อยด้วยเอนไซม์ 19.4% ส่วนการทดลองที่มีการควบคุมค่า pH ในช่วงที่มีการผลิตตัวทำละลาย พบว่าที่ pH 5.5 มีการผลิตตัวทำละลายสูงสุด 20.08 g/L นอกจากนี้ยังพบว่าที่การควบคุมค่า pH ที่สูงกว่า 6.0 ขึ้นไปจะมีการผลิตกรดอินทรีย์เป็นส่วนใหญ่ และมีการผลิตตัวทำละลายเพียงเล็กน้อยเท่านั้น และยังพบว่าการควบคุมค่า pH ที่ 5.25 มีการผลิตอะซิโตนสูงสุดถึง 6.78 g/L ทั้งนี้การทดลองที่มีการควบคุม pH ให้ความเข้มข้นสุดท้ายของตัวทำละลายสูงกว่าที่ไม่มีการควบคุมค่า pH ประมาณ 1.5 เท่า จากผลของความเข้มข้นของแป้งมันสำปะหลังที่ใช้ในการทดลองในช่วง 20-80 g/L พบว่าความเข้มข้นของแป้งมันสำปะหลังที่ 60 g/L มีการผลิตตัวทำละลายสูงสุด คือ 14.33 g/L การใช้ความเข้มข้นเริ่มต้นของแป้งมันสำปะหลังที่ต่ำกว่า 30 g/L จะทำให้เกิดการผลิตกรดอินทรีย์มากกว่าการผลิตตัวทำละลาย สำหรับผลของการใช้แหล่งไนโตรเจนที่ต่างกันในการผลิตตัวทำละลาย พบว่าการใช้ยีสต์สกัดจากยีสต์ที่ใช้แล้วในอุตสาหกรรมการผลิตเบียร์เป็นแหล่งไนโตรเจน ทำให้มีการผลิตตัวทำละลาย 18.46 g/L ซึ่งใกล้เคียงกับได้กับการหมักที่ใช้ยีสต์สกัดทางการค้า ซึ่งผลิตตัวทำละลายได้ 20.86 g/L

กรณีที่ใช้กลีเซอรอลเป็นแหล่งคาร์บอนในการหมัก พบว่าเชื้อ *Cl. acetobutylicum* JCM 7289 สามารถผลิตผลผลิตได้หลากหลาย เช่น บิวทานอล เอทานอล และอะซิโตน อย่างไรก็ตามกลีเซอรอลบางส่วนยังสามารถถูกเปลี่ยนไปเป็น 1,3 โพรเพนไดออล, กรดบิวทิริกและ กรดอะซิติก ตามลำดับ โดยพบว่า การใช้กลีเซอรอลที่ความเข้มข้นมากกว่า 40 g/L มีการผลิตตัวทำละลายเพียง 5.92 g/L ซึ่งน้อยกว่าการใช้กลูโคส (13.85 g/L) ถึง 2 เท่า ทั้งนี้เนื่องจาก วิถีการเกิดกระบวนการสันดาปของกลีเซอรอลที่ทำให้เกิดการสร้างตัวทำละลายตัวอื่น เช่น 1, 3 โพรเพนไดออล เป็นต้น จึงส่งผลให้การผลิตของตัวทำละลาย อะซิโตน บิวทานอล และเอทานอลลดลง จากการศึกษาผลของความเข้มข้นพบว่า ความเข้มข้นของกลีเซอรอลมีผลต่อการเจริญเติบโตของเซลล์และการผลิตตัวทำละลาย โดยการใช้กลีเซอรอลที่ความเข้มข้นเริ่มต้นสูงกว่า 40 g/L เชื้อจะไม่สามารถใช้กลีเซอรอลได้หมด ยิ่งไปกว่านั้นการใช้กลีเซอรอลที่ความเข้มข้นสูงๆ จะมีผลทำให้ประสิทธิภาพในการใช้กลีเซอรอลลดลงอีกด้วย ทั้งนี้ยังพบว่าที่ความเข้มข้นเริ่มต้นของกลีเซอรอลที่ 60 g/L หรือมากกว่าส่งผลให้การเจริญเติบโตของเซลล์ลดน้อยลง จากผลการพิจารณากระบวนการหมักที่มีการควบคุม pH สามารถสรุปได้ว่า ที่ pH 5.5 มีการผลิตตัวทำละลายสูงสุด คือ 11.66 g/L และกระบวนการหมักที่มีการควบคุม pH สามารถผลิตตัวทำละลายได้มากกว่ากระบวนการหมักที่ไม่มีการควบคุมค่า pH สำหรับผลของการใช้แหล่งไนโตรเจนที่แตกต่างกันนั้น สามารถสรุปได้ว่ากระบวนการหมักโดยใช้ยีสต์สกัดจากยีสต์ที่ใช้แล้วเป็นแหล่งไนโตรเจน มีการผลิตตัวทำละลายเท่ากับ 10.34 g/L ซึ่งเทียบเท่ากับการใช้ยีสต์สกัดทางการค้าที่มีการผลิตตัวทำละลายเท่ากับ 11.66 g/L

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

APICHAI SAWISIT : ACETONE-BUTANOL-ETHANOL (ABE) PRODUCTION FROM CASSAVA AND GLYCEROL BY *CLOSTRIDIUM ACETOBUTYLICUM*. THESIS ADVISOR : ASST. PROF. SUNTHORN KANCHANATAWEE, Ph.D., 111 PP.

ABE FERMENTATION/*CLOSTRIDIUM ACETOBYTYLICUM*/CASSAVA/ GLYCEROL

The main objective of this study was to demonstrate the feasibility of using cassava materials and glycerol as a carbon source supplemented with spent brewer's yeast extract as a nitrogen source for acetone, butanol and ethanol (ABE) fermentation by *Clostridium acetobutylicum* TISTR 1462 in batch culture. The solvents production was performed with different pH controlled strategies (pH 4.5-6.5). The effects of cassava starch concentrations on the solvents production were investigated in the range of 20~80 g/L as well as the effects of different types of carbon sources and nitrogen sources. The results showed that *Cl. acetobutylicum* TISTR 1462 was capable of producing solvents efficiently from cassava materials, comparable to when glucose was used. The batch experiment with uncontrolled pH of cassava starch resulted in 14.33 g/L of total solvents as compared with 15.39 g/L of total solvents when glucose was used. Moreover, it was found that enzymatic pretreatment of the gelatinized cassava starch yielding maltose and glucose prior to the fermentation did not improve solvents production as compared with direct fermentation of the gelatinized starch, while the lower solvents production (19.48 %) was observed when cassava materials was hydrolyzed with acid prior to the

fermentation. In the experiment with pH controlled during solventogenic phase, the highest total solvents production (20.08 g/L) was obtained with a controlled pH of 5.5. At a controlled pH 6.0 or higher, the fermentation produced mainly organic acids with a small amount of solvents. It was also found that the highest acetone production (6.78 g/L) was obtained with a controlled pH 5.25. Using the appropriated pH control strategy, the final solvents concentration obtained was almost 1.5 times higher than that obtained under fermentation with uncontrolled pH. Within the range of cassava starch concentration investigated (20-80 g/L), the highest total solvents production (14.33 g/L) was obtained at 60 g/L initial cassava starch concentration. The fermentation performance using initial cassava concentrations lower than 30 g/L were acidogenic rather than solventogenic. For the effect of various nitrogen sources, it revealed that the fermentation performance using spent brewer's yeast extract as a nitrogen source resulted in 18.46 g/L solvents production, comparable to that obtained in fermentation using commercial yeast extract (20.86 g/L).

When glycerol was used as carbon substrate, *Cl. acetobutylicum* JCM 7289 was able to produce relatively great variety of products including butanol, ethanol acetone; however, a varying fraction of glycerol was also converted to 1,3 propanediol, butyric acid and acetic acid, respectively. More than 40 g/L glycerol was utilized, and only 5.92 g/L total solvents were produced, two times lower in concentration as when glucose was used as substrate (13.85 g/L). It could be due to its metabolic route that favors the formation of other solvents such as 1, 3 propanediol, diminishing the production of ABE. According to the result of glycerol concentrations, it had an effect on cell growth and solvents production. When initial glycerol concentration was higher than 40 g/L, residual glycerol was left at the end of

the fermentation. Moreover, the efficiency of glycerol utilization at high initial glycerol concentration was low. In addition, the initial concentration of glycerol at 60 g/L or higher the cell growth rate was retarded. On the fermentation with controlled pH, the result revealed that the highest total solvents production (11.66 g/L) was obtained with a controlled pH of 5.5. Furthermore, total solvents production with controlled pH was higher than that obtained in fermentation without controlled pH. Considering the effect of various nitrogen sources, it revealed that the fermentation performance using spent brewer's yeast extract as a nitrogen source resulted in 10.81 g/L total solvents production, comparable to that obtained with fermentation using the commercial yeast extract (11.66 g/L).

School of Biotechnology

Academic Year 2010

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Co-Advisor's Signature_____

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Apichai Sawisit

CONTENTS

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

ABE	=	Acetone-Butanol-Ethanol
ATP	=	Adenosine 5’-tri-phosphate
BYE	=	Spent brewer’s yeast extract
BTU	=	British thermal unit
CYE	=	Commercial yeast extract
CoA	=	Coenzyme A
°C	=	Degree Celsius
g	=	Gram (s)
g/L	=	Gram (s) per Liter
g/mL	=	Gram (s) per milliliter
h	=	Hour (s)
L	=	Liter (s)
M	=	Molar
mg	=	Milligram (s)
mg/L	=	Milligram (s) per Liter
min	=	Minute (s)
mL	=	Millitre (s)
mm	=	Millimetre (s)
mmHg	=	Millimetre (s) of mercury
MW	=	Molecular weight
NAD	=	Nicotinamide adenine dinucleotide (Oxidized form)

LIST OF ABBREVIATIONS (Continued)

NADH	=	Reduced form of Nicotinamide adenine dinucleotide
OD_{600}	=	Optical density at 600 nm
Pa	=	Pascal
rpm	=	Revolutions per minute
RVP	=	Reid vapor pressure
STP	=	Standard Temperature and Pressure
μL	=	Microlitre (s)
% (w/v)	=	Percentage weight by volume
% (v/v)	=	Percentage volume by volume

CHAPTER I

INTRODUCTION

1.1 Significance of the study

In recent years, high crude oil price and increasing concern over global warming have stimulated efforts towards biosynthesizing fuels from renewable resources (Ezeji *et al*., 2005; Qureshi *et al*., 2008). Ethanol, a traditional biofuel, is not an ideal fuel due to its high hygroscopicity and low energy density, which increase the difficulty involved in expense of its storage and distribution. Compared to ethanol, butanol, which is less hygroscopic and less volatile, has an energy density that is closer to gasoline (Lee *et al*., 2008 a). Moreover, butanol can be used as a chemical feedstock in the plastic and flavor industries (Campos *et al*., 2002). Therefore, increasing attention has been paid to butanol production.

Butanol is one of a product in clostridia fermentation which known as acetone-butanol-ethanol (ABE) fermentation. Clostridia, a group of Gram-positive, spore-forming, obligate anaerobes bacteria, naturally possess pathways that allow the conversion of sugar into solvents, A number of different clostridia have been isolated and their abilities in relation to butanol production varied, but *Clostridium acetobutylicum* is the species that is the most often used for the industrial production of butanol (Jones and Woods. 1986). Butanol can be produced from renewable agricultural resources including molasses, agricultural biomass, wood hydrolysate, starchy materials (corn, wheat, rice, rye, and cassava starches), dairy industry waste,

etc. (Campos *et al*., 2002). The economics of butanol production is affected by raw materials used, the type of bioreactor, the butanol recovery techniques, byproduct credit, yield, solvent concentration, and productivity (Qureshi *et al*., 2001). The main constraint on the economic feasibility of acetone-butanol-ethanol (ABE) production is the cost of raw material. About 60-70% of the total production cost in ABE fermentation is the cost of raw materials (Madihah *et al*., 2001a; Madihah *et al*., 2001b). Thus, the availability of an inexpensive raw material is essential if solvent fermentation is to become economically viable. Most abundantly available, cost-effective raw materials used for fermentation industry are starchy materials. In Thailand, cassava represents an alternative cheap carbon source for fermentation processes that is attractive in both economic and geographical considerations. Cassava is widely grown for its enlarged starch-filled roots, which contain nearly the maximum theoretical concentration of starch on a dry weight basis among food crops. Besides that, cassava is able to grow in poor soils on marginal lands with minimal amounts of fertilizer, pesticides, and water (Yu and Tao, 2009). Therefore, cassava is a promising crop for biofuel production from renewable resources. Another potential alternative substrate is glycerol, this compound is a secondary product of several processes, namely the conversion of fatty oils (sunflower, rape seed) to bio-fuel, which yield 10% (w/v) glycerol (Yazdani *et al*., 2007; Rehman, *et al*., 2008). This has resulted in a sharp decrease of glycerol cost by 10-flod (Rehman *et al*., 2008), hence, there is a great advantage to use glycerol for the various chemical production and bio-fuel from economical and environmental standard point.

In ABE fermentation, bacteria have complex nutritional requirements especially nitrogen source. The most common and the most effective supplement with

yeast extract were generally reported (Madihah *et al*., 2001 b). However, yeast extract is an expensive substance. Therefore, it is attractive to develop a more economical method for ABE production, using materials as a cheaper nitrogen source. Brewer's yeast extract is nutritious waste products of the alcoholic beverage and therefore could be considered as feasible, inexpensive alternative nitrogen source for ABE fermentation.

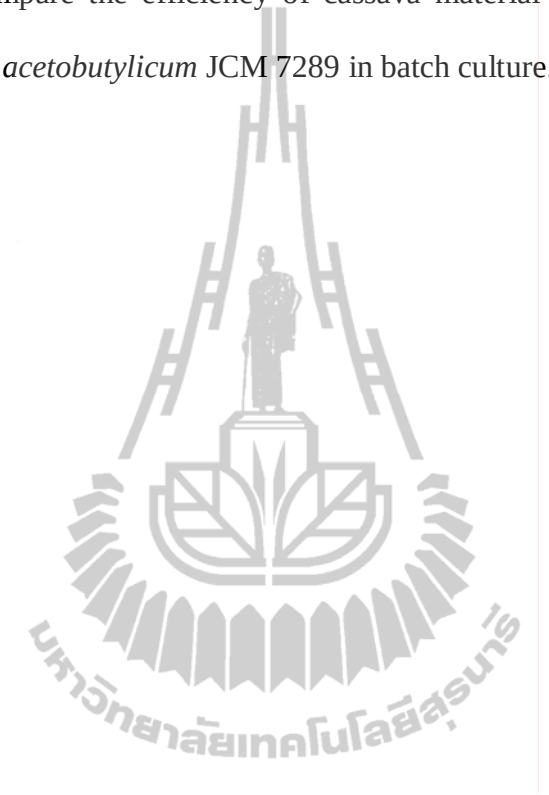
The physiological characteristics and biosynthetic pathways of microbial cell can be altered by the manipulation of the culture pH. It have been reported that culture pH plays an important role in the induction of solventogenesis and this is related to the level of undissociated butyric acid in the culture (Monot *et al*., 1984) and the intracellular levels of dissociated butyrate (Huang *et al*., 1986; Jones and Woods, 1986). The ideal pH control strategy for stimulating solvent production of *Cl. acetobutylicum* has not yet clearly understood. Hence, the improvement of a suitable culture pH that enhances solvent production of cassava materials and glycerol needs to be investigated. This study focused on the development of an efficient and economical process of cassava materials and glycerol fermentation to solvents by *Cl. acetobutylicum* that involves development of pH control strategy for improvement of solvent production and optimization of medium with a view to replace expensive ingredients; such as, yeast extract by renewable lower cost materials.

1.1 Research objectives

(1) To investigate the feasibility of *Cl. acetobutylicum* TISTR 1462 to produce solvents (acetone, butanol and ethanol) using cassava materials as the main substrate supplementing with spent brewer's yeast extract as a nitrogen source in batch culture.

(2) To optimize production conditions of *Cl. acetobutylicum* JCM 7289 to produce solvents using glycerol as carbon source and spent brewer's yeast extract as nitrogen source in batch culture.

(3) To compare the efficiency of cassava material and glycerol in solvents production by *Cl. acetobutylicum* JCM 7289 in batch culture.



CHAPTER II

REVIEW OF THE LITERATURES

2.1 Butanol

Butanol (1-butanol) also commonly known as butyl alcohol, *n*-butanol or methylopropane, is a linear 4-carbon aliphatic alcohol (primary alcohol) having the molecular formula of C_4H_9OH (MW 74.12 g/mol). Butanol is a colorless, flammable, slightly hydrophobic liquid with a distinct banana-like aroma and strong alcoholic odor. In direct contact it may irritate the eyes and skin. Its vapor has an irritant effect on mucous membranes and a narcotic effect in the higher concentrations. It is completely miscible with most common organic solvents, but only sparingly soluble in water (Lee *et al*., 2008a; Dürre, 2008). Other chemicals in the same alcohol family include methanol (1-carbon), ethanol (2-carbon), and propanol (3-carbon) (Kristin, 2007). Table 2.1 summarizes the distinctive characteristics of butanol over other fuels.

2.2 Butanol as Fuel

One of the major superior roles of biobutanol (bio-based butanol) is its appliance in the next generation of motor-fuels. While ethanol has received most of the attention as a fuel additive for many reasons (Hansen *et al*., 2005; Niven, 2005), butanol could be a better direct option due to its own intrinsic physical and chemical properties (Huber *et al*., 2006) and energy content as compared to ethanol (Table 2.1). This means that butanol consumption is close to that of pure gasoline

whereas ethanol-gasoline blends are consumed much faster to obtain the same power input. Additionally, butanol can be mixed with common gasoline at any percentage ratio (Lee *et al*., 2008b) in a similar way as with existing gasoline-ethanol blends (e.g., 23% in Brazil and 10% in United States and some parts of Europe). Also, butanol usage does not require any modifications in car engines or substitutions, producing similar mileage performance to gasoline. Despite this small increase in biofuel consumption the emissions of CO, hydrocarbons and NOx pollutants were drastically reduced. This has a tremendous positive impact on the global environment.

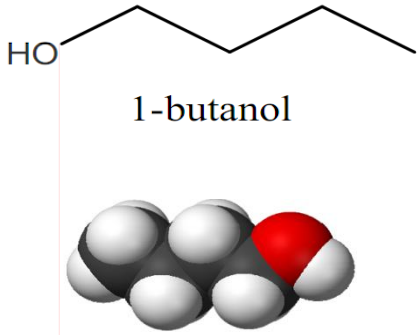
Other important advantages over ethanol include: (a) the lower volatility (less explosive). Butanol has a Reid Vapor Pressure (RVP) 7.5 times lower than ethanol (Lee *et al*., 2008b); (b) it does not readily adsorb moisture (lower hygroscopicity), so is less affected by weather changes; (c) less corrosive (Dürre, 2007); (d) is safer than ethanol because of its high flash point and lower vapor pressure; (e) it has a higher octane rating; (f) butanol has approximately 30% more energy/BTU accumulated per gallon (around 110.000 BTU per gallon, as opposed to ethanol, which has 84.000 BTU per gallon); and (g) complete miscibility with gasoline and diesel fuel. These allow butanol to be a much safer fuel that can be dispersed through existing pipelines and filling stations (Lee *et al*., 2008) with simple integration into the present fuel delivery and storage infrastructures (pipelines, storage tanks, filling stations, etc.). On the other hand, ethanol can only be added shortly prior to use. The vapor pressure of butanol (4 mmHg at 20°C) is 11 times lower than ethanol (45 mmHg at 20°C) enabling it to be directly added to gasoline without regarding evaporation emissions and consequent related complications. Also, the physical and chemical properties of butanol makes possible the blending with gasoline with no phase separation in the

presence of water (less readily contaminated with water) than other biofuel/gasoline blends. However, the viscosity of butanol is twice of that of ethanol and 5-7 times that of gasoline (Wackett, 2008). Other physical properties of butanol, such as density and heat capacity, are somewhat comparable to that of ethanol (Table 2.1).

2.3 Main applications of Butanol

Besides the expected role as engine-biofuel, butanol is actually an important bulk chemical with a broad range of industrial uses. Almost half of the worldwide production is used in the form of butyl acrylate and methacrylate esters used in the production latex surface coatings, enamels, nitrocellulose lacquers, adhesives, sealants, elastomers, textiles, super absorbents, flocculants, fibers, and plastics. Other important butanol-derived compounds are butyl glycol ether, butyl acetate and plasticizers. Compounds of minor applicability are butyl amines and amino resins. Butanol and derived compounds are excellent diluents in paint thinners, hydraulic and brake fluid formulations. It is also used as solvent in the perfume industry and for the manufacturing of antibiotics, vitamins and hormones. Other applications include the manufacture of safety glass, detergents, flotation aids (e.g., butylxanthate), deicing fluids, cosmetics (eye makeup, nail-care products, shaving and personal hygiene products. It is also commonly used as extracting agent and in food and flavor industries) (Lee *et al*., 2008b; Dürre 2008).

Table 2.1 Physical and chemical properties of butanol (adapted from Davis and Morton III, 2008; Lee *et al*., 2008b).

Properties	**Butanol**	**Chemical Structure**
Melting point (°C)	- 89.3	
Specific gravity	0.810-0.812	
Ignition temperature (°C)	35-37	
Auto-ignition temperature (°C)	343-345	
Flash point (°C)	25-29	
Relative density (water: 1.0)	0.81	
Critical pressure (hPa)	48.4	
Critical temperature (°C)	287	
Explosive limits (vol. % in air)	1.4-11.3	
Water solubility 9.0 mL/100 mL	(7.7 g/100 mL at 20°C)	
Relative vapor density (air: 1.0)	2.6	
Vapor pressure (kPa at 20°C)	0.58	

	Butanol	**Gasoline**	**Ethanol**	**Methanol**
Boiling point (°C)	117-118	27-221	78	64.7
Density at 20°C (g/mL)	0.8098	0.7-0.8	0.7851	0.7866
Solubility in 100 g of water	immiscible	immiscible	miscible	miscible
Energy density ($MJ.1^{-1}$)	27-29.2	32	19.6	16
Energy content/value (BTU/gal)	110000	115000	84000	76000
Air-fuel ratio	11.2	14.6	9	6.5
Heat of vaporization (MJ/kg)	0.43	0.36	0.92	1.2
Liquid Heat capacity (Cp) at STP (kJ/k-mol.°K)	178	160–300	112.3	81.14
Research octane number	96	91-99	129	136
Motor octane number	78	81-89	102	104
Octanol/Water Partition Coefficient (as $logP_{o/w}$)[a]	0.88	3.52±0.62	-0.31	-0.77
Dipole moment (polarity)	1.66	n.a.	1.7	1.6
Viscosity (10^{-3} Pa.s)	2.593	0.24-0.32	1.078	0.5445

[a]Log P is a measure of hydrophobicity (lipophilicity) and is similar to polarity. These published values were obtained from Hansch *et al*. (1995) for the three alcohols. In gasoline the Log P was roughly estimated as the average weight of main representative components.

2.4 Chemical Synthesis of Butanol

Butanol has been made industrially using three major chemical processes: Oxo synthesis, Reppe synthesis, and crotonaldehyde hydrogenation (Figure 2.1). In oxo synthesis (hydroformylation), carbon monoxide and hydrogen are added to an unsaturation using metal catalysts such as Co, Rh, or Ru substituted hydrocarbonlyls (Lee *et al*., 2008b). Aldehyde mixtures are obtained in the first reaction step, which is followed by hydrogenation for the production of butanol. Depending on the reaction conditions such as pressure, temperature and type of catalyst, different isomeric ratios of butanol can be obtained. In the Reppe synthesis, propylene, carbon monoxide and water are reacted together in the presence of a catalyst (Bochman *et al*., 1999) generating a mixture of *n*-butaraldehyde and isobutaraldehyde where the former is reduced to *n*-butanol (Wackett, 2008). The Reppe process directly produces butanol at low temperature and pressure. However, this process has not been commercially successful since it requires expensive technology. Until a few decades ago, the common route for butanol synthesis was from acetaldehyde using crotonaldehyde hydrogenation. The process consists of aldol condensation, dehydration, and hydrogenation (Bochman *et al*., 1999). Although rarely utilized nowadays, it may again become significant in the future. While other processes rely completely on petroleum, the crotonaldehyde hydrogenation process provides an alternative route from ethanol which can be produced from biomass. In this case, ethanol is dehydrogenated to form acetaldehyde from which the synthesis can proceed (Swodenk, 1983).

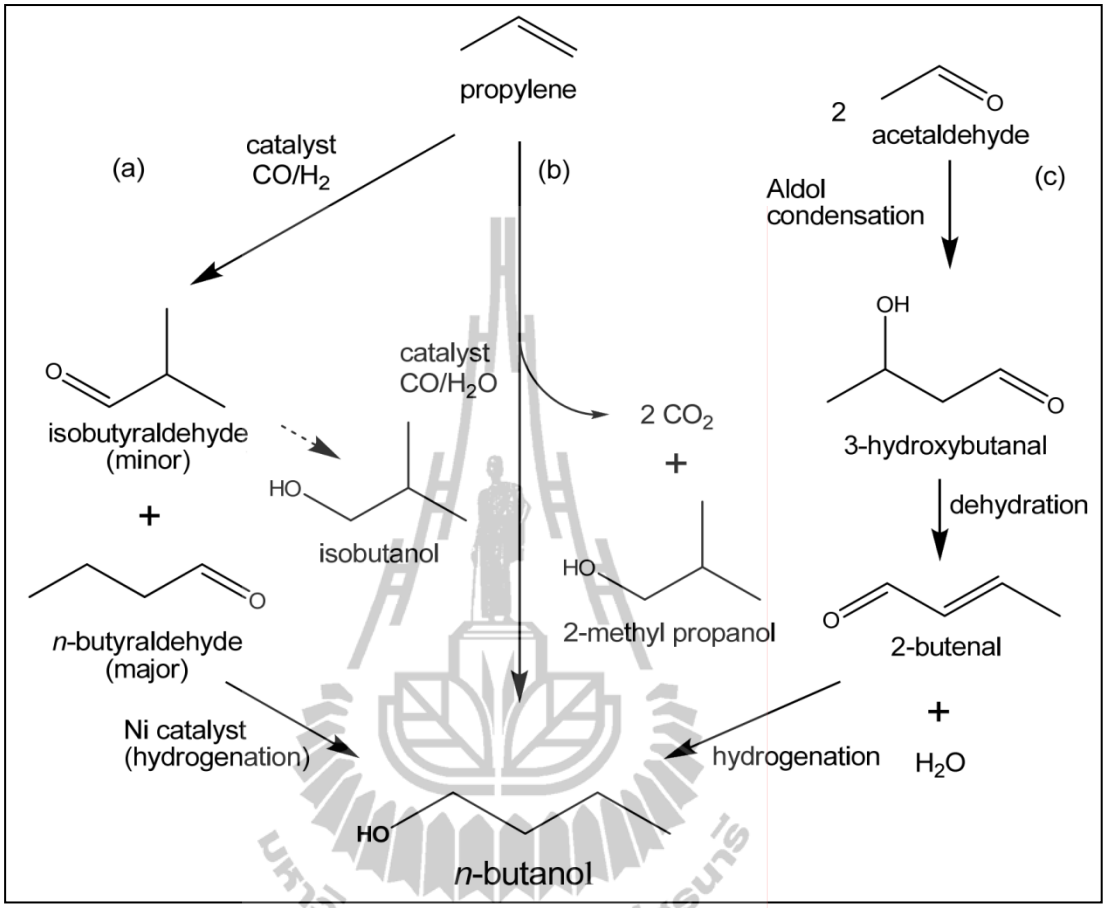


Figure 2.1 Industrial syntheses of butanol and secondary by-products. Chemical routes: (a) Oxo synthesis, (b) Reppe process, and (c) crotonaldehyde hydrogenation (adapted from Lee, 2008a ;Wackett, 2008).

2.5 Butanol fermentation

Butanol also can be produced via the fermentation process called ABE fermentation which is a process that utilizes bacterial fermentation to produces acetone, butanol and ethanol from biomass. It is well known process and it was primary used to produce acetone during World War II. The process is strictly anaerobic, which means that it must not have any oxygen present. Anaerobic

environment is usually maintained by bubbling nitrogen gas. The process produces acetone, butanol and ethanol in a ratio of 3:6:1 (Jones and Woods, 1986). It usually uses a strain of bacteria from Clostridium family. *C. acetobutylicum* is the most well known strain, although *C. beijerinckii* is used for this process, with very good results (Lee *et al*., 2008).

2.6 History of the ABE fermentation

The history in development of the industrial of ABE fermentation process has been well documented (Jones and Woods, 1986; Schuster *et al*., 1998; Badr *et al*., 2001; Ezeji *et al*., 2000b; Lee *et al*., 2008b). The microbial production of butanol was first recovered by Pasteur in 1861. An addition product of this fermentation, known as acetone, was reported by Schardinger in 1905 (Jones and Woods, 1986). Due to the shortage of natural rubber around the turn of the century, the ABE fermentation process was developed, since butanol was considered as a precursor of butadiene, the starting material for synthetic rubber. The British Company Strange and Graham Co., Ltd, became interested and recruited the services of Perking, Weizmann, Fernbach and Schoen in 1910 to study the formation of butanol by microbial fermentation (Lee *et al*., 2008b). This led to the remarkable development of microbial process for butanol production. Weizman succeeded in isolating an organism, later named *Cl. acetobutylicum*, which was able to produce butanol and acetone from starchy materials in better yields than the organism of Ferbanch which utilized potatoes. The Fernbach's process was patented in 1912 and the Weizmann's process in 1915.

The World War I in 1914 caused a sharp increase in the industrial demand for acetone, sine acetone was used to dissolve cordite in the manufacture of explosives.

The Weizmann process was recognized by the British government and a production plant was built at the Royal Naval Cordite Factory at Poole, but production was subsequently moved to the USA and Canada. However, at the end of the war, all these plants were closed down due to reduced demand for acetone. At this time, the automobile industry developed rapidly and required large amounts of solvent (butyl acetate) for nitrocellulose lacquers. This allowed the microbial production of butanol to become of importance again (Lee *et al*., 2008).

The industrial production of ABE by fermentation was carried on until after World War II. It then started to decline due to the unprecedented growth of the petrochemical industry and the increased use of grains and molasses for human and animal consumption. In the 1960's, the industrial production in Europe and North America ceased due to the unfavorable economics. The ABE Fermentation Plant in South Africa was able to continue operating until its closure in 1981 (Jones and Woods, 1986).

The sudden rise in crude oil price in 1973, and again in 1979, triggered a renewed interest in the biotechnological production of the butanol and acetone. It has not let to a process which can compete with the synthetic process from petrochemicals, at least not in the western world. Acetone is currently produced by either the cumene hydroperoxide process or the catalytic dehydrogenation of isopropanol.

2.7 The ABE fermentation process

2.7.1 Microorganisms

Members of genus *Clostridium* are Gram-positive, spore-forming rods

that are anaerobic. These motile bacteria are ubiquitous in nature. Some species of *Clostridium* secrete their active form powerful exotoxins that are responsible for diseases such as tetanus, botulism, and gas gangrene. However, non-pathogenic clostridia have attracted great interest in the 20th century because of their solvent-forming properties (e.g *Cl. acetobutylicum* and *Cl. beijerinckii*). They are capable of converting a range of carbohydrates to end-products such as acetone, butanol, ethanol (ABE fermentation). In certain fermentation, butanol and acetone or the main solvent where in other process only acetone and ethanol are formed. However, two species have been developed for solvent production. They are *Cl. acetobutylicum* and *Cl. beijerinckii*. *Cl. acetobutylicum* that ferment starch, hexoses, or pentoses to butanol, acetone and ethanol in the general ratio of 6:3:1 (Qureshi, 2001; Blaschek, 2001; Lee *et al*., 2008) respectively, *Cl. beijerinckii* ferments hexose or starch and produces largely butanol and a smaller quantity of isopropanol and ethanol.

Cl. acetobutylicum is Gram-positive in growing cultures but Gram-negative in older cultures. Individual vegetative cells of *Cl. acetobutylicum* are straight rod-shaped bacillus ranging in size of 0.5-1.5×1.5-6 μm (Robinson, 2000) (Figure 2.2a). They are typically strictly anaerobes (oxygen free), heterofermentative, spore-forming and motile by peritrichous flagella. During sporulation, cells swell markedly and store granulose, a polysaccharide based material that serves has carbon and energy source during solventogenesis. Spores are oval and subterminal (Figure 2.2b). The optimum growth temperature is 37°C, while the optimum temperature for solvent production is 35^{o}C (McNeil, 1985; Kristiahsen, 1985). The optimum pH values for growth and acids production is 6.5, while a pH of less than 5.3 is often required for solvent production (Monot *et al*., 1983). Active motile cells are regarded

as a prerequisite for good solvent production. Division occurs by transverse fission resulting in long chains of organism which break apart into single cells in liquid medium during vigorous fermentation as well as biotin and 4-aminobenzoate are usually required as growth factors. ABE-clostridial strains are generally classified into four distinct groups based on their biochemical and genetic characteristics (Woods, 1995). The best known groups are the mesophiles *Cl. acetobutylicum* and *Cl. beijerinckii* (formerly known as *Cl. butylicum*) and one of the most documented strains in ABE-fermentation research studies (Karakashev *et al*., 2007).

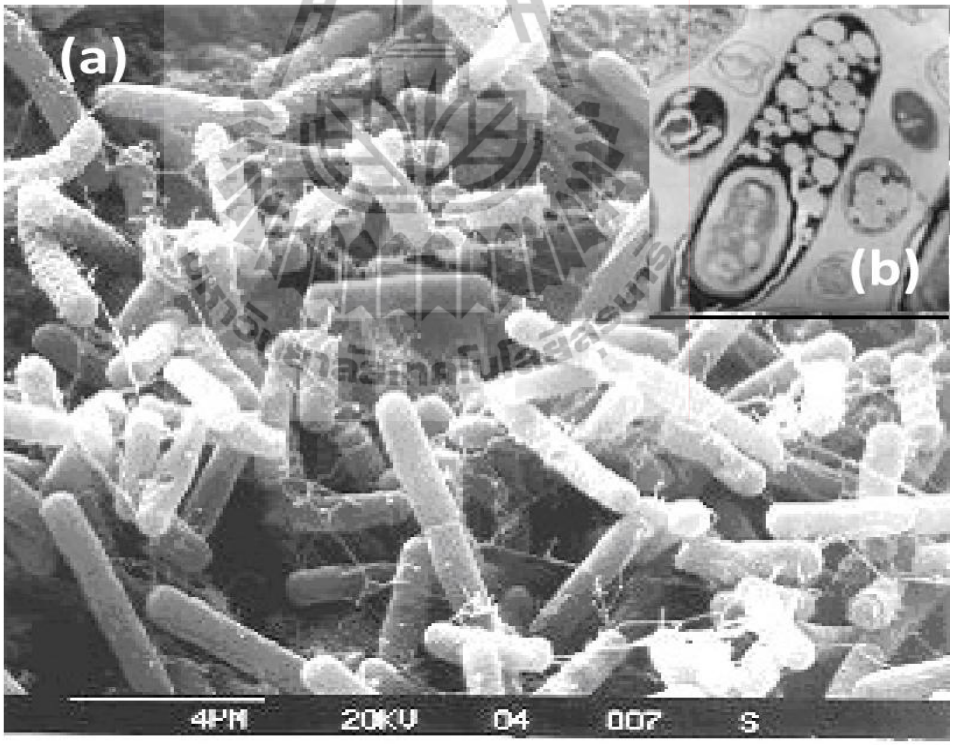


Figure 2.2 Scanning Electron Micrographs (SEM) of *Cl. acetobutylicum* (also called the "Weizman organism") showing the different stages of spore formation: vegetative cells (a) and spore formed cells (b). Image (a) was taken from (Jones *et al*., 1982) and image (b) was taken from (Long *et al*., 1983).

2.7.2 Course of fermentation

The progress of a typical batch fermentation process using *Clostridium acetobutylicum* is now well documented (Jones and Woods, 1986; Schuster *et al*., 1998; Badr *et al*., 2001). It is characterized by two distinct phases which correspond to the two-stage mechanism of product formation. Initially, it is an acidogenic phase where active logarithmic growth of the organism occurs, producing acetic and butyric acids over the period of 7-18 h. These cause a decrease in the pH value of the culture. Eventually, the growth rate decreases and the second phase, known as the solventogenic phase, occurs from 18 h though to 36-60 h. The pH value of the culture rises slightly due to a decrease in growth rate and acids being assimilated and metabolized to solvents. The pH breakpoint is where the acidogenic phase switches to the solventogenic phase (Lee *et al*., 2008b). It has been suggested that the uptake of acid functions as a detoxification process in response to an unfavorable environment, resulting in neutral solvents being produced. These solvents are less toxic to the cell than the previously synthesized organic acids (Jones and Woods, 1986). Hydrogen and carbon dioxide gases are released throughout the fermentation with gas evolution being maximal during the acidogenic phase.

Sugar is utilized throughout the fermentation. The solvents yield approximates 30% (solvents produce/weight sugar utilized). However, slight differences in yields can be observed depending on strains and cultural conditions used (Jones and Woods, 1986; Lee *et al*., 2008). Typical solvent productivities of batch fermentation processes are in the range of 0.2-0.6 g/L/h depending on the operating conditions (Yu *et al*., 1985).

Schuster *et al*. (1998) reported that *Cl. acetobutylicum* showed marked

change in the cell morphology during the course of the cultivation (Figure 2.3). During early growth and the acid production phase, only rod-shaped cells, which sometimes formed chains, were observed. Later, at or just prior to the solvent shift, clostridial forms appeared, containing granulose. As the fermentation proceeded, the cellular granulose content reached a peak, which coincided in most experiments with the maximum solvent productivity, after which it decreased.

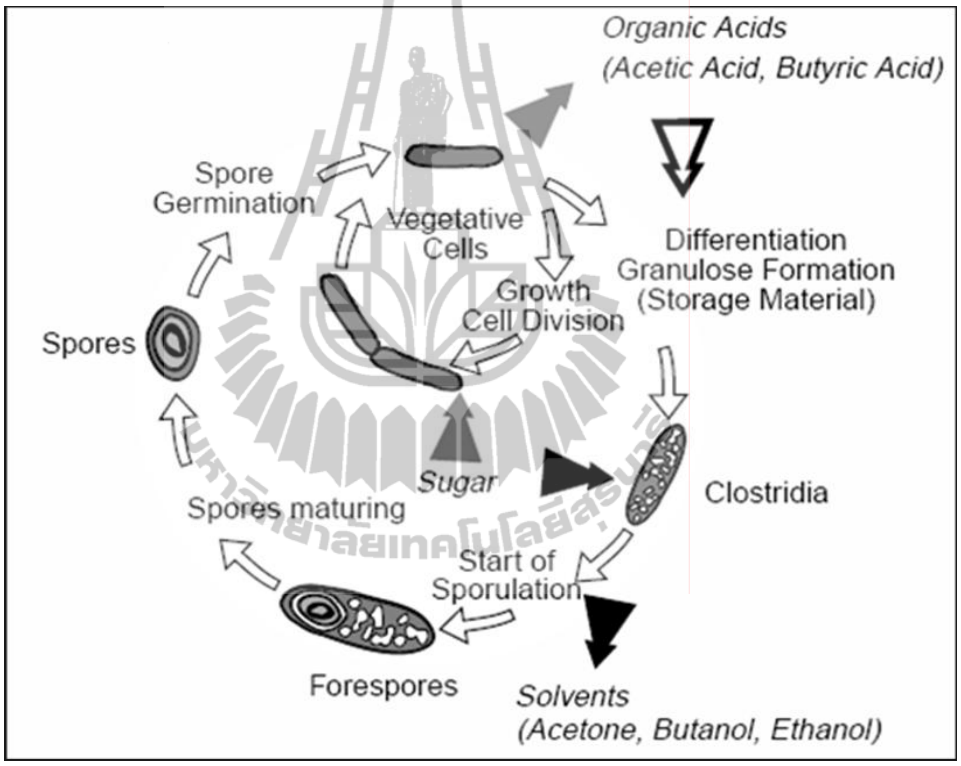


Figure 2.3 Cell physiological cycle of *Cl. acetobutylicum* (Source: Schuster *et al*., 1998).

2.7.3 Biochemistry of the fermentation

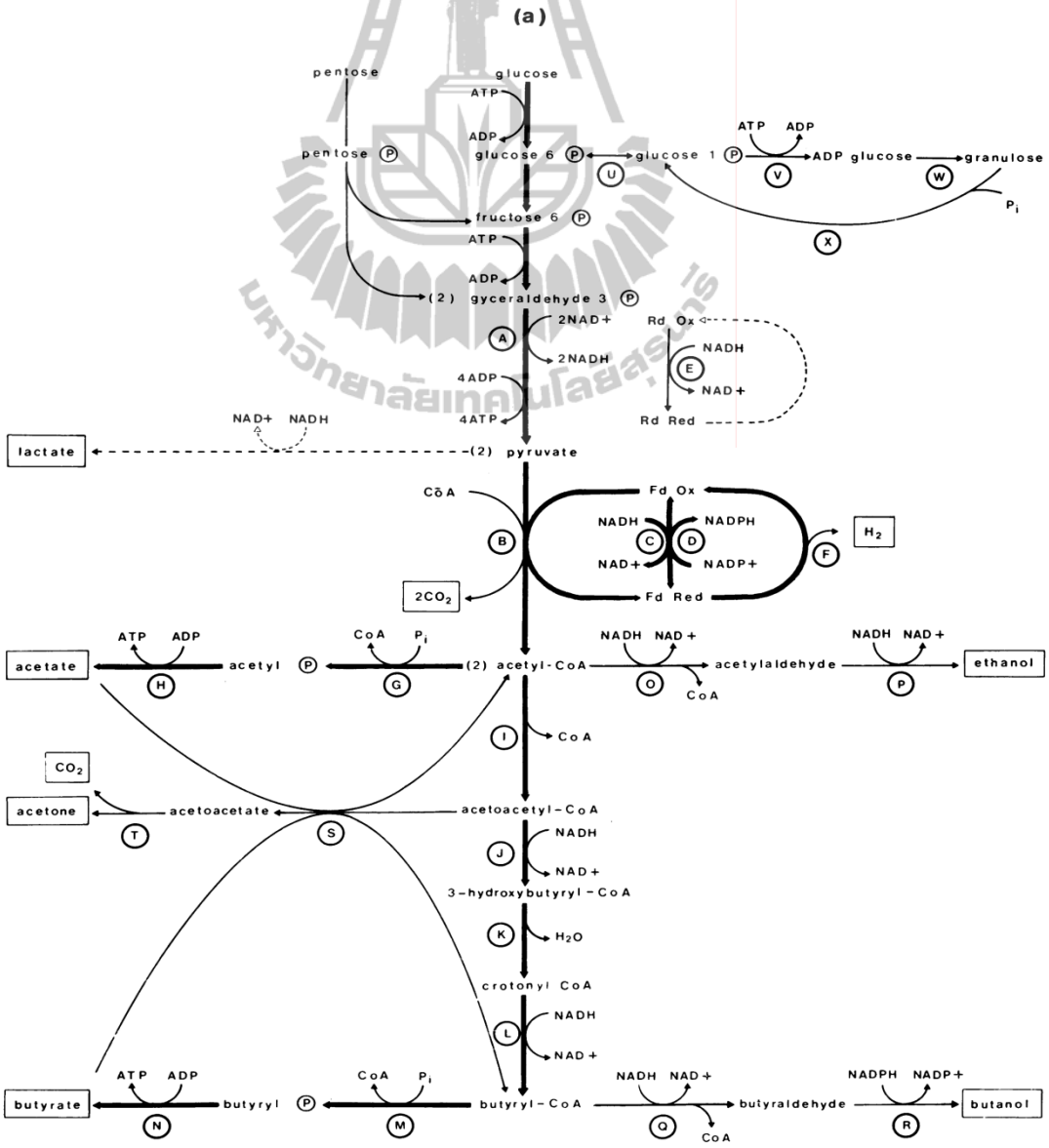
2.7.3.1 Carbon flow

In typical batch cultures of solvent-producing clostridia can be divided into two distinct growth phases, acidogenic phase (Figure 2.4a) and

solventogenic phase (Figure 2.4b). An acidogenic phase (acid-producing phase), mainly acetate, butyrate, hydrogen and carbon dioxide are produced as metabolic end-products which result in a decrease in the pH of the culture medium. The fermentation entered the solventogenic phase when growth reached stationary phase. During this phase, the metabolism of cells undergoes a shift to produce solvents, n-butanol, acetone/isopropanol, and ethanol, along with H_2 and CO_2, are produced by reassimilation of organic acids of previously produced acid end-products which caused the increment of pH.

The main biochemical pathways involved in the conversion of carbohydrate to fatty acids, solvents, carbon dioxide, and hydrogen by *Cl. acetobutylicum* (Figure 2.4a, b) are now well investigated and firmly established. Hexose sugars are metabolized via the Embden-Meyerhof glycolytic pathway to 2 mol of pyruvate, 2 mol of ATP and 2 mol of NADH + H^+, from 1 mol of hexose. Pentose sugars are metabolized by way of the pentose phosphate pathway, leading via a series of steps, to fructose-6-phosphate and glycoceraldehyde-3-phosphate which then enter the EMP pathway (Jones and Woods, 1986). Pyruvate, resulting from glycolysis, is further oxidized by pyruvate-ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to form acetyl-CoA, CO_2 and reduce feredoxin. The acetyl-CoA formed by this phosporoclastic reaction serves as the precursor for all the fermentation products. Two molecules of acetyl-CoA are converted to acetoacetyl-CoA, which is further converted via the cyclic system, leading to the formation of butyrate. As the culture pH falls due to accumulation of acids, acetoacetyl-CoA is diverted from the normal cyclic mechanism, to yield acetoacetate, which is then converted to acetone and CO_2 via the acetoacetate decarboxylase system. This later

step is irreversible (Jone and Woods, 1986). Diversion of the cyclic system to produce acetone prevents further butyric acid formation and eliminates the two steps generating NAD^+. To regenerate NAD^+ the organism must reconvert butyrate to butyryl-CoA, and then reduce the latter to butanol. In addition, further sugar utilization is forwarded to butanol rather than hydrogen production. Ethanol is also produced from acetyl-CoA by two steps of reactions. Acetyl-CoA is converted to acetaldehyde by acetaldehyde dehydrogenase, and then to ethanol by ethanol dehydrogenase. This result in the oxidation of 2 mol of NADH + H^+ to NAD^+.



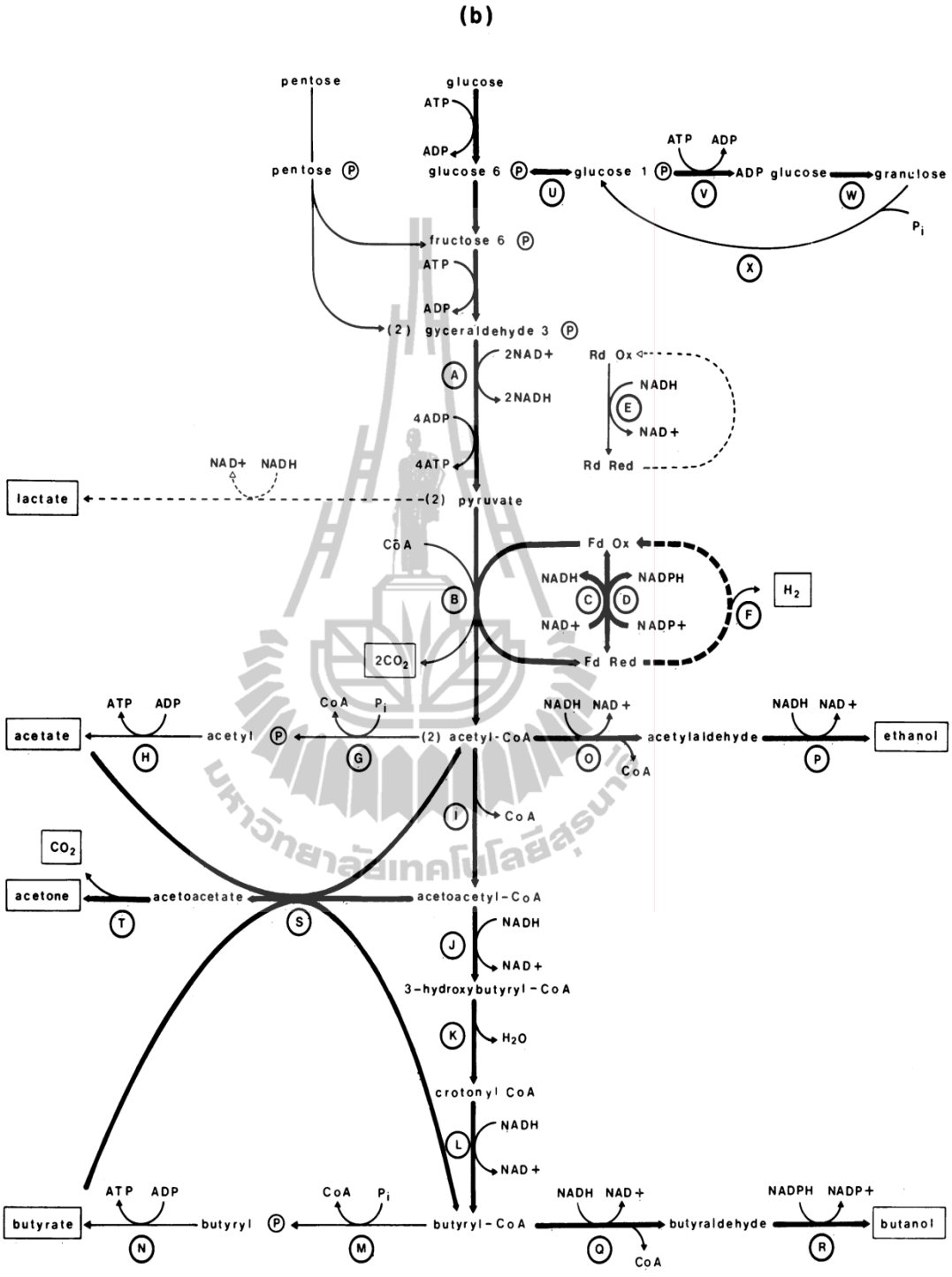


Figure 2.4 Biochemical pathways in *Cl. acetobutylicum*. Reactions which predominate during the acidogenic phase (a) and the solventogenic phase (b) of the fermentation are shown by thick arrows (Source; Jones and Woods, 1986).

Enzymes are indicated by letters as follows: (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; (i) thiolase (acetyl-CoA acetyltransferase); (J) 3-hydroxybutyryl-CoA dehydrogenase; (K) crotonase; (L) butyryl-CoA dehydrogenase; (M) phosphate butyltransferase (phosphotransbutyrylase); (N) butyrate kinase; (O) acetaldehyde dehydrogenase; (P) ethanol dehydrogenase; (Q) butyraldehyde dehydrogenase; (R) butanol dehydrogenase; (S) acetoacetyl-CoA:acetate/butyrate:CoA transferase; (T) acetoacetate decarboxylase; (U) phosphoglucomutase; (V) ADP-glucose pyrophosphorylase; (W) granulose (glycogen) synthase; (X) granulose phosphorylase.

2.7.3.2 Electron flow

During the acid-producing growth phase, there is an excess of reducing quivalents because only a portion of reducing equivalents produced by glycolysis is consumed during the reduction of acetoacetyl-CoA to butyryl-CoA (Figure 2.5). This excess in reducing equivalents is relieved by disposing of the electrons in the form of hydrogen gas by hydrogenase (Figure 2.5). During the solvent-producing growth phase, despite the presence of additional routes for the disposal of electrons via aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) reactions, which consume one mole of NAD(P)H per mole of substrate converted to product, there is still excess in reducing equivalents because the solvent-producing clostridia also produce acetone and the production of acetone does not involve a reduction reaction.

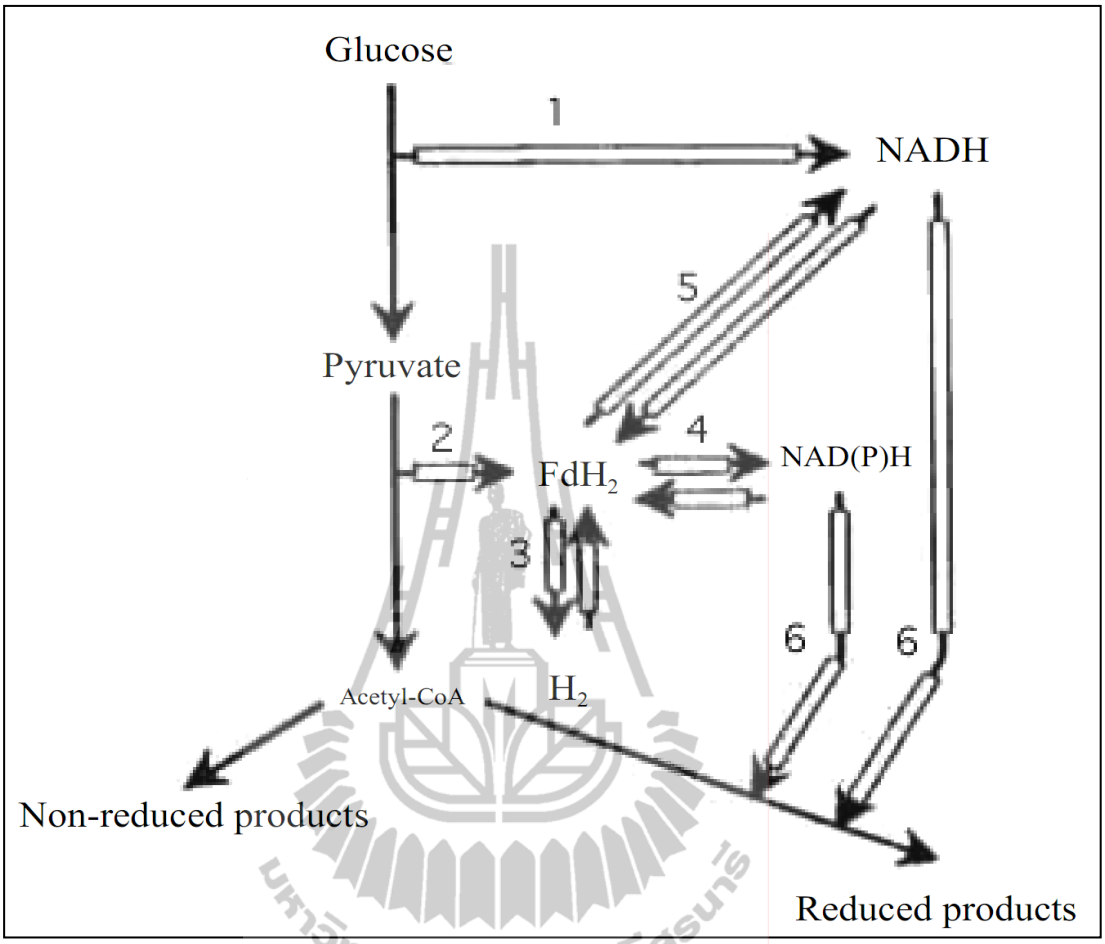


Figure 2.5 Pathways of electron flow (boxed arrows) in solvent-producing clostridia under non-nitrogen fixing growth conditions. Enzymes catalyzing the numbered reactions are as follows (reaction number in parenthesis): glyceraldehyde 3-phosphate dehydrogenase (1); pyruvate:Ferredoxin (Fd) oxidoreductase (2); hydrogenase (3); NADPH:Fd oxidoreductase (4); NADH:Fd oxidoreductase (5); NAD(P)H-dependent dehydrogenases (6). (Source: Mitchell, 1998).

2.7.4 Factors affecting the fermentation

The factor involved in the transition from the acidogenic to the solventogenic phase appears to be more complex than once thought. Several

investigations have been undertaken in batch and continuous culture to understand the way in which the production of solvents is initiated and maintained, but the exact reason for the transition is not clearly understood (Lee *et al*., 2008b). Although these studies brought a wealth of new information, it is evident that no single factor specifically induces solvent production. Hence, all of factor implicated in solventogenesis will be covered in this section.

2.7.4.1 Substrate source and concentration

The initial sugar concentration plays an important role in the ABE fermentation. At low (less than 20 g/L) initial sugar concentration, the fermentation tends to be acidogenic, which little solvent being formed (Lee *et al*., 2008b). However, at higher initial concentration (up to 60 g/L) the process becomes progressively more solventogenic (Madihah *et al*., 2001). At concentration above 80 g/L, considerable sugar remain unfermented, probably due to product inhibition (Madihah *et al*., 2001), while 120 g/L the fermentation activity of culture is negligible (Qadeer *et al*., 1980) due to substrate inhibition.

The mechanism of sugar transport is not fully understood. However, it is believed that phosphotransferase systems are responsible for uptake of glucose and fructose (Lee *et al*., 2008). It is likely that other substrates of *Cl. aecetobutylicum* are taken up by symport mechanisms driven by the trans-membrane proton gradient. Disaccharide such as sucrose or maltose might then be cleaved by an appropriate phosphorylase, and the free glucose can be converted to glucose-6- phosphate by hexokinase (Jones and Woods, 1986).

2.7.4.2 Temperature

The temperature of the fermentation can affect overall yield, solvent ratios, and rate of solvent production. In the molasses fermentation, solvent yields remained fairly constant at temperature between 30°C and 33°C but decrease at 37°C (Jones and Woods, 1986). Similar results were observed in a synthetic medium by McNeil and Kristiahsen, (1985) when they investigated the effect of temperature upon growth rate and solvent productivity in batch culture of *Cl. acetobutylicum*. They found that the total solvent yield decreases with increasing temperature, possibly because of a reduction in acetone production. It appeared that the yield of butanol was not affected by the temperature. In terms of total solvent yield and productivity, the optimum fermentation temperature was reported to be around 35°C.

2.7.4.3 Oxygen

Cl. acetobutylicum is an obligate anaerobe. Optimal growth occurs at negative culture redox potential, E_h, in the range of -250 mV to -400 mV. Exposure of an anaerobic culture to oxygen for a short period of time is not lethal. However, if cultures are exposed to high dissolved oxygen concentrations (40 to 60 µM), the rate of glucose consumption decrease while growth, DNA, RNA and protein syntheses are halted. Under aerobic condition, the organism has been reported to be drained of reducing power, and the production of butyrate, but not acetate, ceased. In addition, there was a marked fall in the level of intracellular ATP. These consequences of oxygenation were all reversible and growth and metabolism resumed when cell were returned to anaerobic conditions (Jones and Woods, 1986).

2.7.4.4 pH and acid end–products

The culture pH plays a crucial role in determining the outcome of sugar metabolism. A number of recent studies have reported that culture maintained at high pH values tend to produce mainly acids, whereas culture maintained at low pH values produce mainly solvents. However, the pH range over which solvent synthesis is likely to occur varies widely depending on the strain and culture conditions used may occur is pH 3.8 to 5.5 (Lee *et al*., 2008b). Although, the industrial strain *Cl. acetobutylicum* P262 can produce good levels of solvents at values approaching pH 6.5 (Jones and Woods, 1986).

The weak organic acids, acetic and butyric acids, produced as the end-products of metabolism are, by nature, toxic to the cell, and are able to partition in the cell membrane in their undissociated form (Jones and Woods, 1986). At sufficiently high acid concentration, the pH gradient across the membrane collapses, resulting in the total inhibition of cell metabolic functions within the cells. At lower concentrations, the accumulation of acid end products, and the associated decrease in pH value, result in a decrease in growth rate it eventually halted, although substrate utilization and cellular metabolism continue (Zhu and Yang, 2004). Hence, it has been suggested that the switch to solventogenesis is a detoxification mechanism of the cell to remove the inhibitory effect when acid end-products reach toxic levels (Hartanis *et al*., 1984; Long *et al*., 1984). In this way, the onset of the solventogenesis is usually associated with a lowered pH of the medium and a critical level of the undissociated form of the acids (Lee *et al*., 2008b). Thus, the trigger concentration of butyrate has been demonstrated to be at lower pH than at the high pH values (Jones and Woods, 1986). It is now generally accepted that the effect of pH is really an effect

of the undissociated butyric acid concentration, and this is likely to be the essential factor in the regulation of solvent production.

2.7.4.5 Nutrient concentration

In the ABE fermentation, in both batch and continuous culture, it is not generally accepted that carbon source limitation is the determination to solvent production (Long *et al*., 1984; Monot *et al*., 1983). Under carbon source limitation, the amount of acid end-product generated is insufficient to achieve the threshold concentration to induce solvent production.

1) Nutrient concentration in batch culture

Many reports in the literature regarding nutrient effect on the regulation of solvent production are often difficult to interpret. Although initial nutrient concentrations are usually described, the nutritional status of the culture during and after the fermentation is often not (Maddox, 1989). According to Maddox (1989), nutrient limitation in batch culture can be defined as a situation where cellular growth is restricted (terminated) due to the exhaustion of an essential nutrient.

Monot *et al*. (1983) reported that strong solvent production can be achieved after exhaustion of nitrogen from the medium. In contrast, Long *et al*. (1984) concluded that nitrogen-limited culture did not produce solvent. Their results indicate that there must be a minimum nitrogen concentration remaining after the growth phase to induce solventogenesis. However, the experiments performed by Roos *et al*. (1985), where the ratio of nitrogen to glucose was varied, suggested that an excess of nitrogen is detrimental to solvent production, and that as the ratio of nitrogen to glucose decrease, the rate of solvent production may be enhance by a

lowered availability of nitrogen. Hence, the possible role of nitrogen limitation in solvent production remains to be clarified.

2) Nutrient concentrations in continuous culture

In recent years, continuous culture of *Cl. acetobutylicum* has been widely use as a research tool to define parameter responsible for changes in the physiology and activity of this microorganism. The fundamental areas of ABE fermentation such as the effect of medium component and acid end-products on solvent production, the influence of temperature, culture pH, dilution rate, maximum attainable solvent concentration and yield and the stability of a continuous culture with regard to solvent production have been widely investigated. (Lee *et al*., 2008b).

There have been conflicting reports with respect to the role of some nutrient concentration in regulation of solvent production. In addition, some reports are difficult to interpret as it is not always clear whether a nutrient-limited condition has, in fact, has been attained. Direct comparison of the results is even more difficult because of differences in the strain used, medium composition, and fermentation conditions. However, it is now generally accepted that no single growth-limiting factor specifically induces solvent production in continuous culture, and each factor must be considered in interaction with other important environmental parameters, such as culture pH, dilution rate, etc. Nevertheless, some nutrients have been shown to be more suitable for growth limitations and production of solvents in high yields than others (Ezeji *et al*., 2005).

2.7.4.6 Product inhibition

One of the limiting factors with respect to the ABE process

is product inhibition, with butanol being the most inhibit product (Jones and Woods, 1986; Lee *et al*., 2008b). Cell metabolism usually continues until solvent production reaches inhibitory levels of around 20 g/L. These dilute product concentrations, which in turn, restrict the concentration of sugar that can be fermented, contribute to the high costs of product recovery. Thus, improvement in this area is critical for the economic viability of the overall process.

Butanol, acetic acid and butyric acid have been reported to be the most inhibitory of the fermentation products (Moreira *et al*., 1981; Kuhn and Linden, 1986). Growth was totally inhibited at a butanol concentration of 12 to 16 g/L, whereas cell growth was inhibited by 50% when Butanol was added at 7 to 13 g/L. The concentrations of acetic acid and butyric acid where 50% inhibition of cell growth occurred, were reported to be at 8.0 g/L and 6.0 g/L, respectively (Assobhei *et al*., 1998). Hence, this inhibitory effect to cell growth caused by butanol, acetic acid and butyric acid occurred at levels commonly observed during the fermentation. On the other hand, acetone and ethanol do not have inhibitory effects at the concentrations normally attained during fermentation (Jones and Woods, 1986).

2.8 Utilization of starchy material for ABE fermentation

Cl. acetobutylicum can utilize a great variety of sugars (Jones and Woods, 1986; Qureshi and Blaschek, 2001; Lee *et al*., 2008b). Traditional substrates such as corn, wheat, millet and rye were originally utilized as a raw material for the commercial ABE fermentation, but due to their high cost, alternative cheaper substrate are now being studies including starchy materials (sago, potato and tapioca starch) (Madihah *et al*., 2001); agricultural waste (Jesses *et al*., 2002); agricultural

residues (Ezeji *et al*., 2007) and dried distiller's grain and soluble (DDGS) (Ezeji, 2008; Blaschek, 2008). The ability of *Clostridium* spp. to produce amylolytic enzymes such as amylases, pullulanase and glucoamylase permits direct fermentation of gelatinized starch to ABE products. (Jones and Woods, 1986; Nimcevic *et al*., 1998; Madihah *et al*., 2001). Utilization of various types of starchy material for ABE production by *Clostridium* spp. has been examined by several researchers as shown in Table 2.2.

The reported work (Nimcevic *et al*., 1998) showed that, very good solvents formation rates were observed when *Cl. beijerinckii* NRRL B592 was cultivated on whole potato media and they found that, no enzymatic hydrolysis of potato starch was necessary.

Madihah and co-worker (2001) reported that *Cl. acetobutylicum* P262 can be utilized various starchy substrates to produce solvents. It shows that the highest total solvents productivity (0.26 g/L/h) was obtained when sago starch was used. However, it was not significantly different for fermentation using sago starch. While the total solvents productivity in the fermentation of potato starches was about 2-fold lower compared with fermentation using corn and sago starch. These studies suggest that starch can be a useful substrate for producing solvent, but depend on different types of starch

Ezeji and co-worker (2003) was evaluated fermentability of packing peanuts, a batch fermentation was run with 80 g/L of packing peanuts using *Cl. beijerinckii* BA101 in P2 medium. The initial and final concentrations of starch were 69.6 and 11.1 g/L, respectively. This resulted in utilization of 58.5 g/L of starch and a solvent yield of 0.32. During 110 h of fermentation, 18.9 g/L of ABE was produced, resulting

in a productivity of 0.17 g/L/h. The concentration of acids was low at 0.2 g/L at 110 h. The results proved that packing peanuts were successfully utilized by *Cl. beijerinckii* BA101 to produce ABE. However, productivity was low at 0.17 g/L/h.

In 2008, Madihah and co-workers revealed that high butanol production from sago starch by *Cl. saccharobutylicum* P262 could be achieved by using a simple pH control strategies during solventogenic phase. Under this appropriate condition, the overall productivity (0.7 g/L/h) was improved 1.4 times, and the final total solvent concentration (27.9 g/L) was improved by approximately 2 folds, when compare with fermentation without pH control.

In 2009, Gu and co-worker evaluated that using cassava medium supplemented with ammonium acetate, acetic acid and butyric acid were generated in abundance and then efficiently re-utilized by *Cl. acetobutylicum* EA 2018 to produce more acetone and butanol.

Recently, Thang *et al.* (2010) demonstrated that *Cl. saccharoperbutylacetonicum* N14 which is a hyperamylolytic strain can be produced solvents efficiently from cassava starch, which was comparable to when glucose was used as a substrate in batch culture. They showed that the highest total solvents (20.5-21 g/L) and solvents yield based on glucose consumed (0.41-0.46) was obtained. These results were comparable with those reported in other work where corn starch and potato starch were used (Nimcevic *et al*., 1998; Ezeji *et al*., 2005). Moreover, these results are much higher than those reported by Madihah *et al*. (2001) when a solvents yield of 0.20-0.34 was observed in direct fermentation of cassava starch by *Cl. acetobutylicum* P262.

Table 2.2 Summary of the literature describing the production of ABE by *Clostridium* spp. using starchy material as a substrate in batch fermentation.

Substrate	Microorganism	Total solvents (g/L)	Total ABE productivity (g/L/h)	Total ABE yield (g solvent/g glucose)	References
Potato starch	*Cl. beijerinckii* NRRL B592	18.9	0.42	-	Nimcevic *et al*., 1998
Potato starch	*Cl. acetobutylicum* P262	4.62	0.06	0.14	Madihah *et al*., 2001
Corn starch	*Cl. acetobutylicum* P262	11.87	0.18	0.36	Madihah *et al*., 2001
Sago starch	*Cl. acetobutylicum* P262	11.03	0.26	0.33	Madihah *et al*., 2001
Tapioca starch	*Cl. acetobutylicum* P262	6.74	0.16	0.20	Madihah *et al*., 2001
Starch-based packing peanuts	*Cl. beijerinckii* BA101	21.7	0.20	0.37	Ezeji *et al*., 2002
Degermed corn	*Cl. beijerinckii* BA101	24.8	0.34	0.42	Campos *et al*., 2002
Corn starch	*Cl. beijerinckii* BA101	20.0	0.28	0.49	Ezeji *et al*., 2005
Sago starch	*Cl. saccharobutylicum* DSM 13864	16.38	0.59	0.35	Liew *et al*., 2006
Sago starch	*Cl. saccharobutylicum* P262	27.9	0.70	0.48	Madihah *et al*., 2008
Cassava starch	*Cl. acetobutylicum* EA 2018	19.4	-	-	Gu *et al*., 2009
Cassava starch	*Cl. saccharoperbutylacetonicum* N14	21.0	0.44	0.41	Thang *et al*., 2010

Note: (-): no data.

2.8.1 Cassava

Cassava is grown for its enlarged starch-filled roots, which contains nearly the maximum theoretical concentration of starch on a dry weight basis among food crops. Fresh roots contain about 30% starch and very little protein (Yu and Tao, 2009). Tapioca (*Manihot esculenta* Crantz) is known by various names in different regions of the world. It is called “tapioca” or “cassava”, and “tapioca” is the name given to the starch. Cassava starch, a dominant source of starch in Thailand, possesses a strong film, clear paste, good water holding property, and stable viscosity. Unlike other starch sources, such as corn, rice and wheat, tapioca roots contain high starch content and a very low quantity of impurities. The Thai tapioca starch industry has over fifty years experience resulting in highly developed processing technology being used by most Thai manufacturers (Sriroth, 1998). The most important characteristics of native Thai tapioca starch is white in color, absence in unpleasant odor, possesses high ratio of amylopectin to amylose (80:20), and provides a high peak viscosity which is very useful in many applications. This characteristic can be further improved by modification (Sriroth *et al.*, 1999). Cassava root can be used to produce cassava chips, cassava pulp, and cassava starch, which are in high demand throughout the world. Thailand, Indonesia, and Brazil are the most prominent exporters of cassava starch, with their production accounting for 95% of the world’s supply.

Cassava chip is one form of the cassava root preservation. The roots deteriorate quickly from the internal heat generated from high respiration rate of the tissues (Ikujenlola and Opawale, 2007) and subsequent infection and rotting by microbes. Therefore, manufacturers prefer to convert cassava into more stable forms to prolong the shelf life of the product. Processing the roots by chipping and drying is

one of the ways of adding value, reducing the cyanogenic glucoside content and improving its storage (Chijindu *et al*., 2008).

Cassava pulp is the solid, moist by-product of cassava starch manufacture, and it represents approximately 10 to 15% of the original root weight. As cassava starch production increases, so does the large volume of waste by-product generated. In Thailand, at least 1 million tons of pulp is generated annually. After drying, some of the by-product is used to produce fertilizer or is included in diets for ruminants and swine. However, an abundance of byproduct still remains.

2.9 Glycerol

Glycerine or glycerol is a clear, odorless, viscous liquid. It is an alcohol with three hydroxyl (OH) groups, giving the synonyms as 1,2,3-trihydroxypropane or 1,2,3-propanetriol. Glycerol preferably called to indicate its alcohol character (trihydric alcohol $C_3H_5[OH]_3$), containing the trivalent radical "glyceryl" (C_3H_5). It has the chemical formula as $C_3H_8O_3$ (Figure 2.6) Glycerol is soluble in water and alcohol but insoluble in ether, benzene, or oils. Refined glycerin is mostly pure glycerol, with the salt, methanol and free fatty acids removed. Glycerol is chemically stable under normal storage and handling conditions. It has no self-reactivity, spontaneous combustibility, or explosive properties. However, it may become explosive when in contact with strong oxidizing agents such as potassium chlorate or potassium permanganate. At low temperatures, glycerol sometimes forms crystals which tend to melt at 17.9°C. Liquid glycerol boils at 290°C under normal atmospheric pressure. Its specific gravity is 1.26 g/cm^3 and its molecular weight is 92.09 g/mol.

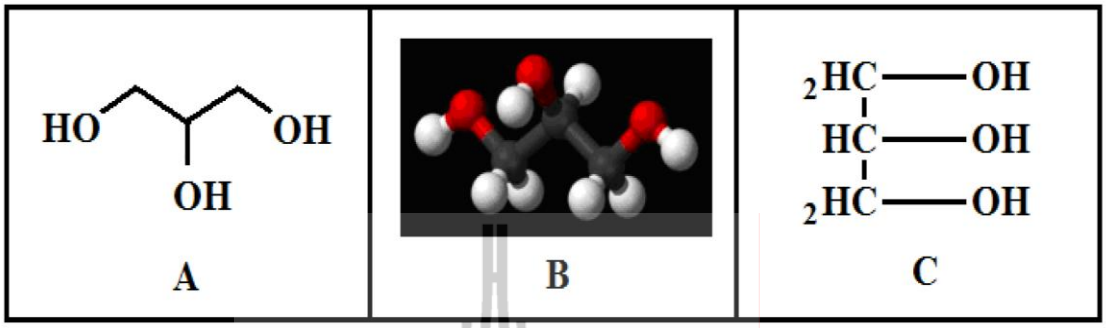


Figure 2.6 Structure model glycerol (A), 3D model (B), and structure formula (C)

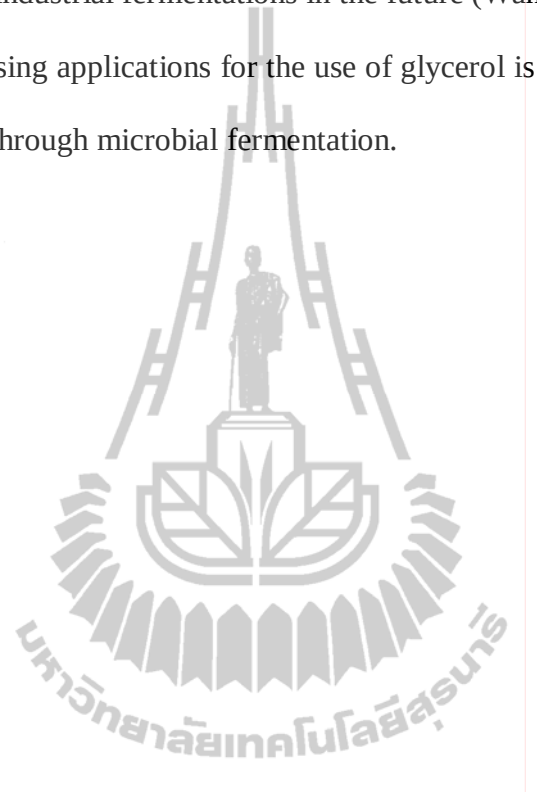
Source: Wikipedia Foundation Inc (2011).

Crude glycerin is a low-value byproduct of the transesterification reaction conducted during production of biodiesel. It is mixed with varying amounts of soap, alcohol, catalyst, and water. This mixture can be in a liquid or solid form, and ranges in color from transparent and light yellow to opaque and dark brown. From a single stage base catalyzed reaction using waste frying oil feedstock, the amount of actual glycerol in the mixture is typically around 40% with the majority of the rest of it being soap. For every 9 kg of biodiesel produced about 1 kg of a crude glycerol by-product is formed (Dasari *et al*. 2005). There are techniques to reduce the water and FFA content. The most common of which that is applicable at small scales is a two stage, acid-base method that uses sulfuric acid during the pretreatment stage.

2.9.1 Main application of glycerol

Glycerol is present in many applications in the cosmetic, paint, automotive, food, tobacco, pharmaceutical, pulp and paper, leather and textile industries. It also is used as a feedstock for the production of various chemicals (Wang *et al*., 2001). New applications are being evaluated in the food industry, the

polyglycerol and polyurethane industry, the field of wood stabilizers and production of small molecules, such as dihydroxyacetone, glyceric and hydroxypyruvic acids and glycerol carbonate (Silva *et al*., 2009). Glycerol has also been considered as a feedstock for new industrial fermentations in the future (Wang *et al*., 2001). Thus, one of the many promising applications for the use of glycerol is its bioconversion to high value compounds through microbial fermentation.



CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cassava

Cassava starch was purchased from Korat starch Industry, Thailand. The cassava starch was stored in desiccator until used.

Cassava chip was purchased from Korat starch Industry, Thailand. The cassava chip was grounded into flour using a Cross-Beater mill (Glen Mill Corp., Maywood, NJ) equipped with a 0.2 mm mesh screen. The cassava chip flour was stored in desiccator until used

Cassava pulp was obtained from Korat starch Industry, Thailand. Dry cassava pulp was prepared by heating at 55°C for 24 h followed by grinding and sieving through a 0.2-mm mesh screen (ZM-100; Retsch, Haan, Germany). The cassava pulp flour was stored in desiccator until used.

3.1.2 Glycerol

Glycerol used was an analytical grade that obtained from Fluka, Switzerland.

3.1.3 Spent brewer's yeast extract

Spent brewer's yeast extract was prepared according to Saksinchai *et al*. (2001). The method as follow; the spent brewer's yeast slurry (approximately

18-20% (w/v) solids) was diluted to 15% (w/v) solids with sterilized water and autolysate for 20 h at 50°C. The autolysate was then heated to 85°C and kept at this temperature for 15 min to activate residual enzyme activity. It was then centrifuged 4000 rpm for 15 min. The clear supernatant was poured off and concentrated to 25% (w/v) in a vacuum evaporator (NESLAB Instruments, Inc., U.S.A.) at 60°C and 300 mbar. The concentrated was subsequently spray dried by spray-dryer (GEA Niro, Denmark) with inlet and outlet temperature of 180°C and 90°C, respectively. The resulting powder (Moisture content 5.5%) was store at room temperature in a dessicator.

3.2 Microorganisms

Two different *Cl. acetobutylicum* stains were carried out in this study. The first stain, *Cl. acetobutylicum* TISTR 1462 was purchased from the culture collection of Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. *Cl. acetobutylicum* JCM 7289 (similar to DSM 1731) and was obtained from Japan Collection of Microorganisms, Tsukuba, Ibaraki, Japan. All microbial stains were maintained as a spore suspension in sterilized water at 4°C.

3.3 Culture media

In this study, culture medium for ABE production is Clostridial media (P2 medium) as reported by Madihah *et al*. (2001) which comprised the component as followed

	Chemical	**Concentration (g/L)**
Buffer:	KH_2PO_4	0.75
	K_2HPO_4	0.75
	CH_3COONH_4	2.2
Minerals:	$MgSO_4.7H_2O$	0.4
	$MnSO_4.H_2O$	0.01
	$FeSO_4.\ 7H_2O$	0.01
	NaCl	1.0
Vitamins:	*p*-aminobenzoic acid	0.001
	Biotin	0.0008
	Yeast extract	5.0

The P2 medium was used in all fermentation experiments. The culture medium was sterilized at 121°C for 15 min and cooled down to room temperature. Vitamin solutions (*p*-aminobenzoic acid and biotin) was sterilized filtered through a 0.20 µm membrane filter and then added to the medium after sterilization.

3.4 Chemical reagents

All of chemicals used in the cassava and glycerol fermentation by *Cl. acetobutylicum* to produce ABE products as follow:

Names of Chemical Reagents	**Company**
Agar	VECHAVIT, THAILAND
Ammonium acetate	MERCK, GERMANY
Biotin	FLUKA, CHINA
Cooked meat medium	FLUKA, CHINA
Dipotassium hydrogen phosphate	AJAX, AUSTRALIA
Dinitrosalicylic acid	FLUKA, SWITZERLAND
Ferrous sulphate heptahydrate	AJAX, AUSTRALIA
Glucose	FLUKA, SWITZERLAND
Glycerol	WAKO, JAPAN
Malachite green oxylate	AJAX, AUSTRALIA
Manganese sulphate, monohydrate	AJAX, AUSTRALIA
Magnesium sulphate heptahydrate	FLUKA, SWITZERLAND
Maltose	FLUKA, CHINA
p-aminobenzoic acid	AJAX, AUSTRALIA
Potassium dihydrogen phosphate	FLUKA, CHINA
Reinforced clostridia agar	AJAX, AUSTRALIA
Sodium chloride	VECHAVIT, THAILAND
Sodium hydroxide	J.K. BAKER, USA
Sulfuric acid	FLUKA, CHINA
Yeast extract	VECHAVIT, THAILAND

3.5 Equipment

All of equipment used in the cassava fermentation by *Cl. acetobutylicum* TISTR 1462 to produce ABE products as follow:

Names of Equipment	**Company**
Analytical Balance	PRESICA, USA
Autoclave Model ACV-3167	HIRIYAMA, JAPAN
Gas chromatograph	KONIK, SPAIN
High performance liquid chromatography	AGILENT, JAPAN
Hot Air Oven	MEMMERT, GERMANY
Hot plate	GIBTHAI, THAILAND
Incubator	EN400 NUVE, TURKEY
Laminar Air Flow Cabinet	AUGUSTA, THAILAND
Micropipette 100,1000,5000 μL	BRAND, GERMANY
pH Meter	CONSORT, SWITZERLAND
Rotary evaporator vacuum	NESLAB, U.S.A.
Refrigerated Centrifuge Super TT21	SORVALL, USA
Spectrophotometer	ANALYTIK JANA, THAILAND
Spray dryer	GEA NIRO, DENMARK
Vortex Mixer	VORTEX-2-GENIE, TAIWAN
Hammer miller	CROMTON, USA

3.6 Methods

3.6.1 Proximate analysis

The proximate composition (ash, fat, and crude fiber) of the cassava starch, cassava pulp and cassava chip were evaluated using the standard Association of Official Analytical Chemists (AOAC) method (Chales *et al*., 2005). The crude protein content was calculated by converting the nitrogen content determined by the micro-Kjeldahl method (N x 6.25) (Vadivel and Janardhanan, 2001). Moisture content was determined according to the standard Association of Official Analytical Chemists method (Oboh *et al*., 2002). Carbohydrate content was calculated by difference (Vadivel and Janardhanan, 2001).

3.6.2 Enzymatic hydrolysis of starch

3.6.2.1 Cassava starch and cassava chip

Two enzymes, α-amylase (Sigma-Aldrich) from *Bacillus subtilis* with an activity of 12,000 U/g and Glucoamylase (Sigma-Aldrich) from *Aspergillus niger* with an activity of 6,000 U/g were used. The method for hydrolysis of cassava hydrolysate was as follows: 90 g of cassava starch was suspended in water. The volume of starch suspension was 400 mL. The liquefaction was carried out by adding 0.3% (v/v) of α-amylase to the slurry at pH 6.0 and incubated at 95°C for 2 h. Saccharification was carried out by adding 0.5% (v/v) of glucoamylase at pH 4.5 and maintained at 58°C for 15 h. The reducing sugar present in the enzymatic hydrolysate was measured using the 3, 5-dinitrosalicyclic acid (DNSA) method (Jesse *et al*., 2002). A standard curve was prepared using standard glucose solution. A blank containing 1 mL of deionized water was heated for 5 min in boiling water with 3 mL

of DNSA reagent. This was used to zero the spectrophotometer. In order to prepare a standard curve, standard solution containing anhydrous D-glucose (0.1-1.0 mg/mL in deionized water) were heated with 3 mL of DNSA reagent for 5 min in a boiling water both followed by measuring of optical density at 540 nm and plot of glucose concentration (as reducing sugar) versus optical density relationship was established. In order to measure the reducing sugar concentration in the hydrolysate cassava, 250 mg of hydrolysate was suspended in 15 mL of deionized water in a 50 mL breaker. One milliliter of the diluted suspension was heated prior to measuring optical density as mention above (with DNSA reagent). The reducing sugar was read from the standard curve using least squares linear regression (see appendix B). However, reducing sugar (glucose and maltose) present in the enzymatic hydrolysate was also determined by the HPLC.

3.6.2.2 Cassava pulp

Enzymatic hydrolysis of cassava pulp were prepared as follow; raw material (1 g dry weight in 4 mL deionized water) was first treated with cellulase (Spezyme CP, Genencor, USA, 5000 U/g) 1.41 U/g dry matter to break down cellulose and release starch granules at 50°C for 24 h. Then, 4.8 U/g of α-amylase (Sigma-Aldrich) from *Bacillus subtilis* with an activity of 12,000 U/g was added into the cassava pulp slurry and the mixture was incubated at 100°C. Subsequently, 4.8 U/g of glucoamylase (Distillase L-400, Genencor, USA, 5000 U/g) was added into liquefied cassava pulp slurry and the mixture was incubated at 60°C. The concentration of reducing sugar liberated from cassava chip was determined by DNS method as mention in previous section.

3.6.3 Acid hydrolysis of cassava pulp

The acid hydrolysate of cassava pulp was prepared by modified method of Thongchul *et al*. (2010) as follow; Cassava pulp were hydrolyzed by diluted hydrochloric acids (1 g of the dried matter was mixed with 9 mL of 1 M HCl) and incubated at 121°C for 15 min. After hydrolysis, the solid residue was separated by filtration through a thin layer cloth. The pH of hydrolysate was adjusted to pH 10 with $Ca(OH)_2$. The resulting precipitate was removed by centrifugation (1,500 rpm for 15 min) and then re-acidifiled to pH 6.0 (Nigam, 2000) followed by further centrifugation to discard the sediment. The reducing sugar present in the hydrolysate was analyzed by DNS method in parallel with HPLC method.

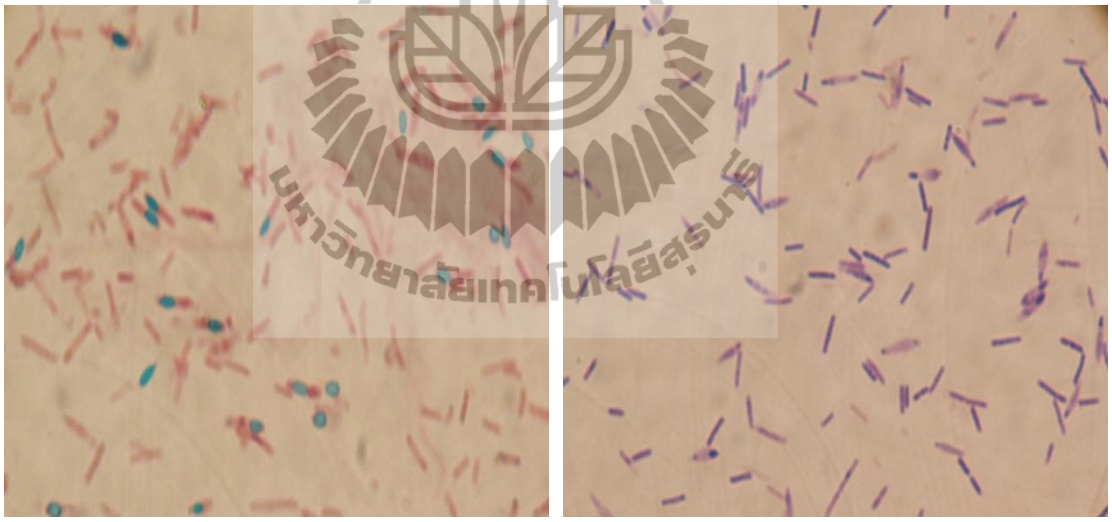
3.7 Preparation of Clostridia spore and culture maintenance

Spore stock culture was revived by heat shocking an aliquot of 0.2 mL in 20 mL Cooked Meat Medium (Sigma chemical) (Ezeji *et al*., 2007) (see the composition of Cooked Meat Medium in appendix A), supplement with glucose (10 g/L), at 75^{o}C for 2 min, followed immediately by cooling in iced water for 1.5 min and then, this culture was incubated anaerobically at 35^{o}C for 17-22 h until vigorous gassing was observed.

Slant of Reinforced clostridial Agar (RCA, HiMedia laboratories, India) (see the composition of RCA in appendix A) were streaked with the revived culture and incubated anaerobically at 35^{o}C. Spore formation was regularly monitored by Bartholomew and Mittwer's spore straining technique using malachite green (Nimcevic *et al*., 1998). After 42 days incubation the sporulated culture were aseptically scraped off into distilled water and dispensed at 2 ml into sterile screw-

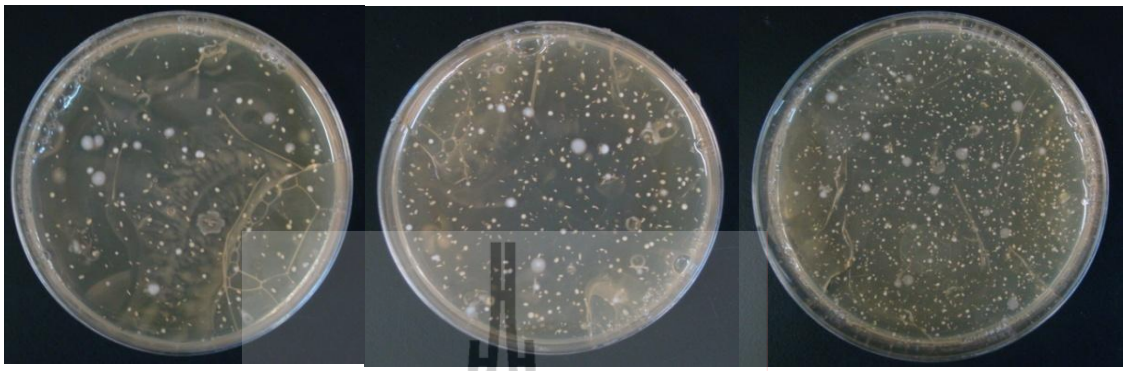
crapped test tube (16x100 mm). In addition, Gram staining was evaluated in order to avoid any contaminations. The spore counted by heamocytometer was in the range 1 x 10^7 and 1 x 10^8 spores per mL. This stock spore suspension was stored at 4^{o}C and, was used for inocula preparation throughout this study.

The spores were stained by Bartholomew and Mittwer's spore straining technique using malachite green and observed under a microscope to verify the presence of spores. Pour plates of the spore suspension in RCM with 1.5% agar were also done to check the viability of the cells. Growth was confirmed after incubation of the plates in an anaerobic jar at 35^{o}C for 24 h.



(a) (b)

Figure 3.1 Morphological characteristic of *Cl. acetobutylicum* under 100x total magnification (a) Green cell is showing the *Cl. acetobutylicum* spores stained with malachite green and (b) Showing Gram positive rod-shaped of *Cl. acetobutylicum.*

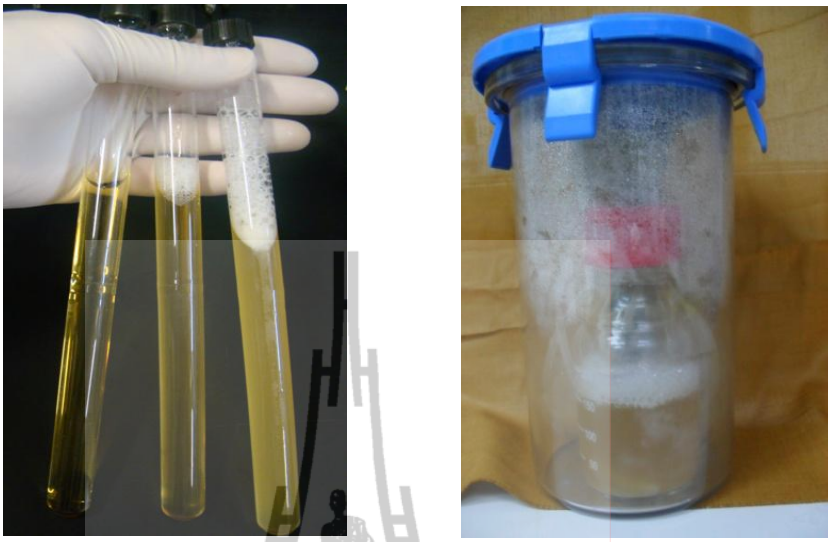


(a) (b) (c)

Figure 3.2 Pour plate colonies of *Cl. acetobutylicum* after 24 h of incubation. (a) 0.2 mL spore suspension; (b) 0.5 mL spore suspension; (c) 1.0 mL spore suspension.

3.8 Inoculum preparation

Cultures were grown in 2 stages as follows: An aliquot of 0.2 mL stock culture was inoculated into 20 mL of Cooked Meat Medium, heat shocked at 75^{o}C for 2 min, and then rapidly cooled in iced water for 1.5 min. The culture was incubated anaerobically at 35^{o}C for 17-22 h until highly motile cells are observed. Two milliliters of the culture were then transferred to 100 mL of appropriate medium (identical to that used in the subsequent main fermentation), and incubated at 35^{o}C for approximately 18 h. A portion of this culture was then used to inoculate the main fermentation [10% (v/v) inoculum].



(a) (b)

Figure 3.3 Regrowing of *Cl. acetobutylicum* cells from spores. (a) 20 mL of cooked meat media before inoculation (left), after inoculation (middle) and during exponential growth (right); (b) 100 mL of appropriate medium after 18 h of incubation.

3.9 Fermentation culture conditions

3.9.1 250-mL Erlenmeyer flask culture

The fermentation was carried out in 250 mL Erlenmeyer flask with a screw-cap tube arm. In order to maintain a closed system, butyl rubber stoppers were used to cover the mouth of the flask while rubber septa were used for the side arm. Culture transfers from system to system were performed using sterile syringes (Terumo®) to maintain culture anaerobicity. The culture media used were autoclaved at 121^{o}C and 15 psi for 15 min after which the vessel was flushed with filter-sterilized oxygen-free nitrogen gas to promote anaerobic condition. System anaerobicity was confirmed by an anaerobic indicator strip (GasPak™). All culture incubations were

done at 35°C. The agitated at 100 rpm was applied only in the fermentation with controlled pH.

(a) (b)

Figure 3.4 Fermentation set-up for controlled pH (a) and with uncontrolled pH experiments (b).

3.9.2 2-L fermentation apparatus

The basic fermenter was a Microferm Labolatory Fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey, USA.) equipped with 2-L glass vessel with 1.5-L working volume. Continuous pH measurement and one-way control of pH were performed using an automatic pH control system consisting of a pH controller module, a pump module. Where necessary, potassium hydroxide solution (3 M KOH) was used to control the pH.

The fermenter vessel, containing medium, was removed from the autoclave and attached to the fermenter apparatus while still hot (80-90°C). Cooling was initiated by means of cold water flowing though hollow baffles, controlled by an electronic thermostat, and oxygen-free nitrogen gas was flushed across the medium

surface. Surface flushing with nitrogen gas was continued after inoculation until good gassing due to the microbial growth.

Figure 3.5 A 2-L bioreactor apparatus for the pH controlled experiment.

In this study, there were two main substrates used: cassava materials and glycerol. In order to optimize the fermentation process to enhance solvents production in batch culture, the overall experiments of this research were:

1) The experiment to investigate effect of different pH control values. Various pH control strategies were applied to fermentation of cassava material and glycerol to solvents. These included: (i) fermentation without pH control for the entire fermentation process when the initial medium pH was adjusted to 6.0, and (ii) fermentation with pH control (initial medium pH 6.0). The medium pH was adjusted during solventogenic phase (4.5, 5.0, 5.5, 6.0 and 6.5) with 50 g/L glucose and 5.0 g/L yeast extract as carbon source and nitrogen source, respectively.

2) The experiments to investigate effect of different types of carbon sources (cassava materials and their hydrolysate as well as glycerol) at concentration of 50 g/L with 5.0 g/L yeast extract as a nitrogen source.

3) The experiment to investigate the effect of carbon source concentration (ranging from 20-80 g/L) with 5.0 g/L yeast extract as a nitrogen source.

4) The experiment to investigate the effect of the different nitrogen source (commercial yeast extract and spent brewer's yeast extract) was carried out with optimum concentration of carbon source that obtained from the previous experiment as a carbon source.

For all experiments, when the temperature was 34^{o}C-37^{o}C, the vitamin solution was added into the medium. The initial medium pH was adjusted to 6.0 using potassium hydroxide solution (3M), temperature was maintained at 35^{o}C, and the agitation speed was set at 100 rpm (in order to avoid any sedimentation) and the fermenter was inoculated with 10% (v/v) with active motile cells prepared as described in Section 5. Five milliliters of fermentation sample were collected twice daily over a period of 5 days for further analysis.

3.10 Analytical methods

Sample was collected at appropriated time intervals and centrifuged at 15,000 rpm for 10 min. The supernatant and the precipitant (cells) were used for further analysis.

3.10.1 Determination of cell concentration

Cell concentration (g cell dry weight per 1 ml of fermentation broth) of the fermentation broth using glucose as a substrate was estimated by the optical density method as cell dry weight using a predetermined correlation between optical density at 600 nm wavelength and cell dry weight (Assobhei *et al.*, 1998) (see the correlation line between optical density at 600 nm wavelength and cell dry weight of each *Cl. acetobutylicum* in appendix B), while cell concentration of starch fermentation was determined using a modified method of Tang *et al.*, 2010. A portion of 20 μL α-amylase was added to 1 mL of culture medium and then incubated at 90°C for 2 h to hydrolyze starch in the medium to soluble dextrin. Samples, which were contained in pre-weighted Eppendorf tubes, were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was discharged and the remaining solid was redissolved with water and once centrifuged. After discharging the supernatant, the cells, which was free from starchy substances, was dried at 105°C for 4 h for determination of dry cell weight. Fresh medium was used as a blank sample.

3.10.2 pH Measurement

All pH measurement was performed by using a pH meter E520 (Metrohm A.G., Hersau, Switzerland) which was calibrated prior to using with pH 4.0 and pH 7.0 buffers.

3.10.3 DAPI/PI staining permeability assay

The physiological bacterial state was examined by a permeability assay using two nucleic acid stains, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI)

and propidium iodide, (PI) (Sigma-Aldrich). DAPI was used as specific DNA fluorochrome to stain all DNA bacteria cells (viable and non viable) since it was able to cross uncompromised cell membranes and bind DNA. In contrast, PI is an indicator of the membrane integrity since it cannot cross the cell membrane unless it has been damaged or compromised. If the cell membrane is damaged, PI enters the cell and binds to the nucleic acids (Carneiro *et al*., 2009; Pablos *et al*., 2011). Therefore, the combination of both stains assesses the physiological state of the bacteria by establishing a relationship between staining and membrane integrity. DAPI stock solution was made up to a concentration of 5 mg/5 mL, in sterile deionized water and 0.5 mL of 2.5% glutaraldehyde. PI stock solution was made up to a concentration of 25 mg/25 mL in sterile deionised water. Both of them were stored at 3-5ºC in the dark.

A volume of 80 µL of DAPI and PI (1 mg/mL) was added to 4 mL of the suspension sample during the photocatalytic experiment, incubated in the dark at room temperature for 10 min and filtered through 0.22 µm polycarbonate membrane filters (Millipore). The filters were placed on glass slides, covered with a cover slip and visualised under immersion oil with a 100xobjective on a Leica DMI 4000B microscope fitted with a fluorescence attachment ebq-100 mc-L and coupled with a Canon Power Shot S80 digital camera. For each sample, three different areas from the inner part of the filter (to avoid edge effects) were randomly chosen, taking two images in each of them using a filter with excitation at 340-380 nm and suppression at 425 nm, and a filter with excitation at 515-560 nm and suppression at 590 nm to visualise DAPI-stained cells and PI-stained cells, respectively.

3.10.4 Determination of fermentation products

The concentration of solvents (acetone, butanol and ethanol) was determined by gas chromatography (GC) equipped with a flame ionization detector (FID). Separations of solvents were achieved by using a capillary column and H_2 as the carrier gas. The column temperature was held at 115°C for 8 min and then programmed at 5°C/min to 170°C with a 10 min final hold. Temperature of the detector and injector were set at 270 and 220°C, respectively. Sample was injected as 2 µL aliquots. Glycerol concentration was determined using glycerol test kits (Boehringer Mannheim, Germany) according to the instructions of the manufacturer.

Reducing sugar (glucose and maltose) resulting from starch hydrolysis during the fermentation and organic acids (acetic and butyric acid) were determined using high performance liquid chromatography (HPLC) with RI detector (Model 1200 series, Agilent technology). Separations of sugars were obtained using MHM-monosaccharide column (Merck) and 4 mM sulfuric acid as the mobile phases. The temperature of the column was operated at ambient temperature with a flow rate of 0.4 mL/min.

Starch concentration of the samples was determined using a modified method of Tang *et al*., 2010. A portion of 20 µL α-amylase was added to 1 mL of culture medium and then incubated at 90°C for 3 h to hydrolyze starch in the medium to soluble dextrin. After that, 8,880 µL of 0.1 M acetate buffer pH 4.5 and 100 µL of glucoamylase were added to the solution and then incubated at 58°C for 4 h. The solution was allowed to cool down to room temperature and then transferred to a 10-mL volumetric flask followed by filling it with distilled water to the volume. Glucose concentration of this solution was determined using the HPLC method. Starch

concentration in fermentation broth was calculated as follows:

Starch concentration (g/L) = Glucose concentration (g/L) × 10^{a} × 0.9^{b}

where *a* is dilution factor and *b* is correction factor for glucose to starch.

3.10.5 Kinetic analysis

Batch fermentation productivity was calculated as total ABE concentration (g/L) divided by fermentation time (h). Fermentation time was defined as the time period when a maximum ABE concentration was reached. ABE yield, was calculated as total ABE produced (g) divided by total carbohydrates (as starch) utilized (g).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Optimization of solvents production by *Clostridium acetobutylicum* TISTR 1462 using cassava material as a carbon source in batch culture

4.1.1 Chemical composition of cassava materials

The composition of the cassava used in this study based on the standard Association of Official Analytical Chemists (AOAC) method (Charles *et al*., 2005) is presented in Table 4.1. It was shown that the higher crude fiber content was observed in cassava pulp followed by cassava chip and cassava starch which was 14.08, 2.05 and 0.17%, respectively. In comparison with the starch content in cassava materials, it revealed that the lower starch content (58% w/w) was obtained in cassava pulp which related to the higher fiber content when compared to cassava chip (85% w/w) and cassava starch (99.04% w/w). In addition, the composition of protein, fat, ash and other components in each cassava materials were varied differently. Agblevor *et al*. (2003) reported that the chemical composition of cassava material was varies and depending on the growing location, season, harvesting methods, as well as method of analysis It has been reported that *Cl. acetobutylicum* was not able to efficiently hydrolyze fiber rich component that presents in the form of lignocellulosic materials (Ezeji *et al*., 2007) due to lacking of ability to produce cellulase for

hydrolyze lignocellulosic materials. However, the ability of producing amylolytic enzyme such as amylase, glucoamylase and pullulanase of *Cl. acetobutylicum* has been determined. For this reason, cassava pulp and cassava chip are starchy-rich lignocellulosic residue, which contains approximately 58 and 85% starch (by weight, dry basis), respectively would be considered as a potential carbon source for *Cl. acetobutylicum* to produce solvents but it must be hydrolyzed to simple sugar prior to the fermentation.

Table 4.1 Comparison of several components of cassava materials.

Samples	Main components (%, w/w)						
	Fat	**Protein**	**Ash**	**Crude fiber**	**Moisture content**	**Carbohydrate**	**Starch content**
Cassava starch	0.21±0.01	1.31±0.01	0.13±0.08	0.17±0.01	5.04±0.17	93.14±0.12	99.04±0.02
Cassava chip	0.52±0.06	1.42±0.03	1.92±0.02	2.05±0.07	8.26±0.13	85.83±0.15	85.85±0.17
Cassava pulp	0.41±0.01	1.77±0.23	1.87±0.03	14.08±0.03	7.56±0.13	74.31±0.24	58.74±0.15

Values represent the mean of duplicate experiment ± SD between samples.

4.1.2 Effect of controlled pH during solventogenic phase on solvents production

In order to determine the precise influence of a controlled difference pH during solventogenic phase (pH 4.5-6.5) on *Cl. acetobutylicum* TISTR 1462 metabolism, the experiments were performed in 2-L fermenter with 50 g/L glucose and 5 g/L commercial yeast extract as a carbon and nitrogen sources, respectively. The fermentation with uncontrolled pH was also performed as a control experiment. The profile of glucose fermentation by *Cl. acetobutylicum* TISTR 1462 under anaerobic condition with uncontrolled pH was shown in Figure 4.1. The transition phase from acidogenic phase to solventogenic phase was clearly shown. An acidogenic phase was observed during the first 24 h of the fermentation where *Cl. acetobutylicum* TISTR 1462 grew rapidly with production of organic acids (acetic acid and butyric acid) which caused reduction in culture pH from 6.0 to 4.75 and only small amount of solvents was produced. The fermentation entered the solventogenic phase when growth reached stationary phase (after 36 h). During this phase, the metabolism of cells undergoes a shift to produce solvents by reassimilation of organic acids which caused the increment of pH.

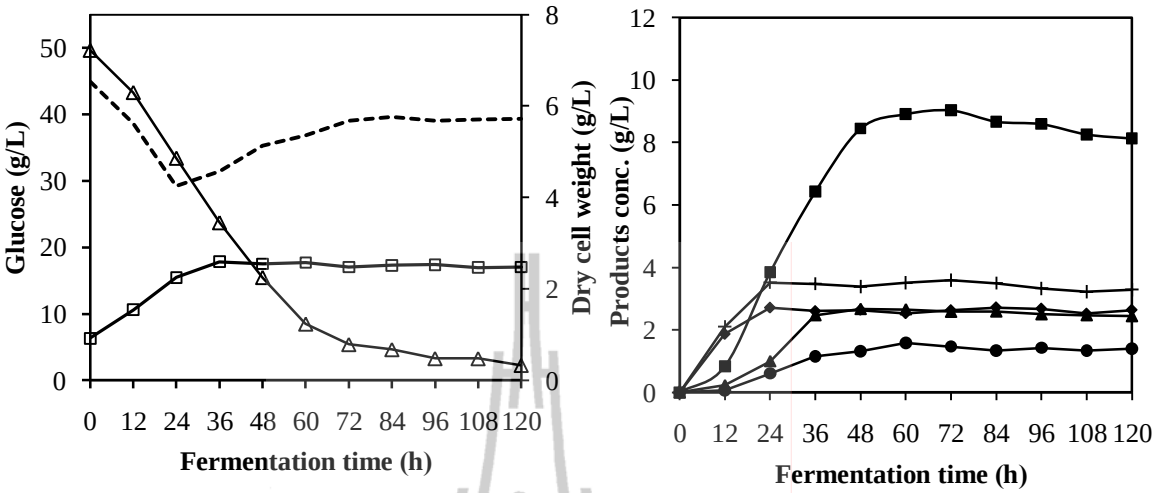


Figure 4.1 Time course of glucose fermentation to solvents by *Cl. acetobutylicum* TISTR 1462 under without controlled pH in batch culture. Glucose concentration used was 50 g/L. Symbols represent: (Δ) glucose, (□) dry cell weight, (---) pH, (■) butanol, (▲) acetone, (●) ethanol, (♦) acetic acid, (+) butyric acid.

For the state of cell development as shown in Figure 4.2, DAPI-stained cells appear blue fluorescent, whereas PI-stained cells appear red fluorescent. The combination of both stains allows the assessing of the membrane integrity along the reaction. Blue cells represent the living cell or not badly damaged bacteria, whereas red cells correspond to bacteria with seriously damaged membrane or dead cell. From Figure 4.2, the results revealed that the number of blue cells (living cell or undamaged cell) decreased whereas the number of red cells (dead cell or damaged cell) increased when the fermentation time increased from 24 to 72 h, which corresponded to the optical density at 600 nm which was reached its maximum at 36 h prior to decreasing (Figure 4.1). In addition, the higher number of red cells was observed at 72 h, which

corresponded to the highest total solvents production, this may be caused by products inhibition. Roos *et al*. (1985) reported that butanol was the most inhibitor of the fermentation products. Growth was totally inhibited at butanol concentration 12 to 16 g/L, whereas cell growth was inhibited by 50% when butanol was added at 7 to 13 g/L.

The typical time course of glucose fermentation to solvents by *Cl. acetobutylicum* TISTR 1462 was shown in Figure 4.3. *Cl. acetobutylicum* TISTR 1462 can produce a large amount of solvents over a wide range of pH (4.5-6.5). This clearly demonstrated that both cellular growth and solvent production were strongly pH dependent. The maximum solvents production (20.08 g/L) was obtained when the pH during solventogenic phase was controlled at pH 5.5 which corresponded to the highest butanol and ethanol production (Table 4.2.). The production of solvents increased with an increasing pH from 4.5-5.5 during solventogenic phase. At a controlled pH 6.0 or above the fermentation were produced mainly organic acids with a small amount of solvents. This was similar to the result obtained by *Cl. bejerinckii* NCIMB 8052 which shown much better growth and solvents production at pH 5.5 than pH 5.0 and no growth at pH 4.0 (Stephens *et al*., 1985). The industrial molasses-fermenting strains, e.g. *Cl. acetobutylicum* P262, are generally regarded as producing solvents at a higher pH range (Jones and Woods, 1986), and for mutant *Cl. acetobutylicum* B18 which was generated from *Cl. acetobutylicum* NRRL B643, a pH optimum of 5.5 in batch culture was demonstrated as well as *Cl. saccharobutylicum* P262 which shown a optimum solvents production at pH 5.5 when the controlled pH during solventogenic phase was applied to use (Madihah *et al*., 2008). On the other hand, a narrow pH optimum around 4.5 and high tolerance to

acid condition which had been found for *Cl. acetobutylicum* strain DSM 1731 (Monot *et al*., 1984). There were sufficient indications that at least one of the investigated characteristics, the optimum pH range for the solvents production, was in accordance with the classification obtained from nucleic acid data (Bahl *et al*., 1982).

For acetone production, the highest production of acetone (6.78 g/L) was observed at pH 5.25 (Table 4.2). It has been reported that the highest acetone production at pH 5.25 was responded to the highest specific activity of thiolase which required for enhancement of acetone production in fermentation using sago starch as a main substrate by *Cl. saccharobutylicum* P262 (Madiha *et al*., 2008). In comparison to the fermentation with uncontrolled pH, total solvents production (20.08 g/L) was almost 1.5 times higher than that obtained during fermentation with uncontrolled pH (15.30 g/L). The production of butanol, acetone and ethanol under these optimum fermentation conditions was 14.43, 4.91 and 0.74 g/L, respectively. High level of butanol formation correlated to the physiological roles of acetoacetate decarboxylase and butanol dehydrogenase. Acetoacetate decarboxylase was responsible for the onset of solventogenesis and butanol dehydrogenese which ensured continued butanol production (Madiha *et al*., 2008).

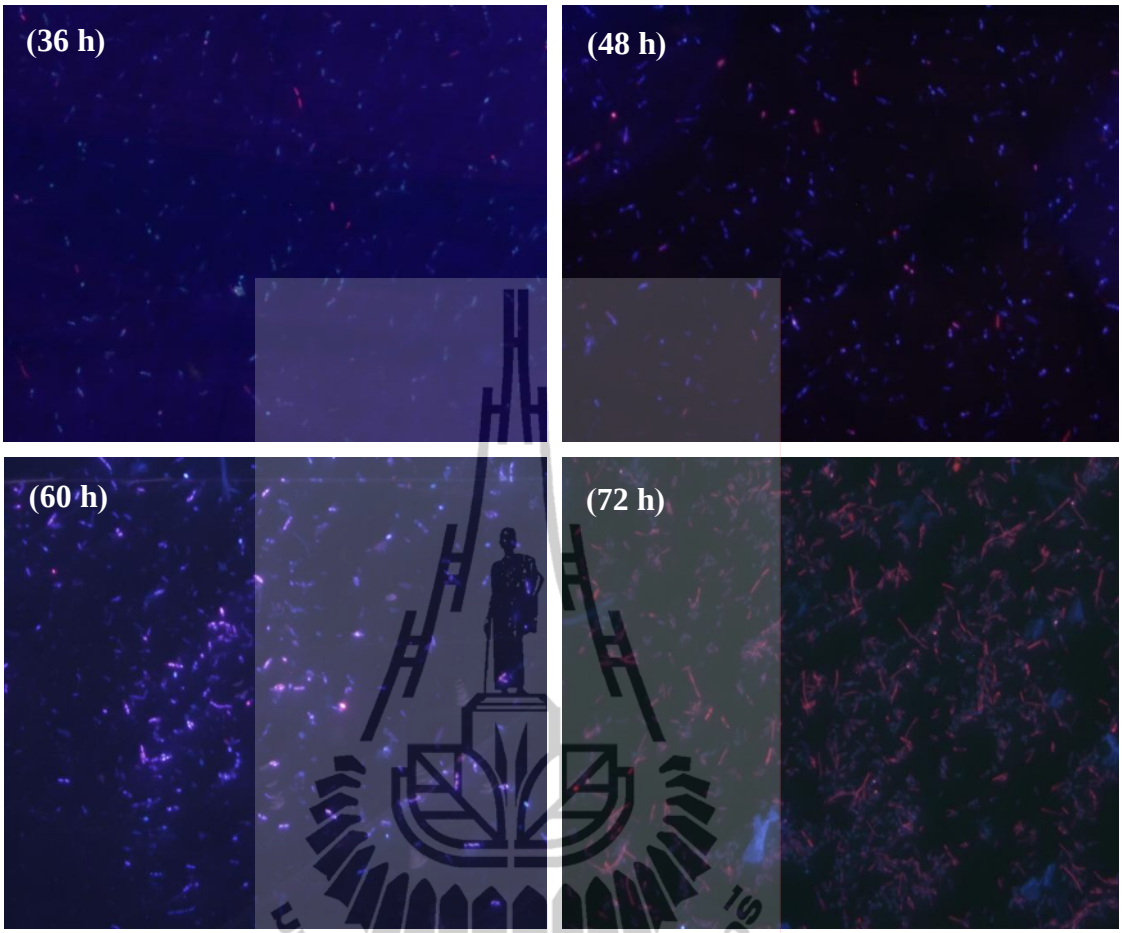


Figure 4.2 The stage of cell development under fluorescent microscopy after dual staining with 1 mg/mL 4, 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) of *Cl. acetobutylicum* TISTR 1462 during 50 g/L glucose fermentation. Red cells representing the dead cell stained with PI and blue cells representing the living cell stained with DAPI.

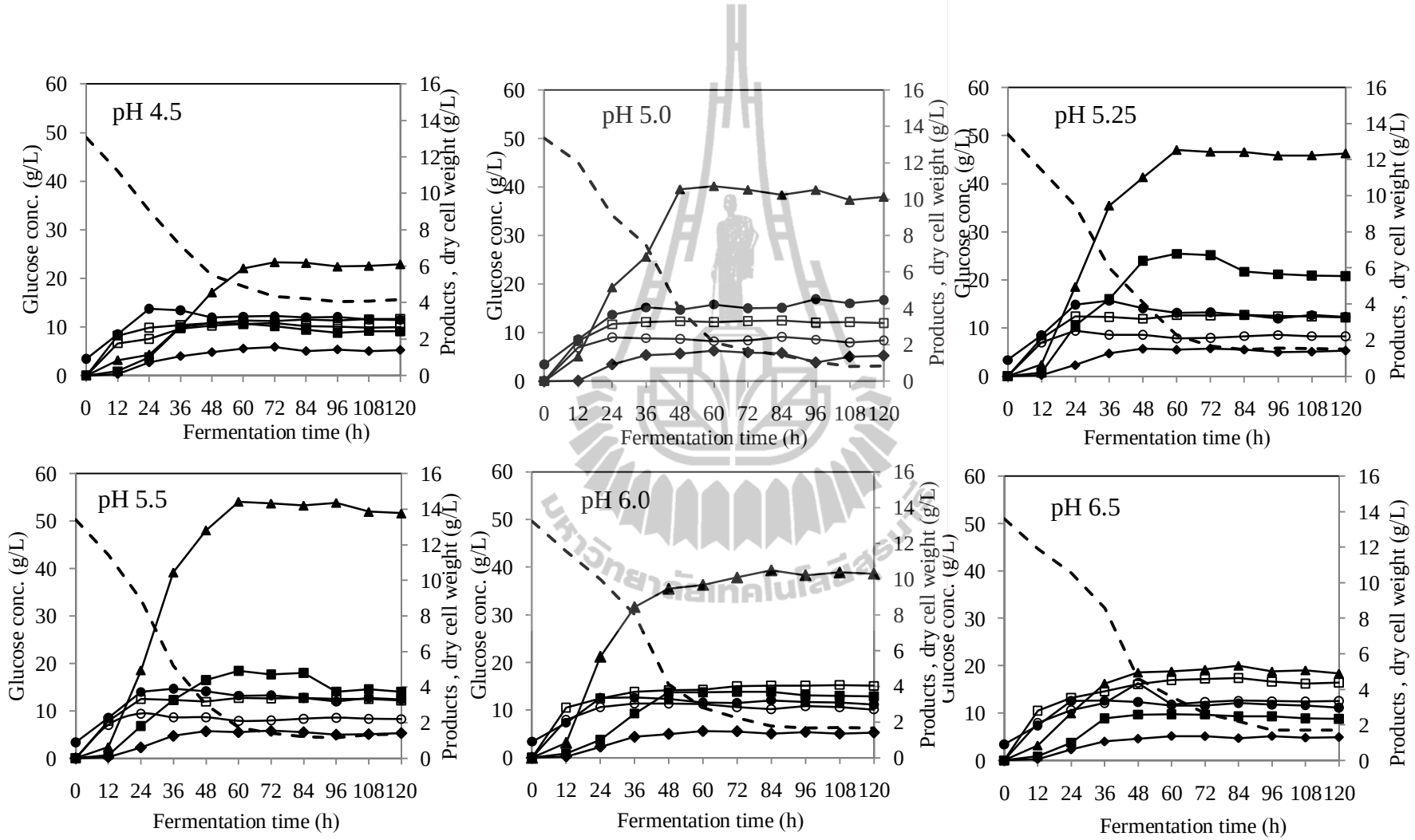


Figure 4.3 Time course of glucose fermentation to solvents by *Cl. acetobutylicum* TISTR 1462. Glucose concentration used was 50 g/L. Symbols represent: (----) glucose, (■) acetone, (▲) butanol, (♦) ethanol, (●) dry cell weight, (□) butyric acid**.** (○) acetic acid.

Table 4.2 Performance of *Cl. acetobutylicum* TISTR 1462 with 50 g/L glucose at different controlled pH during solventogenic phase.

Kinetic parameters	pH						
	Uncontrolled pH	**4.50**	**5.00**	**5.25**	**5.50**	**6.00**	**6.50**
Initial glucose conc. (g/L)	50.20±0.38	51.30±0.45	50.04±0.22	49.96±0.07	50.07±0.33	51.03±0.47	49.86±0.22
Glucose utilized (g/L)	47.98±0.44	40.68±0.28	45.68±0.46	48.97±0.56	48.75±0.17	41.22±0.33	41.38±0.27
Max. dry cell weight (g/L)	1.61±0.12	1.59±0.07	2.31±0.20	2.53±0.09	3.27±0.12	3.01±0.07	3.36±0.14
Max. butyric acid conc. (g/L)	2.72±0.24	2.98±0.26	3.26±0.40	3.69±0.56	3.38±0.67	3.48±0.28	4.63±0.35
Max. acetic acid conc. (g/L)	2.50±0.17	1.81±0.24	3.23±0.09	3.24±0.34	3.12±0.08	3.43±0.34	3.54±0.22
Total organic acids conc.(g/L)	5.22±0.24	4.79±0.26	5.54±0.28	6.22±0.28	6.50±0.37	6.44±0.28	8.17±0.19
Max. butanol conc. (g/L)	11.37±0.26	6.22±0.17	10.72±0.13	12.53±0.40	14.43±0.44	10.48±0.56	5.34±0.33
Max. acetone conc. (g/L)	3.59±0.02	2.71±0.02	3.54±0.05	6.78±0.11	4.91±0.09	3.68±0.07	2.49±0.15
Max. ethanol conc. (g/L)	0.34±0.02	0.37±0.01	0.47±0.02	0.53±0.02	0.74±0.02	0.37±0.10	0.25±0.07
Total solvents conc. (g/L)	15.30±0.22	9.30±0.18	14.73±0.04	19.84±0.11	20.08±0.22	14.53±0.17	8.08±0.24
Fermentation time (h)*	60	72	60	60	60	84	84

Final concentration of organic acids, sugar, and starch were taken when total solvent concentration reached maximum value. Maximum concentration of solvents (acetone, butanol and ethanol) was the highest concentration during the fermentation and the time to reach the maximum values was not the same as for solvents. *Fermentation time was the time taken to reach maximum solvents concentration. Values represent the mean of duplicate experiment ± SD between samples.

Figure 4.4 showed the comparison of yield based on glucose consumed and productivity of total solvents using different controlled pH by *Cl. acetobutylicum* TISTR 1462. The result revealed that using this appropriated pH control strategy (pH 5.5) the overall productivity and yield were 21.21% (0.33 vs. 0.26 g/L/h) and 28.13% (0.41 vs. 0.37 g/L/h) which were higher when compared to the fermentation with uncontrolled pH. This study demonstrated that the pH control strategies were important for improvement of solvents production during fermentation by *Cl. acetobutylicum* TISTR 1462.

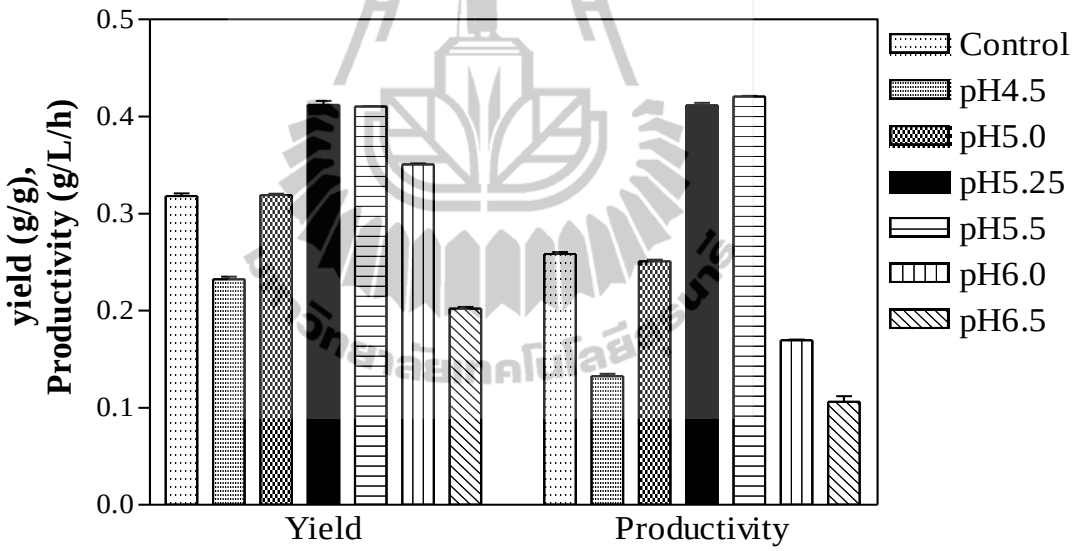


Figure 4.4 Comparison of yield based on glucose consumption and solvents production with 50 g/L glucose fermentation by *Cl. acetobutylicum* TISTR 1462 at different controlled pH in batch culture.

4.1.3 Effect of cassava starch concentration on solvents production

To evaluate the fermentation characteristics of cassava starch by *Cl. acetobutylicum* TISTR 1462, a control fermentation experiment was performed using glucose as a substrate. Fermentation products and cell growth as well as pH

values during batch fermentation by *Cl. acetobutylicum* TISTR 1462 grown in P2 medium containing 60 g/L cassava starch were shown in Figure 4.5. A typical time course of solvent fermentation by *Cl. acetobutylicum* TISTR 1462 using gelatinized cassava starch was similar to glucose fermentation as shown in Figure 4.1 and 4.3. During the early stage of growth, cassava starch was hydrolyzed to maltose and glucose by the action of amylase and glucoamylase secreted out into the medium by *Cl. acetobutylicum* TISTR 1462. The process depended strongly on the activity of α-amylase and glucoamylase secreted by the solvent-producing-strain at the prevailing fermentation (Madihah *et al*., 2000). Knowledge on the properties of solvent-producing-strain was important for the improvement of the process. Direct fermentation of cassava starch could be divided into two phases, acidogenic phase and solventogenic phase. An acidogenic phase was observed during the first 24 h of the fermentation where cell biomass and organic acids (acetic acid and butyric acid) which caused reduction in culture pH from 6.0 to 4.75 were rapidly produced and only small amount of solvents was produced. The fermentation entered the solventogenic phase when growth reached stationary phase (after 36 h). During this phase, the metabolism of cells undergoes a shift to produce solvents by reassimilation of organic acids which caused the increment of pH.

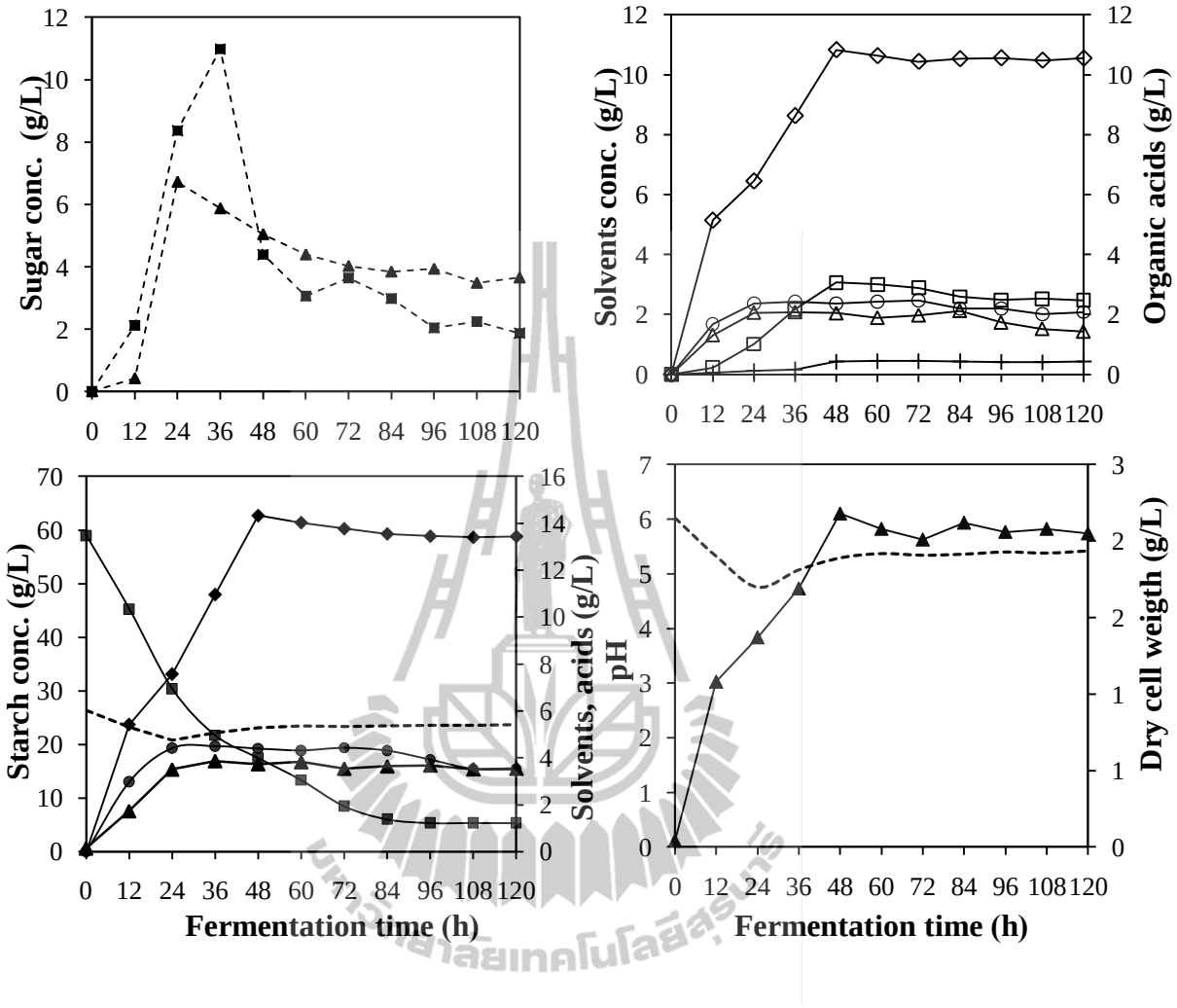


Figure 4.5 Time course of direct fermentation of gelatinized cassava starch to solvents by *Cl. acetobutylicum* TISTR 1462. Cassava starch concentration used was 60 g/L. Symbols represent: (--▲--) glucose, (--■--) maltose, (◊) butanol, (□) acetone, (+) ethanol, (○) butyric acid, (Δ) acetic acid, (----) pH, (■) starch, (▲) dry cell weight, (♦) total solvents, (●) total organic acids.

In order to investigate the effect of cassava starch concentration on ABE fermentation, different experiments were carried out using starch concentrations varying from 20 to 80 g/L. Gelatinized cassava starch exhibited pseudoplastic

behavior and its apparent viscosity increased drastically with increasing of cassava starch concentration (data not show). Experiments using 90 g/L starch were also evaluated but it was very difficult to carry out fermentation since high pseudoplastic behavior and apparent viscosity of medium increased drastically. In general, the fermentation profiles using different concentration of cassava starch were similar to Figure 4.5. The performance of solvents fermentation by *Cl. acetobutylicum* TISTR 1462 using different cassava starch concentrations was summarized in Table 4.3. The highest total solvents production was observed when 60 g/L cassava starch was used which resulted in 14.33 g/L followed by 50 g/L and 70 g/L which resulted in 13.14 and 11.92 g/L, respectively. The maximum dry cell weight attained increased from 20 to 40 g/L starch and was slightly different for fermentation of 40 to 80 g/L starch. Total solvents production increased drastically from 20 to 60 g starch and the decrease in the solvents production was observed at starch concentration above 60 g/L. When starch concentration increased from 40 to 80 g/L, glucose and maltose concentration in medium increased correspondingly, from 0 to 10.41 g/L for glucose and from 0.13 to 2.83 g/L for maltose. At the same time, the residual concentration of starch increased from 3.04 to 12.78 g/L indicating that the amount of starch utilized during fermentation decreased from 89.87% to 68.82%. A similar result was also observed in the fermentation of *Cl. acetobutylicum* P262 using sago starch as a main carbon source to produce solvents (Madihah *et al.*, 2000; Madihah *et al.*, 2001). They concluded that reduction in fermentation performance with high starch concentrations may lead to an increase in apparent viscosity, which in turn, limited the mass transfer for enzymatic hydrolysis and microbial reaction.

Table 4.3 Performance of *Cl. acetobutylicum* TISTR 1462 at different concentrations of starch.

Parameters	Different concentrations of cassava starch (g/L)						
	20	**30**	**40**	**50**	**60**	**70**	**80**
Final glucose conc. (g/L)	0.00±0.00	0.00±0.00	0.00±0.00	0.23±0.11	1.65±0.21	6.86±0.11	10.41±0.06
Final maltose conc. (g/L)	0.00±0.00	0.00±0.00	0.13±0.24	0.51±0.37	1.07±0.07	2.12±0.35	2.83±0.22
Final starch conc. (g/L)	2.02±0.20	3.04±0.38	3.42±0.35	3.03±0.45	5.35±0.12	10.05±0.46	12.78±0.18
Utilized starch (%)	94.90±0.17	89.87±0.22	91.13±0.12	92.54±0.08	86.88±0.08	73.83±0.11	68.82±0.06
Max. dry cell weight (g/L)	1.09±0.02	1.85±0.12	2.29±0.20	2.20±0.02	2.18±0.06	2.29±0.12	2.34±0.04
Max. acetic acid conc. (g/L)	2.13±0.24	1.17±0.36	1.45±0.19	2.23±0.13	2.06±0.32	1.56±0.45	1.47±0.53
Max. butyric acid conc. (g/L)	3.47±0.42	2.38±0.38	2.22±0.44	2.52±0.35	2.56±0.20	2.32±0.08	1.87±0.11
Total organic acids conc.(g/L)	5.60±0.12	3.55±0.16	3.67±0.25	4.75±0.18	4.62±0.16	3.88±0.13	3.34±0.18
Max. acetone conc. (g/L)	0.79±0.22	2.45±0.25	1.86±0.16	2.81±0.20	3.06±0.25	3.37±0.11	2.72±0.23
Max. ethanol conc. (g/L)	0.18±0.04	0.22±0.01	0.32±0.04	0.32±0.06	0.34±0.02	0.37±0.01	0.30±0.06
Max. butanol conc. (g/L)	2.23±0.34	6.88±0.46	9.24±0.26	10.01±0.18	10.93±0.22	8.18±0.45	8.64±0.38
Total solvents conc. (g/L)	3.20±0.12	9.55±0.23	11.42±0.11	13.14±0.08	14.33±0.09	11.92±0.21	11.66±0.11
Fermentation time (h)*	72	60	48	48	48	60	72

Final concentration of organic acids, sugar, and starch were taken when total solvent concentration reached maximum value. Maximum concentration of solvents (acetone, butanol and ethanol) was the highest concentration during the fermentation and the time to reach the maximum values was not the same as for solvent. Yield was calculated base on the potential glucose in medium with assumption that 1 g starch was converted to 1.1 g glucose and 1 g maltose was assumed to produce 1.053 g glucose (Liew *et al.*, 2006). *Fermentation time was the time taken to reach maximum solvents concentration. Values represent the mean of duplicate experiment ± SD between samples.

In fermentation using 30 g/L of cassava starch, the residual concentration of starch was 3.04 g/L, indicating that 89.87% starch (29.66 g/L) was utilized during the fermentation. During this fermentation, neither glucose nor maltose was detected. This indicated that at starch concentration as low as 30 g/L, the strain hydrolyzed starch to glucose slower than they utilized glucose for fermentation. It was possible that at a starch concentration of 30 g/L and lower, fermentation could be deficient in substrate.

From Figure 4.6, it was found that total solvents yield decreased from 0.32 to 0.19 with the increase of starch concentration from 30 to 80 g/L. It was also found that total solvents productivity was decreased from 0.28 to 0.16 g/L/h with the increase of starch concentration from 60 to 80 g/L. This result implied that solvents production using high cassava starch concentration may be affected by the mass transfer rate, which was a function of broth viscosity, with higher viscosity increasing diffusion of metabolized substances. Thang *et al*. (2010) explained that amylolytic activities during active growth were apparently sufficient to hydrolyze high starch concentration to fermentable sugars, high viscosity of fermentation broth resulted in a reduction in solvent production.

Considering butanol concentration, the maximum concentration of butanol in direct fermentation of cassava starch was always lower than 16.0 g/L (Table 4.3). Furthermore, the concentration of butyric acid, which was important for the onset of butanol production (Monot *et al.*, 1983), drastically decreased when starch concentration increased from 40 to 50 g/L. In this case, maximum butyric acid concentration dropped from 2.52 to 2.22 g/L. On the other hand, the production of solvent was not proportional to the amount of organic acids accumulated in the

culture. For instance, slightly higher total acids (4.75 g/L) was accumulated in the fermentation using 50 g/L initial cassava starch as compared with the fermentation using 60 g/L initial cassava starch (4.62 g/L), whereas solvent production (13.14 g/L) was lower than the latter (14.33 g/L). It was reported that solvent production was not stimulated by the high amount of acetic and butyric acid accumulated during the fermentation but high solvent production was much more related to the low amount of undissociated butyric acid during the onset of solvent production (Madihah *et al*., 2001).

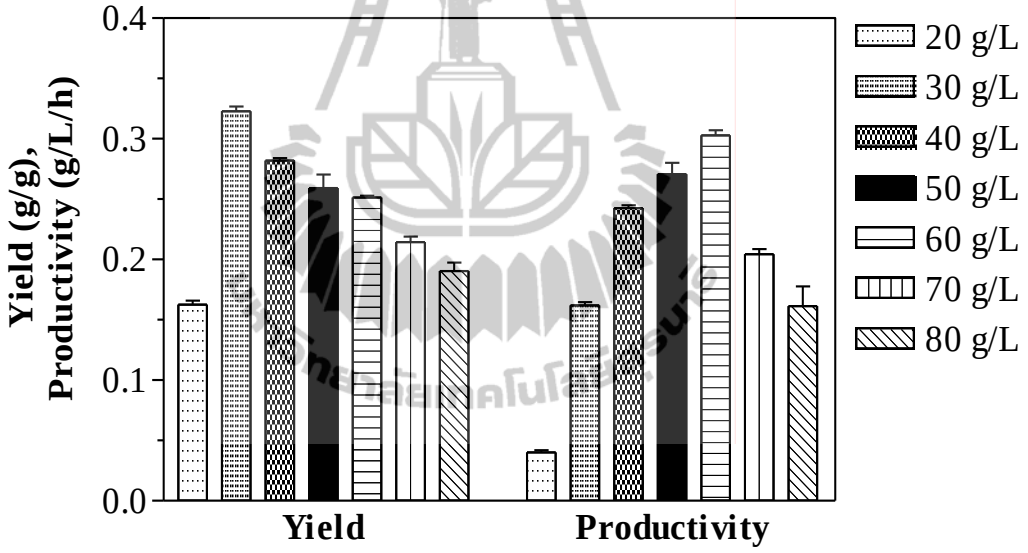


Figure 4.6 Comparison of solvent yield based on glucose consumption and solvents production of different cassava concentrations during fermentation by *Cl. acetobutylicum* TISTR 1462 in batch culture.

4.1.4 Effect of different types of nitrogen source on solvents production

The investigation into the effects of different nitrogen sources, commercial yeast extract and spent brewer's yeast extract, on the performance of *Cl. acetobutylicum* TISTR 1462 was performed using the optimum concentration of

cassava starch for solvents production which obtained from the previous experiment (60 g/L) as a carbon source. An appropriate pH control was also applied in these experiments. In order to compare results obtained in these studies, the concentration used of each yeast extract was fixed at 5 g/L and control fermentation experiment was conducted without adding yeast extract. The result was shown in Figure 4.7, the higher solvents production (20.86 g/L) was obtained with supplementing fermentation medium with commercial yeast extract as compared with those using spent brewer's yeast extract (18.46 g/L). In addition, using commercial yeast extract and spent brewer's yeast extract as a nitrogen source were greatly influenced solvent production when compared with the control. Moreover, maximum cell concentration of the control was around 2 folds lower than that using commercial yeast extract and spent brewer's yeast extract. It was well known that organic nitrogen source such as yeast extract provide various amino acids, vitamins, minerals and growth factors that promote good growth of microorganisms (Madihah *et al*., 2001).

Considering to the fermentation that using brewer's yeast extract as a supplemented nitrogen source, it has been reported that brewer's yeast extract could be used as an easier supplemented nitrogen source for growth and sporulation of *Bacillus thuringiensis kurstaki* (Saksinchai *et al*., 2001). From this result, it was more likely that the cost of commercial yeast extract could be reduced when the fermentation medium was replaced by spent brewer's yeast extract. This finding indicated that cassava starch fermentation supplemented with spent brewer's yeast extract was the most promising substrate for commercial ABE production.

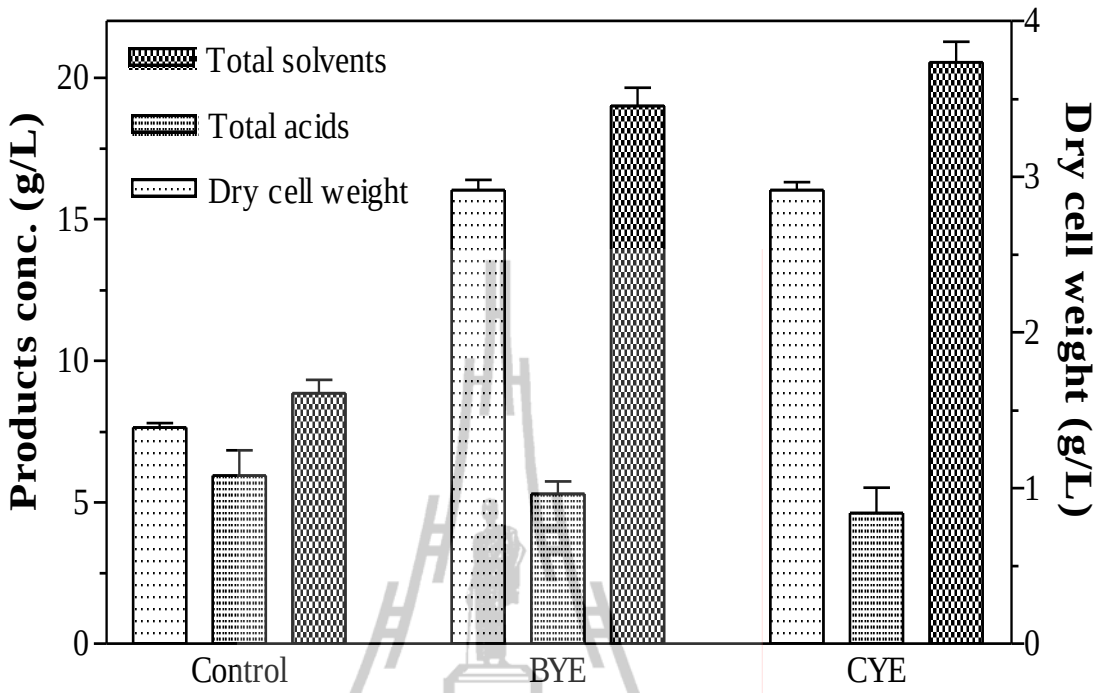


Figure 4.7 Comparison of different nitrogen sources on solvents production during fermentation by *Cl. acetobutylicum* TISTR 1462 with controlled pH 5.5 in batch culture. (BYE, Spent brewer's yeast; CYE, Commercial yeast extract and Control, without yeast extract).

4.1.5 Effect of different types of cassava materials on solvents production

To investigate the fermentability of different cassava material on solvents production, fermentation trials were conducted with around 70 g/L of cassava material and their mixture derived from enzymatic hydrolysis or acid using *Cl. acetobutylicum* TISTR 1462 in P2 medium. This medium contained 60 g/L total starch, which was equal to the medium containing 66 g/L glucose with an assumption that the all starch would be converted to glucose. The controlled appropriate pH (pH 5.5) for solvents production was also applied in these experiments. In order to

compare the results obtained in these studies, control fermentation was conducted in which glucose was used as a substrate. The effect of different types of carbon sources including glucose on solvents production was summarized in Table 4.4. The results showed that *Cl. acetobutylicum* TISTR 1462 was capable of producing solvents efficiently from cassava materials and which was comparable to when glucose was used.

In order to compare the efficiency of cassava materials with non hydrolyzed treatment, the results revealed that the highest total solvents production (21.20 g/L) was obtained when gelatinized cassava starch was used followed by gelatinized cassava chip (19.11 g/L). Considering to cassava pulp, it cannot directly use in fermentation to produce solvent, due to its fiber rich component as shown in Table 4.4 It has been reported that *Cl. acetobutylicum* were not able to efficiently hydrolyze fiber rich component that present in the form of lignocellulosic materials (Ezeji *et al*., 2007), due to lack of ability to produce cellulase for hydrolyze lignocellulosic materials. For this reason, cassava pulp were starchy-rich lignocellulosic residue which contained approximately 58% starch (by weight, dry basis) must be hydrolyzed to simple sugar prior to the fermentation.

In term of pretreatment comparison between enzymes, acid and direct gelatinized (without hydrolysis pretreatment), it was found that the total solvents production using enzymetic hydrolysate and gelatinized cassava materials were slightly different within the range of 19 to 21 g/L. Similar result was obtained by Nimcevic *et al*., 1998, which reported that, very good solvents formation rates were observed when *Cl. beijerinckii* NRRL B592 was cultivated on whole potato media and they found that, no enzymatic hydrolysis of potato starch was necessary. It could

be concluded that enzymatic pretreatment of gelatinized cassava materials yielding maltose and glucose prior to the fermentation did not improve solvents production as compare to direct fermentation of gelatinized cassava materials. In other hand, the lower solvents production (19.48%) was obtained when cassava materials was hydrolyzed with acid prior to the fermentation (21.20 g/L vs. 17.07 g/L). This may be caused by growth inhibitors formed during acid hydrolysis treatment. Many problems associated with acid hydrolysis of carbohydrate are already known, for example, the formation of toxic compounds such as furfural derivatives, aliphatic acids and phenolic compounds, has been reported (Purwadi *et al*., 2004; Lee *et al*., 2009; Thongchul *et al*., 2010). Nevertheless, the acid treatment has been proven to be a fast and cost-effective method for producing monomeric sugars from polysaccharides. Thus, acid-treated cassava pulp for solvents production would be recommended as inexpensive and efficient processes when the fermentation condition was optimized in this medium. This study suggested that all cassava materials could be potential renewable substrate for production of solvents (up to 19 g/L total solvents) when partial hydrolysis treatment was carried out prior to fermentation.

Table 4.4 Performance of *Cl. acetobutylicum* TISTR 1462 at different types of starch materials.

Parameters	Glucose	Gelatinized cassava starch	Enzyme hydrolysate cassava starch	Gelatinized cassava chip	Enzyme hydrolysate cassava chip	Enzyme hydrolysate cassava pulp	Acid hydrolysate cassava pulp
Initial substrate conc. (g/L)	66.02±0.05	60±0.02	65.78±0.09	60±0.01±0.01	65.46±0.06	66.22±0.02	64.72±0.06
Final substrate conc. (g/L)	13.24±0.24	11.21±0.55	11.87±0.34	12.53±0.46	11.75±0.42	12.83±0.33	17.77±0.36
Max. dry cell weight conc. (g/L)	2.65±0.26	2.78±0.05	2.75±0.06	2.70±0.12	2.72±0.06	2.78±0.16	2.54±0.12
Substrate utilized (%)	79.95±0.03	83.02±0.23	81.96±0.32	81.02±0.34	82.05±0.52	80.63±0.44	72.54±0.44
Final acetic acid conc. (g/L)	0.53±0.08	0.66±0.21	1.04±0.35	1.35±0.24	1.26±0.09	1.45±0.27	1.06±0.12
Final butyric acid conc. (g/L)	0.08±0.05	1.12±0.05	2.22±0.12	2.04±0.15	1.89±0.26	2.34±0.16	2.34±0.23
Max. acetone conc. (g/L)	5.45±0.32	4.89±0.11	4.38±0.44	4.05±0.23	4.12±0.38	4.22±0.22	3.86±0.34
Max. butanol conc. (g/L)	15.76±0.54	15.65±0.07	15.14±0.34	14.67±0.34	15.09±0.26	14.92±0.31	12.86±0.27
Max. ethanol conc. (g/L)	0.58±0.02	0.66±0.05	0.34±0.02	0.39±0.02	0.53±0.05	0.52±0.02	0.35±0.02
Total solvents conc. (g/L)	21.79±0.27	21.20±0.32	19.86±0.31	19.11±0.35	19.74±0.32	19.66±0.34	17.07±0.13
Fermentation time (h)*	60	48	48	48	60	60	60
Solvent yield (g solvents/g glucose)	0.41±0.03	0.39±0.12	0.37±0.07	0.36±0.01	0.37±0.03	0.37±0.06	0.36±0.12
Solvent productivity (g solvents/L/h)	0.36±0.01	0.44±0.01	0.41±0.02	0.40±0.02	0.33±0.02	0.33±0.01	0.28±0.01

Final concentration of organic acids, sugar, and starch were taken when total solvent concentration reached maximum value. Maximum concentration of solvents (acetone, butanol and ethanol) was the highest concentration during the fermentation and the time to reach the maximum values was not the same as for solvent. Yield was calculated base on the potential glucose in medium with assumption that 1 g starch was converted to 1.1 g glucose and 1 g maltose was assumed to produce 1.053 g glucose (Liew *et al.*, 2006). *Fermentation time was the time taken to reach maximum solvents concentration. Values represent the mean of duplicate experiment ± SD between samples.

According to the comparison of starchy materials as shown in Table 4.5, the results from batch fermentation with uncontrolled pH of cassava starch using *Cl. acetobutylicum* TISTR 1462 was lower when compared with batch fermentation with uncontrolled pH of sago starch (19.60 versus 14.33 g/L). On the other hand, the total solvent of cassava starch (20.06 g/L) was higher than that obtained from sago starch when the pH was controlled at 5.5. This result indicated that utilizing pH control strategy enhanced solvent production by *Cl. acetobutylicum* TISTR 1462. Therefore, these approaches may be easily applied to use in industrial solvent fermentation process. Regarding to acetone production, it was interesting to note that acetone produced in fermentation using glucose was higher than that produced in fermentation using cassava starch and hydrolysate cassava starch. This fact led to a slightly higher solvent concentration when glucose was used instead of cassava starch and hydrolysate cassava starch. However, the main difference among fermentations using different types of starch was the fermentation time. While fermentation using both cassava starch and cassava chips was finished at 48 h, fermentation using corn and sago starches was terminated at 66 and 77 h, respectively. This resulted in a lower solvent productivity when corn and sago starches were used instead of cassava starch or cassava chips. The explanation for this observation may be due to the difference in physico-chemical properties of starches. Corn starch has an average granule size of 15 µm (Ma *et al*., 2004) and contains approximately 28% amylose (Ahmad *et al*., 1999). Sago starch has an average granule size of 30 µm (Wang *et al*., 2006) and contains 24–31% amylose (Ahmad *et al*., 1999; Sandhu and Singh, 2007), while average granule size of cassava starch is 13–15 µm (Rao and Tattiyakul, 1999) and amylose content is about 18.6–23.6% (Defloor *et al*., 1998). The smaller granule sizes improve

the digestibility by enzymes because smaller granules have a greater surface area and are more rapidly digested by amylases. The relationship between granule size and digestibility was previously reported (Cone *et al*., 1990).

Table 4.5 Performance of *Clostridium* spp. at different types of substrate.

Kinetic parameters	Types of substrate					
	Glucose[a]	Cassava starch[a]	Cassava chip[a]	Cassava starch[b]	Corn starch[c]	Sago starch[d]
Initial substrate conc. (g/L)	66.02±0.05	60±0.02	60±0.01±0.01	60±0.12	50.00	60.00
Final substrate conc. (g/L)	13.24±0.24	11.21±0.55	12.53±0.46	10.46±0.24	11.46	17.06
Max. dry cell weight conc. (g/L)	2.65±0.26	2.78±0.05	2.70±0.12	2.48±0.06	2.60	2.08
Substrate utilized (%)	79.95±0.03	83.02±0.23	81.02±0.34	84.24±0.07	78.60	no data
Final acetic acid conc. (g/L)	0.53±0.08	0.66±0.21	1.35±0.24	0.76±0.34	0.60	0.43
Final butyric acid conc. (g/L)	0.08±0.05	1.12±0.05	2.04±0.15	0.66±0.36	0.00	0.54
Max. acetone conc. (g/L)	5.45±0.32	4.89±0.11	4.05±0.23	3.06±0.33	4.00	1.67
Max. butanol conc. (g/L)	15.76±0.54	15.65±0.07	14.67±0.34	10.93±0.17	16.20	16.00
Max. ethanol conc. (g/L)	0.58±0.02	0.66±0.05	0.39±0.02	0.34±0.08	0.50	0.34
Total solvents conc. (g/L)	21.79±0.27	21.20±0.32	19.11±0.35	14.33±0.11	20.70	17.99
Fermentation time (h)*	60	48	48	48	66	77
Solvent yield (g solvents/g glucose)	0.41±0.03	0.39±0.12	0.36±0.01	0.26±0.05	0.48	0.37
Solvent productivity (g solvents/L/h)	0.36±0.01	0.44±0.01	0.40±0.02	0.24±0.01	0.31	0.23

Final concentration of organic acids, sugar, and starch were taken when total solvent concentration reached maximum value. Maximum concentration of solvents (acetone, butanol and ethanol) was the highest concentration during the fermentation and the time to reach the maximum values was not the same as for solvent. Yield was calculated base on the potential glucose in medium with assumption that 1 g starch was converted to 1.1 g glucose and 1 g maltose was assumed to produce 1.053 g glucose (Liew *et al*., 2006). *Fermentation time was the time taken to reach maximum solvents concentration. Values represent the mean of duplicate experiment ± SD between samples.

[a]Batch fermentation using a controlled pH 5.5 by *Cl. acetobutylicum* TISTR 1462 (This work).
[b]Batch fermentation with uncontrolled pH by *Cl. acetobutylicum* TISTR 1462 (This work).
[c]Batch fermentation with uncontrolled pH by *Cl. saccharobutylicum* P262 (Thang *et al*., 2010).
[d]Batch fermentation with uncontrolled pH by *Cl. acetobutylicum* P262 (Madihah *et al*., 2001).

4.2 Optimization of solvents production by *Clostridium acetobutylicum* JCM 7289 using glycerol as a sole carbon source in batch culture

4.2.1 Effect of glycerol concentration on solvents production

The different concentrations of glycerol were varied determined (20-80 g/L). All fermentation experiments were conducted in 250 mL Erlenmeyer flasks with a screw-cap tube arm. In order to compare the results obtained in these studies, control fermentation experiment was performed in which glucose was used as a substrate. The growth of *Cl. acetobutylicum* JCM 7289 on P2 medium with glycerol as a carbon source in batch culture, showed a drastic change in products pattern compared with that during glucose fermentation as shown in Figure 4.8, more than 40 g/l glycerol was utilized, and only 6.02 g/L total solvents were produced which was two times lower in concentration when glucose was used as substrate (13.85 g/L). This could be due to its metabolic route that favors the formation of other products, such as 1,3 propanediol, diminishing the solvents production. It has been demonstrated that glycerol was more reduced substrate than glucose, for the same amount of carbon glycerol metabolism liberates twice as much NADH as glucose. The reducing equivalent excess provided by the conversion of glycerol must be oxidized through the NADH uptake pathways (Vasconcelos *et al*., 1994; Amans *et al*., 2001).

In *Klebsiella*, *Citrobacter*, *Clostridium* and *Enterobacter*, glycerol is metabolized both oxidatively and reductively (Zhu *et al*., 2002) as shown in Figure 4.9. In oxidatively pathway, an NAD^+-dependent glycerol dehydrogenase catalyzes

the oxidation of glycerol to dihydroxyacetone (DHA), which is then phosphorylated to dihydroxyacetone phosphate (DHAP) via a DHA kinase. A triosphosphate isomerase catalyzes the transformation of DHAP to glyceraldehyde-3-phosphate, which enters the glycolytic pathway. The reducing pathway, involving a coenzyme B12-dependent glycerol dehydratase catalyzes the transformation of glycerol to 3-hydroxypropionaldehyde, which is reduced to 1,3-propanediol via an NAD^+-dependent 1,3-propanediol dehydrogenase (Amans *et al*., 2001). This second metabolic pathway maintains the redox balance of the cell and is necessary while the microorganism is using glycerol as a carbon and energy source. The final 1,3-propanediol product is highly specific for glycerol fermentation and cannot be obtained from any other anaerobic conversion (Homann *et al*., 1990; Deckwer, 1995).

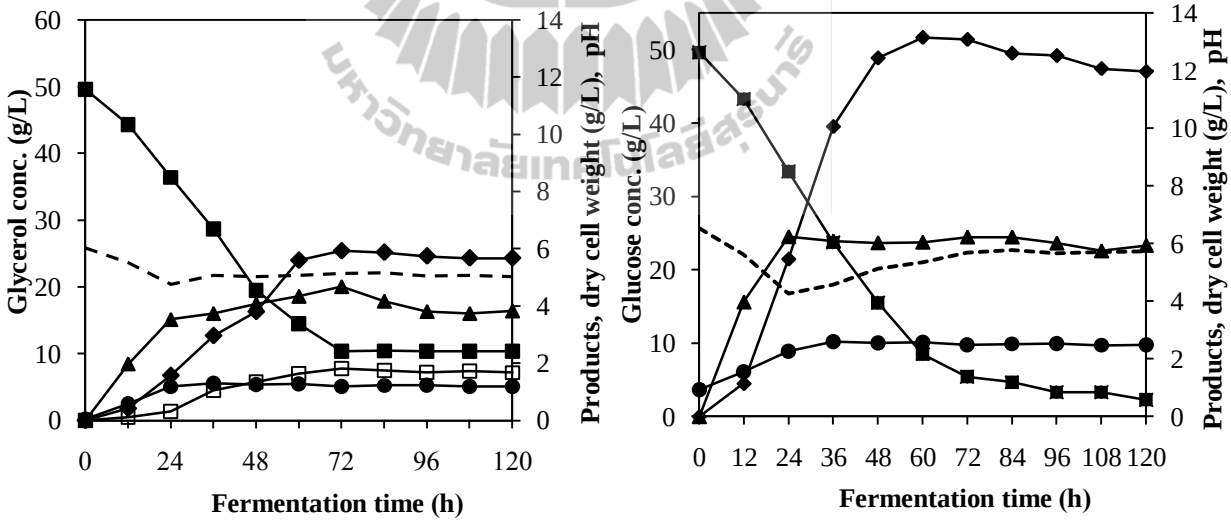


Figure 4.8 Fermentation profiles of glucose and glycerol in solvents production by *Cl. acetobutylicum* JCM 7289 in batch cultures. Initial substrate concentration used was 50 g/L. (A) Glycerol fermentation, (B) Glucose fermentation. Symbols represent: (■) glycerol and glycerol, (----) pH, (▲) total acids, (♦) total solvents, (●) dry cell weight, (□) 1,3 propanediol.

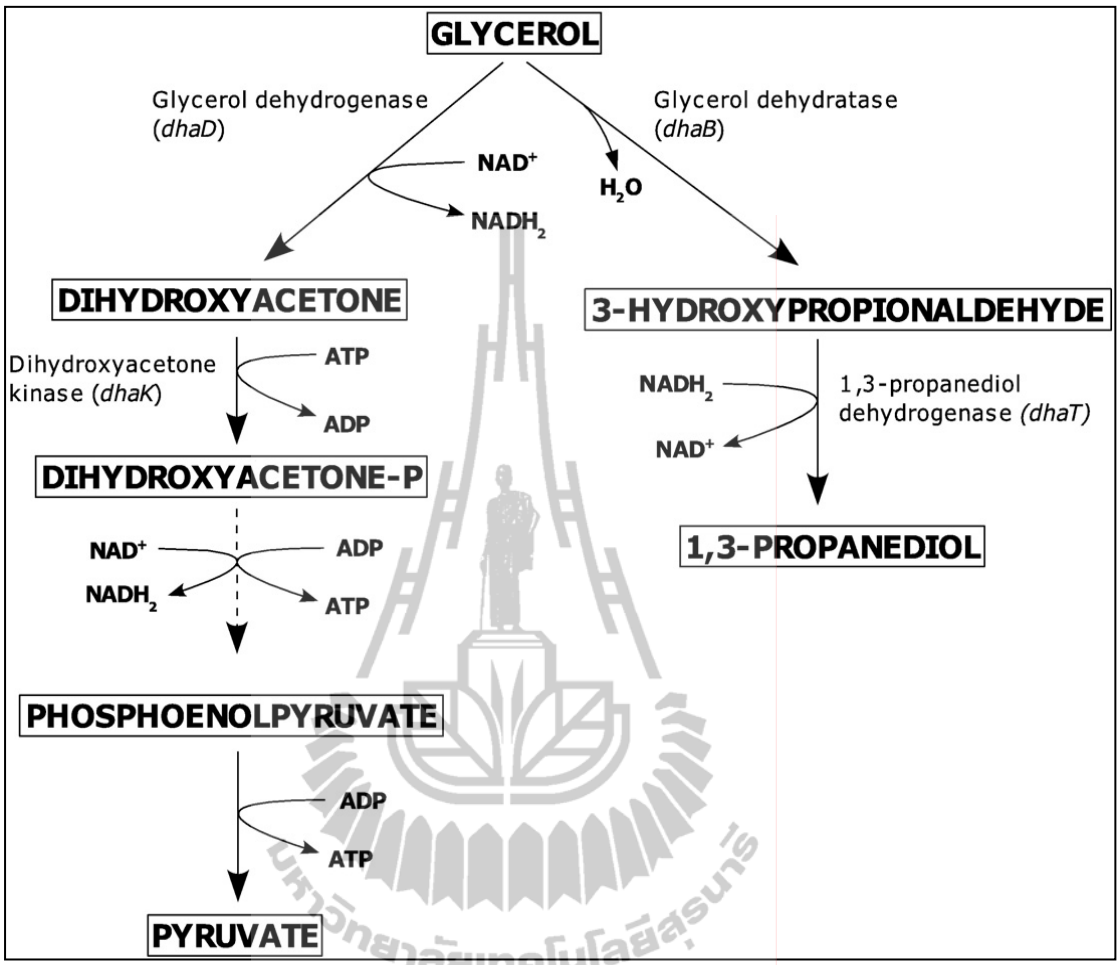


Figure 4.9 Fermentative patterns of glycerol dissimilation dependent on 1,3 propanediol formation. The key enzymes of the *dha* regulon and respective genes related to glycerol metabolism are shown. Pyruvate will be reduced to different organic compounds dependent on microorganism and fermentation conditions, regenerating NAD+ (adapted from Silva *et al*., 2009; Biebl, 2001).

The effect of glycerol concentration was shown in Figure 4.10, the resulted revealed that solvents production increased as the initial glycerol concentration increased from 20 to 50 g/L. Decreasing in solvents production was

observed when initial glycerol concentration above 60 g/L. The maximum solvents production was obtained when 50 g/L initial glycerol concentration was used which resulted in 6.02 g/L total solvents production. It was also found that glycerol concentration at 30 g/L or lower the fermentation performance was mainly organic acids with small amount of solvents production. Interestingly, the concentration of 1,3 propanediol was increased with increasing glycerol concentration. Similar result was observed in glycerol fermentation by *Cl. pasteurianum* DSM 525 which demonstrated that most of glycerol was fermented to butanol and ethanol, but propanediol formation increased with increasing glycerol concentration (Biebl, 2001).

In term of glycerol consumption, it showed that glycerol was utilized completely within 60 h of incubation when 20 and 30 g/L glycerol was used, but it had residue glycerol left at the end of fermentation when more than 40 g/L glycerol was used, as cell growth was inhibited at the high glycerol concentration (Figure 4.10b). In addition, the efficiency of glycerol utilization at higher initial glycerol concentration was slower and glycerol remained in the culture.

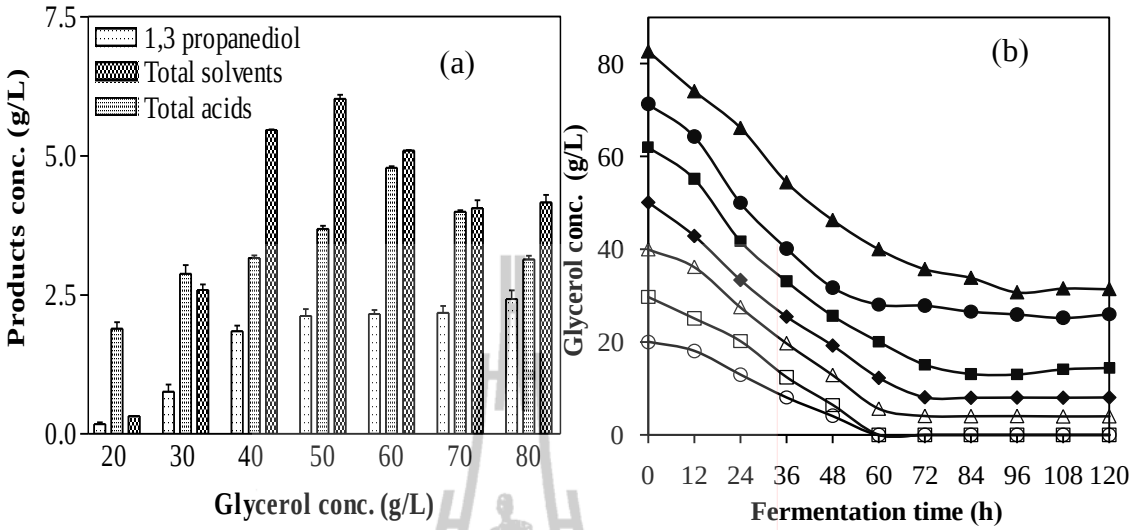


Figure 4.10 Influence of glycerol concentration on fermentation products by *Cl. acetobutylicum* JCM 7289 in batch cultures. (a) fermentation products, (b) glycerol consumption. Symbols represent: (○) 20 g/L, (□) 30 g/L, (Δ) 40 g/L, (♦) 50 g/L, (■) 60 g/L, (●) 70 g/L, (▲) 80 g/L.

4.2.2 Effect of controlled pHs on solvents production

To improve solvents production using glycerol as a carbon source, the optimum concentration of glycerol for solvents production that obtained from the previous experiment (50 g/L) was used. The experiment was performed at various controlled pH values ranging from 4.5 to 6.5 in batch culture. In order to compare the fermentation performance, control fermentation experiment was performed with uncontrolled pH. The results revealed that *Cl. acetobutylicum* JCM 7289 was able to grow over a wild pH range (4.5 to 6.5) as shown in Figure 4.11. At a controlled pH below 5.5, Most of glycerol were fermented to solvents (butanol and ethanol) but solvents production was decreased when a controlled pH above 6.0 was applied. It was also found that dry cell weight was decreased when pH was above 6.0, whereas, 1,3 propanediol formation was increased with increasing pH. Similar results were

observed in the growth of *Cl. acetobutylicum* on glycerol in a chemostat culture, which showed that the reduced growth rate of *Cl. acetobutylicum* was observed when pH was decreased from 6.6 to 5.6 and lower. It suggested that the enzymes involved in glycerol metabolism or their synthetic systems were pH sensitive. This was probably of particular importance in the pathway leading to 1,3-propanediol synthesis, since the relative yield of 1,3-propanediol was reduced to a greater extent than the growth rate (Huang *et al*., 1986).

Figure 4.11 also showed that the highest total solvents production (10.55 g/L) was obtained when controlled pH 5.5 was applied which was about two times higher in concentration than the fermentation with uncontrolled pH (5.92 g/L). These results indicated that the controlled pH values in glycerol fermentation by this *Cl. acetobutylicum* enhanced the solvents production. Dabrock *et al*. (1992) reported that *Cl. pasteurianum* DSM 525 was not able to grow over a wide pH range as it was for *Cl. acetobutylicum*, the pH had almost no effect on the production of solvents (ethanol and butanol) which were produced at low concentrations (2 to 5 mM) over the pH range from 4.8 to 7.0. In addition, glycerol fermentation by *Cl. pasteurianum* DSM 525 using constant pH values between 4.5 to 7.5 did not revealed significant differences in product formation except for an increased in the ethanol content starting at pH 6.5 (Dabrock *et al*., 1992).

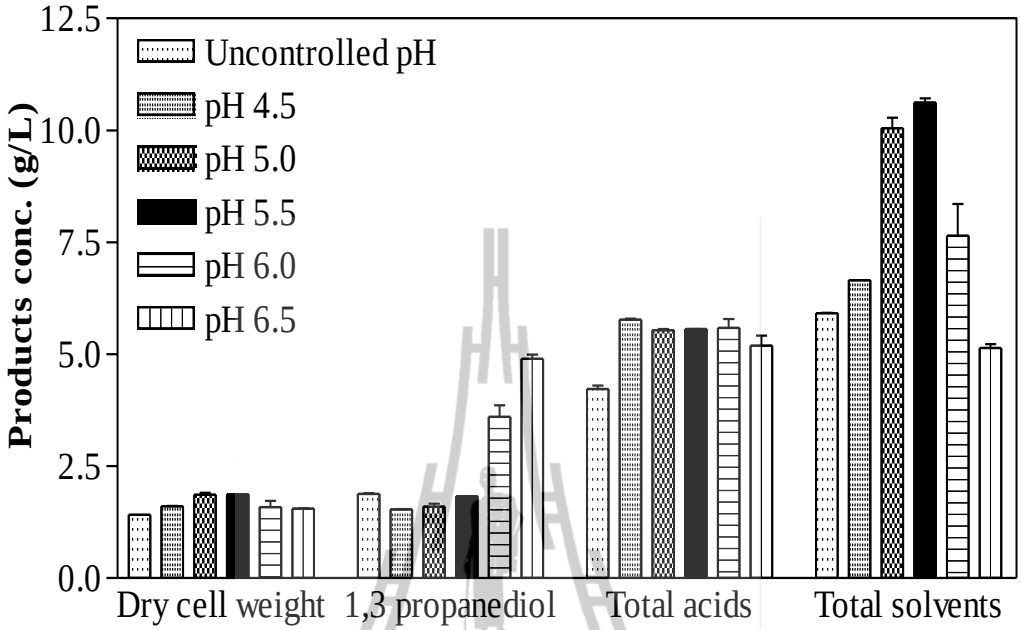


Figure 4.11 Effect of controlled pH on fermentation products by *Cl. acetobutylicum* JCM 7289 using glycerol as a carbon source in batch culture.

4.2.3 Effect of commercial and spent brewer's yeast extract in solvents production

To investigate the efficiency of different nitrogen sources, commercial yeast extract and spent brewer's yeast extract on solvents production, glycerol fermentation by *Cl. acetobutylicum* JCM 7289 was performed using an optimum concentration of glycerol for solvents production which obtained from the previous experiment (50 g/L) as a carbon source. An appropriate pH control (pH 5.5) was also applied in these experiments. In order to compare results obtained in these studies, the concentration used of each yeast extract was fixed at 5 g/L and control fermentation experiment was conducted without yeast extract. The result revealed that the higher solvents production (10.63 g/L) was obtained with supplementing the fermentation medium with commercial yeast extract as compared to those using spent brewer's

yeast extract (8.46 g/L) (Figure 4.12). In addition, using commercial yeast extract and spent brewer's yeast extract as a nitrogen source were greatly influenced solvents production when compared with the control. Considering to the maximum dry cell weight, it was shown that the maximum dry cell weight of the control was around 2 folds lower than that using commercial yeast extract and spent brewer's yeast extract. This result was similar to the fermentation of cassava starch by *Cl. acetobutylicum* TISTR 1462 from our previous studied which showed that good growth of *Cl. acetobutylicum* TISTR 1462 and higher solvents production was observed when supplementing the fermentation medium with brewer's yeast extract as compared with the fermentation without yeast extract. This result was acceptable since yeast extract contained various amino acids, vitamins, minerals and growth factors that promote the growth of *Clostridium* spp. (Madihah *et al*., 2001; Tran *et al*., 2010).

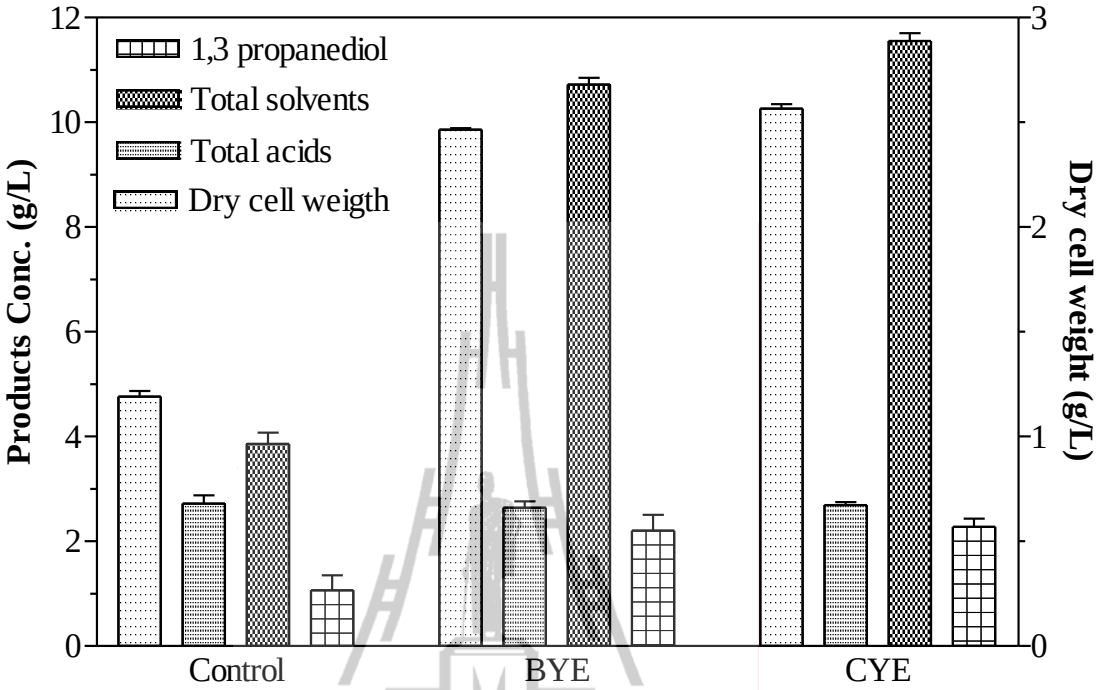


Figure 4.12 Effect of different nitrogen sources, commercial yeast extract and spent brewer's yeast extract on solvents production by *Cl. acetobutylicum* JCM 7289 using 50 g/L glycerol as a carbon source in batch culture with a controlled pH 5.0. (BYE, Spent brewer's yeast; CYE, Commercial yeast extract and Control, without yeast extract).

4.2.4 Effect of different carbon sources on solvents production

To investigate the effect of carbon source including glucose, glycerol and cassava starch on solvents production, the fermentation of *Cl. acetobutylicum* JCM 7289 was conducted in a 2-L fermenter with working volume of 1.5-L. An appropriate controlled pH (pH 5.5) was also applied. In order to compare the results, the concentration used for each carbon source was fixed at 50 g/L. The result was summarized in Table 4.6, *Cl. acetobutylicum* JCM 7289 can utilize all substrates as a

carbon source to produce solvents. The solvents production obtained from each carbon sources were 20.43, 20.08 and 11.66 g/L for cassava starch, glucose and glycerol, respectively. This showed that cassava starch and glucose favored solvents production more than glycerol by this clostridial strain.

Considering the fermentation of glycerol, *Cl. acetobutylicum* JCM 7289 was able to form a relatively great variety of products including butanol, ethanol, acetone, 1,3 propanediol, butyric acid and acetic acid as shown in Table 4.6. Butanol was the main fermentation product accompanied by ethanol. Biebl (2001) explained that more glycerol was converted to butanol than to 1,3 propanediol, due to the fermentation to butanol was the energetically preferred pathway, but that formation of 1,3 propanediol was necessary for the reducing equivalent balance in any case. However, as glycerol was more reduced than the cell mass formed along with the fermentation products, additional reducing equivalents were released and need an acceptor. This was why a certain amount of glycerol always had to be reduce to 1,3 propanediol.

In term of acetone production, there was insignificant different between the fermentation using glucose and cassava starch as the maximum acetone concentration of glucose and cassava starch were 4.91 and 4.89 g/L, respectively. It was interesting to note that acetone produced in fermentation using glycerol was drastically lower when compared with fermentation using glucose and cassava starch as a carbon source. Vasconcelos *et al*. (1994) demonstrated that glycerol was more reduced substrate than glucose, for the same amount of carbon glycerol metabolism liberated twice as much NADH. The reducing equivalent excess provided by the conversion of glycerol must be oxidized through the NADH uptake pathways like, 1,3

propanediol or alcohol production (butanol and ethanol) (Amans *et al*., 2001). This reason was confirmed by Meyer *et al*. (1986) with a CO-gassed continuous culture. They elucidated that acetone formation did not necessarily have to accompany a sustained butanol production. Acetone formation required no reduction energy, and it appeared that the cellular control mechanisms avoid its production in order to maximize the regeration of NAD(P) in the reduced nucleotide-consuming pathway. Moreover, the enzymes associated with acetone production, CoA-transferase and acetoacetate decarboxylase which found in glycerol fermentation by *Cl. acetobutylicum* were presented at a level 10 times lower than in alcohol-and acetone producing culture grown on glucose at pH 4.4. This finding inferred that these enzymes might poorly expressed and thus explained why acetone was slightly produced.

Table 4.6 Comparison of different carbon sources on solvents production by *Cl. acetobutylicum* JCM 7289 using a controlled pH 5.5 in batch culture.

Kinetic parameters	**Glycerol**	**Glucose**	**Cassava starch**
Initial substrate conc. (g/L)	50.12±0.07	50.07±0.12	50.00±0.36
Substrate consumed (g/L)	35.49±0.22	48.75±0.57	47.04±0.79
Max. dry cell weight conc. (g/L)	2.08±0.12	2.67±0.07	2.55±0.15
Max. butyric acid conc. (g/L)	2.78±0.32	3.38±0.27	3.19±0.09
Max. acetic acid conc. (g/L)	2.65±0.15	3.12±0.26	2.76±0.15
Total organic acids conc.(g/L)	5.43±0.23	6.50±0.21	5.95±0.12
Max. butanol conc. (g/L)	6.28±0.33	14.43±0.45	14.66±0.27
Max. acetone conc. (g/L)	0.25±0.22	4.91±0.17	4.89±0.28
Max. ethanol conc. (g/L)	2.33±0.09	0.74±0.02	0.88±0.12
Total solvents conc. (g/L)	11.66±0.22	20.08±0.18	20.43±0.22
1,3 propanediol conc. (g/L)	2.26±26	0	0
Fermentation time* (h)	60	48	60
Solvent yield (g solvents/g glucose)	0.33±0.01	0.41±0.07	0.43±0.02
Solvent productivity (g solvents/L/h)	0.19±0.02	0.42±0.02	0.34±0.01

Final concentration of organic acids, sugar, and starch were taken when total solvent concentration reached maximum value. Maximum concentration of solvents (acetone, butanol and ethanol) was the highest concentration during the fermentation and the time to reach the maximum values was not the same as for solvent. *Fermentation time was the time taken to reach maximum solvents concentration. Values represent the mean of duplicate experiment ± SD between samples.

CHAPTER V

CONCLUSION

The main objective of this study was to investigate the feasibility of *Clostridium acetobutylicum* TISTR 1462 to produce solvents (acetone, butanol and ethano) using cassava materials as the main substrate supplementing with spent brewer's yeast extract as a nitrogen source in batch culture. The results showed that *Cl. acetobutylicum* TISTR 1462 can utilize cassava materials which were abundant and relatively inexpensive carbon source as a potential substrate to produce solvents. The results also revealed that the enzymatic pretreatment of gelatinized starch prior to the fermentation was not necessary, due to no improvement in solvents production when compared with direct fermentation of gelatinized starch. In addition, the lower solvents production (19.48 %) was obtained when cassava materials was hydrolyzed with acid prior to the fermentation (21.20 g/L vs. 17.07 g/L). This may be caused by growth inhibitors formed during acid hydrolysis treatment. However, the acid treatment has been proven to be the fast and cost-effective method for producing monomeric sugars from polysaccharides. Thus, acid-treated cassava pulp for solvents production would be recommended as inexpensive and efficient processes when the fermentation condition was optimized in this medium. High performance of solvent fermentation could be achieved using cassava starch with batch fermentation employing a very simple pH control strategy (pH 5.5). Using these appropriate pH control strategy, the final solvent concentration obtained was almost 1.5 times higher

than that obtained during fermentation with uncontrolled pH. For the comparison of different types of nitrogen source, the result revealed that using spent brewer's yeast extract in solvent production resulted in 18.46 g/L which was comparable with that obtained with the fermentation using commercial yeast extract (20.86 g/L). From this result, it was more likely that the cost of the commercial yeast extract cloud be reduced when the fermentation medium was replaced by the spent brewer's yeast extract. This finding indicated cassava materials could be potential renewable substrate for production of solvents (up to 19 g/L total solvents) when partial hydrolysis treatment was carried out prior to fermentation. Moreover, cassava material fermentation using a control pH 5.5 supplemented with spent brewer's yeast extract was the most promising substrate for commercial ABE production. Since the cassava materials were relatively abundant biomass in Thailand.

The second objective was to investigate the achievable of utilizing glycerol as the main substrate to produce solvents by *Cl. acetobutylicum* JCM 7289 in anaerobic batch fermentation. *Cl. acetobutylicum* JCM 7289 could utilize glycerol as substrate to produce solvents. However, a varying fraction of glycerol was also converted to 1,3 propanediol, butyric acid and acetic acid. More than 40 g/l glycerol was utilized, and only 5.92 g/L total solvents were produced which was two times lower in concentration when glucose was used as substrate (13.85 g/L). It could be due to its metabolic route that favored the formation of other solvents such as 1, 3 propanediol, diminishing the production of solvent. According to the result of glycerol concentrations, it has an effect on cell growth and solvents production. When initial glycerol concentration was more than 40 g/L, there was residue glycerol left at the end of fermentation. Moreover, the efficiency of glycerol utilization at high initial

glycerol concentration was low. In addition, the initial concentration of glycerol at 60 g/L or above the cell growth rate was retarded. Considering the fermentation with controlled pH, the result revealed that the highest total solvents production (12.89 g/L) was obtained with a controlled pH of 5.5. Moreover, total solvents production with controlled pH was higher than that obtained in fermentation without controlled pH. These results indicated that the pH controlled was influential for enhancement of solvents production during fermentation by this *Cl. acetobutylicum* JCM 7289. For the effect of various nitrogen sources, it revealed that the fermentation performance using spent brewer's yeast extract as a nitrogen source resulted in 10.81 g/L total solvents production which was comparable to that obtain with fermentation using commercial yeast extract (11.66 g/L). From the physiological point of fermenting glycerol, the fermentation described was interesting as it combined the two pathway of butanol and of 1,3 propanediol formation in one organism the latter pathway serve to regenerate NADH, which was released during biomass formation but also become the major route in the anaerobic utilization of glycerol by this *Cl. acetobutylicum* JCM 7289.

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APPENDICES

APPENDIX A

CHEMICAL COMPOSTITION

1. Cooked Meat Medium

Beef heart extract	30 g/L
Meat peptone	20 g/L
D (+) glucose	2 g/L
Sodium chloride	5 g/L

2. Reinforced Clostridial Agar

Casein enzymic hydrolysate	10 g/L
Beef extract	10 g/L
Yeast extract	5 g/L
Dextrose	5 g/L
Sodium chloride	3 g/L
Sodium acetate	1 g/L
Soluble starch	0.5 g/L
L- cystein hydrochloride	0.5 g/L
Agar	15 g/L

APPENDIX B

STANDARD CALIBRATION CURVE

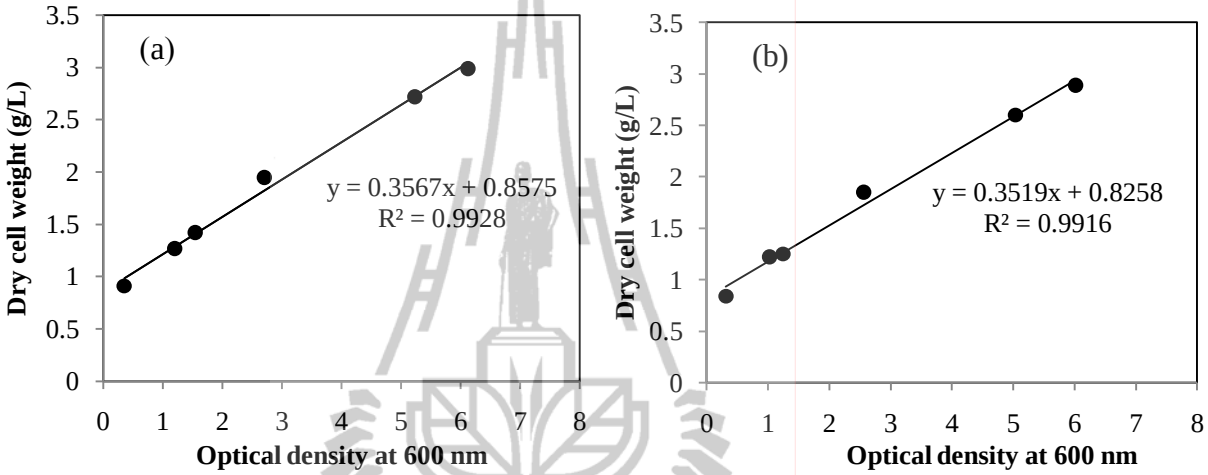

Figure 1B Correlation lines between optical density (OD) and biomass concentration (dry cell weight), (b) *Cl. acetobutylicum* TISTR 1462, (a) *Cl. acetobutylicum* JCM 7289

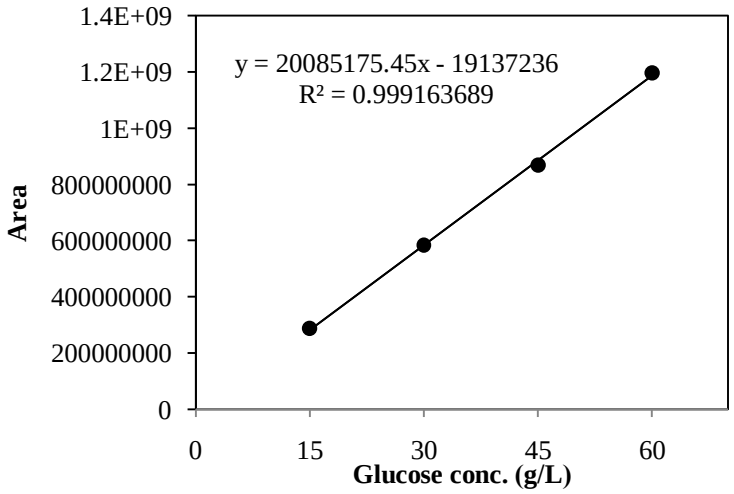

Figure 2B Standard calibration curve of glucose using high performance liquid chromatography (HPLC)

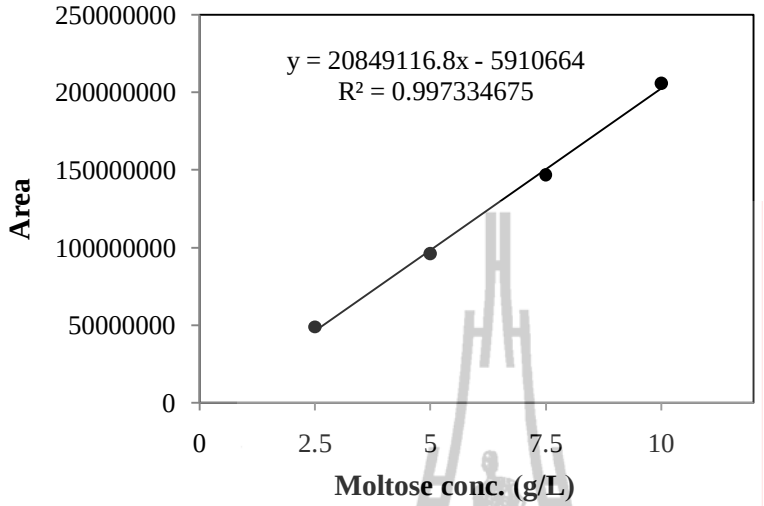

Figure 3B Standard calibration curve of maltose using high performance liquid chromatography (HPLC).

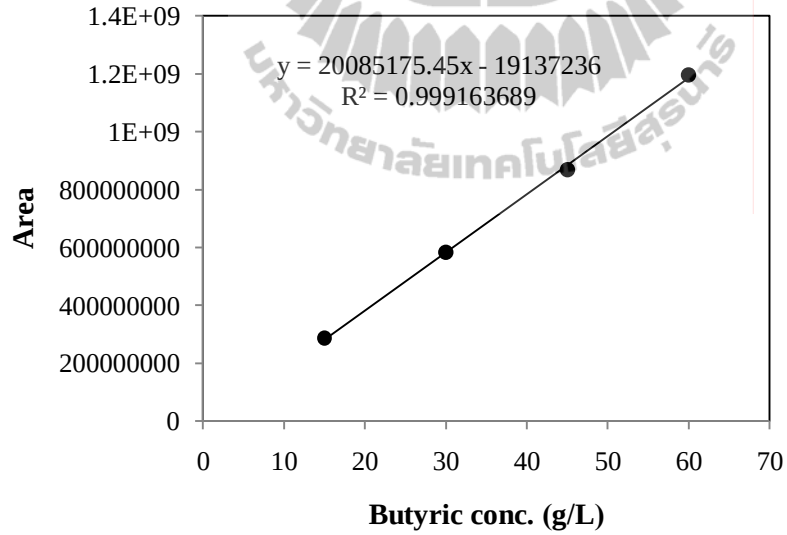

Figure 4B Standard calibration curve of butyric acid using high performance liquid chromatography (HPLC).

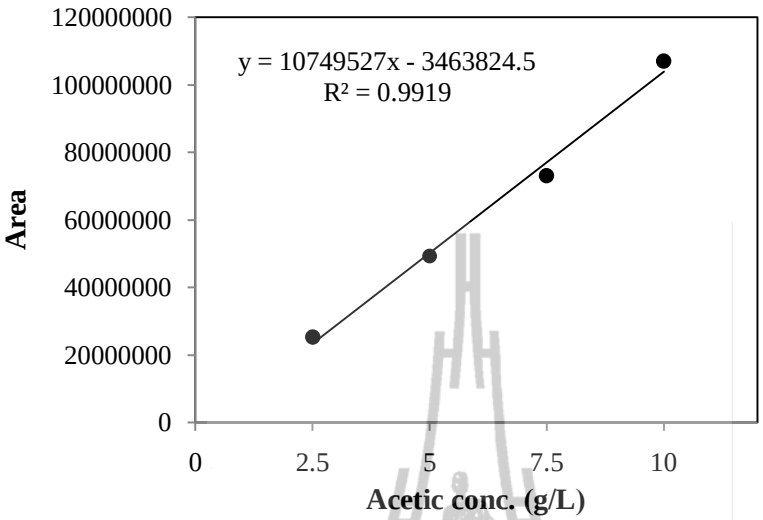

Figure 5B Standard calibration curve of acetic acid using high performance liquid chromatography (HPLC).

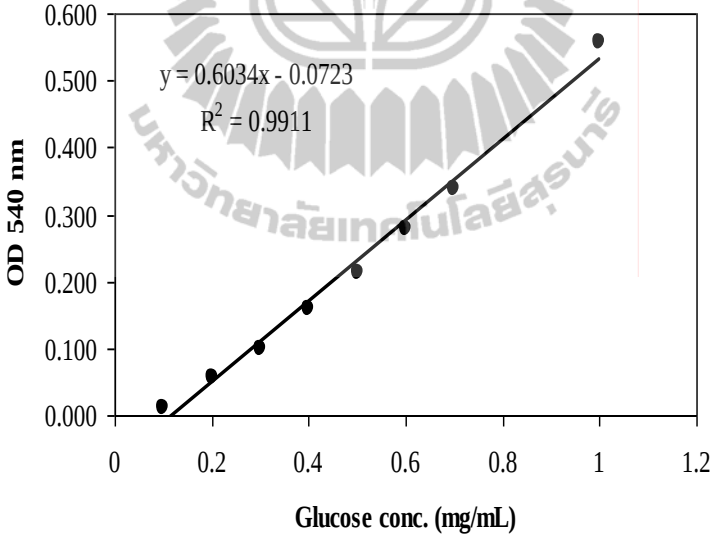

Figure 6B Standard calibration curve of sugar concentration by measurement density using a spectrophotometer at 540 nm by DNSA method.

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Acetone-Butanol-Ethanol (ABE) Production from Cassava by *Clostridium acetobutylicum* TISTR 1462

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Abstract

Acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* TISTR 1462 using different pH related conditions were investigated. The ABE production was performed with different pH controlled strategies (pH 4.5-6.5) and 50 g/L glucose as a carbon source. The effect of cassava starch concentrations on the solvents production were investigated at 20~80 g/L as well as the effect of different types of carbon sources and nitrogen sources were also evaluated. The results in batch culture showed that *Cl. acetobutylicum* TISTR 1462 was capable of producing solvents efficiently from cassava starch, which was comparable to when glucose was used. The batch experiment with uncontrolled pH of cassava starch resulted in 14.33 g/L of total solvents as compared to 15.39 g/L of total solvents when glucose was used. Moreover, it was found that enzymatic pretreatment of gelatinized cassava starch yielding maltose and glucose prior to the fermentation did not improve solvents production as compare to direct fermentation of gelatinized starch. In the fermentation

with pH controlled during solventogenic phase, the highest total solvents production (20.08 g/L) was obtained with a controlled pH of 5.5. At a controlled pH 6.0 or above the fermentation were produced mainly organic acids with a small amount of solvents. It also found that the highest acetone production (6.78 g/L) was obtained with a controlled pH 5.25. Using the appropriated pH control strategy, the final solvents concentration obtained was almost 1.5 times higher than that obtained under fermentation with uncontrolled pH. At the range of cassava starch concentration investigated (20-80 g/L), the highest total solvents production (14.33 g/L) was obtained at 60 g/L initial cassava starch concentration. The fermentation performance using initial cassava concentrations lower than 30 g/L were acidogenic rather than solventogenic. For the effect of various nitrogen sources, it revealed that the fermentation performance using spent brewer's yeast extract as a nitrogen source resulted in 18.46 g/L solvents production which was comparable to that obtained in fermentation using commercial yeast extract (20.86 g/L).

Keywords: Acetone-Butanol-Ethanol production, Cassava starch, *Clostridium acetobutylicum*

BIOGRAPHY

Mr. Apichai Sawisit was born on May 20, 1985 at Roi-Et, Thailand. He obtained his Bachelor degree of Science in Food Technology from Department of Food Technology, Faculty of Agricultural, Ubon Ratchathani University, Ubon Ratchathani, in 2007. After graduation, he decided to further study for master degree in the field of Bioprocess engineering at school of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakorn Ratchasima, Thailand. During study, he received a scholarship from Tsukuba University to do some part of his research in Japan from April 5 to July 4, 2010. During this time, he had experience to practice skills in improvement of ABE fermentation from glycerol by *Clostridium* spp. He research topic was Acetone-Butanol-Ethanol **(**ABE**)** Production from Cassava and Glycerol by *Clostridium acetobutylicum*. The results from part of this study have been presented as oral presentation at the 1st Payap University Research Symposium 2011 on February 16th, 2011, Chiang Mai, Thailand. His work is going to be published in the proceeding of the 1st Payap University Research Symposium 2011, organized by Payap University and Upper Northern Research Administration Network in the title of "Acetone-Butanol-Ethanol **(**ABE**)** Production from Cassava by *Clostridium acetobutylicum* TISTR 1462", 2011.