Production of Acetone-Butanol-Ethanol from Volatile Fatty Acid and Decanter Cake Hydrolysate by *Clostridium* spp.

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บทคัดย่อ

ในปัจจุบันการผลิตบิวทานอลผ่านกระบวนการผลิต อะซิโตน บิวทานอล เอทานอล (เอบีอี) โดยเชื้อ *Clostridium* spp. มีความสำคัญมากขึ้น เนื่องจากราคาของเชื้อเพลิงฟอสซิลที่มีปริมาณจำกัดมีแนวโน้มสูงขึ้นอย่างต่อเนื่อง ซึ่งบิวทานอลมีข้อดีกว่าเอทานอล คือ ให้ค่าพลังงานที่สูงกว่าและมีการกัดกร่อนน้อยกว่า ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อผลิตบิวทานอลจากกรดไขมันระเหยง่าย (ส่วนที่ 1) และจากกากตะกอนดีแคนเตอร์ของโรงงานสกัดน้ำมันปาล์มโดยใช้เชื้อที่มีการเจริญและเชื้อที่ไม่มีการเจริญแต่มีกิจกรรมในการเปลี่ยนกรดไขมันไปเป็นบิวทานอล (ส่วนที่ 2) นอกจากนี้ยังศึกษาการประยุกต์ใช้เซลล์ตรึงรูปเพื่อการผลิตบิวทานอล (ส่วนที่ 3)

ในส่วนที่ 1 เนื่องจากเชื้อที่ไม่มีการเจริญแต่มีกิจกรรมในการเปลี่ยนกรดไขมันไปเป็นบิวทานอลมีข้อดีคือ สามารถเลี้ยงในอาหารที่ไม่เติมแหล่งไนโตรเจนได้ จึงได้ทำการศึกษาการผลิตบิวทานอลโดยเลี้ยงเชื้อ *Clostridium* spp. 6 สายพันธุ์คือ *C. acetobutylicum* DSM 1731, *C. butyricum* TISTR 1032, *C. beijerinckii* TISTR 1461, *C. acetobutylicum* TISTR 1462, *C. acetobutylicum* JCM 1419 และ *C. beijerinckii* JCM 1390 ในอาหารที่ไม่เติมแหล่งไนโตรเจนและเติมกรดบิวทิริกเป็นแหล่งคาร์บอนเพียงอย่างเดียว พบว่าเชื้อ *Clostridium* spp. โดยเฉพาะ *C. beijerinckii* TISTR 1461 สามารถเปลี่ยนกรดบิวทิริกไปเป็นบิวทานอลได้โดยตรง และเชื้อมีการผลิตบิวทานอลเพิ่มขึ้นเมื่อมีการเติมน้ำตาลกลูโคสเป็นสับสเตรทร่วม โดยให้ปริมาณบิวทานอลสูงสุดเท่ากับ 10.18 กรัมต่อลิตร คิดเป็นร้อยละ 88 ของปริมาณเอบีอีทั้งหมด และคิดเป็นผลผลิตบิวทานอลเท่ากับ 0.68 C-mol butanol/C-mol carbon sources นอกจากน้ำตาลกลูโคสแล้วน้ำตาลโมเลกุลเดี่ยวชนิดอื่นๆ ได้แก่ กาแลคโตสและไซโลส และน้ำตาลโมเลกุลคู่ ได้แก่ มอลโตส, ซูโครส และแลคโตส สามารถใช้เป็นสับสเตรทร่วมกับกรดบิวทิริกได้ นอกจากนี้ยังพบว่าเชื้อยังสามารถเปลี่ยนกรดอะซิติกไปเป็นบิวทานอลได้เช่นกัน จากการศึกษาครั้งนี้แสดงให้เห็นว่ามีความ

เป็นไปได้ที่จะใช้เชื้อ *Clostridium* ในการผลิตบิวทานอลจากกรดไขมันระเหยง่ายและน้ำตาลได้โดยไม่ต้องเติมแหล่งไนโตรเจน

ส่วนที่ 2 จากการศึกษาการใช้กากตะกอนดีแคนเตอร์ซึ่งเป็นวัสดุเศษเหลือจากโรงงานสกัดน้ำมันปาล์มเพื่อเป็นสับสเตรทในการผลิตบิวทานอล พบว่ากรดไนตริกเหมาะสมที่จะใช้ย่อยกากตะกอนดีแคนเตอร์ให้เป็นน้ำตาล แต่เนื่องจากกระบวนการย่อยด้วยกรดทำให้เกิดสารยับยั้งการเจริญของเชื้อ จึงทำการกำจัดสารยับยั้งโดยใช้สารดูดซับ พบว่าถ่านกัมมันต์สามารถลดสารยับยั้งได้ดีที่สุดและทำให้เกิดการสูญเสียน้ำตาลน้อยที่สุด เมื่อนำไฮโดรไลเสตจากกากตะกอนดีแคนเตอร์ที่ผ่านการย่อยและกำจัดสารยับยั้งมาใช้ในการผลิตบิวทานอลโดยเชื้อ *C. beijerinckii* TISTR 1461 พบว่าเชื้อผลิตบิวทานอลได้ 3.42 กรัมต่อลิตร ผลผลิตเท่ากับ 0.28 โมลคาร์บอนของบิวทานอลต่อโมลคาร์บอนของสับสเตรทและมีผลผลิตต่อเวลาเท่ากับ 0.048 กรัมต่อลิตรต่อชั่วโมง ในอาหารที่มีการเติมแหล่งไนโตรเจน ในขณะที่การใช้ไฮโดรไลเสตที่ได้จากการย่อยกากตะกอนดีแคนเตอร์เป็นแหล่งคาร์บอนร่วมกับกรดบิวทิริกในอาหารที่ไม่เติมแหล่งไนโตรเจน พบว่าเชื้อ *C. beijerinckii* TISTR 1461 สามารถผลิตบิวทานอลได้ 6.94 กรัมต่อลิตร ผลผลิตเท่ากับ 0.47 โมลคาร์บอนของบิวทานอลต่อโมลคาร์บอนของสับสเตรทและมีผลผลิตต่อเวลาเท่ากับ 0.096 กรัมต่อลิตรต่อชั่วโมง ซึ่งการศึกษาครั้งนี้แสดงให้เห็นว่ากากตะกอนดีแคนเตอร์สามารถใช้เป็นวัตถุดิบในการผลิตบิวทานอลได้ทั้งจากเชื้อที่มีการเจริญและเชื้อที่ไม่มีการเจริญ

ส่วนที่ 3 เนื่องจากการตรึงเซลล์เป็นเทคนิคที่ทำให้สามารถผลิตผลิตภัณฑ์ชีวภาพได้อย่างมีประสิทธิภาพและต่อเนื่อง จึงทำการศึกษาการตรึงเซลล์โดยใช้วัสดุเศษเหลือจากต้นปาล์ม ได้แก่ กะลาปาล์ม, เส้นใยปาล์ม, ทะลายปาล์มเปล่า และทางปาล์ม เพื่อผลิตบิวทานอลโดยเชื้อ *C. beijerinckii* TISTR 1461 โดยใช้อาหารที่ไม่เติมแหล่งไนโตรเจนที่มีกรดบิวทิริกและน้ำตาลกลูโคสเป็นแหล่งคาร์บอนร่วม พบว่าการตรึงเซลล์บนทางปาล์มโดยใช้ปริมาณที่เหมาะสมของทางปาล์มที่ร้อยละ 7 โดยน้ำหนักต่อปริมาตร ทำให้เชื้อสามารถผลิตบิวทานอลได้สูงสุดคือ 9.98 กรัมต่อลิตร ในรอบที่ 1 ของการหมัก อย่างไรก็ตามในรอบที่ 2 และ 3 ของการหมักพบว่า เชื้อผลิตบิวทานอลได้น้อยลงเท่ากับ 8.31 กรัมต่อลิตร และ 4.95 กรัมต่อลิตร ตามลำดับ จากการศึกษาการใช้กระบวนการผลิตแบบสองขั้นตอนโดยนำเซลล์ตรึงรูปกลับมาเลี้ยงซ้ำอีกครั้งในอาหารที่มีแหล่งไนโตรเจนเป็นเวลา 24 ชั่วโมง ก่อนนำไปหมักในขั้นตอนที่สองเพื่อผลิตบิวทานอล พบว่าการหมักแบบสองขั้นตอนนี้ทำให้เชื้อสามารถผลิตบิวทานอลได้สูงขึ้นและคงที่ โดยให้การผลิตบิวทานอลเท่ากับ 11.17 กรัมต่อลิตร, 10.81 กรัมต่อลิตร และ 11.52 กรัมต่อลิตรในรอบที่ 1, 2 และ 3 ตามลำดับ

Thesis title	Production of Acetone-Butanol-Ethanol from Volatile Fatty Acids and Decanter Cake Hydrolysate by *Clostridium* spp.
Author	Mr. Sawang Loyarkat
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ABSTRACT

In present time, butanol production via acetone-butanol-ethanol (ABE) fermentation by *Clostridium* spp. has become more attractive, due to the limited supply of petroleum and fossil fuels that causing of oil prices to increase continuously. Butanol is better than ethanol because it has higher energy content and is far less corrosive. This study aimed to produce butanol from volatile fatty acids (Part I) and decanter cake waste from palm oil mill by the use of growing cells and cells in stationary phase (Part II). The immobilized cells were also applied to produce the butanol (Part III).

Part I, the cells in stationary phase were used because they can produce butanol in a cost-effective medium (nitrogen-free medium). Six *Clostridium* spp. Include *C. acetobutylicum* DSM 1731, *C. butyricum* TISTR 1032, *C. beijerinckii* TISTR 1461, *C. acetobutylicum* TISTR 1462, *C. acetobutylicum* JCM 1419 and *C. beijerinckii* JCM 1390 were incubated in a nitrogen-free medium (non-growth medium) containing only butyric acid as a sole precursor for a direct conversion to ABE. Cells in stationary phase of *Clostridium* spp., especially *C. beijerinckii* TISTR 1461, could convert butyric acid to butanol via their sole solventogenic activity. This activity was further enchanced in the presence of glucose as a co-substrate. In addition to glucose, other monosaccharides (i.e. galactose, xylose) and disaccharide (i.e. maltose, sucrose, lactose) could also be used as a co-substrate with butyric acid. However, among the organic acids tested, only butyric acid and acetic acid could be converted to butanol. The highest butanol production of 10.18 g/L, the highest butanol

ratio 88% in the ABE products, and the highest butanol to substrate ratio of 0.68 C-mol butanol/C-mol carbon sources were achieved by this strategy. This study has shown that it is possible to use cells in stationary phase of *Clostridium* spp. to directly produce butanol from volatile fatty acids and sugars without use of medium containing costly nitrogen source.

Part II, the decanter cake waste from palm oil mill was used as a renewable substrate for butanol production. Decanter cake was mostly hydrolyzed to fermentable sugar by nitric acid. The detoxification of the hydrolysate using activated-charcoal effectively removed inhibitory by-product with less sugar loss. A butanol production from detoxified hydrolysate was performed using growing and cells in stationary phase of *C. beijerinckii* TISTR 1461. By growing cells of *C. beijerinckii* TISTR 1461, 3.42 g/L of butanol was produced with a yield of 0.28 C-mol butanol/C-mol substrate and productivity 0.048 g/L/h. When the detoxified hydrolysate was used as a co-substrate with butyric acid for direct conversion to butanol by cells in stationary phase, 6.94 g/L of butanol was produced with a yield of 0.47 C-mol butanol/C-mol substrate and productivity 0.096 g/L/h. This study has shown that decanter cake could serve as low-cost substrate for butanol production by both growing and cells in stationary phase.

Part III, since immobilization technique is a promising approach to efficiently and continuously produce bioproducts, a immobilization system using palm tree wastes (i.e. oil palm shell, palm press fiber, empty fruit bunch, and oil palm frond) as support materials was developed for cells immobilization of *C. beijerinckii* TISTR 1461. The immobilized cells were used for butanol production in a repeated-batch fermentation using a nitrogen-free medium containing only butyric acid and glucose as carbon sources. The immobilized cells on oil palm frond at optimal amount of 7% (w/v) produced the highest butanol of 9.98 g/L in the 1st batch fermentation. However, in the 2nd and 3rd batch fermentation the immobilized cells produced lower amount of butanol at 8.31 g/L and 4.95 g/L, respectively. The two-stage fermentation was then developed to solve this problem. After being used for one batch, the immobilized cells were re-incubated in a nitrogen-rich medium for 24 h before re-used in the second batch of butanol production. This two-stage process was repeated

three times. The butanol production by this strategy remained high and constant at 11.17 g/L, 10.81 g/L and 11.52 g/L in the 1st, 2nd and 3rd cycles, respectively.

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LIST OF ABBREVIATION AND SYMBOLS

TYA	=	Tryptone-yeast extract-acetate medium
CPO	=	Crude Palm Oil
FFB	=	Fresh Fruit Bunch
DC	=	Decanter Cake
WSH	=	Wheat Straw Hydrosate
OPS	=	Oil Palm Shell
DDGS	=	Distiller Dry Grain and Soluble
PBRs	=	Packed Bed Reactors
FBB	=	Fibrous Bed Bioreactor
EFB	=	Empty Fruit Bunch
PPF	=	Palm Press Fiber
OPF	=	Oil Palm Fiber
SLC	=	Soluble Lignin Degradation Compound
HMF	=	5-Hydroxymethyl Furfural
RSM	=	Response Surface Methodology
CCD	=	Central Composite Design
SEM	=	Scanning Electron Microscope
EMP	=	Embden-Meyerhof-Parnas Pathway
VFAs	=	Volatile Fatty Acids
YE	=	Yeast Extract
WP	=	Whey Protein
ISP	=	Isolated Soy Protein
PVA	=	Polyvinyl alcohol

CHAPTER 1

INTRODUCTION

In present time, butanol production via acetone-butanol-ethanol (ABE) fermentation by clostridia has become more attractive, due to the limited supply of petroleum and fossil fuels that causing of oil prices to increase continuously Butanol is better than ethanol when blending with gasoline. It has lower pressure, higher energy contents and a much lower affinity for water (7.8%) in comparison with ethanol (100%) giving its greater tolerance to moisture and water contamination. Moreover, butanol is far less corrosive than ethanol and can be shipped and distributed through existing pipeline and filling stations. Butanol production from sugars such as glucose is a biphasic fermentation, the first phase is the acidogenic phase in which sugar is metabolized into organic acids by growing cells. During this phase two molecules of carbon are lost as carbon dioxide in the course of the pyruvate-ferredoxin oxidoreductase reaction. The second phase is the solventogenic phase in which the organic acids are reassimilated to produce butanol along with acetone and ethanol (Shinto *et al*., 2007). The loss of carbon flow toward cell growth and the formation of these by-products made the butanol yield from sugars so low, typically only 0.15 g-butanol/g-glucose (Maddox, 1989). This low butanol yield has made the conventional butanol production by growing cells uneconomical. It has been reported that cells in stationary phase could be used to directly convert glucose and butyric acid to butanol without losing the carbon flow toward cell growth and this lead to a higher yield of butanol. In addition, the use of cells in stationary phase is promising because they can produce butanol in a cost-effective medium (nitrogen-free medium) (Al-shorgani *et al*., 2012). An important factor in butanol production is the high cost of fermentation substrate that contributes to >60% of the total production cost of biobutanol (Jones and Woods, 1986). Corn or molasses cannot be used as substrates because their values as food and feed increase their cost and make them uneconomical for butanol production. While a number of low-cost substrates have been previously evaluated (Qureshi *et al*., 2001; Qureshi and Maddox, 2005), recently lignocellulosic materials are of interest for use as low-cost renewable substrates. This

is especially so for those generated from the agricultural industry because they are abundant, renewable and readily available (Qureshi *et al*., 2010a; Qureshi *et al*., 2010b).

Aside from substrate, another limiting factor in ABE production is butanol toxicity and inhibition (Ezeji *et al*.,2010; Gu *et al*., 2011). Many *in situ* butanol recovery techniques, including pervaporation, liquid–liquid extraction, adsorption, and gas stripping, have been reported to relieve butanol inhibition and enhance the efficiency of ABE fermentation (Vane, 2008). Among them, gas stripping is an effective technique with low energy requirement and easy to integrate with the fermentation process. (Ezeji *et al*., 2004; Lu *et al*., 2012; Qureshi *et al*., 2007; Xue *et al*., 2012).

The palm oil industry shows important role in Thailand's economy. Palm oil occupies 70% of the Thai vegetable oil in market, and is estimated to be substance 40,000 million baht per year and has an average annual growth rate of 15% during the last decade (Chavaparit *et al*., 2006). Most palm oil mills are located in the southern part of the country. All 71 palm oil mills, had a more or less similar production process (wet process) and the crude palm oil (CPO) yield was about 17-20% of fresh fruit bunch (FFB). However, the amount of pollutant produced during palm oil extraction is higher than palm oil produced. The use of decanter system is an effective solution to meet the zero emission targets in palm oil mills. The three phase decanter system separates sludge from oil clarifier into oil, sludge and decanter cake (DC). The sludge which contains a low concentration of pollutant can be easily treated and recycled into the mill for fresh fruit bunch extraction. In practice, the mill neglect and leave the DC for natural degradation (Razak *et al*., 2012). According to the waste to wealth through biotechnology concept, DC, which is composed of cellulose and hemicellulose, can be utilized as the substrate for production of enzyme such as cellulase, sugar and biofuel.

Currently, most decanter cake is used as fertilizer and animal feed while its utilization as a source of biofuel production is minimal. This biomass should be investigated in terms of its potential for bioconversion to butanol. Firstly, cellulose and hemicellulose in the decanter cake should be hydrolyzed into their corresponding monomers (sugars) for utilization by *Clostridium* spp. Although several methods for

hydrolyzing lignocellulose have recently been proposed, two commonly used processes are acid and enzymatic hydrolysis. During the hydrolysis process the main toxic byproducts such as furfural, 5-hydroxymethylfurfural (HMF), and phenolic compounds from soluble lignin are generated depending on the chemicals, temperature and time employed. Several studies have been conducted to minimize their negative effects on fermentation, such as attempting to minimize their formation during hydrolysis, detoxifying the hydrolysate (Wang and Chen, 2011; Ezeji *et al*., 2007a) converting these inhibitors into non-inhibitory products (Pienkos and Zhang, 2009) and isolating or developing species of microorganisms that can tolerate these inhibitors (Taherzadeh and Karimi, 2007).

Immobilized cell system has been suggested as an effective means for improving ABE production, since it is potential to increase process productivity, ease to separate cell mass from the bulk liquid, reduce risk of contamination, better operational stability and cell viability for several cycles of fermentation and reduce production costs. The most suitable carriers for cell immobilization are entrapment in calcium alginate bead because these techniques are simple, cost effective and non-toxic (Behera *et al*., 2010). There is also a surge in the attempts toward discovery out a renewable and biodegradable carrier which is basically not synthetic, easy to use, inexpensive and available naturally in abundance. The advantages accruable from such biomaterials (sawdust, wood chips/shavings, rice husks, cotton towels, and straw) are reusability, freedom from toxicity problems, mechanical strength for necessary support, long time operation, high fermenting activity and opening of spaces within the matrix for growing cells (Guénette and Duvnjak, 1996; Das *et al*., 1993; Shukla *et al*., 1989; Huang *et al*., 2004; Forberg and Haggstrom, 1985).

In this study, the growing cells of several *Clostridium* spp. were prepared in a nitrogen-rich medium and inoculated into a nitrogen-free medium for performing butanol production by the cells in stationary phase. The most suitable strain for this butanol producing strategy was selected for further study. Various sugars were also evaluated for their effects on butanol production by the cells in stationary phase. The decanter cake, a waste from palm oil mill, was hydrolyzed and used as a low-cost substrate for ABE production by *Clostridium* spp. The ABE production from decanter cake hydrolysate by a suitable *Clostridium* sp. was

optimized in both conventional mode and solventogenesis mode. Moreover, the continuous ABE production by immobilized *Clostridium* sp. was performed to increase the productivity.

Objectives of the study

1. To study the growth and ABE production of *Clostridium* spp. in nitrogen-rich medium and nitrogen-free medium.
2. To optimize the chemical hydrolysis of decanter cake and detoxification of the hydrolysate using various methods.
3. To study ABE production from decanter cake hydrolysate by *Clostridium* spp. and optimize the medium component for ABE production from decanter cake hydrolysate.
4. To optimize the process for ABE production by immobilized cells.

CHAPTER 2

LITERTURE REVIEWS

1. Butanol

Butanol (IUPAC nomenclature, 1-butanol; CAS no. 71-36-3) also commonly known as butyl alcohol, n-butanol or methylolpropane, refers to a four-carbon alcohol with a formula of C_4H_9OH (MW 74.12 $g \cdot mol^{-1}$). There are four possible isomeric structures for butanol, from a straight-chain primary alcohol to a branched-chain tertiary alcohol. Butanol is a colorless, igneous, slightly hydrophobic liquid with a distinct banana-like aroma and strong alcoholic odor. It is completely miscible with most common organic solvents and has less solubility in water. (Lee *et al*., 2008b; Dürre, 2008). Different chemicals in the same alcohol family include methanol (1-carbon), ethanol (2-carbon), and propanol (3-carbon) (Kristin, 2007).

1.1 Butanol as fuel

One of the major distinguished roles of biobutanol (bio-based butanol) is its appliance in the next generation of motor-fuels. Whereas, 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 1). This means butanol consumption is close to that of pure gasoline whereas ethanol-gasoline blends are consumed much faster to obtain the same power input. In addition, butanol can be mixed with common gasoline at any percentage ratio (Shota *et al*., 2008) 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). Besides, butanol has been demonstrated to work in vehicles designed for use with gasoline without modification, producing similar mileage performance to petrol. Other important advantages over ethanol include: (a) the lower volatility (less explosive), butanol has a lower Reid vapor pressure than ethanol (7.5 times) (Yang, 2008); (b) it does not readily adsorb moisture (lower hygroscopicity), so is less affected by weather changes; (c) less corrosive (Dürre, 2007); (d) it 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. This allows butanol to be a much safer fuel that can be dispersed through existing pipelines and filling stations (Yang, 2008) with simple integration into the present fuel delivery and storage infrastructure (pipelines, storage tanks, filling stations, etc.). Ethanol, on the other hand, can only be added shortly prior to use. However, the viscosity of butanol is two times 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 those of ethanol (Table 1).

Table 1. Specification of alcohol and conventional fossil fuel

	Gasoline	Diesel	Methanol	Ethanol	Butanol
Molecular formula	C4–C12	C12–C25	CH_3OH	C_2H_5OH	C_4H_9OH
Cetane number	0–10	40–55	3	8	25
Octane number	80–99	20–30	111	108	96
Oxygen content (% weight)	–	–	50	34.8	21.6
Density (g/mL) at 20 °C	0.72–0.78	0.82–0.86	0.796	0.790	0.808
Autoignition temperature (°C)	~300	~210	470	434	385
Flash point (°C) at closed cup	−45 to −38	65–88	12	8	35
Lower heating value (MJ/kg)	42.7	42.5	19.9	26.8	33.1
Boiling point (°C)	25–215	180–370	64.5	78.4	117.7
Stoichiometric ratio	14.7	14.3	6.49	9.02	11.21
Latent heating (kJ/kg) at 25 °C	380–500	270	1109	904	582
Flammability limits (%vol.)	0.6–8	1.5–7.6	6.0–36.5	4.3–19	1.4–11.2
Saturation pressure (kPa) at 38 °C	31.01	1.86	31.69	13.8	2.27
Viscosity (mm^2/s) at 40 °C	0.4–0.8 (20 °C)	1.9–4.1	0.59	1.08	2.63

Source: Freeman *et al*. (1988)

1.2 Biological of butanol production

Butanol is produced along with acetone and ethanol by (Acetone – Butanol – Ethanol, ABE fermentation) metabolic activities of the *Clostridium* species broadly observed in acid producing and solvent producing phases. The metabolic pathways of clostridia based on the degradation of hexose to pyruvate by the

Embden–Meyerhof–Parnas pathway detected hexokinase, glucose-6-phosphate isomerase, and pyruvate kinase activities in different groups of clostridia (i.e., saccharolytic, proteolytic, cellulolytic, or nitrogen-fixing), which were all capable of degrading carbohydrates. Pentose sugars can also be metabolised via the pentose phosphate pathway (Gheshlaghi *et al.*, 2009). Pyruvate is transformed into acetyl-CoA by pyruvateferredoxin oxidoreductase in the next part of the metabolism, which is the keystone in the production of primary metabolites. Bacteria grow exponentially in the first phase of fermentation (acid-producting phase) along the formation of acids (commonly acetic acid and butyric acid), leading to decrease of pH to 4.5 (Gheshlaghi *et al.*, 2009). In this phase, glycolysis pathway is active to produce pyruvate consuming glucose, which is changed to acetyl-CoA (Figure 1). Acetyl-CoA is the major precursor for production of acetic acid, butyric acid, ethanol, butanol and acetone anaerobically. Acetic acid and butyric acid are produced in acidogenesis phase through two related steps from acetyl-CoA and butyryl-CoA, respectively. Phosphate acetyltransferase and acetate kinase enzymes are complicated for acetate synthesis, while phosphate butyltransferase and butyrate kinase are active for butyrate production. Four enzymes, namely thiolase, b-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase catalyze the production of butyryl-CoA from acetyl-CoA (Jones and Woods, 1986; Jiang *et al.*, 2009).

At the final stage of acidogenesis phase, acid production slows down due to effect of low pH. To compensate the negative effect of low pH, organism changes its metabolic activity from acidogenesis phase to solventogenesis phase. In this phase, acetic acid and butyric acid are consumed as substrates for biosynthesis of acetone and butanol while no growth is observed (Dürre, 2007). On the other hand, metabolic activity of this complex phase shifting differs from organism to organism. For example, conversion of butyrate to butanol is hardly observed in the case of *C. saccharoperbutylacetonicum* N1-4 in lack of glucose in the media. Interestingly, highest yield of butanol (0.671 mol-butanol/mol-butyrate) from butyrate was detected in presence of glucose in the media (Tashiro *et al.*, 2007). The acetyl-CoA and butyryl-CoA are the key intermediates in producing ethanol and butanol (Figure 1).

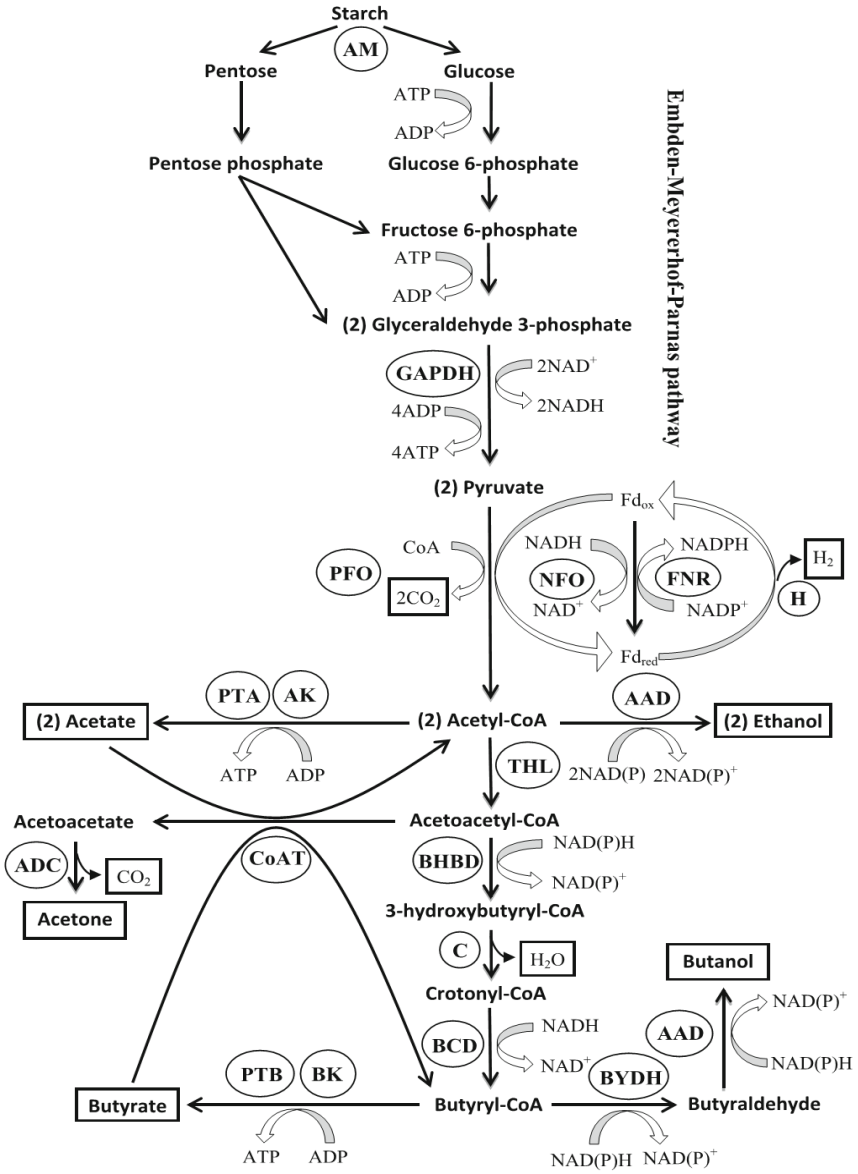

Figure 1. Metabolic pathways in *C. acetobutylicum* for the acidogenic and solventogenic phase.

Source: Jones and Woods, 1986

Enzymes are abbreviated as follows: AM = amylase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; PFO = pyruvate:ferredoxin oxidoreductase, NFONADH-ferredoxin oxidoreductase, FNR NADPH = ferredoxin oxidoreductase, H = hydrogenase; PTA = phosphotransacetylase, AK = acetate kinase, THL = thiolase (acetyl-CoA acetyltransferases), BHBD = 3-hydroxybutyryl-C dehydrogenase, crotonase = (3-hydroxbutyryl-CoA-dehydratase), BCD = butyryl-CoA dehydrogenase, CoAT = CoA-transferase, ADC = acetoacetate decarboxylase, BK = butyrate kinase, PTB = phophotransbutyrylase, AAD = alcohol/aldehyde dehydrogenase, BYDH = butyraldehyde dehydrogenase.

These pathways produce acetaldehyde and butyraldehyde respectively, as intermediate in the presence of two sets of dehydrogenases. The reduction of butyryl-CoA to butanol is mediated by butyraldehyde dehydrogenase and butanol dehydrogenase (Gheshlaghi *et al.*, 2009; Sillers *et al.*, 2008). Six enzymes (thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase, and butanol dehydrogenase) and consistent seven genes (*thiL*, *hbd*, *crt*, *bcd*, *etfA*, *etfB*, and *adhe*) were identified for the transformation of acetyl-CoA to butanol (Figure 1). This multi-gene expression was examined in many organisms (e.g. *Escherichai coli*, *Saccharomyces cerevisiae*, and *Pseudomonas putida*) aiming improvement of butanol productivity (discussed in subsequent section) (Huang *et al.*, 2010; Inui *et al*., 2008; Nielsen *et al.*, 2009; Sillers *et al.*, 2008). In both *C. acetobutylicum* and *C. beijerinckii*, the activity of butanol dehydrogenase was NADPH dependent rather than NADH dependent (Dürre, 1998). Ethanol could be produced individually under certain adverse media conditions. On the other hand, ethanol formation was observed in noticeable amount in comparison to acetone and butanol. The uptake of organic acids (butyric and acetic acids) was attached with formation of acetone for butanol synthesis resulting from complex mechanism of metabolic pathways (Figure 1). However, mechanism of complex regulatory aspects of butanol synthesis associated with acetone formation is still an open question (Jones and Woods, 1986; Papoutsakis, 2008; Jiang *et al.*, 2009). The optimal cultivation conditions for *Clostridium* species are as follows: temperature 35–37 ºC, pH in the range of 4.5–7.0, and atmosphere of pure CO_2 or N_2 or mixture of N_2 and CO_2 in the mole ratio of 1:9 (Zigová et al., 1999). The process of the production of acetone, butanol, and ethanol, also known as ABE fermentation, has been known for more than a hundred years. The traditional batch fermentation process, however, suffers from two problems which restrict its commercial development. These are its low productivity and the severe product inhibition which accompanies the process, so that the ABE concentration in the broth rarely exceeds 20 g/L (Qureshi and Maddox, 1995). Nowadays, the ABE fermentation process continues to receive attention as a source of fuel and chemical feedstocks based on renewable resources; one way to resolve the fermentation problems is the use of immobilized microorganisms.

Three major and final products of ABE fermentation: acetone, butanol, and ethanol have widespread commercial uses. Butanol can be used as an intermediate in chemical synthesis and as a solvent in a wide variety of chemical and textile industry applications. Moreover, butanol has been considered as a potential fuel or fuel additive (Lee *et al*., 2008a). Almost half the worldwide production is converted into acrylate and methacrylate esters (Dürre, 2008). Acetone is widely used as a solvent in industrial and household products, including paints and nail polish removers. Nowadays, acetone is produced by chemical synthesis, by the cumene (isopropylbenzene) route (Bedia *et al*., 2010). Ethanol has been the focus of technological development in renewable fuel production for its compatibility with gasoline and existing infrastructures (He *et al*., 2009). On the other hand, it is known that ethanol is only produced in small amounts following metabolic pathways in *Clostridium* (Zigová et al., 1999). The microorganism *C. acetobutylicum* naturally produces the solvents: acetone, butanol, and ethanol at a mole ratio of approximately 3:6:1, hence the final yields of ethanol are not of great interest. Excellent yields of ethanol were reported only by mutants, e.g. by *C. acetobutylicum* hbd:int with the blocked butyrate/butanol (C_4) metabolic pathway, resulting in an ethanol yield from glucose of 0.38 g/g (Lehmann and Lütke-Eversloh, 2011).

ABE fermentation is specific for several representatives of *Clostridium* species: *Clostridium acetobutylicum*, *C. beijerinckii*, *C. aurantibutyricum*, *C. carboxidivorans*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (Ezeji *et al*., 2007; Lee *et al*., 2008; Sormutai *et al*., 1996; Liou *et al*., 2005). It should also be noted that *C. beijerinckii* NCIMB 8052, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* were originally designated as *C. acetobutylicum* (Lee *et al*., 2008). *C. acetobutylicum*, as the main producer of products of ABE fermentation, is a strictly anaerobic, heterofermentative, Gram-positive bacterium, in which the metabolism involves two phases. The first is the acidogenic phase, during which the acids-forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products. This acidogenic phase usually occurs during the exponential growth phase (Lee *et al*., 2008a; Huang *et al*., 2004). The second phase is the solventogenic phase, during which the acids are re-assimilated and used in the production of acetone, butanol, and ethanol at pH < 5 (acids are produced

during acidogenesis at higher pH), (Lee *et al*., 2008a; Zigová *et al*., 1999). The transition from the acidogenic to the solventogenic phase is the result of a dramatic change in gene expression pattern.

2. Characteristics of decanter cake (DC) and its applications

Decanter cake (DC) is a pasty-type by-product derived from mechanical extraction of crude palm oil (Afdal *et al*., 2011). Table 2 shows characteristics of decanter cake (DC). It is slightly different from palm oil mill effluent or palm oil sludge as it is process by a specific processing machine. The decanter cake is produced after passing through a process of decanting, centrifuging and then drying within the machine system (Southworth, 1985). Haron and Mohammed (2008) reported that a mill with 90 t/hr fresh fruit bunch processing capacity will produce about 160–200 tonnes of decenter cake. DC from palm oil mills can be utilized in several different ways. For example, it can be mixed with inorganic fertilizers and converted into commercial grade pellet animal feed. To sell this by-product the decanter sludge has to be dried into a cake with moisture content below 10% through the use of low pressure steam from the boiler with a temperature of 210°C or horizontal dryer. The temperature of the dryer exhaust gases is about 100°C. The dry decanter product can be converted into commercial grade animal feed pellets (Chavalparit *et al*., 2006; Schmidt, 2007). However, the use of this technology has not yet been commonly practical (Schmidt, 2007).

Table 2. Characteristics of decanter cake (DC)

Parameter	Yahya *et al*. (2010)	Seephueak *et al*. (2011)	Afdal *et al*. (2012)	Razak *et al*. (2012)
pH	-	-	4.62 - 4.95	4.08 ± 0.02
Moisture (%)	76.38	-	-	76.46 ± 0.8
Cellulose (%)	-	-	-	21.61
Hemicellulose (%)	-	-	-	3.94
Lignin (%)	-	-	-	30.66
Ether extract (%)	-	58.88	2.10 - 3.51	
Crude protein (%)	-	6.66	11.58 - 11.87	-
Dry matter (%)	-	92.50	26.11- 27.19	-
Ash (%)	-	0.18	5.70 - 6.21	22.25
Carbon (%)	51.70	-	-	55.17
Nitrogen (%)	2.38	-	-	2.80
Carbon/Nitrogen	21.72	-	-	19.70
Sulfur (%)	0.39	0.16	-	0.30
Phosphorus (%)	-	0.19	-	0.20
Potassium (%)	2.39	0.71	-	1.40
Calcium (%)	1.02	0.74	-	0.90
Sodium (%)	-	0.02	-	-
Magnesium (%)	0.80	0.37	-	0.30
Boron (mg/kg)	-	-	-	9.00
Manganese (mg/kg)	-	111.19	0.41 - 0.45	38.00
Copper (mg/kg)	-	29.33	4.28 - 8.80	59.00
Iron (mg/kg)	-	2,700.12	12.60 - 13.59	4,438.00
Zinc (mg/kg)	-	33.08	-	30.00
Nickel (mg/kg)	-	-	0.94 - 0.97	-
Chromium (mg/kg)	-	-	0.81 - 0.29	-

3. Hydrolysis of lignocellulosic biomass and detoxification

Lignocellulosic biomass consist mainly of cellulose (35–50% of dry weight), hemicellulose (25–35%) and lignin (10–25%) (Cherubini, 2010). Cellulose is a polysaccharide consisting of a linear chain of several hundred to many thousands of β(1→4) linked D-glucose units, usually existing in a form of highly ordered crystalline structure, which is water insoluble and hinders hydrolysis. Hemicellulose is a branched polymer and contains typically sugars such as D-galactose, D-glucose, D-mannose, D-xylose and L-arabinose. Not only regular sugars can be found in hemicellulose, but also their acidified form, for instance glucuronic acid and galacturonic acid can be present. Lignin contains phenyl propane units forming a three dimensional complex structure with chemical bonds between other lignins, hemicelluloses and celluloses (Taherzadeh and Karimi, 2007). Lignin protects the cellulose in the cells wall and complicates enzymatic hydrolysis due to its resistance against chemical and enzymatic degradation (Jørgensen *et al*., 2007).

In general, non-cellulosic biomass such as starch require an acidic or enzymatic hydrolysis step before fermentation. Lignocellulosic biomass instead require additional pretreatment before hydrolysis and fermentation. This is because of the complex structure of lignocellulosics including cellulose crystallinity, cellulose protection by lignin, and low accessibility of enzymes to the lignocellulosic compounds. The sheathing by hemicelluloses and the degree of hemicellulose acetylation additionally hinder the biomass processing (Hsu, 1996).

3.1 Acid hydrolysis

Acid hydrolysis is mainly performed with diluted or concentrated solutions of sulfuric (H_2SO_4) or hydrochloric acid (HCl). Dilute acid pretreatment (acid concentration of 0.5–5 wt%) is operated at high pressures (around 1MPa), at lower temperatures (120–160 °C) in a batch process with high solids loading (10–40 wt% dry substrate/reaction mixture) or in a continuous process at high temperatures of 180–200 ◦C with low solids loading such as 5–10 wt% (Sun and Cheng, 2002). Dilute acid hydrolysis maybe performed in two or more stages to avoid the degradation of monosaccharides and the formation of inhibitors. Different type of reactors such as

percolation, plug flow, shrinking-bed, batch and countercurrent reactor have been applied for pretreatment of lignocellulosic materials (Taherzadeh and Karimi, 2007). Concentrated acids (30-40%) are not preferred because they are corrosive and must be recovered to make the pretreatment economically feasible (Sun and Cheng, 2002). Acid is added either by mixing with the biomass in a reactor, by acid percolation through the biomass bed, or by spraying acid onto the biomass. Heating of acid and biomass mixture may be done indirectly through the reactor walls or directly as a steam injection (Mosier *et al*., 2005). Reactors must be resistant to corrosion and for economic feasibility acids have to be recovered after the hydrolysis (von Sivers and Zacchi, 1995). Acid pretreatment may require the use of an alkali to neutralize the hydrolysate. In addition, such acids must be recovered after hydrolysis to make the process economically feasible.

Reaction time is another important factor in the hydrolysis processes. If the hydrolysis time is longer than 1 h, xylose concentration would decrease due to the degradation of hemicelluloses into byproducts (Cruz *et al*., 2000). Palmqvist and Hahn-Hagerdal (2000) reported the formation of main products and by-products during the hydrolysis process as follows: cellulose can be degraded into glucose, and hemicelluloses can be degraded into xylose, mannose, acetic acid, galactose, and glucose. At high temperatures and pressures, glucose and xylose will be degraded into furfural and 5-hydroxymethylfurfural (5-HMF), respectively. When furfural and 5-HMF are further degraded, formic acid and levulinic acid are formed. In addition, phenolic compounds are generated from the partial breakdown of lignin. Besides these compounds, other substances are formed during hydrolysis, namely syringic, vanillic, caproic, caprylic, pelargonic, and palmitic acids, which are toxic to fermenting microorganisms.

3.2 Alkaline hydrolysis

Some bases can be used for the pretreatment of lignocellulosic biomass, and the effect of alkaline pretreatment depends on the lignin content of the biomass (McMillan, 1994). Alkali pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Moiser *et al*., 2005). Alkali pretreatment can be carried out at ambient conditions, but pretreatment times are on the order of hours or days rather than minutes or seconds. Compared with acid processes, alkaline processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated. Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatment agents. Of these four, NaOH been studied the most (Elshafei *et al*., 1991; Soto *et al*., 1994; Fox *et al*., 1989; MacDonald *et al*., 1983). However, calcium hydroxide ($Ca(OH)_2$) is a low cost alkaline physicochemical pretreatment that has been reported to enhance the digestibility of lignocellulosic biomass. It is possible to recover calcium from an aqueous reaction system as insoluble calcium carbonate by neutralizing it with inexpensive carbon dioxide; the $Ca(OH)_2$ can subsequently be regenerated using established lime kiln technology. The process of lime pretreatment involves slurrying the lime with water, spraying it onto the biomass material, and storing the material in a pile for a period of hours to weeks. The particle size of the biomass is typically 10 mm or less. Elevated temperatures reduce contact time.

Lime pretreatment removes amorphous substances (e.g., lignin and hemicellulose), which increases the crystallinity index. Chang *et al*. (2000) reported correlations between enzymatic digestibility and three structural factors: lignin content, crystallinity, and acetyl content. They concluded that (1) delignification and deacetylation remove parallel barriers to enzymatic hydrolysis, (2) crystallinity significantly affects initial hydrolysis rates but has less of an effect on ultimate sugar yields; and (3) extensive delignification is sufficient to obtain high digestibility regardless of acetyl content and crystallinity. In addition, alkali pretreatments remove acetyl and the various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose surface (Kim and Holtzapple, 2006). Therefore, alkaline pretreatment can play a significant role in exposing the cellulose to enzyme hydrolysis.

Table 3. Plant cell wall derived inhibitors profile from different lignocellulosic biomass

Lignocellulose hydrolyzate	Inhibitors profile (g/L)	Ref.
Sugarcane bagasse	Furan derivatives, 1.89; Phenolic compounds, 2.75; Acetate, 5.45	Chandel *et al*. (2007)
Saccharum spontaneum	Furfural, 1.54 ± 0.04; Phenolic compounds, 2.01 ± 0.08	Chandel *et al*.(2011)
Soft wood	Acetate, 5.3; Furfural, 2.2	Qian *et al*. (2006)
Wheat straw	Furfural, 0.15±0.02; Aceate, 2.70±0.33	Nigam (2001)
Rice straw	Acetate, 1.43; 5-HMF, 0.15; Furfural, 0.25	Baek and Kwon (2007)
Spruce wood	Phenolics, 0.44 ± 0.05; Furfural, 1.0 ± 0.1; 5-HMF,3.3 ± 0.2; Acetate, 5.0 ± 0.4; Levulinic acid, 0.2 ± 0.1;	Alriksson *et al*. (2010)
Corn stover	Acetate, 1.48; Furan derivatives, 0.56; Phenolic compounds, 0.08	Cao *et al*. (2009)

Lime has been used to pretreat wheat straw (85 °C for 3 h) (Chang and Nagwani, 1998), poplar wood (150 °C for 6 h with 14 atm of oxygen) ((Chang and Nagwani, 2001), switchgrass (100 °C for 2 h) (Chang *et al*., 1997), and corn stover (100 °C for 13 h) (Karr and Holtzapple, 1998). Karr and Holtzapple (2000) showed that pretreatment with $Ca(OH)_2$ increased the enzymatic hydrolysis of corn stover by a factor of 9 compared to that of untreated corn stover. The optimal pretreatment conditions were determined to be a lime loading 0.075 g of $Ca(OH)_2$/g of dry biomass, a water loading of 5 g of H_2O/g of dry biomass, and heating for 4 h at 120 °C. It was suggested that that pretreatment with lime can lead to corn stover polysaccharide conversions approaching 100%.

Lignin removal increases enzyme effectiveness by eliminating nonproductive adsorption sites and by increasing access to cellulose and hemicellulose. Kim and Holtzapple (2006) pretreated corn stover with excess

$Ca(OH)_2$ (0.5 g of $Ca(OH)_2$/g of raw biomass) in non-oxidative (in the presence of nitrogen) and oxidative (in the presence of air) conditions at 25, 35, 45, and 55 °C. The oxidative treatment and additional consumption of lime (up to 0.17 g of $Ca(OH)_2$/g of biomass) can extensively delignify. Both non-oxidative and oxidative conditions at 55 °C removed approximately 90% of the acetyl groups in 1 week at all temperatures studied. Delignification process depended on the reaction temperature and the presence of oxygen. During this process, lignin and hemicellulose would be selectively removed, but cellulose was not affected by this pretreatment at mild temperatures of 25-55 °C. On the other hand, the degree of crystallinity of cellulose slightly increased from 43% to 60% because amorphous components such as lignin and hemicellulose were removed. Kong *et al*. (1992) reported that alkalis can remove acetyl groups from hemicelluloses (mainly xylan), thereby reducing the steric hindrance and enhancing carbohydrate digestibility by the hydrolytic enzymes. They also concluded that the sugar yield in enzymatic hydrolysis is directly related to acetyl group content.

Dilute NaOH treatment of lignocellulosic biomass has been found to cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization and in crystallinity, separating of structural linkages between lignin and carbohydrates, and disrupting the lignin structure. Bjerre *et al*. (1996) reported that the digestibility of NaOH-treated hardwood was reported to increase from 14% to 55% with a decrease of lignin content from 24-55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26%. Dilute NaOH pretreatment was also found to be effective for the hydrolysis of straws with relatively low lignin contents of 10-18%. Chosdu *et al*. (1993) used a combination of irradiation and 2% NaOH for pretreatment of corn stalk, cassava bark, and peanut husk. The glucose yield of corn stalk was only 20% in untreated samples and this increased to 43% after treatment with electron beam irradiation at a dose of 500 kGy and 2% NaOH, but the glucose yields of cassava bark and peanut husk were only 3.5% and 2.5%, respectively.

3.3 Detoxification of inhibitory compounds

During the pretreatment and hydrolysis of lignocellulosic biomass, a great amount of compound that can seriously inhibit the subsequent fermentation are formed in addition to fermentable sugars. The sensitivity to the inhibitors varies with the microorganisms and within different strains of microorganism. The inhibitors substances are generated as a result of the hydrolysis of the lignocellulosic biomass include furan derivatives (furfural, 5-HMF), aliphatic acids (acetic, formic and levulinic acids), and phenolic compounds (Larsson *et al*., 1999). The inhibitors affecting the enzymatic hydrolysis include formic acid inhibiting cellulases and xylanases, and vanillic acid, syringic acid and syringaldehyde inhibiting xylanases activity (Panagiotou and Olsson, 2007). In addition, glucose and cellobiose inhibit the activity of β-glucosidases and cellulases. Before enzymatic hydrolysis increases the sugar yield were increased after washing of the pretreated biomass with water (Tengborg *et al*., 2001).

Phenolic compounds, i.e. syringaldehyde, ferulic, cinnamaldehyde and ρ-coumaric acids are considered the major lignocellulosic originated inhibitors in butanol fermentation and need to be removed in the upstream processing (Ezeji and Blaschek, 2008). Phenolic compounds can increase the membrane flexibility of microorganisms and thus affect negatively the cells integrity and operation (Heipieper *et al*., 1991). The net effects of different inhibitors may have a great impact on the fermentation, but these effects are more difficult to determine.

The concentration of inhibitors in the hydrolyzates may be decreased by the optimization of the upstream processing. The significant additional costs would be required to prevent their formation. . There is a need to identify and understand the mode of action of inhibitory compounds present in different hydrolyzates. Because this relates to the development of fermentation and detoxification methods for inhibitors removal. Most of the detoxification methods are effective only for a certain inhibitor or a class of inhibitors. Since detoxification increases the costs of the production process, it is then crucial to carefully evaluate the need of removing the inhibitors. In addition, it is important to consider the costs, effectiveness and the integration of the detoxification step into the whole process.

There are several detoxification methods such as physical (evaporation, membrane mediated detoxification), chemical (neutralization, overliming, activated charcoal treatment, ion exchange resins, and extraction with ethyl acetate) and biological detoxification (laccase, lignin peroxidase), in-situ detoxification, in-situ microbial detoxification etc. Table 4 summarizes the hydrolyzate detoxification using various non-biological methods employed to the variety of lignocellulosic hydrolyzates. Each method represents its specificity to eliminate particular inhibitor from the hydrolyzate. The effect of evaporation on the removal of fermentation inhibitors has been summarized in Table 4.

3.3.1 Physical methods

1) Evaporation

The evaporation under vacuum can remove volatile compounds such as acetic acid, furfural and vanillin from lignocellulosic hydrolyzate. However, this method retains the concentration of non-volatile toxic compounds (extractives and phenolic compounds) in the hydrolyzates. Palmqvist *et al*. (1996) observed the removal of most volatile fraction (10% v/v) from willow hemicellulosic hydrolyzate by rotary evaporation. Wilson *et al*. (1989) found a reduction in the concentration of acetic acid, furfural and vanillin by 54%, 100% and 29%, respectively, compared with the concentrations in the hydrolyzate. Larsson *et al*. (1999) studied the removal of furfural (90%) and 5-HMF (4%) using vacuum evaporation from wood hemicellulose hydrolyzate. The enhanced production of xylitol was reported from hemicellulosic hydrolyzate after removal of acetate, furfural and other volatile compounds (Converti *et al*., 2000).

The sugarcane bagasse hydrolyzate that was vacuum evaporated followed by activated charcoal treatment revealed 89% removal of furfural (Rodrigues *et al*., 2001) with partial elimination of acetic acid. Zhu *et al*. (2011) applied the complex extraction to detoxify the pre-hydrolyzate corn stover using mixed extractant (30% trialkylamine-50% n-octanol−20% kerosene). The detoxification resulted into removal of 73.3% acetic acid, 45.7% 5-HMF and 100% furfural.

Table 4. Different detoxification strategies (Non-biological) applied to lignocellulose hydrolyzates for the removal of fermentation inhibitors

Lignocellulosic Hydrolysae	Detoxification methods	Changes in hydrolyzate composition	Ref.
Sugarcane bagasse	Neutralization	NA	Chandel *et al*. (2007)
Saccharum spontaneum	Overliming ($Ca(OH)_2$)	Removal of furfurals (41.75%), total phenolic compounds (33.21%), no effect on acetic acid content. Reduction of reducing sugars (7.61%)	Chandel *et al*. (2011)
Oak wood	Activated charcoal	Removal of phenolic compounds (95.40%)	Converti *et al*. (1999)
Wheat straw	Ion exchange-D 311 + over-liming	Removal of furfurals (90.36%), phenolic compounds (77.44%) and acetic acid (96.29%)	Zhuang *et al*. (2009)
Wheat straw	Ethyl acetate + Overliming ($Ca(OH)_2$)	Removal of furfurals (59.76%), phenolics (48.23%) and acetic acid (92.19%)	Zhuang *et al*. (2009)
Aspen	Rotary evaporation	Removal of acetic acid (54%), furfural (100%) and vanillin (29%)	Wilson *et al*. (1989)
Spruce wood	Dithionite and sulfite	No major change in composition of hydrolyzates	Alriksson *et al*. (2010)
Corn stover	Membrane based organic phases alamine 336	Removal acetic acid (60%)	Grzenia *et al*. (2008)

Source: Anuji *et al*. (2011)

2) Membrane separations

Membrane separations that use adsorptive microporous membranes with surface functional groups may eliminate the cell wall derived inhibitors from the lignocellulose hydrolyzates. The feed is being pumped through the membrane pores that bind to the solute predominantly by convection. This process can reduce the processing time. The drop in the pressure for flow through adsorptive membranes changes significantly compared to the typical packed beds. Wickramasinghe and Grzenia (2008) found that the performance of membrane assisted system for acetic acid removal from the biomass hydrolyzates was better than ion-exchange resins. Various diluted organic phases (alamine 336, aliquat 336) have been used for the removal of acetic acid (60%) from corn stover hydrolyzates (Grzenia *et al*., 2008). Later, Grzenia *et al*. (2010) used the membrane extraction for removal of inhibitors from sulfuric acid derived hemicellulosic hydrolyzate of corn stover. Extraction of sulphuric, acetic, formic and levulinic acid as well as 5-HMF and furfural was removed when alamine 336, octanol and oelyl alcohol used in the organic phase. Thus, the adsorptive membranes may offer significant improvements over traditional ion-exchange resins.

3.3.2 Chemical methods

1) Neutralization

The neutralisation of acidic nature of hemicellulosic hydrolyzates is necessary step before using hydrolyzates for fermentation. $Ca(OH)_2$ or sodium hydroxide (NaOH) are most used for neutralization of hydrolyzates (pH 6.0-7.0). Through this process, furfurals and phenolics can be removed by precipitation to the some extent. Table 4 summarizes the neutralization effect on the removal of fermentation inhibitors from lignocellulose hydrolyzates.

2) $Ca(OH)_2$ over-liming

$Ca(OH)_2$ over-liming with a combination of high pH and temperature has been used for detoxification of dilute sulfuric acid-pretreated hydrolyzates (Chandel *et al*., 2007; Martinez *et al*., 2000). This process could help to remove volatile inhibitory compounds such as furfural and 5-HMF from the hydrolyzate but causing a sugar loss (~10%) by adsorption (Chandel *et al*., 2011; Martinez *et al*., 2000; Ranatunga *et al*., 2000). The dried $Ca(OH)_2$ that is added in acidic hydrolyzates

is then converted into gypsum which can be used as plaster of paris having many commercial values. The effect of overliming on the removal of fermentation inhibitors from the variety of lignocellulose hydrolyzates have been summarized in Table 4.

3) Activated charcoal treatment

The detoxification usingactivated charcoal is a cost effective with high capacity to absorb compounds without affecting levels of sugar in hydrolyzate (Canilha *et al*., 2008; Chandel *et al*., 2007; Mussatto and Roberto, 2004). The effectiveness of activated charcoal treatment depends on pH, contact time, temperature and the ratio of activated charcoal taken versus the liquid hydrolyzate volume (Prakasham *et al*., 2009).

4) Ion exchange resins

Ion exchange resins treatment has been known to remove lignin-derived inhibitors, acetic acid and furfurals from the hydrolyzate. However, the ion-exchange resins based separation of fermentative inhibitors may not be cost effective (Lee *et al*., 1999). Because it would provide most effective means of inhibitor separation only when the hydrolyzate being adjusted to a pH of 10 which requires significant quantities of base chemicals (Ranjan *et al*., 2009). It has been reported that the anion treatment also helps to remove most inhibitors (i.e. levulinic, acetic, formic acids, and furfural and 5-HMF). Villarreal *et al*. (2006) investigated the effect of four different ion exchange resins (cation and anion) for the detoxification of *Eucalyptus* hemicellulosic hydrolyzates for the improved xylitol production by *Candida guilliermondii*. The ion exchange detoxification drastically enhanced the fermentability of the hydrolyzate. Total 32.7 g/L of xylitol was achieved after 48 h fermentation, which correspond to 0.68 g/L/h volumetric productivity and 0.57 g/g xylitol yield factor (Villarreal *et al*. 2006). Chandel *et al*. (2007) observed that ion exchange resins diminished furans (63.4%) and total phenolics (75.8%) from sugarcane bagasse acid hydrolyzates. Although the ion exchanges resins is effective, however is not cost effective that reflects its limited feasibility in commercial industrial purpose in lignocellulosics derived products synthesis. Table 4 summarizes the effect of different ion exchange resins treatment on detoxification of lignocellulose hydrolyzate.

5) Extraction with ethyl acetate

Ethyl acetate has been found that it could extract the fermentation inhibitors and this led to an increased ethanol yield in fermentation by *Pichia stipitis* from 0 to 93% of that obtained in the reference fermentation (Wilson *et al*., 1989). The extraction procedure could eliminate acetic acid (56%), and total furfural, vanillin and 4-hemicelluloses hydrolyzate with the extraction using ethyl acetate or diethyl ether (Cruz *et al*. 1999). Ethyl acetate extraction has been shown to increase the rate of glucose consumption (Clark and Mackie, 1984). The low molecular weight phenolic compounds were suggested to be the most inhibiting compounds in the ethyl acetate extract (Zhuang *et al*., 2009). Pasha *et al*. (2007) detoxified the *Prosopis juliflora* hydrolyzate with $Ca(OH)_2$ overliming in conjunction with ethyl acetate. In these studies, the ethanol yield of 0.459 ± 0.012 g /g, productivity of 0.67 ± 0.015 g/L/h and fermentation efficiency of 90% after fermentation of this detoxified sugar syrup with fusant *S. cerevisiae* VS3 (Table 4).

3.3.3 Biotechnological routes for detoxification

Biotechnological methods encompass the application of living microorganisms and/or the microbial enzymatic applicability for the detoxification of lignocellulose hydrolyzates due to concerns of feasibility and affordability of physico-chemical treatments. The biological methods of detoxification are more feasible, environmental friendly, with fewer side-reactions and less energy requirements (Parawira and Tekere, 2011.). The microorganisms and/or the enzymes have potential to alter the chemical nature of inhibitors in hydrolyzates. However, the slow reaction time of microbial/ enzymatic detoxification and the loss of fermentable sugars make them unattractive (Yang and Wyman, 2008). Therefore, there is a need to explore the biotechnological routes of detoxification towards process economization.

1) Microbial pretreatment of lignocellulosics

It has been reported that the lignocellulosic substrate after pretreatment with microorganisms could easily be hydrolyse into fermentable sugars requiring less acid load, lower temperature, and less hydrolysis time (Kuhar *et al*., 2008). The microorganisms can degrade only lignin and leave cellulose and hemicelluloses behind with loose bonding of both in the raw material. The maximum de-polymerization of carbohydrate polymers into simple sugars with less fermentation

inhibitors can be achieved by the microbial mediated pretreatment. The pretreated lignocellulose substrate when hydrolyzed leads to maximum sugar recovery with minimum inhibitors in short period of time by eliminating the requirement of detoxification step (Liang *et al*., 2010; Kuhar *et al*., 2008; Keller *et al*., 2003).

2) Microbial acclimatization

The adaptation of microorganisms to lignocellulosic hydrolyzates can improve the product yields (Silva and Roberto, 2001; Sene *et al*., 2001). Acclimatization of fermentative microorganisms to the hydrolyzates containing inhibitors prior to fermentation did improve the productivities (Parawira and Tekere, 2011).The microorganisms can adapt to a variety of fermentation media when multiple essential nutrients or compounds along with suitable carbon and nitrogen sources are supplementedMicroorganisms, *P. stipitis*, *Candida shehatae* and *Pachysolen tannophillus* are being explored to overcome inhibition and improve fermentation ability of xylose-containing hydrolyzates by adaptation (Tomas-Pejo *et al*., 2010; Zhu *et al*., 2009; Martin *et al*., 2007). Parekh *et al*. (1987) employed *P. stipitis* on a steam-stripped hardwood hydrolyzate that improved ethanol production (46%), with >90% xylose utilization compared with steam-unstripped hardwood hydrolyzate. Microorganism *P. stipitis* NRRL Y-7124 adapted on wheat straw hemicellulosic hydrolyzates, showed improved ethanol production (yield, 0.41 ± 0.01 g/g) equivalent to 80.4 ± 0.55% theoretical conversion efficiency (Nigam, 2001). The adaptation of *Candida guilliermondii* to rice straw hemicellulose hydrolyzate for xylitol production was found to be an effective and inexpensive method to alleviate the inhibitory effect of toxic compounds on the xylose- to-xylitol bioconversion (Silva and Roberto, 2001).

3) In-situ microbial detoxification

The in-situ detoxification of impurities can be performed at a higher rate that may economize the overall production in the same vessel. Several attempts were made to detoxify the lignocellulose hydrolyzates directly using wild (yeasts, fungi, bacteria) and/or recombinant microorganisms expressing the laccase or peroxidases. Under in-situ microbial detoxification, the microorganisms are being grown in lignocellulose hydrolyzate to detoxify the inhibitory substances by transforming their chemical nature (López *et al*., 2004). Palmqvist *et al*. (1997) studied the use of

Trichoderma reesei to degrade the inhibitors from willow hemicellulosic hydrolyzate and found that this process directly assisted improvements in ethanol productivity by three fold and yield four fold. Larsson *et al*. (1999) also detoxified the dilute-acid hydrolyzate of spruce by *T. reesei* and found that this was most efficient compared to anion exchange, over-liming, and treatment with laccase enzyme, however, with higher consumption of fermentable sugars (35%). A fungal isolate, *Coniochaeta ligniaria*, (NRRL30616), was also reported to metabolize furfural, 5-HMF, aromatic and aliphatic acids, and aldehydes present in corn stover hydrolyzate (Nichols *et al*., 2008). The microbial mediated detoxifications are been considered effective than the soft rot fungi resulted into less sugar consumption with shorter incubation time. In addition, , five bacteria related to *Methylobacterium extorquens*, *Pseudomonas* sp., *Flavobacterium indologenes*, *Acinetobacter* sp., *Arthrobacter aurescens*, and fungus *C. ligniaria* C8 (NRRL30616) have been isolated (López *et al*., 2004). They were capable of depleting toxic compounds from defined mineral medium containing a mixture of ferulic acid, 5-HMF, and furfural as carbon and energy sources. Organism *C. ligniaria* C8 (NRRL30616) was effective in removing furfural and 5-HMF from corn stover hydrolyzate.

Okuda *et al*. (2008) investigated the biological detoxification of a waste house wood (WHW) hydrolyzate by thermophilic bacterium *Ureibacillus thermosphaercus*. Chromatographic analysis confirmed that *U. thermosphaercus* degraded the furfural or 5-HMF present in the synthetic hydrolyzates, and the phenolic compounds present in the WHW hydrolyzates. The bacterium grows rapidly and consumes less than 5% fermentable sugars. In another example of in-situ detoxification, Tian *et al*. (2009) isolated yeast strains namely Y1, Y4 and Y7 and evaluated their efficiency for ethanol production after in-situ detoxification of hydrolyzates. Strains Y1 and Y4 yielded 0.49 g and 0.45 g ethanol/g glucose, equivalent to maximum theoretical values of 96% and 88.2%, respectively. Further, attempts are underway to detoxify the lignocellulose hydrolyzates through direct application of microorganisms in the hydrolyzate. Table 5 summarizes the microorganism used for in–situ detoxification of lignocellulose hydrolyzates.

Table 5. Different detoxification strategies (biological) applied to lignocellulose hydrolyzates for the removal of fermentation inhibitors

Lignocellulose Hydrolysae	Enzyme/ Microorganisms	Changes in hydrolyzate composition	References
Sugarcane bagasse	Laccase	80% removal of phenolics	Martin *et al*. (2002)
Lignocellulose hydrolyzate	Peroxidase from *C. cinereus* IFO 8371	100% removal of ρ coumaric acid, ferulic acid, vanillic acid and vanillin	Cho *et al*. (2009)
Spruce	Lignin residue	53% removal of phenolics and 68% removal of furans	Bjorklund *et al*. (2002)
Willow	*T. reesei*	Considerable removal of phenolics, furans and weak acids	Palmqvist *et al*. (1997)
Corn stover	*Coniochaeta ligniaria*	80% Removal of furfural and 5-HMF	López *et al*. (2004)
Sugarcane bagasse	*Issatchenkia Occidentalis* CCTCC M 206097	Reduction of syringaldehyde (66.67%), ferulic acid (73.33%), furfural (62%), and 5-HMF (85%)	Fonesca *et al*. (2011)
Spruce	Continuous fermentation	Elimination of detoxification step; improved ethanol yield, 0.42-0.46 g/g	Purwadi *et al*. (2007)
Willow	High cell density fermentation (10 g/l dry weight)	High ethanol productivity even in un-detoxified hydrolyzate	Palmqvist *et al*. (1996)

Source: Anuji *et al*. (2011)

4) Alterations in fermentation conditions

The presence of lignocellulose inhibitors in fermenting medium affects the ethanol and biomass productivities as microorganism take more incubation times to convert into products (Chandel *et al*., 2007; Nilvebrant *et al*., 2001; Zaldivar *et al*., 2001). Usually the ethanol productivity is determined by cell-specific productivity and cell mass concentration, cumbersome by lignocellulose-derived inhibitors. To overcome by inhibitors, high cell-mass inocula are effective to tolerate the stress of inhibitory substances (Purwadi *et al*., 2007). The ethanol productivity has been increased by maintaining the initial cell-mass at higher density (Brandberg *et al*., 2007). By altering the initial cell density, the increased production of ethanol (0.44 g/g) was reported at initial cell density (10 g/l dry weight) (Palmqvist *et al*., 1996). The ethanol productivity in fed-batch fermentation was limited by the feed rate that in turn, was limited by the cell-mass concentration (Taherzadeh *et al*. 1999).

In continuous fermentation, the ethanol productivity also depends upon the rate of dilution. Since the microbial growth rate is known to decrease by the inhibitors, the productivity in continuous fermentation of lignocellulosic hydrolyzates remains low (Lee *et al*., 1996; Palmqvist *et al*., 1998). Purwadi *et al*. (2007) has achieved the ethanol yield of 0.42-0.46 g/g sugar utilized from the crude hydrolyzates of spruce wood as carbon source under continuous fermentations using the flocculating *S. cerevisiae* CCUG 53310. Cellular recirculation strategy was employed in the fermentation of an enzymatic hydrolyzate of spruce (Palmqvist *et al*., 1998), and in fermentation of bagasse hydrolyzate (Ghose and Tyagi, 1979).

4. Lignocellulosic substrate for ABE production

4.1 Wheat straw

Wheat straw has been used as a fermentation substrate for the production of acetone, butanol and ethanol has been described by Qureshi *et al*. (2007). In their report, wheat straw was ground to fine particles, re-suspended in a solution of 1% (v/v) sulfuric acid and autoclaved. The commercial cellulases, glucosidases and xylanases were added to hydrolyze the sugar polymers to soluble sugars. The cultivation of *C. beijerinckii* NCP260 in obtained wheat straw hydrolyzate (WSH) as substrate and supplemented with nutrients, yielded 0.4 g

ABE/g sugar utilized, and end concentrations of acetone and butanol of 11.9 g/L and 12 g/L, respectively. The results were similar to the results on synthetic medium with glucose as carbon source. Additionally, the WSH was fermentable without detoxification steps, in contrast to other lignocellulosic hydrolyzates, where inhibiting concentrations of toxic compounds (furfurals, phenolic compounds or organic acids) were present and needed to be detoxified process to the fermentation (Claassen *et al*., 2000, Ezeji and Blaschek, 2008, Maddox and Anne, 1983, Walton and Martin, 1979)

4.2 Barley straw

Qureshi *et al*. (2010a) studied on the use of barley straw hydrolyzate have also been successfully used as the substrate for butanol production by *C. beijerinckii* BA 101. The culture did not accumulate more than 7.1 g/L total ABE, which was lower than the use of wheat straw hydrolyzate suggested that this substrate is toxic to the culture. To reduce this, different biotechnological strategies were applied to lignocellulose hydrolyzates for the removal of fermentation inhibitors potential toxicity effect. The barley straw hydrolyzate was subjected to a number of treatments including dilution with water, mixing with wheat straw hydrolyzate (1 : 1 ratio) and treating with lime (overliming). Treatment of the hydrolyzate with lime produced superior results (production of 26.6 g/L ABE with a productivity of 0.39 g/L/h) and demonstrated that this detoxification method is effective in removing fermentation inhibitors from the culture medium.

4.3 Corn stover

Corn stover is another potential substrate and is available in abundance in the mid-western region of USA. Approximately 2.20×10^{11} dry kg of stover could be available for the production of biofuels. The resultant maize stover hydrolyzate was subjected to ABE fermentation using *C. beijerinckii* P260. The hydrolyzate did not support any cells growth or fermentation, suggesting that it contained fermentative inhibitors. In order to circumvent this inhibitor problem, the maize stover hydrolyzate was treated with lime as in case of barley straw hydrolyzate and fermented using *C. beijerinckii* P260. Additionally, the dilution approaches were also applied. All three techniques were successful in reducing toxicity or removing inhibitors. The overlimed hydrolyzate resulted in the production of 26.3 g/L ABE with a productivity of 0.31 g/L.h (Qureshi *et al*., 2010b). These and other studies suggest that the development of

cultures capable of tolerating inhibitors would be of great benefit. In another report on the production of ABE from maize stover hydrolyzate (65% total sugars), 25.6–25.8 g/L ABE was produced from this substrate (Parekh *et al*., 1988). No inhibition from inhibitors was reported.

4.4 Corn fiber

Corn fiber, a by-product of the maize wet milling process, has been used to produce butanol after hydrolysis using dilute sulfuric acid pretreatment and enzymatic saccharification (Qureshi *et al*., 2008). Since the hydrolyzed maize fiber contained inhibitors that arrested cells growth and fermentation, it could not be fermented directly. The hydrolyzate was treated with lime ($Ca(OH)_2$; overliming) followed by passing through a column packed with adsorbent resin XAD-4 (trade name). The inhibitors were removed and the resultant hydrolyzate was fermented successfully. In the fermentation, 9.3 g/L ABE was produced using the treated hydrolyzate. Since, maize fiber contains 60–70% carbohydrates, 4.7106 dry tonnes of maize fiber would result in the production of 1.22106 tonnes of ABE (0.733106 tonnes of butanol). In another report, maize fiber xylan was used to produce ABE (Qureshi *et al*., 2006) in an integrated process from which toxic butanol was removed simultaneously.

4.5 Distiller dry grain and soluble (DDGS)

DDGS, a by-product from a corn-based ethanol facility, were pretreated using three different methods (diluted acid, hot water and ammonium fiber expansion) and enzymatically hydrolyzed. The acid-pretreated material after detoxification by overliming were fermented to acetone, butanol, and ethanol by five different strains. Best results were obtained with the hot-water pretreated DDGS, with end concentrations of total solvents between 10 and 13 g/L for all strains tested. High yields of solvents, above 0.30 g ABE/g sugar consumed, were obtained for all sugars present in the hydrolyzates, with total sugar consumption, showing the versatility of solvent-producing strains (Ezeji and Blaschek, 2008).

4.6 Switch grass

The switch grass hydrolyzate was fermented into ABE without any additional treatment and it resulted in low production of 1.5 g/L ABE. This was speculated that fermentation inhibitors were present in the hydrolyzate. In order to

produce ABE, hydrolyzate was diluted with water and the culture was able to produce 14.6 g/L ABE (Qureshi *et al*., 2010a). In this case and the treatment with lime remained unsuccessful and no ABE was produced from switch grass hydrolyzate treated in this manner.

5. ABE production by solventogenesis phase

In conventional mode of ABE production from sugar such as glucose is biphasic fermentation, the first phase is the acidogenesis phase and the second phase is solventogenesis phase. However, two molecule of carbon are lost as carbon dioxide in the course of pyruvateferredoxinoxidoreductase reaction from pyruvate produced in the sugar metabolism which results in the decreased yield of butanol to carbon less than the maximum theoretical yield of 0.667 C-mol/C-mol (Shinto *et* al., 2007). In numerous studies, the positive effect of acetate or butyrate as fermentation medium components was confirmed for various *Clostridium* strains (Soni and Jain, 1997; Chen and Blaschek, 1999a; Lee *et al*., 2008b; Yusof *et al*., 2010). *C. saccharoperbutylacetonicum* N1-4 has a superior capability to reutilise formed acids or to consume supplied acids. The highest reported butanol productivity, in solventogenic clostridia (7.99 g/L/h), was obtained in a continuous high-cells density system using *C. saccharoperbutylacetonicum* N1-4; the cells-recycle system was operated at a dilution rate of 0.85 h-1 by feeding a nitrogen-free substrate containing glucose and butyric acid (20 and 10 g/L, respectively). The culture was regenerated every 4 h by a 15 min supply of fresh growth supporting medium containing nitrogen and 0.01 M methyl viologen as an electron carrier that inhibits hydrogen formation (Baba *et al*., 2011).

The results of Baba *et al*. (2011) were confirmed by another research group that studied the influence of varying glucose/butyric acid ratios on butanol yield with the same *Clostridium* strain in a nutrient-limited medium. The glucose/butyric acid ratio (20/10 g/L) resulted in a yield of 0.99 mol of butanol per mol of consumed mixed substrate and used cells in stationary phase. Moreover, two metabolic pathways of butyrate consumption, starting either by reaction with acetoacetyl-CoA or by butyrate phosphorylation (see Figure 1), were hypothesised (Al-Shorgani *et al*., 2012). The main weakness in this approach, where butyric acid is fed as a co-substrate

together with carbohydrate, is the price of butyric acid. To overcome this, a two-step process was developed using subsequently *C. tyrobutyricum* for butyric acid production and *C. acetobutylicum* for 1-butanol production (Huang *et al*., 2004).

If cultured *C. saccharoperbutylacetonicum* N1-4 was continuously fed with a glucose (20 g/L) /lactic acid (5 g/L) mixture during pH controlled fed-batch fermentation, the concentration of 1-butanol and butanol productivity reached values of 15.5 g/L and 1.76 g/L/h, respectively. The efficient conversion of lactic acid into 1-butanol was confirmed by lactic acid radioactive labeling and the determination of 1-butanol by mass-spectrometry (Oshiro *et al*., 2010). In contrast to the positive effects of acetic, butyric and lactic acids on 1-butanol production, the profoundly adverse effect of formic acid, which accumulates to 0.5–1 mM, on *C. acetobutylicum* DSM 1731 cells during pH-uncontrolled glucose fermentation was demonstrated (Wang *et al*., 2011). Formic acid was suspected to be one of the most deleterious agents, and to have caused the so-called acid crash i.e., the state when clostridial fermentation ended prematurely before switching to the solventogenic phase. Wang *et al*., (2011) acknowledged that the potential harmful effects of formic acid on ABE fermentation were investigated and theorized as early as in 1931 (Wynne, 1931).

Table 6 A comparison of ABE production from agricultural residues.

Substrate	Total ABE (g/L)	Productivity (g/L/h)	Yield (g/g)	Ratio 0f A:B:E	References
Wheat straw	25	0.42	0.60	10.8:10.9:1	Qureshi *et al* (2007)
Wheat straw	13.38	0.19	0.51	5.9:10.5:1	Qureshi *et al.* (2008a)
Barley straw	26.64	0.39	0.43	4:10.5:1	Qureshi *et al.* (2010a)
Rice straw	3.0	0.017	1.04	2.2:17.2:1	Ranjan and Moholkar (2012)
Corn fiber	9.3	0.10	0.39	13.5:32:1	Qureshi *et al.* (2008b)
Corn stover	26.27	0.31	0.44	2.1:3.8:1	Qureshi *et al.* (2010b)
DDGS	10.4	0.14	0.34		Ezeji and Blaschek (2008)
Switch grass	14.61	0.17	0.39	6.1:13.5:1	Qureshi *et al*. (2010b)

6. Factor effecting ABE production

6.1 pH

The pH of the medium has been recognized to exert a significant influence on the acetone-butanol fermentation. This was due to the sensitivity of the solventogenic clostridia to the pH. The ability of cells to maintain an intracellular pH value near neutrality during the fermentation of sugars is subject to stress imposed by the fermentation products (Huang *et al*., 1986). The extent that how the cells can cope with the stress is very important because any change in intracellular pH would lead to alteration of cellular functions (Huang *et al*., 1986; Konings and Veldkamo, 1983). The assumption has been that a low pH is a prerequisite for solvent production (Jones and Woods, 1986; Kim *et al.*, 1984). *C. acetobutylicum* cultures maintained at relatively high pH produced more acid and less solvent than cultures maintained at low pH. *C. beijerinckii* was reported that it was capable of producing acetone-butanol in a medium in which the pH is maintained at pH 7 throughout the fermentation, although such production is dependent on supplementation of the medium with acetate and butyrate at high concentrations (Holt *et al.*,1984). The fact that high concentrations of acetate and butyrate are required to trigger solventogenesis at pH 7 focuses attention on the effect of the undissociated forms of these acids on the cells culture. Several related parameters such as acidic pH, high weak acid concentrations, and high cells densities appear to be involved in triggering solvent production (George and Chen, 1983).

A decrease in the pH of the medium normally associated with the onset of acetone-butanol production. The pH decrease is linked to the accumulation of acid end-products, which are in undissociated form (Jones and Woods, 1986). The pH can decrease to below 4.5 during the early part of fermentation in poorly buffered media due to sudden termination of solventogenesis, and a phenomenon known as “acid crash” may occur. The acid crash is associated with a rapid termination of solventogenesis after the switch has occurred and when the combined concentration of undissociated acetic and butyric acids exceeds a critical threshold value in the fermentation broth (Maddox *et al*., 2000). The acid crash is not the culture degeneration, which occurs as a result of an apparent failure in switching from the acidogenic to the solventogenic phase and takes place over a period of time as might

occur during continuous fermentation. As a result, increasing the buffering capacity of the growth medium can be a simple method for achieving a high concentration of the less toxic butyrate ion before inhibitory levels of undissociated butyric acid are reached. This then increased growth and carbohydrate utilization, as well as provided a greater amount of butyrate to serve as a precursor for butanol production (Bryant and Blaschek, 1988).

During the acetone-butanol fermentation by *C. beijerinckii* BA101 in P2 medium (Qureshi and Blaschek, 1999) the fermentation was initiated by growing the culture in P2 medium with a pH value near neutrality and then allowing the pH to fall to the optimum value (pH 5.0 to 5.5) for triggering solventogenesis. *C. beijerinckii* BA101, when grown in P2 medium, is able to maintain the optimum pH value for acetone-butanol production after the onset of solventogenesis and requires no external pH adjustment (Qureshi and Blaskchek, 1999; Ezeji *et al*., 2003).

6.2 Sugar concentration

A high sugar concentration (160 g/L) was found to be toxic to *C. beijerinckii* BA101 (Ezeji *et al*., 2003). This was due to the substrate inhibition. The effect of a high initial sugar concentration within the tolerable limits of the microorganism appears to have a more profound effect on the lag phase duration of the culture than on solvent production. The lag phase for cell growth at high glucose concentrations of 158 g/L was as long as 23 h. Qureshi and Blaschek (2001) demonstrated that at a glucose concentration ranging from 60 to 102 g/L, the maximum cells concentration (1.74 g/L dry weight) could be achieved.. *C. beijerinckii* BA101 differs in osmotolerance when compared to the *C. acetobutylicum* P262 industrial strain (Maddox and Qureshi, 1995) which is able to tolerate more than 250 g/L lactose present in whey permeate (Qureshi and Maddox, 2003).

The effect of substrate on acetone-butanol production by the *C. beijerinckii* BA101 fermentation of industrial liquefied starch varies with the composition and presence of inhibitors in the medium. The presence of inhibitors lowers the sugar tolerance level of *C. beijerinckii* BA101. Therefore, during operation of fermentation processes, attention must be paid to the concentration of inhibitors that are present in the medium in order to determine the appropriate initial sugar concentration to be employed (Ezeji *et al*., 2003).

6.3 Acetate in feed

Degeneration is a phenomenon that solvent-producing clostridia will lose the ability to produce solvents following repeated subculture or continuous cultivation. The addition of sodium acetate to the fermentation medium was found to prevent the degeneration in *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* BA101 (Chen and Blaschek, 1999a). Chen and Blaschek (1999b) studied the effect of acetate on the molecular and physiological aspects of *C. beijerinckii* NCIMB 8052 solvent production and strain degeneration. They found that *C. beijerinckii* NCIMB 8052 grown in modified P2 medium without added acetate produced large amounts of acids and little solvent, consistent with it being a degenerated culture. The addition of 20 mM sodium acetate was able to stabilize solvent production by *C. beijerinckii* NCIMB 8052 and to maintain the cells density, while growth decreased rapidly in the absence of additional acetate (Chen and Blaschek, 1999a; Chen and Blaschek, 1999b). The highest butanol concentrations observed were 0.6, 5.3, and 13.9 g/L for cultures grown in medium containing 0, 20, and 60 mM sodium acetate, respectively (Chen and Blaschek, 1999b).

Chen and Blaschek (1999b) reported that based on RNA and enzyme analyses coenzyme A (CoA) transferase was highly expressed and has higher activity in *C. beijerinckii* NCIMB 8052 grown in medium containing added sodium acetate than that in the absence of sodium acetate. This CoA transferase is an important enzyme responsible for acid reassimilation in solventogenic *Clostridium* spp. In addition, higher acetate kinase- and butyrate kinase-specific activity were also observed when the culture was grown in medium containing added acetate. The increase in solvent production by *C. beijerinckii* NCIMB 8052 grown in medium containing higher concentrations of added acetate might be related to acetate assimilation, which increases the carbohydrate utilization efficiency of the culture. Therefore, acid uptake by CoA transferase would result in lower intracellular acetyl-CoA, which can cause the rate of glycolysis to increase. The higher specific activity of acetate kinase and butyrate kinase enzymes exhibited by *C. beijerinckii* grown in medium containing high concentrations of sodium acetate appears to have contributed to an increase in acid reassimilation, which permits the direct utilization and conversion of the added acetate to solvent (Chen and Blaschek, 1999b).

6.4 Solvent (AB) toxicity

The product inhibition is an important problem associated with the typical ABE fermentation due to solvent toxicity, and although butyric acid is more toxic than butanol, its concentration during a normal solvent-producing fermentation remains low. Butanol toxicity has limited the concentration of sugar solutions for fermentation and resulted in low reactor productivity, low acetone-butanol yield, and a low final acetone-butanol concentration. The solventogenic clostridia are able to carry out cellular functions until the concentration of the solvent reaches inhibitory levels at around 20 g/L during the acetone-butanol fermentation. Higher than this level, further cellular metabolism ceases (Jones and Woods, 1986). The low final acetone-butanol concentration results in increased recovery costs, the need for large-capacity fermenters, and, ultimately, is a major limitation in the industrial production of solvents by the solventogenic clostridia.

Alcohols (including ethanol and butanol) are known to damage the structure of cells membranes and, thereby, inhibit cellular functions and this damage is directly related to the impact on the fluidity of the cells membrane (Baer *et al*., 1987). The autolytic degradation of solvent-producing cells of *C. acetobutylicum* P262 was linked to a toxic level of butanol, which was suggested to be involved in the triggering of the release of a cells-free autolysin during the solventogenic phase (Jones and Woods, 1986; Van der Westhuizen *et al*., 1982; Barber *et al*., 1979). It has been suggested that an increase to 22 to 28 g/L would be required to make biological synthesis economically competitive once again (Woods, 1995; Dürre, 1998). However, in a typical acetone-butanol batch fermentation, acetone-butanol concentrations in excess of 20 g/L over a period of 40 to 60 h are difficult to achieve (Woods, 1995). In order to achieve this acetone-butanol concentration level, it has been further suggested that increasing the tolerance of solvent-producing clostridia cells to butanol might result in the production of higher concentrations of solvents.

To solve solvent toxicity problem, in situ/online butanol removal currently appears to be the most viable path to follow. Several alternative methods, including reverse osmosis (Garcia *et al*., 1986), membrane-based systems such as pervaporation (Groot *et al*., 1984) and perstraction (Qureshi *et al*., 1992) as well as adsorption (Nielson, 1988), liquid-liquid extraction (Evans and Wang, 1988), and gas

stripping (Ezeji *et al*., 2003; Groot *et al*., 1989) have been examined. The application of some of these techniques results in a reduction in butanol inhibition and high sugar utilization (Maddox *et al*., 1995) thereby reducing volumes of the process streams. In such systems, up to 100% utilization of the sugar available in the feed has been demonstrated (Ezeji *et al*., 2003).

6.5 Agitation

Other functions of the bioprocess are to maintain the cells suspension and gas dispersion in broth. It is necessary to reduce the non-uniformities in fluid by removing concentration gradients of nutrients and temperature gradient (promoting heat transfer). Agitation helps in uniformity of aeration, suspension of microbes and nutrients in aerobic fermentation process. The aim of agitation is limited in maintaining homogeneity of nutrient and microbes in anaerobic fermentation.. Homogeneity is necessary for high mass transfer between nutrients and microbes to enhance the rate of fermentation by increased interfacial area. Yerushalmi and Volesskhy (1985) demonstrated the effect of agitation with *C. acetobutylicum* ATCC 824 using semi-solid reinforced clostridium medium (RCM). They found that the specific rate of anaerobic solvent production enhanced on increasing impeller speed from 190–340 rpm with working volume of 10 L. Maximum specific rates of butanol, acetone, and ethanol production were 5.54, 3.85, and 0.8 mmole g/L respectively. They also found that higher impeller speed (higher than 340 rpm) showed low specific rates of production. This phenomenon can be attributed due to competition between solvent production and biomass biosynthesis, and mechanical damage of cells at high shear rates.

6.6 Temperature

Most acetone-butanol producers are mesophilic and display temperature optima for fermentation between 30 and 37°C. At lower temperature, solvent production by *C. acetobutylicum* was decreased, but at elevated temperature acetone yield decreased but butanol yield remained unaffected (McNeil and Kristiansen, 1986). A thermophilic strain capable of producing butanol from cellulose at 50-65°C has also been reported. Optimum temperature for grain fermentation is between 36 and 37°C though the fermentation is normal in the range of 34-41°C. There is usually a loss of acetone and change in the solvent ratio at higher temperature (Beesch, 1952).

6.7 Production of bacteriocin

C. acetobutylicum cells undergo autolysis in the stationary phase of the fermentation. A bacteriocin has been isolated from these fermentations (Finn and Nowrey, 1958). It causes extensive lysis of the culture involving cells associated with solvent production (Barber *et al*., 1979). Release of bacteriocin begins late in the exponential phase of growth (24 h). The substance has been characterized as a glycoprotein of Mr 28 kDa. The autolysin gene appeared to be chromosomal since no plasmid DNA was detected in this *C. acetobutylicum* strain (Webster *et al*., 1981). The relationship between high butanol levels and increased autolytic activity has been established (Van der Westhuizen *et al*., 1982).

6.8 Oxygenation

Many steps have been taken to ensure anaerobic conditions during fermentation, ranging from the use of reducing agents, oxygen-free nitrogen, and gas-proof tubing on the experimental scale to blanket of fermentation gas on the industrial scale. As obligate anaerobes, butanol producing organisms require anaerobic conditions. A low redox potential (below -250 mV) is essential for acetone-butanol fermentation. *C. acetobutylicum* NCIB 8052 can detoxify molecular oxygen by NADH without forming H_2O_2 (O'Brein and Morris, 1971). This organism was able to grow anaerobically and produced solvents at a redox potential of +370 mV poised by potassium ferrocyanide. Vegetative cells survived in the presence of oxygen for several hours. Work of Hongo (1957) with redox dyes showed that higher yields of butanol can be achieved by the addition of 5mM neutral red into the fermentation system. This effect was later ascribed to the presence of neutral red-linked hydrogenase and pyridine nucleotide reductase activity in cells (Lovitt *et al*., 1988).

The mechanism of oxygen toxicity has been investigated in detail (Hongo, 1957) and concluded as follows:

a) Oxygen is itself a toxic agent.

b) Anaerobes require low redox potentials to grow well.

c) Organisms lacking catalase, are killed by H_2O_2 formed by reducing some of the oxygen.

When growth of *C. acetobutylicum* was studied under anaerobic (E_h -400 to -370 mV), aerated (E_h -50 to 0 mV; DO <1 µM)), and aerobic (E_h +100 to

+150 mV; DO 40-50 µM) conditions, the specific growth rates (about 0.6 h^{-1}) of the organism were similar under aerated and anaerobic conditions (O'Brein and Morris, 1971). Exposure of an anaerobic culture to oxygen (40-60 µM) for periods up to 6 h was not found lethal. At high DO levels, the rate of substrate consumption decreased, and growth and net synthesis of DNA, RNA, and protein stopped. However, these consequences of oxygenation were all reversible. There was no evidence to suggest the formation of H_2O_2. Oxygen (40 µM) inhibited growth in a medium poised at-50 mV, whereas growth was normal in an anaerobic environment poised at +370 mV. Biochemical and physiological consequences of oxygenation in acetone-butanol fermenting organisms are

1) Decrease in the rate of substrate consumption.
2) Cessation of growth of the organism.
3) Cessation of net synthesis of DNA, RNA, and protein.
4) Fall in the intracellular ATP level.
5) Increase in NADH oxidase activity.
6) Cessation of butyrate formation.
7) Starvation of energy and draining of reducing power.

7. Butanol recovery techniques

The common online butanol removal techniques, are adsorption, liquid–liquid extraction, perstraction, reverse osmosis, pervaporation, and gas stripping, which could be integrated with ABE fermentation (Table 7) (Zheng *et al*., 2009). In the direction of liquid–liquid extraction, the use of a mixed extractant containing 20% decanol in oleyl alcohol enhanced butanol formation using *C. acetobutylicum* ATCC 4259 in controlled pH conditions while decanol (extractant) alone was toxic for organism. It was interesting to note that decanol held larger butanol distribution coefficient over either oleyl alcohol or the mixture of both. The optimal two opposite consequences (toxicity and distribution coefficient) of extractants exceeded butanol yield of 0.47 (Qureshi and Maddox, 2005; Evans and Wang, 1988). Ishii *et al*. (1984) preferred a mixture of oleyl alcohol and guerbet alcohol as extractant for butanol among 29 organic compounds, based on their non-toxicity towards the cells of *C. acetobutylicum* IAM 19012. In their study, biosynthesis of butanol was 2.5-fold

higher than conventional fermentation processes (without extraction of product) (Ishii *et al*., 1984). However, the main concern of this extractive technique was to optimize the combination of non-toxicity and distribution coefficient, as extractant having significant distribution coefficient was toxic to the bacterial cells and vice versa. Consequently, identification and/or synthesis of any compound or mixture of compounds having non-toxic activity and high distribution coefficient will be advantageous for liquid–liquid extraction of butanol from fermentation broth.

Another viable recovery technique, namely gas stripping, was integrated with batch (Maddox and Qureshi, 1995; Qureshi and Blaschek, 2001; Ezeji *et al*., 2007), fed-batch (Ezeji *et al*., 2007; Ezeji *et al*., 2004), and continuous fermentation processes (Qureshi and Blaschek, 2001). Butanol fermentation integrated with gas stripping was investigated in concentrated sugar broth using *C. acetobutylicum* P262. This process was simple and economical (no need of expensive equipments); and was advantageous over others as it reduces the butanol inhibition without affecting the culture, concentration of nutrients and reaction intermediates. It was also successful to improve the productivities and to reduce the process volume (reactor size) efficiently (Qureshi and Maddox, 1995; Qureshi and Blaschek, 2001). Nitrogen (Qureshi and Blaschek, 2001) and a combination of CO_2 and H_2 (own gases of fermentation) (Qureshi and Maddox, 1995, Ezeji *et al*., 2007; Ezeji *et al*., 2004a) or the mixture of all three were employed on stripping in fermentation broth. However, use of nitrogen gas provided better results than any other gas or combination of gases. Nowadays, fermentation integrated with gas stripping is attracting the attention of researchers in scaling up of biobutanol synthesis.

Table 7. Advances in product recovery to enhance the production of ABE.

Recovery method	Raw material	Bacterial strain	Type of reactor	Max. titer of ABE (without recovery) in g/L	Max. titer of ABE (with recovery) in g/L	Ref.
Gas stripping	Sugar solution	*C. beijerinckii* BA101	Batch	-	69.7	Qureshi and Blaschek. (2001)
	Sugar solution	*C. beijerinckii* BA101	Fed-batch	-	120	Qureshi and Blaschek (2001)
	Glucose	*C. beijerinckii* BA101	Fed-batch	17.7	232.8	Ezeji *et al*. (2004b), Ezeji *et al*. (2003)
	Liquefied corn starch	*C. beijerinckii* BA101	Batch	18.4	23.9	Ezeji *et al*. (2007a)
	Saccharified liquefied corn starch	*C. beijerinckii* BA101	Batch	18.2	26.5	Ezeji *et al*. (2007b)
	Saccharified liquefied corn starch	*C. beijerinckii* BA101	Fed-batch	-	81.3	Ezeji *et al*. (2007b)
	Glucose	*C. beijerinckii* BA101	Batch	17.7	75.9	Ezeji *et al*. (2003)
Pervaporation	Glucose	*C. beijerinckii* BA101	Fed-batch	25.3	165.1	Qureshi and Blaschek, (2000)
	Glucose	*C. acetobutylicum* ATCC 824	Fed-batch	-	154.97	Qureshi *et al*. (2001)

Table 7. Advances in product recovery to enhance the production of ABE. (Cont'd)

Recovery method	Raw material	Bacterial strain	Type of reactor	Max. titer of ABE (without recovery) in g/L	Max. titer of ABE (with recovery) in g/L	Ref.
Pervaporation	Glucose	*C. beijerinckii* BA101	Fed-batch	25.3	165.1	Qureshi and Blaschek, (2000)
	Glucose	*C. acetobutylicum* ATCC 824	Fed-batch	-	154.97	Qureshi *et al*. (2001)
Perstraction	Whey permeate (lactose)	*C. acetobutylicum* P262	Batch	7.72	136.58	Qureshi and Maddox (2005)
	Whey permeate (lactose	*C. acetobutylicum* P262	Fed-batch	7.72	57.8	Qureshi and Maddox (2005)
Adsorption		*C. acetobutylicum*	Fed-batch	13.5	23.2	Qureshi *et al*. (2005)
		C. acetobutylicum	Fed-batch	13.5	59.8	Qureshi *et al*. (2005)
		C. acetobutylicum	Fed-batch	13.5	387.3	Qureshi *et al*. (2005)

8. Overview on fermentation mode

8.1 Batch and fed-batch fermentation of lignocellulosic biomass

One stage and two stage fermentation studies were performed to enumerate the performance of batch and fed-batch process in producing biobutanol from numerous feedstocks (like lignocellulosic and starchy biomass) and various microbes. Qureshi *et al*. (2008a) studied five combinations of pretreatments (acidic and enzymatic hydrolysis) of raw material and fermentation processes using *C. beijerinckii* P260. These five combinations were: (i) fermentation with pretreated wheat straw, (ii) separate hydrolysis and fermentation of wheat straw without

removing sediments, (iii) simultaneous hydrolysis and fermentation of wheat straw without agitation, (iv) simultaneous hydrolysis and fermentation with addition of sugar supplementation, and (v) simultaneous hydrolysis and fermentation with agitation by gas stripping. The results revealed the maximum productivity of butanol (0.31 g/L/h) using a combination process (v) i.e.; simultaneous hydrolysis of wheat straw and fermentation with agitation from gas stripping. Here, monosaccharides, viz. glucose, xylose, arabinose, galactose, and mannose, were identified in hydrolyzate. Qureshi *et al*. (2008a) claimed that the use of simultaneous hydrolysis of wheat straw towards simple sugar and fermentation to butanol was an attractive method than expensive glucose in ABE fermentation processes. Subsequently, hydrolysis of wheat and fermentation were attempted in a single reactor with semi batch mode aiming 100% hydrolysis of wheat straw and its fermentation towards butanol. In this case, productivity was doubled (0.77 g/L/h) in batch fermentation. Further, supplement of sugar feed to the reactor helped in improving the productivity of the fed-batch fermentation (Qureshi *et al*., 2008b).

Prior to batch fermentation process, pretreatment of the raw materials was crucial step to control the concentration of inhibitors in hydrolyzate (product from hydrolysis of raw materials). Two pretreatment processes (acidic and enzymatic) were analyzed with corn fiber hydrolyzate in batch fermentation. Fermentation using corn fiber hydrolyzate, treated with enzymatic method, resulted a higher yield (0.35 g/g) than acidic method as fermentation inhibitors were eliminated in the case of enzymatic method (Qureshi *et al*., 2008c). Likewise, the presence of inhibitors in hydrolyzates of other cellulosic substrates (barley straw, corn stover, and switch grass hydrolyzate) was detected in several studies of biobutanol production using batch fermentation. Fortunately, these inhibitors could be successfully removed by treatment of hydrolyzate with $Ca(OH)_2$ (Qureshi *et al*., 2010a; Qureshi *et al*., 2010b).

Biobutanol syntheses have been also attempted using carbon dioxide as a substrate obtained from coal-derived synthesis gas. In this fermentation study, suitable organism was *Butyribacterium methylotrophicum*. Two stage process were performed. In the first stage, butyric acid and acetic acid formation (acidogenesis) was carried out using *B. methylotrophicum*, and in the second stage, butanol synthesis (solventogenesis) was achieved from acids using *C. acetobutylicum*. Interestingly, it

was found that the composition of final products could be dependent on pH (Worden *et al*., 1991). Batch and fed-batch fermentation processes were limited by several factors such as time consumption in sterilization of bioreactors, re-inoculation, solvent inhibition and low productivity. These limitations were compensated by using continuous fermentation processes.

Table 8. Comparison of batch and fed-batch fermentation processes for butanol production based on raw materials, and yield/productivity

Fermentation process	Strain used	Substrate	Yield (g/g)	Productivity (g/L/h)	Maximum titer of ABE (g/L)	Ref.
Batch fermentation	*C. beijerinckii* P260	Barley straw	0.43	0.39	26.64	Qureshi *et al*. (2010)
	C. beijerinckii P260	Wheat straw	0.41	0.31	21.42	Qureshi *et al*. (2008)
	C. beijerinckii BA101	Corn fibers	0.36–0.39	0.10	9.3	Qureshi *et al*. (2008)
	C. beijerinckii P260	Corn stover and switchgrass (1:1)	0.43	0.21	21.06	Qureshi *et al*. (2008)
	C. beijerinckii P260	Switchgrass	0.37	0.09	14.61	Qureshi *et al*. (2008)
Fed-batch fermentation	*C. beijerinckii* P260	Wheat straw	-	0.36	16.59	Qureshi *et al*. (2008)
	C. saccharoperbutyl-acetonicum N1–4	Synthetic medium with butyric acid	0.49	0.42	16.0	Tashiro *et al*. (2004)

8.2 Fed-batch fermentation using butyric acid

In another study, the effect of feeding butyric acid under pH-stat fed-batch fermentation process using *C. saccharoperbutylacetonicum* was examined (Tashiro *et al*., 2004). The pH-stat fed-batch process was carried out by maintaining a constant pH through controlled addition of butyric acid. This process enhanced the productivity of butanol. The maximum butanol concentration (of 16 g/L) was achieved with a 72% higher productivity than conventional batch process.

8.3 Continuous fermentation

Continuous fermentation processes have several advantages over batch fermentation processes. This includes: fermentation can be operated in a continuous mode (Ezeji and Blaschek, 2007), only one inoculum culture is required in fermentation for long time and drastic reduction of sterilization and inoculation time that lead to a higher productivity. The most common strategies of continuous fermentation including free cells, immobilized cells, and cells recycles, have been attempted (Nimcevic and Gape, 2000; Qureshi and Blaschek, 2004).

8.3.1 Continuous fermentation processes using free cells

In continuous fermentation processes using free cells, cells are free to move within fermentation broth due to the agitation by mechanical agitator or by air lifting. The microbial cells and nutrients can be maintained in the suspension and helps in promoting mass transfer. Ezeji and Blaschek (2007) studied the sustainability of the continuous cultivation of *C. beijerinckii* BA101 in degermed corn based P2 medium for biobutanol fermentation. They reported that butanol production was merely possible in saccharified degermed corn or in combination of saccharified and normal degermed corn, while normal degermed corn showed negative results. In addition, stability of physical and chemical properties of gelatinized corn starch as raw material was examined using free cells and it was noted that the degree of hydrolysis of gelatinized corn starch decreased with time. The physical and chemical properties change adversely during storage time due to retro-gradation (reaction takes place in gelatinized starch to make less digestive structures of starch), resulting low productivity in ABE fermentation (Liew *et al*., 2005; Ezeji *et al*., 2005).

8.3.2 Continuous fermentation using immobilized cells

Fermentation process using immobilized cells is advantageous over free cells continuous fermentation process. For example, cells immobilization allows long survival time of cells (due to lack of mechanical agitation) in solventogenesis phase without frequent cells regeneration. When wood pulp was used as a cell holding material to prevent cell wash out, the solvent (2-propanol plus butanol) productivity from glucose increased from 0.47 to 5.52 g/L/h with the yield of 54 % (Survase *et al*., 2011b). The average butanol concentrations with brick-immobilized cells and suspended cells were 8.07 and 4.56 g/L, respectively. The ABE productivity with immobilized cells was 1.89 times that of suspended cells (Yen and Li, 2011).

To overcome low cell density in traditional ABE fermentation, continuous fermentation processes with cell recycling and retention have been successfully applied (Tashiro *et al*., 2004; Malaviya *et al*., 2012). A systematic comparison of continuous production of ABE using free cell cultivation with and without cell recycling and cell recycling with cell bleeding was performed by Tashiro *et al*., (2004). Continuous fermentation of *C. pasteurianum* MBEL_GLY2 with cell recycling was carried out to get the ABE and butanol productivity of 8.3 and 7.8 g/L/h, respectively, at a dilution rate of 0.9 per h (Malaviya *et al*., 2012). Packed bed reactors (PBRs) are packed with a suitable immobilization material on which a biofilm is formed and used for continuous production of desired metabolites. The reaction rates are much higher as compared to batch reactors. Recently studied immobilization materials in PBRs are listed in Table 10. Qureshi *et al*. (2004) obtained ABE productivity of 16.13 g/L/h at dilution rate of 2 per h with brick immobilized cells during reactor operation for 2302 h.

To increase the sugar utilization, Lienhardt *et al*. (2002) suggested that the reactor effluent should be recycled. A maximum productivity of 12.14 g/l h was achieved by Survase *et al*. (2011b) using a wood pulp immobilized PBR and sugar mixture identical to that of a wood hydrolyzate as feed. Wood pulp as an immobilization material was also reported by Survase *et al*. (2011a) with SEW spent liquor from spruce chips as feed. The highest productivity obtained was 4.86 g/l/h. Napoli *et al*. (2010) used PBR reactor with Tygon rings as an immobilization material and lactose as feed to obtain a maximum productivity of 5 g/L/h.

8.3.3 Improved fermentation processes using free cells

1) Cell recycling and bleeding

Another novel technique, a modified and improved version of free cells continuous fermentation (cells recycling and bleeding) process was examined using high cells density of *C. saccharoperbutylacetonicum*. In this system, membrane module (filtration) was used to recycle the cells into the bioreactor for improvement of cells concentration leading to better butanol productivity. On the other hand, optimization of dilution rate facilitated cells bleeding (i.e., removal of excess concentration of cells) from bioreactor to maintain optimum density of fermentation broth. In such cell recycling system, 10-fold higher cells concentration has been recorded and 6-fold higher yield of butanol (11.0 g/L/h) was observed than conventional continuous fermentation without cells recycling (1.85 g/L/h). This process included advantages like homogeneity of broth that facilitated diffusion over fermentation process in cells-immobilized bioreactor (Tashiro *et al*., 2004)

2) Continuous flash fermentation

A flash fermentation technology was proposed by Mariano *et al*. (2010) to overcome the low productivity hurdle in synthesizing butanol. This innovative technology consisted of three interconnected units, viz. fermenter, cells retention system, and vacuum flash vessel (for continuous recovery of butanol from broth). Simulation analysis (dynamic behavior of the process) revealed that the flash fermentation process could be promising for high productivity of butanol. Moreover, final concentration of butanol was expected to be more than 20 g/L and this process could also be helpful in reduction of distillation costs and benefit environment due to lower quantity of waste water generation. Another mathematical model was formulated base on experimental results to optimize productivity, energy requirement and product purity (Shi *et al*., 2005). The model showed that the performance of the fermentation process can be improved using high feed concentration. Also, high product purity could be achieved using two-vessel partial flash system (first vessel of two to three plates and second as a flash vessel) over two-vessel flash system.

Table 9. Comparison of continuous fermentation processes for butanol production based on raw materials, and yield/productivity

Fermentation process	Strain used	Substrate	Yield (g/g)	Productivity (g/L/h)	Maximum titer of ABE (g/L)	Ref.
(i) Free cell	*C. saccharobutylicum* DSM 13864	Sago starch	0.29	0.85	9.1	Liew *et al*. (2005)
	C. beijerinckii BA101	Degermed corn	-	0.29–0.30	14.28	Ezeji *et al*. (2007)
	C. beijerinckii BA101	Starch and glucose	-	0.42	9.9	Ezeji *et al*. (2005)
(ii) Immobilized cells	*C. acetobutylicum* P262	Whey permeate	3.5–3.6	0.36–1.10	8.6	Qureshi and Maddox (2005)
	C. acetobutylicum 824A	Lactose and yeast extract	-	0.78	1.43	Napoli *et al*. (2010)
	C. acetobutylicum ATCC 55025	Corn	0.42	4.6	12.50 (butanol)	Huang *et al*. (2004)
	C. acetobutylicum P262	Defidered-sweet-potatoslurry	0.20	1.0	7.73	Badr *et al*. (2001)
	C. beijerinckii BA101	Synthetic medium	0.36	12.43	8.8	Qureshi *et al*. (2004)
(iii) Cells recycling and bleeding	*C. saccharoperbutylacetonicum* N1-4	Synthetic medium	-	11.0	8.58	Tashiro *et al*. (2004)

Table 10. Comparison of continuous fermentation processes on immobilization material

Immobilization material	Strain	Substrate	Yield (g/g)	Productivity (g/L/h)	Total ABE (g/L)	Reference
Brick	*C. acetobutylicum* BCRC 10 639	Glucose	0.19	1.21	11.28	Yen and Li (2011)
Wood pulp	*C. beijerinckii* DSM 6423	Glucose	0.29	5.25	9.2	Survase *et al*. (2011c)
Porous PVA	*C. beijerinckii* NCIMB 8052	Glucose + butyric acid	0.44	0.40 (butanol)	13.4 (butanol)	Lee *et al*. (2009)
Wood pulp	*C. acetobutylicum* DSM 792	SEW liquar + glucose	0.27	4.86	7.59	Survase *et al*. (2011a)
Wood pulp	*C. acetobutylicum* DSM 792		0.28	12.14	8.09	Survase *et al*. (2011b)
Corn stalk	*C. acetobutylicum* ATCC 55025	Glucose	0.32	5.06	5.1	Zhang *et al*. (2009)
Tygon™ ring	*C. acetobutylicum* DSM 792	Lactose	0.28	5.0	5.19	Napoli *et al*. (2010)
Brick	*C. beijerinckii* BA 101	Glucose	0.38	1.58	7.9	Qureshi *et al*. (2012)

9. Immobilization technique

Immobilization is one of the most extensively used methods to improve the fermentation process. In the field of biotechnology, it is defined as the technique used for the physical or chemical fixation of cells, organelles, enzymes or other proteins (e.g. monoclonal antibodies) onto a solid support, into a solid matrix, or retained by a membrane, in order to increase their stability and facilitate their repeated or continued use. The immobilization of whole cells is based on the retention of catalytically active cells within the restricted region of a bioreactor. That the

immobilized cells may be alive leads to unique effects in this form of heterogeneous catalysis, taking into consideration the individual conditions of the fermentation process which depended on the selected immobilized microorganism (Karel *et al*., 1985). One example of that specific process is the immobilization of anaerobic microorganisms, which presents new possibilities in fermentation, insufficiently investigated to date. In the immobilization it is more difficult to maintain the cells activity of anaerobic microorganisms than the cells activity of aerobic microorganisms, because it is performed in an anaerobic atmosphere. One of the methods for achieving success in the immobilization of anaerobic microorganisms is to induce sporulation, the process when spores, the most resistant stage of the cell, are formed (Burns and Minton, 2011).

Conventional fermentations with free cells encounter a number of difficulties such as low cells density, nutritional limitations, batch-mode operations with high downstream times, higher costs of microbial recycling and installation, high contamination risks, susceptibility to environmental variations, and limitations of the dilution rate in continuous fermentation due to wash-out (Ramakrishna and Prakasham, 1999; de Vasconcelos *et al*., 2004). The immobilization process eliminates most of the constraints faced by the free-cell systems.

Since the early seventies, when Chibata's group announced their success in operating the continuous fermentation of L-aspartic acid (Tosa *et al*., 1973), numerous research groups have attempted various microbial fermentations with immobilized cells (Ramakrishna and Prakasham, 1999). The advantages of cell immobilization include enhanced fermentation productivity, the feasibility and facilitation of continuous processing (preventing loss of cells), lower costs of recovery and downstream processing (Kourkoutas *et al*., 2004; Najafpour, 2006). Immobilization also improves cell stability and the catalytic effects of biocatalysts and extends their applicability by recycling biocatalysts (Bučko *et al*., 2012). Elimination of the lag phase is important, as also is efficient continuous operation without repeated inoculation (Jiang *et al*., 2009).

Immobilization may improve genetic stability and protects cells against shear forces (Kourkoutas *et al*., 2004; Çaylak and Sukan, 1998). The immobilization process also entails some disadvantages, which are largely based on fermenter design

limitations, such as mass-transfer, growth of cells in the reactor, aeration, and back-mixing (Núñez and Lema, 1987). The type and structure of carriers also have great significance for the stability of the process and appropriate selection of immobilization techniques and supporting materials is required to minimise the disadvantages of immobilization (Lozinsky and Plieva, 1998; Beshay, 2003). However, the industrial use of immobilized cells remains limited hence further application will depend on the development of immobilization procedures that can be readily scaled up (Kourkoutas *et al*., 2004).

There are many ways of distinguishing immobilization techniques. They are most closely identified with classification into three major categories, based on the immobilization method used:

(i) Aggregation (porous carrier adsorption, surface adhesion, self-aggregation);

(ii) Cell-containment behind barriers;

(iii) Entrapment within a porous matrix;

The genera *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Fusobacterium*, *Megasphera*, etc. belong largely within the anaerobic process (Zigová *et al*., 1999). Bacteria of the *Clostridium* genus are classified as rod-shaped, sporeforming, Gram-positive bacteria, lacking the ability to reduce sulphates. The *Clostridium* species are capable of converting a wide range of carbon sources (e.g. glucose, galactose, cellobiose, mannose, xylose, and arabinose) to compounds such as butanol, acetone, 1,3-propanediol, propan-2-ol, ethanol (Yamamoto *et al*., 1998; Ezeji *et al*., 2007; Lee *et al*., 2008a) or hydrogen, a gas known as a highly efficient energy-carrier (Jo *et al*., 2008; Ding *et al*., 2009). Some of these compounds can serve as biofuels directly, while others including butyric acid can be used in the chemical conversion to biofuels (Papoutsakis, 2008).

Several types of ABE fermentation experiments are realised by immobilized cells and their results depend on the techniques used in the immobilization process.

Table 11. Experimental conditions, yields and productivity of ABE fermentation

Fermentation type[a]	Immobilisation[b]	Organism	Dilution rate (h^{-1})	Substrate conc.[c] (g/L)	ABE conc.[d] (g/L)	Yield (g/g)	Productivity (g/L/h)	Ref.
Continuous	N/A free cells)	*C. acetobutylicum* ATCC 824	0.1	Glucose, 50, FM: 50	15.9	0.32	1.5	Jone and Wood, 1986
Batch	N/A free cells)	*C. beijerinckii* BA 101	-	Glucose, 59.8	24.2	0.42	0.34	Ezeji *et al*., 1985
Continuous	FBB	*C. acetobutylicum* ATCC 55025	0.1	Glucose 20, FM: 54	11.3	0.53	5.6	Huang *et al*., 2004
Continuous	Adsorption, bricks	*C. acetobutylicum* BCRC 10639	0.05	Glucose 60, FM: 60	14.3	0.24	-	Yen *et al*., 2011
Continuous, PBR	Adsorption, bone char	*C. acetobutylicum* P-262	0.45	CWP, 60, FM: 60	8.1	0.36	3.6	Qureshi and Maddox, 1995
Continuous, PBR	Adsorption, ceramic bead	*C. acetobutylicum* P-262	0.129	DSPS (starch: 39.7), FM 39.7	7.73	0.19	1.0	Badr *et al*., 2001
Continuous	Entrapment, Ca-alginate	*C. acetobutylicum* DSM 792	1.0	Glucose 60, FM: 60	4.0	0.21	4.02	Frick and Schugerl (1986)
Continuous	Entrapment, carrageenan matrix	*C. acetobutylicum* DSM 792	0.69	Glucose 60, FM: 60	4.0	0.18	2.80	Frick and Schugerl (1986)
Continuous	Entrapment chitosan matrix	*C. acetobutylicum* DSM 792	0.53	Glucose 60, FM: 60	2.7	0.18	1.43	Frick and Schugerl (1986)

Table 11. Experimental conditions, yields and productivity of ABE fermentation (Cont'd.)

Fermentation type[a]	Immobilisation[b]	Organism	Dilution rate (h^{-1})	Substrate conc.[c] (g/L)	ABE conc.[d] (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
Continuous	Adsorption brick	*C. beijerinckii* BA 101	2.0	Glucose 62, FM: 62	7.9	0.38	15.8	Qureshi *et al*., 2000
Batch	Adsorption, Amberlite	*C. pasteurianum* MTCC 116	-	Crude glycerol, 25	B: 8.8	B: 0.35	B: 0.07	Khana *et al*., 2011
Batch	Adsorption, cotton towel	*C. acetobutylicum* B3	-	Glucose, 60	B: 15.6	0.38	1.88	Liu *et al*., 2013
Continuous	Adsorption, wood pulp fibres	*C. acetobutylicum* DSM 792	1.9	Glucose,60	7.19	0.40	13.66	Survase *et al*., 2012
Continuous	Adsorption, wood pulp fibres	*C. acetobutylicum* DSM 792	1.5	SM, 47.8	8.09	0.28	12.14	Survase *et al*., 2012

a) PBR – packed-bed reactor; b) FBB – fibrous-bed bioreactor; c) FM – concentration of feed medium, CWP – casein whey permeate, DSPS – de-fibred sweet potato slurry, SM – sugar mixture (2.3 arabinose, 4.5 galactose, 8.5 glucose, 22.0 mannose, 10.5 xylose (g L^{-1})); d) B – butanol (if only data for butanol production were published)

Source: Dolejš *et al*., 2013.

9.1 Aggregation

Yen *et al*. (2011) developed a process for continuous ABE fermentation with *C. acetobutylicum* immobilized onto bricks. They achieved the high total solvent concentration of 14.3 g/L at a dilution rate of 0.054 h^{-1} and a yield of 0.24 g/g of final solvents, which is no more than with the free-cell system. However, the fermentation process lasted for more than 500 h, during which the immobilized microorganisms continued to produce all the solvents. A further advantage is the low cost of bricks as

the immobilization carrier and, due to method of immobilization (adsorption), there are no nutrient transfer limitations.

Clearly, the highest productivity of 13.66 g/L/h was achieved by Survase *et al*. (2012) during the continuous fermentation with immobilized *C. acetobutylicum*, using wood pulp fibres. Wood pulp fibres are recyclable, biodegradable and in the process presented were inoculated with an activated spore culture, prepared by heat shock at 80 ºC for 10 min from sporulated cells. The major advantage of this type of immobilization derives from the direct contact between nutrients and immobilizedd cell, minimising the diffusion problems; Table 11 shows that adsorption is the method most frequently used for immobilization.

Raw materials from industry and their further utilization by microorganisms are significant areas of interest for biotechnology. One of the raw materials, defibred sweet-potato-slurry (DSPS), was used for ABE fermentation by immobilized *C. acetobutylicum*, with the results of the experiment summarised in Table 11. The DSPS contained 39.7 g of starch and use of the adsorption method (form of aggregation) for immobilization (Badr *et al*., 2001). The use of starch as a substrate leads to a problem with the viscosity of the slurry and, as a consequence, problems with mixing and pumping of the medium (Soni *et al*., 2003). The ceramic beads with immobilized cells improved the mixing properties of starch hydrolyzate. Productivity of 1.0 g/L/h and yield of 0.19 g/g render this type of carbon source and fermentation mode comparable with the free-cell continuous system. This cheap substrate can be used for ABE fermentation rapidly and continuously in the current bioreactor system with immobilized highly active α-amylase-containing *C. acetobutylicum* P-262 (Badr *et al*., 2001).

Casein whey permeate is another raw material used as a substrate for ABE fermentation. The same strain, *C. acetobutylicum* P-262, was immobilized onto bone char and used for continuous fermentation in a packedbed bioreactor. Table 11 shows the yield and productivity to be relatively high in comparison with other immobilized cell fermentations using adsorption and the productivity of 3.6 g/L/h clearly makes this process attractive. When whey permeates is used as the fermentation substrate, it is desirable to maintain its concentration in the reactor

influent at 50–60 g/L. This is because, at lower concentrations, the system becomes acidogenic rather than solventogenic (Qureshi and Maddox, 1995).

9.2 Cell containment behind barriers

Huang *et al*. (2004) immobilized *C. acetobutylicum* in a fibrous-bed bioreactor (FBB) and used it in continuous fermentation mode with medium recirculation, when a butanol productivity of 5.6 g/L/h was obtained.The FBB consisted of a jacketed glass column (total volume: 800 mL) packed with a spiral-wound fibrous matrix (packed volume: 200 mL). Table 11, shows that the yield of solvent products of ABE fermentation is clearly higher than that achieved by other immobilization immobilization techniques and modes of fermentation, when *C. acetobutylicum* was used. In comparison with the yield of solvents (0.53 g/g), achieved by fermentation with free cells of *C. acetobutylicum*, the immobilized system yield is more than 1.5-fold higher. Fermentations using FBB are widely used due to their advantages, which include an efficient and continuous mode of operation without the need for repeated cell inoculation, elimination of the lag phase, good long-term stability, while enabling simplified downstream processing.

9.3 Entrapment within porous matrix

Examples of cell immobilization by entrapment include carrageenan, chitosan, and calcium alginate, as developed by Frick and Schugerl (1986). A high productivity of 4.02 g/L/h was achieved by fermentation using the cell immobilized in Ca-alginate. Table 11 shows a comparison of this productivity and yield of solvents with other fermentations, using other immobilization techniques (e.g. productivity of 4.0 g/L/h achieved by fermentation with bone char adsorption) by Qureshi and Maddox (1995). Despite slight differences in the productivity of solvents between entrapment and other immobilization techniques, entrapment has several advantages. Initially, the process stability, when the immobilized cells are better protected against changes during fermentation (temperature changes, contaminations, carbon source changes) and then the better protection of cells against exposed shear forces. For these reasons, long-lasting fermentations at relatively high dilution rates are feasible (Frick and Schugerl, 1986).

C. beijerinckii is also an excellent producer of butanol, with a biphasic metabolism similar to *C. acetobutylicum*. Some strains of *C. beijerinckii* can also

produce propan-2-ol, instead of ethanol, in a solventogenic phase (Lee *et al*., 2008). *C. beijerinckii* can utilise a large variety of substrates, as is the case for other solventogenic clostridia. With the exception of glucose, the highest concentration of ABE products (19.1 g/L) was achieved when cellobiose (with a starting concentration of 55 g L^{-1}) was used as the carbon source in a batch fermentation with free cells, whereas the lowest amount of ABE (10 g/L) was produced when galactose (with a starting concentration of 55 g/L) was used (Ezeji *et al*., 2007).

An example of immobilization of *C. beijerinckii* BA 101 is its immobilization using clay brick particles. This strain is known as a hyper-butanol-producing culture (Qureshi *et al*., 2000). The hyper-amylolytic/butanolagenic *C. beijerinckii* BA101 strain was generated from *C. beijerinckii* NCIMB 8052 (formerly only *C. acetobutylicum*) using chemical mutagenesis. Clay brick was chosen for its low cost and availability and the relatively simple immobilization process. This consisted of transferring the actively growing culture into the column containing the brick support and allowing the cells to adhere and grow. A continuous feed was allowed after 6 h of cell growth and the reactor was operated for 597 h. In conclusion, a much higher solvent productivity of 15.8 g/L/h (Table 11) was achieved than the best previously reported in other immobilized cell and cell recycle reactors (5.6 g/L/h) (Qureshi *et al*., 2000; Huang *et al*., 2004). Nevertheless, the process is vitiated by the fact that only a fraction of the biomass was in the solventogenic phase and a significant proportion of the biomass was present as inactive spores. Therefore, it is proposed that sporulation be blocked to achieve higher productivity (Lee *et al*., 2008a).

CHAPTER 3

MATERIALS AND METHODS

Materials

1. Microorganisms

Clostridium acetobutylicum DSM 1731 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany. *C. butyricum* TISTR 1032, *C. beijerinckii* TISTR 1461 and *C. acetobutylicum* TISTR 1462 three strains were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand. *C. acetobutylicum* JCM 1419 and *C. beijerinckii* JCM 1390 two strains were obtained from the Japan Collection of Microorganisms, RIKEN BioResource Center, Japan. All strain are maintained at -20°C as a suspension of spore in 25% (v/v) glycerol in microtube. When need, 1 mL stock culture was heat shocked for 3.5 min in 75°C hot water, cooled for 1 min in ice water (Qureshi *et al*., 2001) and subsequent transferred into 100 mL of 40 g/L glucose Tryptone-yeast extract-acetate TYA medium. Before sterilization, the medium in the bioreactor was flushed with sterile oxygen-free nitrogen gas until the resazurin was colorless. The cultivation was incubated for 18 to 24 hours at 37°C under anaerobic condition when the log phase is reached (OD 600 nm of 2.0) (Yokoi *et al*., 2001; Chang *et al*., 2008).

2. Media

Tryptone-yeast extract-acetate (TYA) medium was used for preculture and main culture. The composition of TYA medium was as follows (g/L): glucose, 20; yeast extract, 2; tryptone, 6; CH_3COONH_4, 3; $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01. The pH was adjusted to 6.5 with 1 M NaOH or 1 M HCl.

A nitrogen-free medium (named as TYA–N free medium) was used for conversion of butyric acid to butanol by solventogenesis phase of *Clostridium* spp. The cells were washed several times to remove nitrogen from the preculture medium. The composition was as follows (g/L): glucose, 10-20; KH_2PO_4, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; butyric acid, 1-15. The pH was adjusted to 6.5 (Al-Shorgani *et al*., 2011)

3. Decanter cake

Decanter cake was obtained from Taksin Palm (1971) Co., Ltd, Surat Thani Province, Thailand. Prior to use, the decanter cake was sun dried and then dried in hot air oven at 60 °C until free from moisture and ground to fine powder using mortar and pestle. The substrate was shredded into different particle sizes using a commercial sieve (Analysette, Fritsch, Germany) and different screen meshes. In order to ensure the homogeneity of the sample, the most abundant particle size of this substrate (0.42 mm diameter) was selected for carrying out the experiments.

4. Support matrices for cell immobilization

Four common carriers were used in this study: oil palm shell (OPS), empty fruit bunch (EFB), palm press fiber (PPF) and oil palm frond (OPF). Each was evaluated for butanol production and compared to that of the free cells. The fermentation and cell immobilization processes were performed simultaneously. For cell immobilization, 5 g of each support were placed in a serum bottle before cell inoculation.

5. Instruments

Instruments	Series	Suppliers
Autoclave	SS-325	Tommy, USA
Balance 2 digit	BP 2100 S	Sartorius
Balance 4 digit	PA214	OHAUS, USA
Centrifuge	CF-10	Wise Spin, Korea
Gas Chromatography	7890A	Hewlett Packard, USA
Laminar flow	-	Clean, USA
pH meter	EF-201	Mettler Toledo, China
Spectrophotometer	LIBRA-S22	Biochrom, England
Stirred	MS115	ML, Thailand
Vortex	VM-10	WiseMix, Korea
Water bath	W350	Memmert, Germany

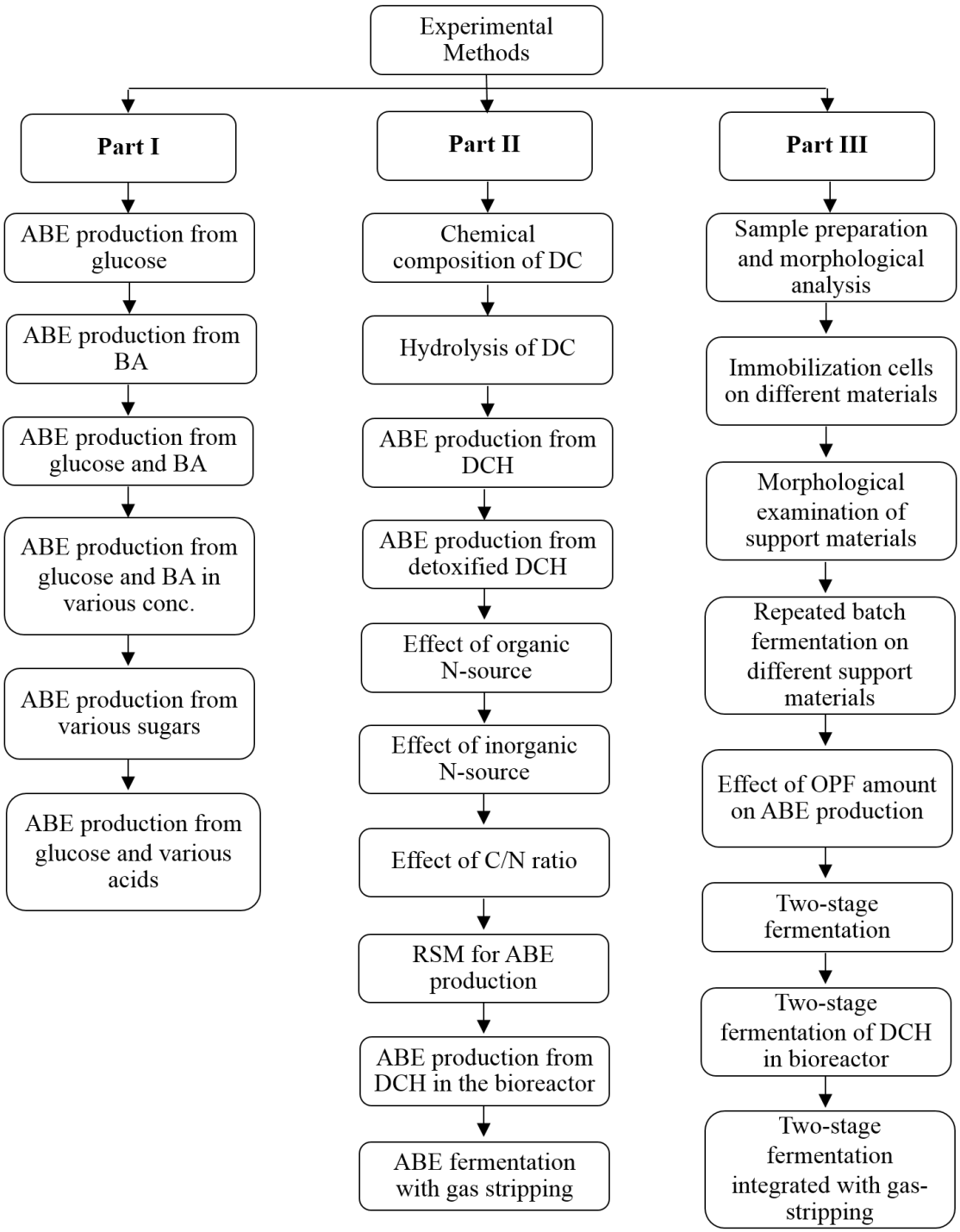

Figure 2. Schematic of the experimental procedure

Experimental Methods

Part I Screening of high butanol production *Clostridium* sp.

1.1 ABE production from glucose

Six strains of *Clostridium* were grown in TYA medium at 37°C for 18-24 h. This culture was used as a starter culture. The medium was sterilized by autoclaving at 121°C for 15 min, and the medium was sparged with oxygen-free nitrogen before use to ensure anaerobic condition. An inoculum of (10% v/v) *Clostridium* spp. were cultivated in TYA medium using 20 g/L glucose as a carbon source in 55 mL serum bottle with a 55 mL working volume. The cultures were incubated at 37°C with agitating in an incubator under anaerobic conditions for 144 h. The sample was taken every 24 h for growth organic acid and ABE analysis. The butanol yield and productivity was calculated. The strain that gave high butanol was selected.

1.2 ABE production from butyric acid by solventogenesis phase

Clostridium spp. were cultivated in TYA N-free medium using only butyric acid as a precursor to be converted to butanol. The culture was carried out as described in 1.1. The ABE production from various concentration was compared.

1.3 ABE production from glucose and butyric acid by solventogenesis phase

Clostridium spp. were cultivated in TYA N-free medium using 10 g/L of glucose and 1 g/L butyric acid as a precursor to be converted to butanol. The culture was carried out as described in 1.1.

1.4 ABE production from glucose and butyric acid in various concentration by solventogenesis phase

Clostridium spp. were cultivated in TYA N-free medium using glucose and butyric acid as a precursor to be converted to butanol. There were 20 sets of experiments using different glucose concentrations of 0, 10, 15 and 20 g/L and butyric acid concentration of 0, 1, 5, 10 and 15 g/L. The culture was carried out as described in 1.1. The ABE production from various sugar was compared.

1.5 ABE production from various sugars by solventogenesis phase

The suitable strain from 1.1 was cultivated in two different modes; conventional mode in TYA medium and solventogenesis mode in TYA N-free medium. The carbon sources used were: monosaccharides (glucose, xylose and galactose), disaccharides (sucrose, lactose and maltose) and sugar alcohols (mannitol, xylitol and inositol). The sugars and butyric acid (BA) concentrations used were 15 g/L. The culture was carried out as described in 1.1. The ABE productions from various sugars was compared.

1.6 ABE production from various acids by solventogenesis phase

The suitable strain from 1.2 was used in this study. The effect of acids on ABE production by solventogenesis phase was investigated using 1, 5, 10 and 15 g/L of either formic, acetic, propionic and butyric acids mixed with 15 g/L of glucose. The culture was carried out as described in 1.1. The strain that gave high butanol was selected.

Part II ABE production from decanter cake hydrolysate (DCH)

2.1 Hydrolysis of decanter cake

2.1.1 Effect of type of chemical used in hydrolysis

Fifty grams of decanter cake was soaked in 500 mL of 500 mM hydrochloric acid (HCl), nitric acid (HNO_3), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), potassium hydroxide (KOH), and calcium hydroxide ($Ca(OH)_2$) in 1-L flask for 15 minutes with agitation at 150 rpm followed by autoclaving at 121°C and 15 psi for 60 min. All the hydrolysate was separated from the mixture by vacuum filtration and measured for total sugar, furfural, 5-HMF and soluble lignin. The chemical that gives highest amount of total sugar was selected.

2.1.2 Effect of concentration of chemical

The concentration of selected conditions was varied at 250, 500, 750, 1,000, 1,250 and 1,500 mM. The hydrolysis were carried at as described in 2.1.1. The concentration that gave highest amount at total sugars was selected.

2.2 ABE production from undetoxified decanter cake hydrolysate

The experiment to investigate the effect of inhibitors from hydrolysis step on ABE production was conducted. DCH at a final concentration of total sugar of 20 g/L was used. The culture was carried out as described in 1.1. The treatment that gave highest butanol was selected.

2.3 Detoxification of decanter cake hydrolysate (DCH)

2.3.1 Activated charcoal

The hydrolysate was separated from the mixture by vacuum filtration, and then concentrated by evaporation at 100 °C. The concentrated hydrolysate of decanter cake was treated with 7.5 % (w/v) of granular activated charcoal at 30 °C with shaking at 150 rpm for 12 hours and no pH adjustment. The activated charcoals was separated from decanter cake hydrolysate by vacuum filtration. All hydrolysates was stored at -20°C and used within 2 weeks. Prior to fermentation, the concentrated hydrolysate was adjusted to pH 6.5±0.5 with $Ca(OH)_2$. The precipitate formed was removed by centrifugation at 8,000 g for 15 minutes (Wang and Chen, 2011).

2.3.2 Overliming

The pH of the hydrolysate from which solid are removed by vacuum filtration was adjusted to 10 with $Ca(OH)_2$ (called overliming). The mixture was heated 90 °C for 30 min with intermittent mixing. This is followed by cooling to room temperature and adjusted pH to 6.5 with HCl. The hydrolysate was then filter by filter paper (Qureshi *et al*., 2010a).

2.4 Effect of organic nitrogen source

The experiment to investigate the effect of type of organic nitrogen source at a concentration of 1 g N/L (nitrogen containing compound were analyzed by the Kjedal method) was carried out using an organic nitrogen source such as yeast extract (9.18 g/L), whey protein (6.78 g/L), isolated soy protein (5.05 g/L) and urea (2.19 g/L). The culture was carried out as described in 1.1. The organic nitrogen source that gave highest butanol was selected.

2.5 Effect of inorganic nitrogen source

The experiment to investigate the effect of different types of inorganic nitrogen source (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, and NH_4COOCH) mixed with the best organic nitrogen source from 2.3.2 was carried out. The culture was carried out as described in 1.1. The inorganic nitrogen source that gave highest butanol was selected.

2.6 Effect of C/N ratio

The experiment to the effect of nitrogen concentration (ranging from 1 to 16 g N/L) was carried out using a mixture of suitable organic and inorganic nitrogen sources. The culture was carried out as described in 1.1. The nitrogen source concentration that gave highest butanol was selected.

2.7 Response surface methodology for ABE production using the detoxified DCH as a co-substrate with butyric acid

In this study, DCH was also used as a co-substrate with butyric acid in a nitrogen-free medium for butanol production. The effect of the concentration of sugar from DCH and butyric acid on butanol production, were investigated using response surface methodology (RSM). A central composite design (CCD) with two variables at three levels was followed to determine the response pattern and also to determine the synergy of the variables. According to this design, 13 runs were conducted containing five replications at the central point for estimating the purely experimental uncertainty variance. The relationship of the variables was determined by fitting a second order polynomial equation to data obtained from the13 runs. A uniform design and analysis of the data were carried out. The response surface analysis was based on the multiple linear regressions taking into account the main, quadratic and interaction effects, in accorded with the following equation:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x^2_i + \Sigma\beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, x_i and x_j represent the variables or parameters, β_0 is the offset term, β_i is the linear effect, β_{ij} is the first order interaction effect and β_{ii} is the squared effect. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Response surface plots

were developed to indicate an optimum condition by using the fitted quadratic polynomial equations obtained.

2.8 ABE production from DCH in the bioreactor

The pre-culture was conducted in a 100 mL serum vial, while a 1000 mL glass bottle (Schott Duran, Germany) was used for the main fermentation. The pre-cultured cells in exponential growth (24 h) were inoculated (10% v/v) into the main fermentation. *C. beijerinckii* TISTR 1461 was cultivated at 37ºC, 120 rpm, and pH 6.0 without pH-control in 700 mL medium contained in the 1000 mL bioreactor.

Media, bioreactor, bottles, vacuum filters, and other materials were sterilized at 121ºC for 15 min. Glucose and DCH were autoclaved separately from the remaining medium components. After autoclaving, glucose and other media components were mixed, capped with a rubber stopper and flushed with filter-sterilized nitrogen gas (passing through a 0.2 µm membrane filter) before inoculation.

2.9 ABE fermentation with gas stripping for butanol recovery

For the batch and two-stage fermentation with gas stripping, gas stripping was initiated from about 36 h by recycling oxygen free N_2 gas through the system to create gas bubbles in the culture using a twin-head peristaltic pump. The ABE vapors were cooled (to 5°C) in a condenser which had been previously fluxed with oxygen free N_2 gas. The stripped ABE was collected into the solvent collector (125 mL flask). To maintain a constant liquid level inside the reactor, O_2-free distilled water was regularly added to the reactor (as some water was lost due to gas stripping). Sample were withdrawn at intervals for amylase, organic acids and ABE analysis (Ezeji *et al*., 2004)

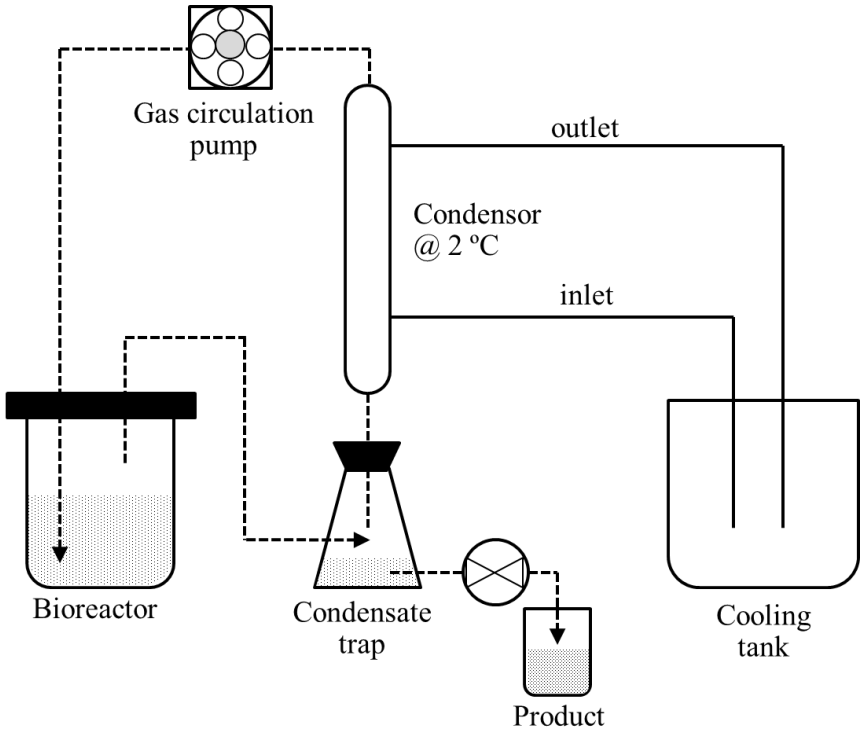

Figure 3. The integrated fermentation system with gas stripping for online butanol recovery.

Part III ABE production by immobilized cells of *Clostridium* spp.

3.1 Sample preparation and morphological analysis

Oil palm shell (OPS), palm press fiber (PPF), empty fruit bunch (EFB) and oil palm frond (OPF) were evaluated for cell immobilization and compared to that of free cells. Each carrier was cut to nominally 1-2 cm length and wash thoroughly with tap water until the washings was clean and colorless. Dry at 50 °C (Singh *et al*., 2013). The morphology of immobilization support matrices were analyzed by scanning electron microscope (SEM). The samples were then washed three times with 0.1 M phosphate buffer saline (PBS) solution and incubated in 2.5% glutaraldehyde in PBS for 2 h at 4 °C. After washing the samples 3 times with 0.1 M PBS, specimens were dehydrated using graded ethanol washes (50, 70, 80, 90, 95, and 100%, 15 min each), and critical-point dried. The samples were gold sputter coated and viewed using an FEI Quanta 400 (SEM-Quanta).

3.2 Cells immobilization

The vegetative growing cells of selected *Clostridium* sp. from 1.2 was harvested by centrifugation at 4,000 rpm for 15 minutes in sterile centrifuge tube washed with sterile 0.85% NaCl. The cell was resuspended in tris-acetate buffer (pH 6.5) and was used for immobilization. The fermentation and cell immobilization processes was performed simultaneously. No additional immobilization process was performed in this study. In the batch of cells immobilized on carier, 5 g of carrier was placed into a serum bottle 100 mL with 25 ml of fermentation medium (including 20 g/l of glucose) was placed in a serum bottle for cell cultivation. The fermentation medium were as described above. During fermentation the sample was taken every 24 h (2 mL) for organic and ABE analysis.

3.3 Repeated-batch fermentation on different support materials

To conduct the solventogenesis phase for ABE production using suitable immobilized cells (from 3.1), batch experiment was carried out in 100 mL serum bottle containing 70 mL of TYA N-free medium containing DCH and butyric acid at suitable concentration from 2.3.5 and 30 mL of immobilized cells (from 3.1). The cultures was incubated at 37°C with agitating in an incubator under anaerobic conditions.The sample was taken every 24 h for organic and ABE analysis. The repeated batch operation was performed, following the study of Yen *et al*. (2011), using a fill and draw operation which was carried out in a serum bottle containing 70 mL of TYA N-free medium containing 15 g/L of DCH and 10 g/L of BA from TYA N-free medium (DCH as the carbon source) and 30 mL of carrier for each batch. The supernatant was removed by syringe at the end of each cycle (keeping the cell-immobilized brick pieces inside the serum bottle). After the removal of the supernatant, 70 mL of fresh fermentation medium was added in order to commence the next cycle. The cultures was incubated at 37°C for 24 h with agitating in an incubator under anaerobic conditions for 120 h. The sample was taken every 24 h for organic and ABE analysis.

3.4 Effect of OPF amount on ABE production

OPF pieces at the optimum size (from Section 3.1.1) were prepared and they were sterilized as previously mentioned. The sterile OPF were added into fresh TYA medium containing different amount of OPF (3, 5 and 7 g). The immobilization was performed as previously described.

3.5 Two-stage fermentation

After a certain period of H_2 production in stage-II, before reaching the plateau of butanol production rate, the immobilized cells were again returned to acidogenic growth condition (stage-I) by decanting the TYA medium and filling the reactor with the N-free medium. After a certain period of time in stage-I when cell were supposed to have acquired a good ability, they were returned back to the solventogenic phase (stage-II).

3.6 Two-stage fermentation of DCH in bioreactor

The experimental setup with a two-stage bioreactor was carried out with OPF as the support material. The bioreactors contained OPF, packed in a nylon mesh basket which served as a carrier material. Fresh medium was continuously introduced into R1 with the help of a peristaltic pump. The bioreactors were inoculated with 10% v/v highly motile cells of *C. beijerinckii* TISTR 1461. Fermentation was allowed to proceed in the batch mode for 24 h, after which fermentation feed medium was continuously pumped into bioreactor. All the bioreactor were maintained at 37 °C and agitated at 150 rpm using a magnetic stirrer.

3.7 Two-stage fermentation integrated with gas-stripping

The experimental setup with a two-stage bioreactor was carried out with OPF as the support material. For the batch and two-stage fermentation with gas stripping, gas stripping was initiated from about 36 h by recycling oxygen free N_2 gas through the system to create gas bubbles in the culture using a twin-head peristaltic pump. The ABE vapors were cooled (to 5°C) in a condenser which had been previously fluxed with oxygen free N_2 gas. The stripped ABE was collected into the solvent collector (125 mL flask). To maintain a constant liquid level inside the

reactor, O_2-free distilled water was regularly added to the reactor (as some water was lost due to gas stripping). Sample were withdrawn at intervals for amylase, organic acids and ABE analysis (Ezeji *et al*., 2004)

Analytical methods

1. Growth measurement

The concentration of *Clostridium* spp. in the fermentation broth was measured at 600 nm (OD600) using an ultraviolet–visible spectrophotometer (Libra S22, Biochrome, UK) (Monot *et al*., 1982).

2. Determination of products

Acetone, butanol, ethanol, acetic acid and butyric acid was analyzed with gas chromatography (Agilent technology 7890A Network GC System) equipped with a HP-INNOWAX column 19091N-133 (30m × 250 μm × 0.25 μm, Agilent technologies) and flame-ionized detector. The oven temperature was programmed to increase from 50 to 180°C and the rate of 10 °C/min. The injector and detector temperature was set to 240 °C. Helium was used as a carrier gas with a flow rate of 1 mL/min (Marino *et al*., 2001).

3. Determination of sugar

Glucose, xylose and arabinose was determined by high-performance liquid chromatography (Agilent 1200 HPLC, Agilent Technologies, USA) with a Rezex ROA Organic Acid H^+ organic acid column (5 μm, 7.8 mm × 300 mm; Phenomenex, Macclesfield, UK) and a reflextive index detector. The mobile phase is HPLC grade water at a flow rate of 0.6 mL/min at 35 °C. The mobile phase is 0.005 M sulfuric acid (Zautsen *et al*., 2008).

4. Determination of furfural and hydroxymethylfurfural (HMF)

Concentration of furfural and 5-hydroxymethylfurfural (HMF) in the decanter cake hydrolyzate was determined with spectrophotometer. The absorbance of furfural and HMF were 276 and 282, respectively. The concentrations of furans in the samples was calculated using standard of known concentration (Zhang *et al*., 2011).

5. Determination of Soluble Lignin Degradation Compounds (SLC) concentrations

SLC was identified by ultraviolet spectra and estimated by the method of Mussatto and Roberto (2006). The pH of samples was distilled properly with pH 12.0 NaOH solution before they was analyzed at 280 nm using a UV spectrophotometer. The SLC concentration was calculated according to the equations

$$SLC = 4.187 \times 10^{-2} (A_{LIG280} - A_{PD280}) - 3.279 \times 10^{-4}$$

$$A_{PD280} = (C_F \varepsilon_F) + (C_{HMF} \varepsilon_{HMF})$$

Where SLC is the soluble lignin degradation compounds concentration (g/L); A_{LIG280} is the absorbance reading at 280 nm after correction for dilution; C_F and C_{HMF} are the concentrations (g/L) of furfural and HMF determined by spectrophotometric method; ε_F and ε_{HMF} are the extinction coefficients (L/g cm) of furfural (146.85) and HMF (114.00).

6. Total sugar

The total sugar present in DCH was estimated by Dubois method (1956) by adding 2 mL of sample and 1.0 mL of 5% phenol solution, and mixing gently by vortexing, making sure that the film of lipid at the bottom of tube is undisturbed. The mixture was added with 5 mL of conc. sulfuric acid, vortexed and then heated for 5 min in a boiling water bath and allowed to be cool for 30 min. The absorbance of the orange color at 490 nm was against a reagent blank. The total sugar concentration was calculated using standard curve of glucose.

7. Calculation of the yield and productivity

The equation for calculation of the yield of butanol to carbon source ($Y_{butanol/carbon}$) was modified from Tashiro *et al*. (2007) as follows:

$$Y_{butanol/carbon} = (C_{butanol} \times 4) / (C_{butyrate} \times 4 + C_{glucose} \times 6)$$

Where $C_{butanol}$ is the production of butanol (mM) and $C_{butyrate}$ and $C_{glucose}$ are the consumption of butyrate (mM) and glucose (mM), respectively.

Butanol yield to glucose utilization (g-butanol /g-glucose) = P/S

Where P is the butanol production (g/L) and S is the glucose utilization (g/L).

Butanol productivity (g/L/h) was calculated as butanol produced (g/L) divided by the time of fermentation.

8. Calculation of C/N ratio

The equation for calculating C/N ratio was as follows:

C/N ratio = Carbon in carbon source concentration (g/L)/nitrogen content in nitrogen source (g/L)

The nitrogen content in each organic nitrogen source was determined by Kjedahl method.

CHAPTER 4

RESULTS AND DISCUSSION

1. Screening of high butanol producing *Clostridium* sp.

A number of systematic studies (Keis *et al*., 2001; Johnson *et al*., 1997) have revealed that the industrial solvent producing clostridia comprise three species (*C. acetobutylicum*, *C. beijerinckii*, and *C. butyricum*). All of the original starch-fermenting strains were found to belong to a single specie of *Clostridium acetobutylicum*. This species is phylogenetically distinct and only very distantly related to the other three more closely related species containing the later generation of sugar-fermenting industrial strains. The majority of these saccharolytic strains have been identified as belonging to the *Clostridium beijerinckii* species which contains at least 17 distinct sub-groups based on genomic DNA/DNA hybridisation and DNA fingerprint patterns. Existing strains belonging to the other saccharolytic species can both be sub-divided into two genomic DNA fingerprint groups (Keis *et al*., 1995).

The aim of this study was to establish media supplemented with glucose that would allow the various strains of solvent-producing clostridia produce high yields of solvents under laboratory conditions The solvent-producing abilities of different strains of *C. acetobutylicum*, *C. beijerinckii*, and *C. butyricum* were compared. In order to obtain results that could be compared directly it was necessary to use a common set of conditions. While the particular set of culture conditions utilized for comparative studies might be close to optimum for some species and strains, it has to be accepted that it is unlikely that the specific conditions used would be optimal for all strains tested.

1.1 ABE production from glucose

To facilitate comparative fermentation studies, a TYA medium that would enable most strain to produce consistent and opposite concentration and yield was required. TYA medium having glucose as a carbon source (Hongo and Murata, 1965) was designated as an adaptable fermentation medium that supports a good

solventogenic fermentation by various strains tested. The control fermentation was conducted using TYA medium containing 20 g/L of glucose. Fermentation was started with the pH of 6.5 and the amount of solvent and acid produced, the media pH and total biomass accumulated in the system were observed during 168 h of fermentation (Figure 4). All strains first entered the acidogenesis phase and this was accompanied by a decrease in the media pH to 4.76 - 5.69 during 24 h after the start of fermentation.

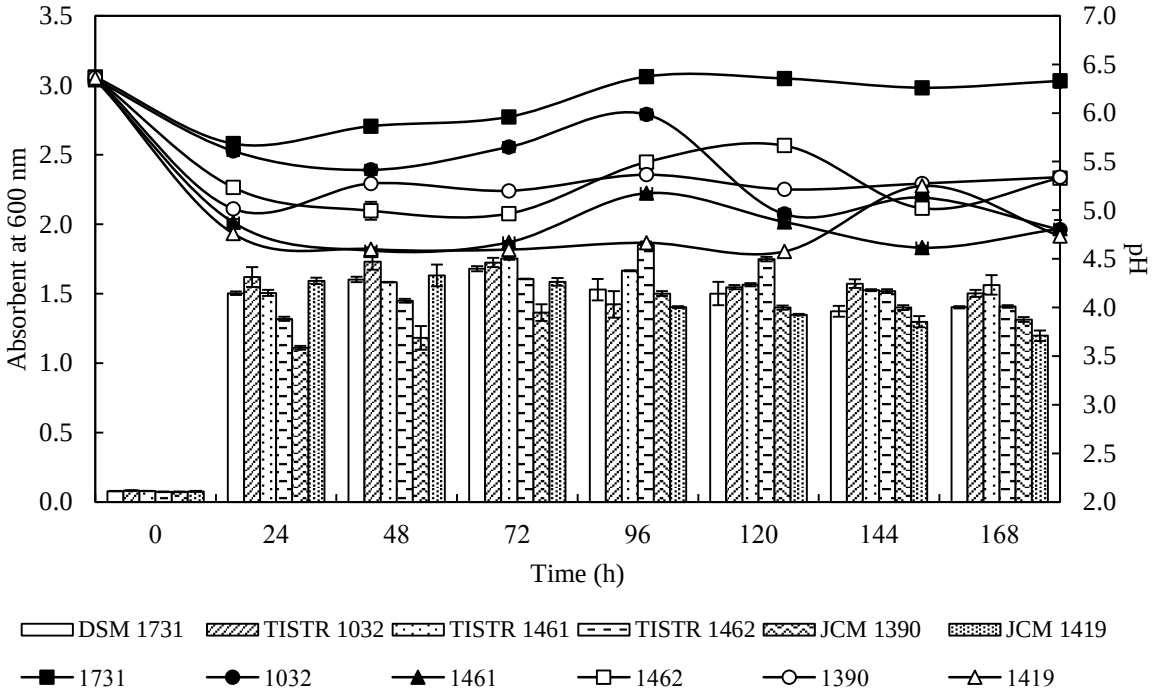

Figure 4. Growth (bars) and pH (lines) profile of pure culture of *Clostridium* spp. incubated using TYA medium containing 20 g/L glucose (◆) *C. acetobutylicum* DSM 1731, (■) *C. butyricum* TISTR 1032, (▲) *C. beijerinckii* TISTR 1461, (●) *C. acetobutylicum* TISTR 1462, (△) *C. beijerinckii* JCM 1390, (○) *C. acetobutylicum* JCM 1419.

Butanol can only be generated when the cells enter sporulation process but this discourages cell growth (Terracciano and Kashket, 1986). Therefore, balancing cell growth and sporulation timing is a key factor to influence the carbon flow towards cell growth or butanol generation. Most importantly, keeping the cells in

pre-sporulation state (an optimum pH at 5.5) is necessary to ensure constant butanol generation and to maximize the carbon conversion to final products, since cells cannot reverse from spores to pre-sporulation (Bramono *et al*., 2011). The biomass on cultivation are presented in absorbent at 600 nm are shown in Figure 4.

Fermentation products in clean supernatant of culture broth of *Clostridium* spp. grown on glucose are shown in Figure 4. Only *C. beijerinckii* TISTR 1461 (Figure 5b) and *C. butyricum* TISTR 1032 (Figure 5d) produced high concentration of butanol above 7 g/L. While other four strains did not perform well in the TYA medium. TYA medium has been selected as a general laboratory culture medium for comparative studies in the literature but was not ideal for all strains.

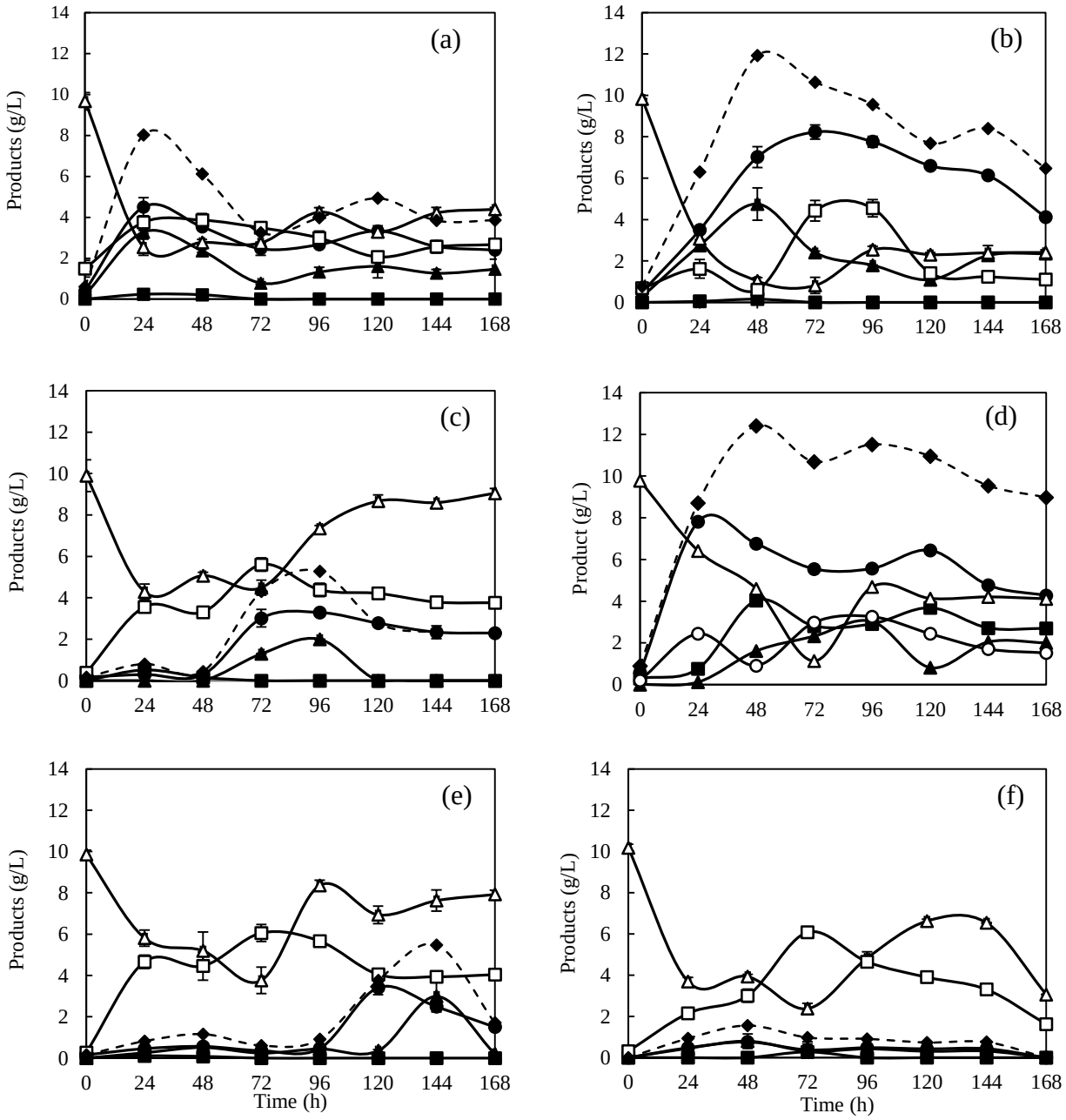

Figure 5. Product formation by pure culture of *Clostridium* spp. incubated in TYA medium using 20 g/L glucose (a) *C. acetobutylicum* DSM 1731, (b) *C. beijerinckii* TISTR 1461, (c) *C. acetobutylicum* TISTR 1462, (d) *C. butyricum* TISTR 1032, (e) *C. acetobutylicum* JCM 1419, (f) *C. beijerinckii* JCM 1390 ((■) acetone ; (●) butanol; (▲) ethanol; (△) acetic acid; (○)butyric acid); (◆) ABE)

1.2 ABE production from butyric acid by solventogenesis phase

As butyric acid is one of the factors required for triggering the onset of solventogenesis, it use as a sole precursor for direct conversion to butanol was investigated. In this study, three species of the most commonly used clostridia for butanol production including *C. acetobutylicum* (Terracciano and Kashket, 1986; Qureshi *et al*., 2001; Dürre and Hollergschwandner, 2004), *C. beijerinckii* (Chen and Blaschek, 1999), and *C. butyricum* (Tran *et al*., 2010) were each cultivated in a nitrogen-free medium. Their cells in stationary phase activities for direct conversion of butyric acid to butanol were tested (Figure 6). The control was the result obtained from cells in stationary phase incubated in a nitrogen-free medium without butyric acid (0 g/L). When 1 g/L of butyric acid was added to the medium, only *C. acetobutylicum* DSM 1731 and *C. beijerinckii* TISTR 1461 produced a significant conversion of butyric acid to butanol and acetone. When the butyric acid was increased up to 5 g/L, the butanol production by *C. acetobutylicum* DSM 1731 was increased up to 0.79 g/L with a butanol ratio of 48 % (Figure 6a). Similarly, the butanol production by *C. beijerinckii* TISTR 1461 was also increased up to 0.83 g/L with a butanol ratio of 52 % (Figure 6c). The consumption of substrate (butyric acid) was also increased with increasing the butyric acid concentration. The butanol yield from substrate was 0.28 and 0.29 C-mol/C-mol for *C. acetobutylicum* DSM 1731 and *C. beijerinckii* TISTR 1461, respectively. However, increasing the butyric acid concentration up to 10 g/L enhanced only the production of acetone but not butanol. This would be the enhanced activity of CoA transferase that produces butanol couple with acetone (Richta *et al*., 2012). At this butyric acid concentration, the ABE production by *C. acetobutylicum* DSM1731 and *C. beijerinckii* TISTR 1461 reached the maximum levels of 1.86 and 1.88 g/L, respectively, but with lower butanol ratios of 34 and 31 %, respectively. When the butyric acid concentration was increased up to 15 g/L, the ABE production by both *C. acetobutylicum* DSM 1731 and *C. beijerinckii* TISTR 1461 were decreased. In the case of *C. butyricum* TISTR 1032 and *C. beijerinckii* JCM1390, the butanol production was increased up to 0.77 and 0.69 g/L, respectively, along with an increase in the butyric acid concentration up to 15 g/L (Figure 6b, d).

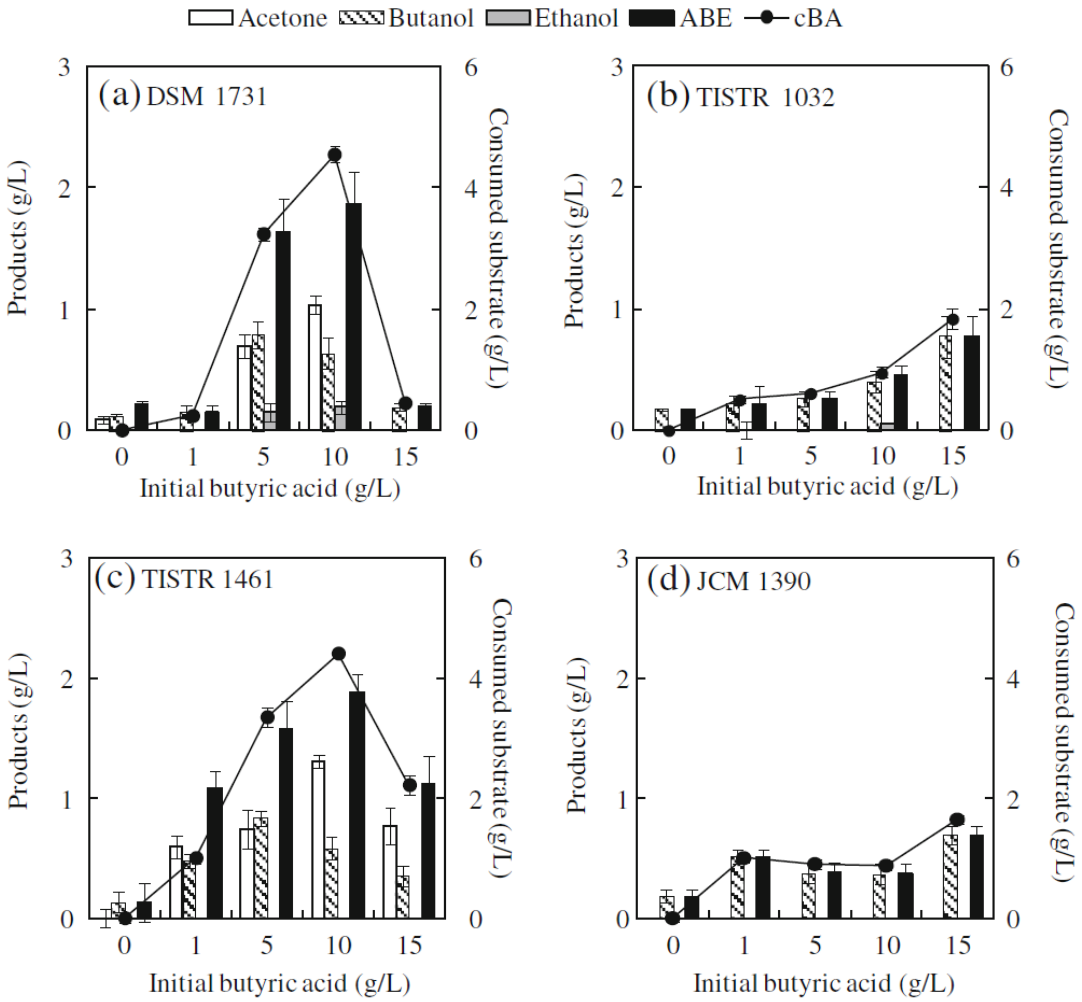

Figure 6. Comparison of ABE production from butyric acid (BA) and consumption of butyric acid (cBA) by cells in stationary phase of *Clostridium* spp. incubated for 72 h in a nitrogen-free medium in the absence of glucose. a *C. acetobutylicum* DSM 1731, b C. butyricum TISTR 1032, c *C. beijerinckii* TISTR 1461, and d *C. beijerinckii* JCM 1390

Al-Shorgani *et al*. (2007) reported that the addition of 1 g/L of butyric acid not only triggered the solventogenesis pathway but also consumed as a substrate to produce butanol. However, when using only butyric acid without glucose, the cells hardly utilized butyric acid and produced only small amount of butanol. One possibility would be an insufficient amount of energy-rich metabolites such as ATP or NADH to support the assimilation of acids during the solventogenesis phase (Shinto *et al*., 2007). It has been reported that the butanol could be efficiently produced from butyric acid when those energy-rich metabolites were supplied by other metabolic processes such as from glucose (Dürre and Hollergschwadner, 2004; Tashiro *et al*., 2004). Therefore, in the study of Al-Shorgani *et al*. (2007) they found that when glucose was added, the cells in stationary phase produced more butanol from butyric acid.

1.3 ABE production from glucose and butyric acid by solventogenesis phase

As mentioned above, since it has been reported that the efficient conversion of butyric acid to butanol requires energy-rich metabolites supplied by glucose metabolism; in this experiment, 10 g/L glucose was added as a co-substrate together with butyric acid in a nitrogen-free medium (Figure 7). In the presence of glucose, all strains produced higher amounts of butanol compared to those without glucose (Figure 6). It should be noted that butanol production was also observed in the nitrogen-free medium containing only glucose as a sole substrate (see Figure 6 at 0 g/L initial butyric acid). Especially by the cells in stationary phase of *C. beijerinckii* TISTR 1461, butanol concentration as high as 2.36 g/L was produced from 10 g/L glucose without the addition of butyric acid (Figure 7c). Using a combination of 10 g/L glucose with 1 g/L butyric acid, the butanol production by *C. beijerinckii* TISTR 1461 was enhanced up to 4.77 g/L. The butanol production by *C. acetobutylicum* DSM 1731 was also enhanced up to 2.29 g/L. However, a lesser effect was observed for *C. butyricum* TISTR 1032 and *C. beijerinckii* JCM 1390. Since the increase in butanol production by *C. beijerinckii* TISTR 1461 (+2.41 g/L) was much higher than the amount of butyric acid added (1 g/L) (Figure 6c), this indicated that the added butyric acid did work well as a triggering factor for solventogenesis (Kalii *et al*., 2006) and enhanced the conventional fermentation of glucose to butanol. When the

butyric acid concentration was increased up to 15 g/L, the butanol production was further enhanced up to the maximum level of 4.84, 6.84, and 5.51 g/L for *C. acetobutylicum* DSM 1731 (Figure 7a), *C. butyricum* TISTR 1032 (Figure 7b), and *C. beijerinckii* JCM 1390 (Figure 7d), respectively, but not for *C. beijerinckii* TISTR 1461. The butanol production by *C. beijerinckii* TISTR 1461 slightly decreased at 15 g/L butyric acid. This could be due to the inhibitory effect of butyric acid at high concentrations (Maddox *et al*., 2000).

The butyric acid consumption was increased with increasing initial butyric acid concentration up to 15 g/L for all strains. However, the glucose consumption decreased when the initial butyric acid was increased higher than 10 g/L. It should be noted that among the four strains tested, *C. beijerinckii* TISTR 1461 utilized all substrates (10 g/L glucose and 10 g/L butyric acid) and produced the highest amount of butanol (7.87 g/L). This gave a butanol yield of 0.54 C-mol/C-mol. It was also of interest to note that in the presence of glucose *C. acetobutylicum* DSM 1731 (Figure 7a) and *C. beijerinckii* TISTR 1461 (Figure 7c) produced butanol four- to five folds higher than acetone. Therefore, the butanol ratios in the ABE product were as high as 80–99 % while those without glucose were only 30–50 %. In the case of *C. butyricum* TISTR 1032 (Figure 7b) and *C. beijerinckii* JCM 1390 (Figure 6d), the butanol ratios were >99 % either with or without glucose. The selectivity for butanol formation could be explained by two different mechanisms for conversion of butyric acid to butanol, the CoA transferase pathway with acetone formation, and the *buk/ptb* pathway without acetone formation (Richter *et al*., 2012). The results suggested that in the presence of glucose, the latter pathway might be more enhanced than the former pathway, and in the case of *C. butyricum* TISTR 1032 and *C. beijerinckii* JCM 1390, the former pathway might have not been used. On the contrary, the only strain that has been used for the direct conversion of butyric acid to butanol, *C. saccharoperbutylacetonicum* N1-4 produced no acetone and ethanol in the absence of glucose but did in the presence of glucose (Al-shargoni *et al*., 2012).

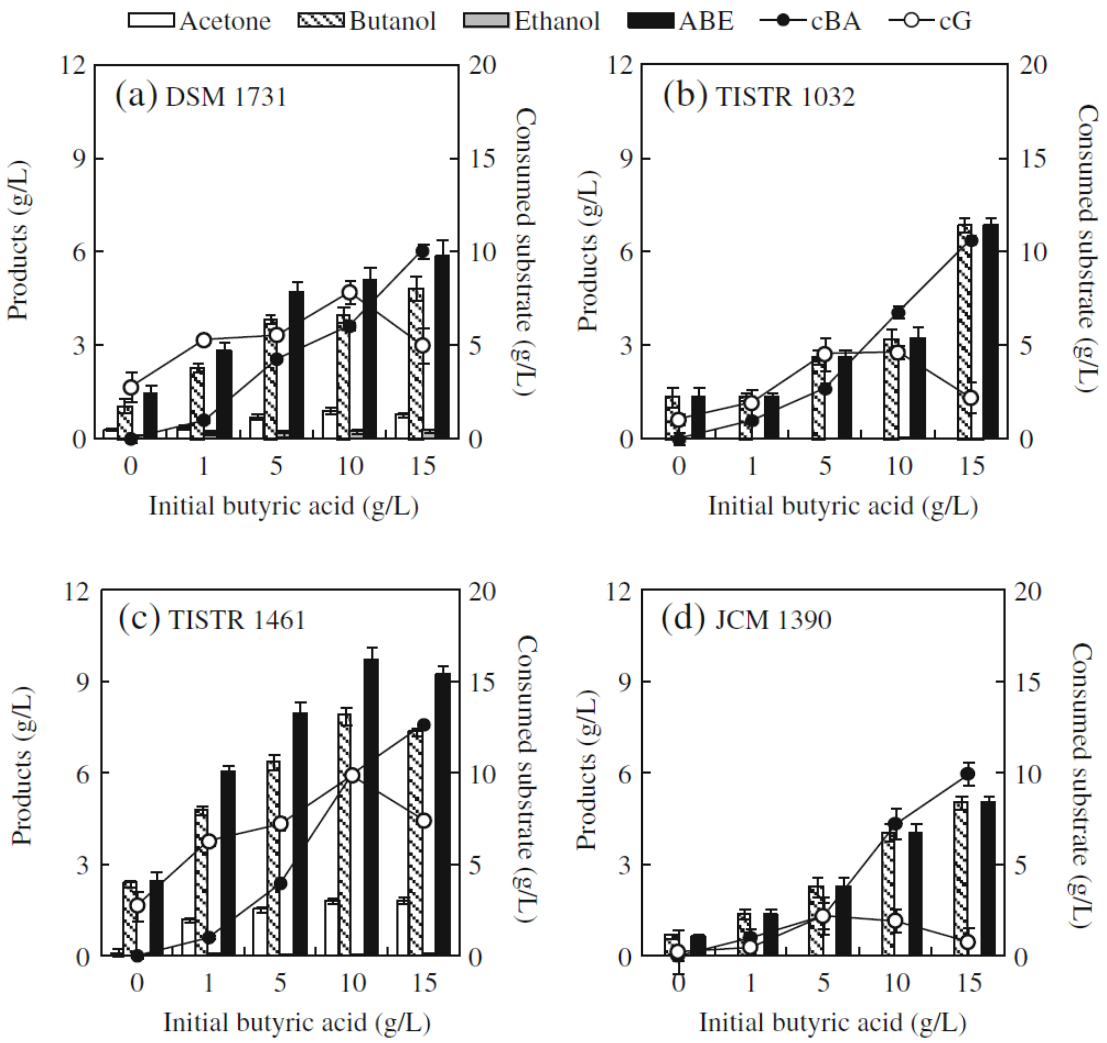

Figure 7. Comparison of ABE production from butyric acid (BA) and glucose (G), consumption of butyric acid (cBA), and consumption of glucose (cG) by cells in stationary phase of *Clostridium* spp. incubated for 72 h in a nitrogen-free medium in the presence of glucose (10 g/L). a *C. acetobutylicum* DSM 1731, b *C. butyricum* TISTR 1032, c *C. beijerinckii* TISTR 1461, and d *C. beijerinckii* JCM 1390

Among the four strains tested, it could be concluded that the cells in stationary phase of *C. acetobutylicum* DSM 1731 and *C. beijerinckii* TISTR 1461 had the high potential for converting butyric acid and glucose to butanol just as they did in the absence of glucose. The results also showed that it was possible to directly convert glucose to butanol by the cells in stationary phase of clostridia except by *C. beijerinckii* JCM 1390. The cells in stationary phase of *C. beijerinckii* JCM 1390 could not produce butanol from glucose as a sole substrate, but it could produce butanol (0.69 g/L) from butyric acid without the addition of glucose (Figure 7d). With the addition of 10 g/L glucose, it produced butanol up to 4.6 from 15 g/L butyric acid (Figure 7d). The less glucose consumption by *C. beijerinckii* JCM 1390 compared with those by other strains suggested that its butanol production might likely originate through the solventogenesis of butyric acid rather than the conventional fermentation of glucose.

1.4 ABE production from glucose and butyric acid in various concentration by solventogenesis phase

To investigate the synergistic effect of glucose and butyric acid concentrations on butanol production by the cells in stationary phase of *C. beijerinckii* TISTR 1461, the concentration of glucose was varied in the range of 0–20 g/L with a combination of butyric acid concentration at 1, 5, 10, and 15 g/L (Figure 8). At 1 g/L butyric acid, an increase of glucose concentration from 10 g/L up to 15 and 20 g/L had no effect on the production of butanol (Figure 8a). Although butyric acid was completely consumed, the glucose consumption was not complete. At 5 g/L of butyric acid, with an increase of glucose concentration from 10 to 15 g/L (Figure 8b), the butanol production increased from 6.34 to 8.34 g/L (a 31 % increase). As 14.2 g/L of glucose and all butyric acid were consumed, a butanol yield obtained was 0.64 C-mol/Cmol. At 10 g/L butyric acid, with an increase of glucose concentration from 10 to 15 g/L (Figure 8c), the butanol production increased from 7.86 to 12.0 g/L (a 35 % increase) with complete substrate utilization. This gave the maximum butanol yield of 0.68 C-mol/C-mol and a high butanol ratio of 88 %.

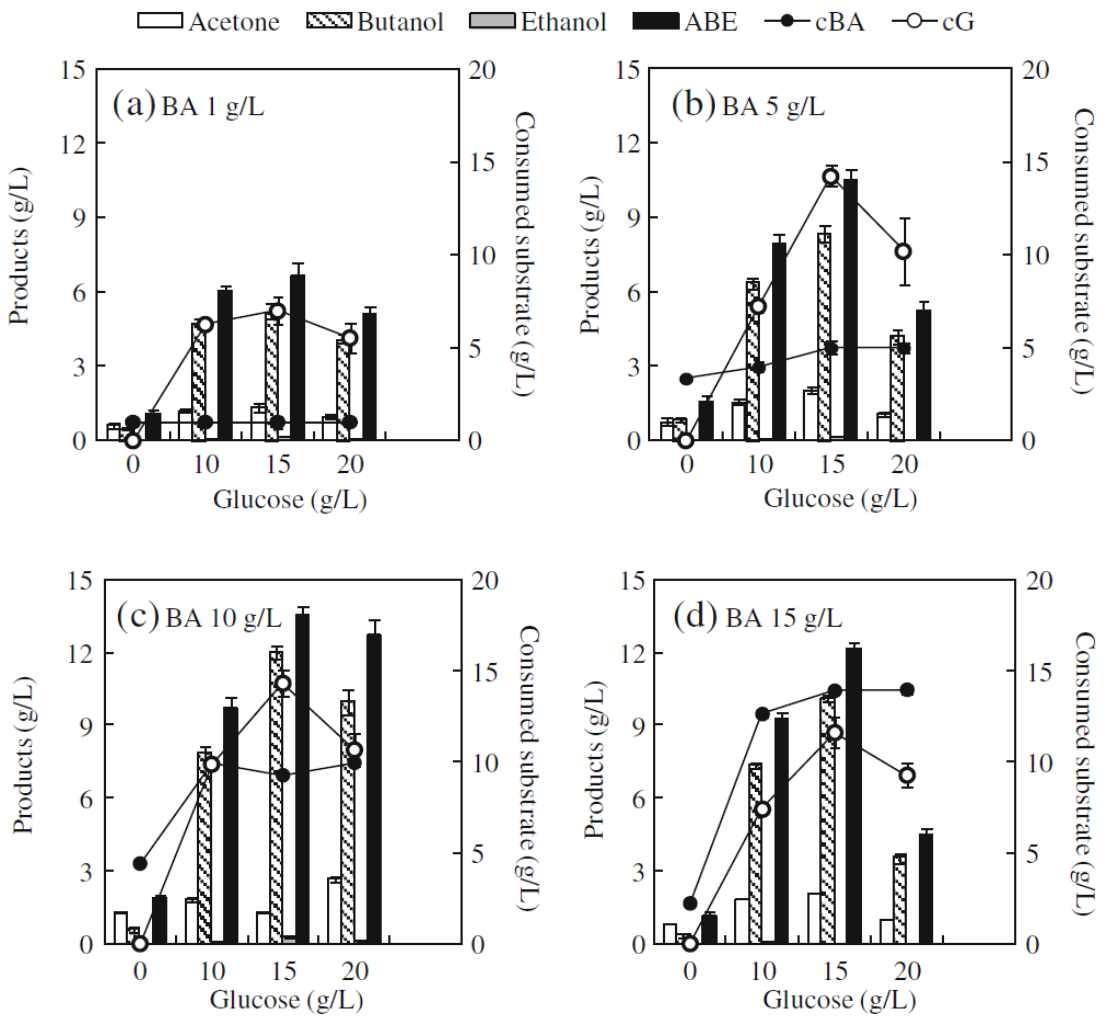

Figure 8. Effect of glucose and butyric acid (BA) concentrations on ABE production, consumption of butyric acid (cBA), and consumption of glucose (cG) by cells in stationary phase of *C. beijerinckii* TISTR 1461 incubated for 72 h in a nitrogen-free medium. a BA 1 g/L, b BA 5 g/L, c BA 10 g/L, and d BA 15 g/L

At a higher butyric acid concentration of 15 g/L, the butanol productions at all glucose concentrations were lower than those at 10 g/L butyric acid. This could be due to an inhibitory effect of high butyric acid concentrations above 10 g/L (Maddox *et al*., 2000). When the glucose concentration was increased up to 20 g/L, the butanol production decreased especially at high concentrations of butyric acid. Typically, the initial sugar concentration that had an adverse effect on butanol production was >100 g/L (Ezeji *et al*., 2004b) However, in the presence of butyric acid at initial time and absence of nitrogen source, the susceptibility of the cells to glucose concentration could be changed.

Table 12 shows the comparison of butanol production using butyric acid as a substrate in this study with those in the literature. Huang *et al*. (2004) used a low concentration of butyric acid (3.5 g/L) as a co-substrate for the continuous production of butanol from glucose by immobilized growing cells of *C. acetobutylicum* ATCC 824. A high butanol yield of 0.68 C-mol/C-mol was obtained. In the study of Lee *et al*. (2008a), they found that when the butyric acid was added at a low concentration to a continuous culture of growing cells of *C. beijerinckii* NCIMB 8052, both butanol production and glucose consumption rates were increased. This was because the butyric acid promoted an earlier shift to the solventogenesis phase. They also found that when using immobilized cells, the butanol yield was improved from 0.388 to 0.713 C-mol/C-mol but the butanol ratio in the ABE was reduced from 75 to 66 %. Richter *et al*. (2012) studied the continuous culture of growing cells of *C. saccharoperbutylacetonicum* N1-4 with a co-feed of butyrate and glucose. They concluded that the slight increase in butanol yield that occurred with the addition of butyrate must have been caused by the stimulation of the conventional ABE fermentation from glucose and not because the added butyrate was converted into butanol.

Baba *et al*. (2011) reported that under a nitrogen source-limited condition, cells survived and had butanol producing ability but there was little proliferation. In their study, a continuous high-cell density system of *C. saccharoperbutylacetonicum* N1-4 was operated at a dilution rate of 0.85 h^{-1} by feeding a nitrogen-free medium containing glucose and butyric acid (20 and 10 g/L, respectively).

Table 12. Butanol production when using butyric acid as a substrate

Strain	Cells	Mode	G/BA (g/L)	$Y_{BtOH/C}$	Butanol (%)	Butanol (g/L)	Reference
C. acetobutylicum ATCC 824	Growing	Continuous	54/3.5	0.68[a] (immo)	66	5.1 (immo)	Huang *et al*., (2004)
C. beijerinckii NCIMB 8052	Growing	Continuous	60/1.58	0.388 (free), 0.713 (immo)	75, 66	7.1 (free), 13.4 (immo)	Lee *et al*., (2008)
C. saccharoper-butylacetonicum N1-4	Growing	Continuous	60/10.52	0.388	-[b]	4.0	Ritcher *et al*., (2012)
C. saccharoper-butylacetonicum N1-4	Non-growing	Continuous	20/10	0.686	82	9.4	Baba *et al*., (2011)
C. saccharoper-butylacetonicum N1-4	Non-growing	Batch	20/10	0.577	20	8.6	Tashiro *et al*., (2007)
C. saccharoper-butylacetonicum N1-4	Non-growing	Batch	20/10	0.99	87	13.0	Al-shagoni *et al*., (2012)
C. beijer-inckii TISTR 1461	Non-growing	Batch	15/10	0.68	88	12.0	This study

The unit of $Y_{butanol/carbon}$ is C-mol/C-mol

G glucose; BA butyric acid

[a] The butanol yield to glucose of 0.42 g/g was converted to 0.68 C-mol/C-mol

[b] Not available

Their system gave the highest butanol yield of 0.686 C-mol/C-mol and a high butanol concentration of 9.4 g/L. This result of Baba *et al*. (2011) was confirmed by Al-Shorgani *et al*. (2012) who studied the influence of varying glucose/butyric acid ratio on butanol yield with the same *Clostridium* strain in a nutrient-limited medium. They reported that the use of a 10 % inoculum of growing cells produced 12.99 g/L butanol from 20 g/L glucose in the presence of 10 g/L butyric acid, while Tashiro *et al*. (2007) produced only 7.5 g/L butanol from the same combination using concentrated washed cells. This study showed that *C. beijerinckii* TISTR 1461 was another strain with a high potential for use to convert butyric acid to butanol since it produced a comparable amount of butanol (12.0 g/L) to that of Al-Shorgani *et al*. (2012) but used a lower amount of glucose (15 g/L).

1.5 ABE production from various sugars by solventogenesis phase

Various sugars, monosaccharides (glucose, galactose, and xylose) and disaccharides (maltose, sucrose, and lactose) were each used as a co-substrate for the direct conversion of butyric acid to butanol by the cells in stationary phase of *C. beijerinckii* TISTR 1461 (Figure 9). The results showed that all sugars could be used as a co-substrate with butyric acid since they gave higher amount of butanol (2–12 g/L depending on the sugar used) than using butyric acid alone (Figure 6c, see BA=10 g/L). Moreover, the use of sugar as a co-substrate also improved the butyric acid consumption and the butanol ratio. Hexose sugars are metabolized via the Embden–Meyerhof–Parnas (EMP) pathway with the conversion of 1 mol of hexose to 2 mol of pyruvate, with net production of 2 mol each of ATP and NADH, while pentose sugars are metabolized via the pentose phosphate (PP) pathway and are converted to pentose-5-phosphate and dissimilated by means of the transketolase (TK)–transaldolase (TA) sequence, resulting in the production of fructose-6-phosphate and glyceraldehyde-3-phosphate (G3P), which then enter the glycolytic pathway (Shinto *et al.*, 2008). Among the sugars tested, glucose was the most suitable sugar for being used as a co-substrate to butyric acid. Since xylose, sucrose, and lactose are the main components of hemicellulosic biomass, molasses, and whey lactose, respectively, the use of these sugars as a co-substrate with butyric acid in a nitrogen-free medium would be another alternative use of these by-products. Although there are several reports of butanol

production from these sugars, the use of these sugars as a co-substrate with butyric acid for butanol production by the cells in stationary phase has not been reported.

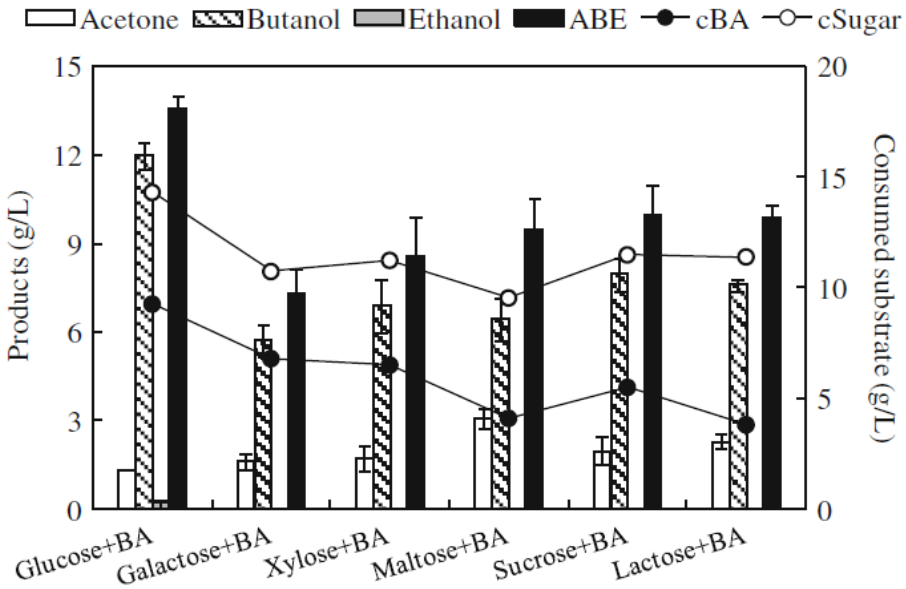

Figure 9. Effect of different sugars on ABE production, consumption of butyric acid (cBA), and consumption of sugar (cSugar) by cells in stationary phase of *C. beijerinckii* TISTR 1461 incubated for 72 h in a nitrogen-free medium containing sugar and butyric acid (BA) at concentrations of 15 and 10 g/L, respectively

1.6 ABE production from various acids by solventogenesis phase

Solid organic waste materials are abundantly present in many agricultural areas. The use of these materials for fuel production would be economically very attractive. The production of fuel from waste materials is considered sustainable as the waste is tuned from an environmental burden into a benefit. Waste materials are, however, often unsuitable for ethanol production as their sugar content is low. In this study, propose butanol production from organic waste materials through biological reduction of volatile fatty acids derived from fermentative biomass acidification. Acidification of waste materials containing lipids, proteins and carbohydrates is a cheap reliable step known from anaerobic digestion. This step within anaerobic digestion produces an effluent containing volatile fatty

acids (VFAs) such as acetic, propionic and butyric acids together with a gas phase containing carbon dioxide and hydrogen (Metcalf and Eddy, 2003). As acidification is a well-known process on the biological reduction of the carboxylic group of a VFA to an alcohol was focused. The aims of this study are to investigate butanol production from VFA using glucose as the co-substrate, to establish a process for high butanol production *C. beijerinckii* TISTR 1461. In addition to butyric acid, several other organic acids including formic, acetic, and propionic acids were tested for their effects on butanol production by the cells in stationary phase of *C. beijerinckii* TISTR 1461 in the presence of 15 g/L glucose (Figure 9). At 1 g/L of organic acid (Figure 9a), butyric acid and acetic acid promoted butanol production from 0.78 g/L of the control (using only 15 g/L glucose) to 3.27 and 3.83 g/L, respectively. All butyric and acetic acids were consumed, but more than 7 g/L of glucose remained. With an increase in the concentration of organic acid up to 5 and 10 g/L (Figure 10b, c), only butyric acid could be efficiently converted to butanol (7.89 and 12.0 g/L, respectively) with complete substrate utilization.

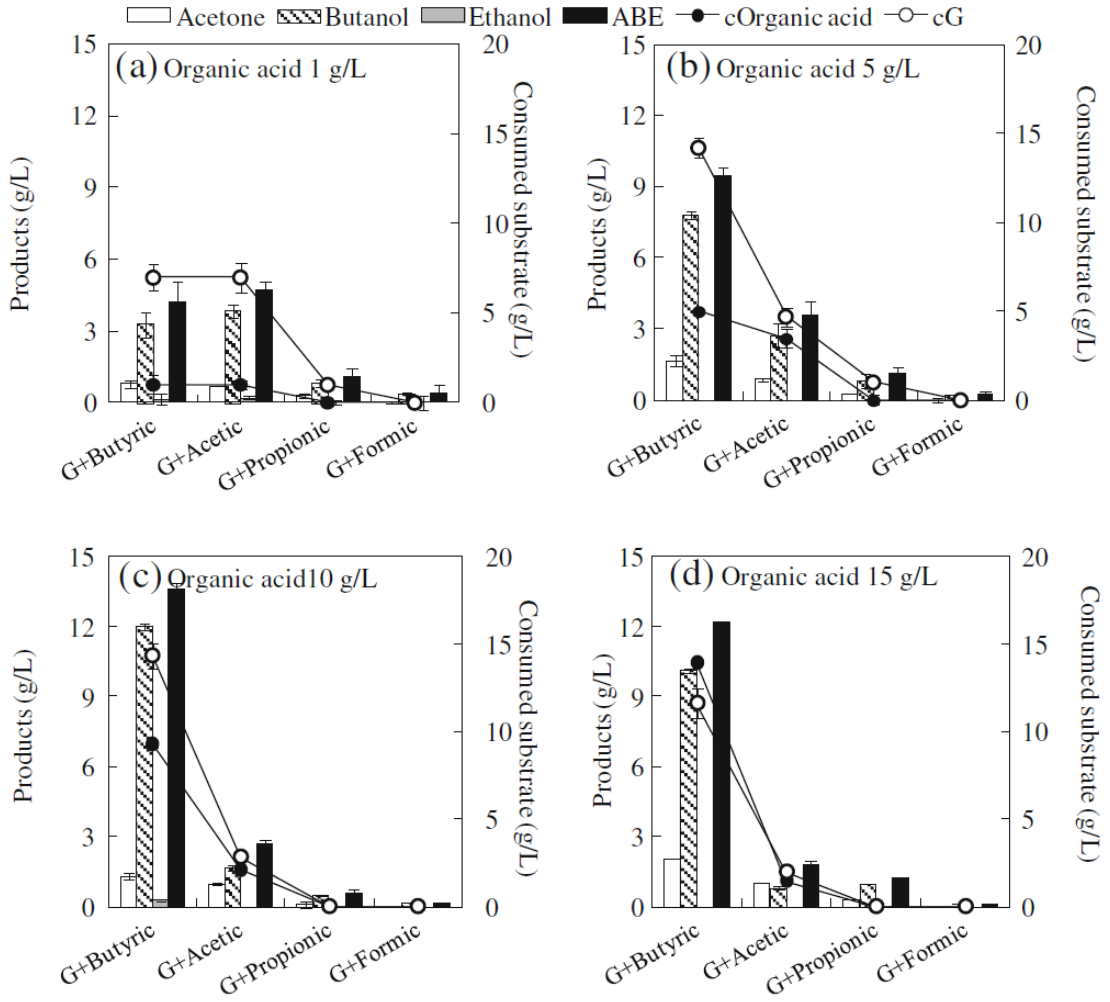

Figure 10. Effect of different organic acids and their concentrations on ABE production, consumption of organic acids (cOrganic acid), and consumption of glucose (cG) by cells in stationary phase of *C. beijerinckii* TISTR 1461 incubated for 72 h in a nitrogen-free medium containing glucose (G) at concentration of 15 g/L. (a) Organic acid at 1 g/L, (b) organic acid at 5 g/L, (c) organic acid at 10 g/L, and (d) Organic acid at 15 g/L

Chen and Blaschek (1999a) had reported the effect of acetate on the solvent production by *C. beijerinckii* NCIMB 8052. Their RNA and enzyme analyses showed that CoA- transferase was highly expressed and has higher activity when the acetate was added. This would have contributed to an increase in acid reassimilation and permitted the direct utilization and conversion of the added acetate to solvent. In this study, although there was a stimulation of butanol production by adding acetic acid at 1 g/L, further increase in acetic acid concentration did not improve the final concentration of butanol. In contrast to the positive effects of acetic and butyric acids on butanol production, the use of propionic and formic acids had an adverse effect. The results indicated that these acids could not be used as a substrate for direct conversion to butanol.

2. Acetone-butanol-ethanol production from decanter cake

In this study, decanter cake was hydrolyzed to fermentable sugars using several chemicals. The decanter cake hydrolysate (DCH) obtained was used as a sole carbon source for butanol production by growing cells of *Clostridium beijerinckii* TISTR 1461. The inhibitors formed during the hydrolysis process were determined and their effects on butanol production before and after detoxification were studied. Then, the butanol production from detoxified DCH supplemented with cheap industrial byproducts as nitrogen sources was evaluated and optimized. In addition, the detoxified DCH was also used as a co-substrate for direct conversion of butyric acid to butanol in a nitrogen-free medium. In addition, gas stripping was employed as an *in situ* recovery method to alleviate butanol toxicity and enhance butanol production and sugar conversion in the fermentation.

The characteristics of DC are summarized in Table 13. The amounts of cellulose and hemicellulose were 18.96 % and 4.68%, respectively. Cellulose content was the same level with that of thr reported by Razak *et al*. (2002). Differences may be related to climate conditions, soil chemical composition (Bendahou *et al*., 2007). High levels of lignin (25.34%), made the conversion of DC into polyoses difficult. Lignin covers the cellulose and hemicellulose structures of DC and reduces the access of cellulases to these structures during the enzymatic hydrolysis process.

Table 13. Composition of decanter cake

Parameter	This study	Seephueak *et al.* (2011)	Razak *et al.* (2012)
Moisture (%)	77.91	-[a]	76.46
Cellulose (%)	18.96	-	21.61
Hemicellulose (%)	4.68	-	3.94
Lignin (%)	25.34	-	30.66
Ash (%)	15.88	-	22.25
Oil and grease (%)	4.87	-	4.30
Nitrogen (%)	2.74	-	2.80
Phosphorus (%)	1.81	0.19	0.20
Potassium (%)	3.97	0.71	1.40
Sodium (%)	0.89	0.02	
Calcium (%)	3.69	0.74	0.90
Magnesium (%)	10.25	0.37	0.30
Iron (%)	7.58	12.60-13.90	0.44
Copper (mg/kg)	3059.50	29.33	59.00
Manganese (mg/kg)	6460.30	111.19	38.00
Chlorine (mg/kg)	19.00	-	-
Sulphur (mg/kg)	0.07	1600.00	-

[a] Not available

2.1 Hydrolysis of decanter cake

In this study, the decanter cake was treated with various commonly used acids (HCl, HNO_3, H_2SO_4) and alkalis (NaOH, KOH, $Ca(OH)_2$) and compared with the use of water (H_2O, the so called hydrothermal process) (Figure 10). Alkali pretreatments have been reported to be effective for lignin solubilization, while showing minor cellulose and hemicellulose solubilization rather than by acid or a hydrothermal processes (Carvalheiro *et al.*, 2008). Among the alkalis used, sodium hydroxide gave the highest total sugar concentration that also indicating its high ability to hydrolyze the lignocelluloses structure of the decanter cake. Although calcium hydroxide (lime) has been reported to remove lignin and acetyl groups from

hemicellulose (Alvira *et al*., 2010), it did not work effectively on the conversion of decanter cake to sugars.

In this study, it should be noted that the optimum condition for each chemical might be different. However, the aim of this study was to select the chemical that gives the maximum production of sugar at the same condition tested. Compared with alkali hydrolysis, acid hydrolysis produced a higher amount of sugar. The main function of the acid was to solubilize hemicellulose and the cellulose fraction of the biomass. Dilute acid hydrolysis is favored for the solubilization of hemicellulose while concentrated acid hydrolysisis for cellulose. This is because of a highly ordered crystalline structure of cellulose (Zheng *et al*., 2009). Among the acids tested, nitric acid gave the highest total sugar of 16.07 g/L which was 1.21-fold and 1.34-fold higher than those using hydrochloric acid and sulfuric acid, respectively (Figure 10a). This would be because nitric acid also solubilized lignin and led to a higher release of sugars (Xiao and Clarkson, 1997). Although sulfuric acid and hydrochloric acid are most widely used (Liu and Wyman, 2004; Esteghlalian *et al*., 1997). Sun and Cheng (2002) reported that concentrated acids including hydrochloric acid and sulfuric acid, are toxic, corrosive, and hazardous, and require corrosion resistant reactors. Besides, such acids must be recovered after hydrolysis to make the process economically feasible. Rodríguez-chong *et al*. (2004) used nitric acid to pretreat sugarcane bagasse, and they confirmed that nitric acid was more efficient than sulfuric acid and hydrochloric acid. Xiao and Clarkson (Xiao and Clarkson, 1997) also found that the addition of nitric acid had a tremendous effect on the acid pretreatment of newspaper. Thus, nitric acid is another strong acid that has been shown to be effective for the hydrolysis of lignocellulosic materials.

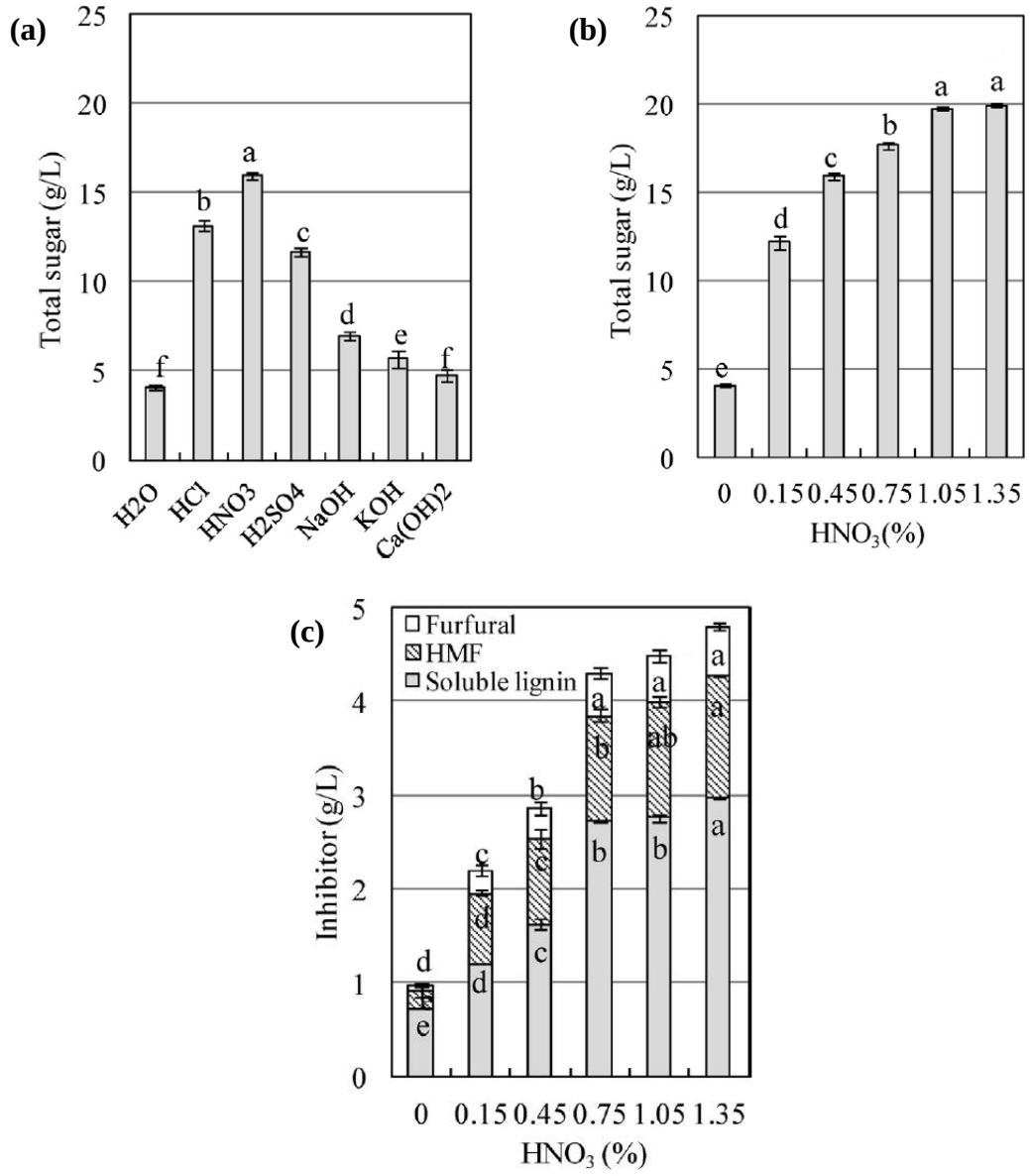

Figure 11. Effect of the chemical agent used for hydrolysis of decanter cake (a) and the effect of nitric acid concentration on the produced sugars (b), and inhibitors (c). Different small letters in the chart indicate significant difference between treatments ($P < 0.05$).

Since nitric acid released most sugars from the decanter cake, the effect of the nitric acid concentration on the production of sugars was then investigated (Figure 11b). When the concentration of nitric acid increased, the amount of solubilized sugars also increased but at a nitric acid concentration higher than 1.05%, the amount of released sugar was not significantly different from that at 1.35%.In addition to sugars, some by-products such as furfural, hydroxylmethylfurfural (HMF), and soluble lignin, were also produced depending on the concentration of nitric acid used. The by-products generated during the hydrolysis process were as follows: cellulose was degraded into glucose, and hemicellulose was degraded into xylose, mannose, acetic acid, galactose and glucose. At high acid concentrations, glucose and xylose would be degraded into furfural and hydroxymethylfurfural (HMF), respectively, and phenolic compounds would be generated from the partial breakdown of lignin (soluble lignin). Among these byproducts, furfural and HMF are thought to damage cell walls and cell membrane and inhibit the activities of glycolytic enzymes and proteins and also inhibit RNA synthesis by microorganisms (Palmqvist and Hahn-hagerdal, 2000).

In the acid hydrolysis process, important factors for forming the by-products are time, temperature and acid concentration. If the reaction time is longer than 1 h, the xylose concentration decreases due to degradation to form toxic byproducts (Cruz *et al*., 2000). Moderate temperatures (<160 °C) have proven adequate for hemicellulose hydrolysis, promoting little sugar decomposition (McMillan, 1994). In this study, the reaction time and temperature were finally fixed at 1 h and 121 ºC, respectively. As shown in Figure 11c by increasing the nitric acid concentration up to 0.75%, furfural, HMF and soluble lignin increased from 0.07, 0.18 and 0.74 g/L (control) up to 0.46, 1.12 and 2.73 g/L, respectively. The use of higher concentrations of acid did not significantly increase the concentrations of these compounds. Considering the amount of sugars and byproducts obtained, the nitric acid at 1.05% therefore was chosen to hydrolyze the decanter cake. The DCH obtained was then concentrated by evaporation before use to achieve a higher sugar concentration (>20 g/L). This resulted in a slight increase in concentrations of furfural, HMF and soluble lignin up to 0.6, 1.25 and 3.25 g/L, respectively. It should be noted that some extent of acetic acid was resulted from the hydrolysis of acetyl

bonds in the hemicellulose content. Its concentration in the hydrolysate increased from 2.97 g/L up to 3.56 g/L after evaporation. It has been reported that acetic acid could be utilized for butanol production (Chen and Blaschek, 1999a). The final sugar compositions of the hydrolysate were mainly glucose 9.96 ± 0.33 g/L (49%) followed by xylose 7.15 ± 0.49 g/L (35%) and arabinose 3.28 ± 0.77 g/L (16%).

Some hydrolysates, for example, corn fiber hydrolysate, did not yield high solvent production even after detoxification (Qureshi *et al*., 2008). Evidently, some biomass feedstocks are more challenging to handle than others. In particular, fermenting the untreated decanter cake hydrolysate has not been successful, and detoxification is necessary. This is because the decanter has a high lignin content, which could result in a high level of phenolic compounds formed during the pretreatment. Phenolic compounds are more toxic than sugar degradation products such as furfural and HMF, and can significantly reduce cell growth and sugar assimilation during fermentation (Palmqvist and Hahn-Hägerdal, 2000). Furfural and HMF at concentrations below 1.0 g/L were reported to be non-toxic; instead, they could stimulate ABE fermentation (Ezeji *et al*., 2007).

2.2 ABE production from undetoxified decanter cake hydrolysate

The decanter cake hydrolysate (DCH) obtained from the previous section was adjusted to pH 6.5 and added with the other nutrients. *C. beijerinckii* TISTR 1461 was cultivated in a DCH based medium with a total sugar concentration of 20 g/L (Figure 12a). The initial concentration of acetic acid was high at approximately 9 g/L (right Y-axis) because ammonium acetate was used as a nitrogen source and some (3.56 g/L) resulted from the hydrolysis of acetyl bonds in the hemicellulose content of the decanter cake. When using DCH as a sole carbon source, *C. beijerinckii* TISTR 1461 produced only butyric acid and no obvious decrease of acetic acid was observed during 2 days of cultivation. At the 5th day of fermentation, *C. beijerinckii* TISTR 1461 produced only 1.35 g/L of butanol and the total acetone and butanol (AB) was only 2.06 g/L. It should be noted that *C. beijerinckii* TISTR 1461 did not produce any detectable level of ethanol. This low butanol and AB productivity could be the result of the toxicity of some derivatives such as furfural and

HMF formed during the acid hydrolysis process, as shown in Figure 9c. Therefore, the DCH needed to be detoxified.

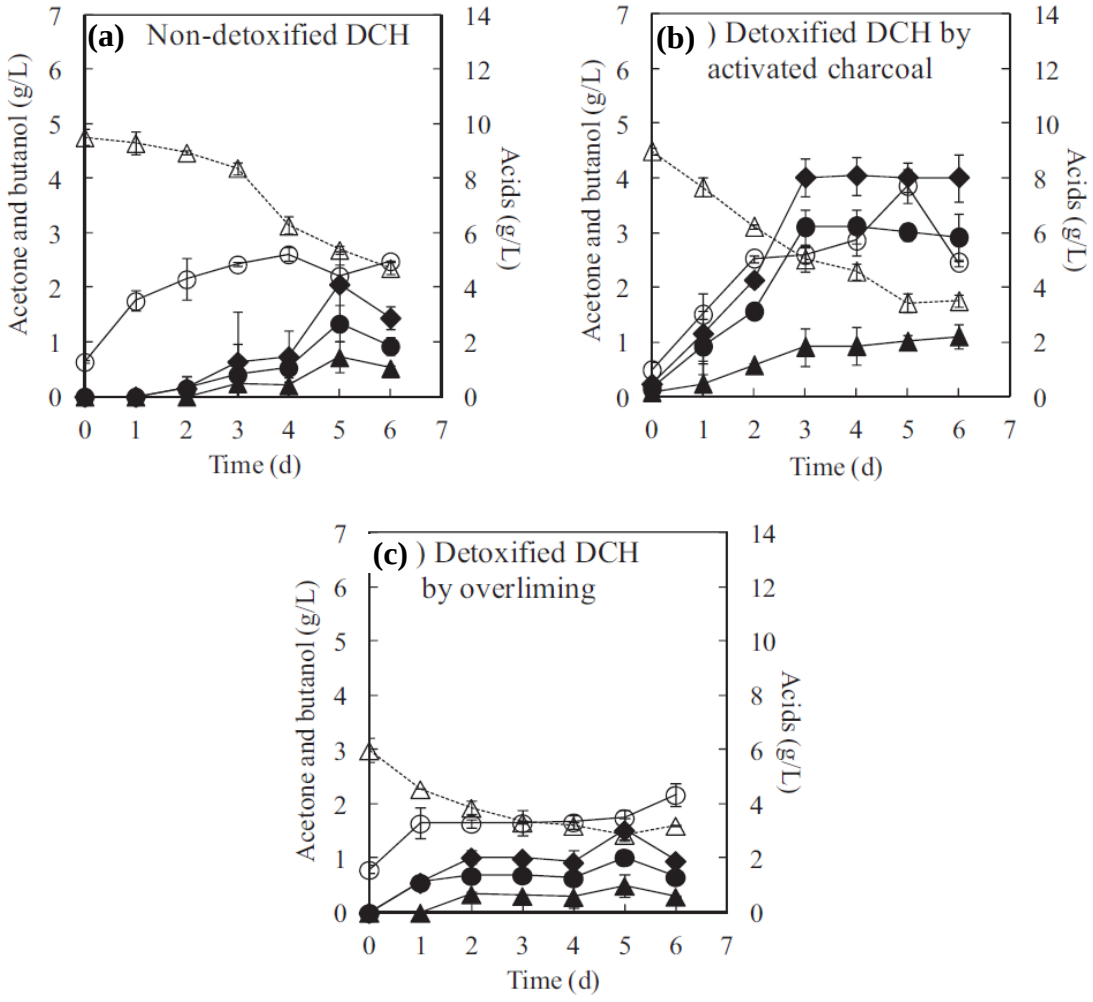

Figure 12. Effect of the detoxification method on formation of products by growing cells of *C. beijerinckii* TISTR 1461. Products formed using non-detoxified DCH (a), DCHdetoxified by activated charcoal (b) and DCH detoxified by overliming (c). (▲) Acetone; (●) butanol; (◆) acetone and butanol (AB); (△) acetic acid; (○) butyric acid.

2.3 ABE production from detoxified decanter cake hydrolysate

Normally, the hydrolysate is neutralized using alkali before use. It has been reported that alkali neutralization somehow removes inhibitors and this could have possible stimulatory effect on the fermenting microorganisms (Persson *et al*., 2002). Other very diverse detoxification methods have been proposed such as: neutralization with lime followed by the addition of activated carbon and filtration; partial removal of furfural and soluble lignin by molecular sieves; vapor stripping for the removal of volatile inhibitors (Olsson and Hahn-Hägerdal, 1996); and alternative biological methods (Khiyami *et al*., 2005; Tian *et al*., 2009) in which a yeast was used for *in situ* detoxification of spruce and birch hydrolysates. Through this strategy, the same yeast cells converted the inhibitors and maintained their concentration at low levels without the need for any other detoxification treatment.

Since activated charcoal is often used to remove compounds from the liquid phase by absorption, it was used to remove inhibitors from DCH in this study. In addition, partly due to economic reasons, DCH was also detoxified using the overliming procedure. The dried calcium hydroxide was added to the acidic hydrolysates and converted into gypsum which can be used as plaster of paris with some commercial value. This method has been demonstrated to help with the removal of volatile inhibitory compounds such as furfural and HMF but it might cause a sugar loss (Chandel *et al*., 2007). As shown in Table 14, each potent inhibitor was reduced by both methods. Furfural was reduced by 60–65% and became lower than 0.25 g/L. HMF was also reduced by 51–57% and became lower than 0.65 g/L. The differences between these two methods are the ability to remove soluble lignin degradation compounds (SLC) and the effect on acetic acid and sugar loss. SLC was removed by 60%when using activated charcoal but only 37% of the SLC was removed when using the overliming method. Acetic acid and sugar loss were 59% and 22%, respectively with the overliming method, while those were only 7.5% and 5.8%, respectively with the use of activated charcoal. Activated charcoal has been known to have a high capacity to absorb compounds without affecting the levels of sugar in hydrolysates (Canilha *et al*., 2008). In addition to less effect on the sugar level, there was also less effect on acetic acid content. This was another advantage of this method since the *Clostridium* could use the acetic acid to produce butanol.

Figure 12b and 11c show the use of treated-DCH for butanol production by growing cells of *C. beijerinckii* TISTR 1461. The cells did consume acetic acid at an early time and produce 3.12 g/L of butanol and 4.04 g/L of AB from the DCH treated with activated charcoal (Figure 12b). In this experiment, *C. beijerinckii* TISTR 1461 also produced only trace amounts of ethanol (<0.1 g/L). This would be a unique characteristic of the *C. beijerinckii* TISTR 1461. Since the overliming method also effectively reduced the concentration of potent inhibitors except SLC (Table 14), *C. beijerinckii* TISTR 1461 produced butanol and AB from the overlimed DCH at an earlier cultivation time than with the non-treated DCH. However, the butanol and AB production using the overlimed DCH was reduced by a half compared to that using the activated charcoal-treated DCH. Since the sugar concentration in the DCH medium was adjusted to be the same at 20 g/L before use, the lower productivity of butanol could be explained by higher SLC concentration or the lower acetic acid concentration. These results indicated that detoxification using activated charcoal was suitable when the hydrolysate was used as a carbon source for butanol production. While the detoxification by overliming that significantly removed acetic acid would be suitable when acetic acid was considered to be an inhibitor of the fermentation.

Studies using activated charcoal detoxification showed that it was effective in removing organic acids at low pH and phenolic compounds at high temperature from lignocellulosic hydrolysates (Rodrigues *et al*., 2001). It was reported that 48.9% color and 25.8% lignin degradation products were removed from rice straw hydrolysate after activated charcoal detoxification (Mussatto and Roberto, 2004). Overliming was reported to be effective in removing inhibitors and improving ABE production from some lignocellulosic biomass such as barley straw and bagasse hydrolysates (Martinez *et al*., 2001; Qureshi *et al*., 2010 a,b), but it did not improve the fermentability of DCH by *C. beijerinckii* in this study due to decreased concentration of total sugar 22% higher than treated with activated charcoal (5.8%) (Table 5). This suggests that each type of biomass is unique in its composition, and the respective hydrolysate requires a specific detoxification in order to be successfully used in the subsequent fermentation.

Table 14. Effect of detoxification method on the removal of potent inhibitors and sugar loss

Potent inhibitors/sugar	Untreated DCH	Activated charcoal-treated DCH	Overliming-treated DCH
Furfural (g/L)	0.60	0.24 (60%)	0.21 (65%)
HMF (g/L)	1.25	0.16 (51%)	0.54 (57%)
SLC (g/L)	3.25	1.30 (60%)	2.04 (37%)
Acetic acid (g/L)	3.56	3.29 (7.5%)	1.46 (59%)
Total sugar (g/L)	20.4	19.2 (5.8%)	15.96 (22%)

Data in parentheses are percent removal of inhibitors and loss of sugar compared to untreated DCH.

2.4 Effect of organic nitrogen source

To reduce the cost of nitrogen sources, all original nitrogen sources in the TYA medium were replaced by nitrogen-containing by-products. These were whey protein (WP), isolated soy protein (ISP) and urea. The results were compared with the medium containing yeast extract (YE) as the sole organic nitrogen source (Figure 13). Each organic nitrogen source was added at 1 g-N/L. It was of interest that the use of whey protein gave the highest butanol production (2.32 g/L) followed by the isolated soy protein (1.18 g/L), urea (0.65 g/L) and yeast extract (0.45 g/L). The highest AB production (3.17 g/L) was also obtained when using whey protein. The organic acids produced when using whey protein and and isolated soy protein were 5.56 and 4.63 g/L, respectively. While those using yeast extract and urea were as high as 8.51 and 8.18 g/L, respectively. In general, factors for better growth would result in a higher amount of acid concentrations and these acids would cause the so called acid crash. This is the state when clostridial fermentation ended prematurely before switching to the solventogenic phase (Wang *et al*., 2011). Another possibility would be an insufficient amount of energy rich metabolites such as ATP or NADH to support the re-assimilation of organic acids during the solventogenesis phase (Shinto *et al*., 2007). The results indicate that yeast extract and urea promoted the growth-associated production of acids while whey protein and isolated soy protein promoted

the further conversion of acids to butanol. Although the use of these alternative nitrogen sources gave lower amounts of butanol (2.32 g/L) compared with the original TYA medium (3.12 g/L), the costs of them are much lower than the original nitrogen sources in the TYA medium.

Supplement the biobutanol fermentation medium with yeast extract is a common practice, as reported in the literature (Fontaine *et al*., 2002; Yan *et al*., 1988; Yu *et al*., 2011). Yeast extract was used as a nitrogen source for cell culture and fermentation process, which is enriched with protein, amino acid, minerals, vitamins, and growth factors that promote the growth of microorganism. It was found that yeast extract has a strong effect on the production of biobutanol and sugar utilization during biobutanol fermentation from spoilage date fruits; the addition of yeast extract significantly increased the production biobutanol (Abd-Alla and Elsadek El-Enany, 2012). Chua *et al*. (2012) investigated the effect of yeast extract on biobutanol production using *Clostridium* G117 and found that increasing the yeast extract addition from 0.4% to 1% enhanced the production of butanol from 8.52 to 8.61 g/L.

Li *et al*. (2012) reported the transcriptional level of *ctfAB* was considerably stimulated after yeast extract addition, and CoA transferase was activated accordingly leading to smooth phase shift occurrence and acceleration of organic acids re-assimilation. CoA-transferase activation determined the butanol production enhancement after the yeast extract addition.

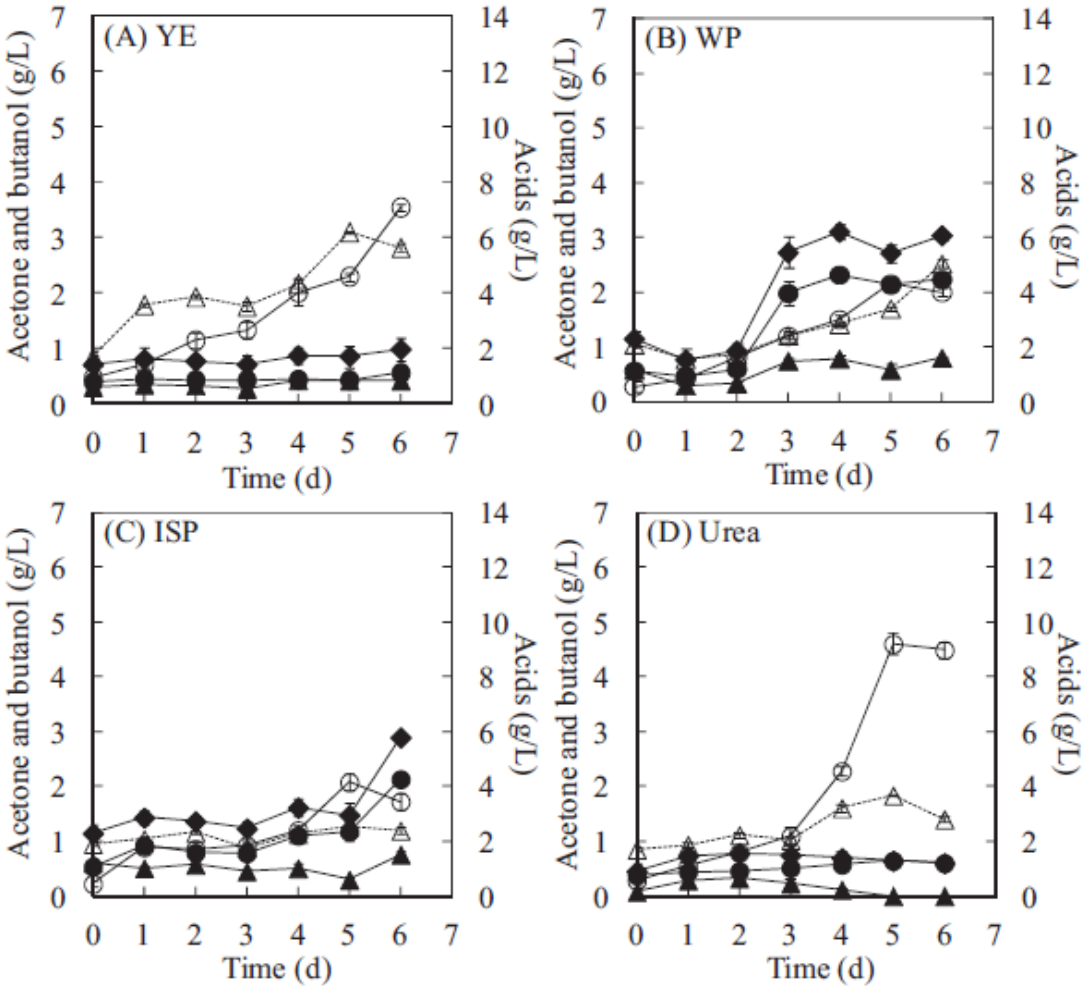

Figure 13. Effect of organic nitrogen sources added together with detoxified DCH on formation of products by growing cells of *C. beijerinckii* TISTR 1461. (A) Yeast extract (YE), (B) Whey protein (WP), (C) Isolated soy protein (ISP) and (D) Urea. (▲) Acetone; (●) butanol; (◆) acetone and butanol (AB); (△) acetic acid; (○) butyric acid.

2.5 Effect of inorganic nitrogen source

Since it has been reported that the anions from an inorganic nitrogen source could be metabolized by *Clostridium* with a positive effect on the conversion of acids to ABE (Welsh *et al*., 1987), the effect of inorganic nitrogen sources were then investigated. Each inorganic nitrogen source, i.e. ammonium chloride, ammonium nitrate, ammonium sulfate, and ammonium acetate, was used at 1 g-N/L in combination with whey protein as an organic nitrogen source. The type of inorganic nitrogen source greatly influenced the production of butanol. The highest butanol production of 2.87 g/L and AB production of 3.54 g/L were obtained when ammonium sulfate was used in combination with whey protein (Figure 14c). The mixture of ammonium sulfate and whey protein made a significant positive contribution toward butanol production but this was not noteworthy for acetone. The use of either ammonium chloride (Figure 13a) or ammonium acetate (Figure 14d) with whey protein enhanced the production of acids but these acids were not efficiently converted to butanol. In contrast, the use of ammonium nitrate negatively affected both acid and AB production (Figure 14b). It has been reported that ammonium acetate is a necessary compound in synthetic medium for the growth of *C. acetobutylicum* and for solvent synthesis (Ladisch, 1991).

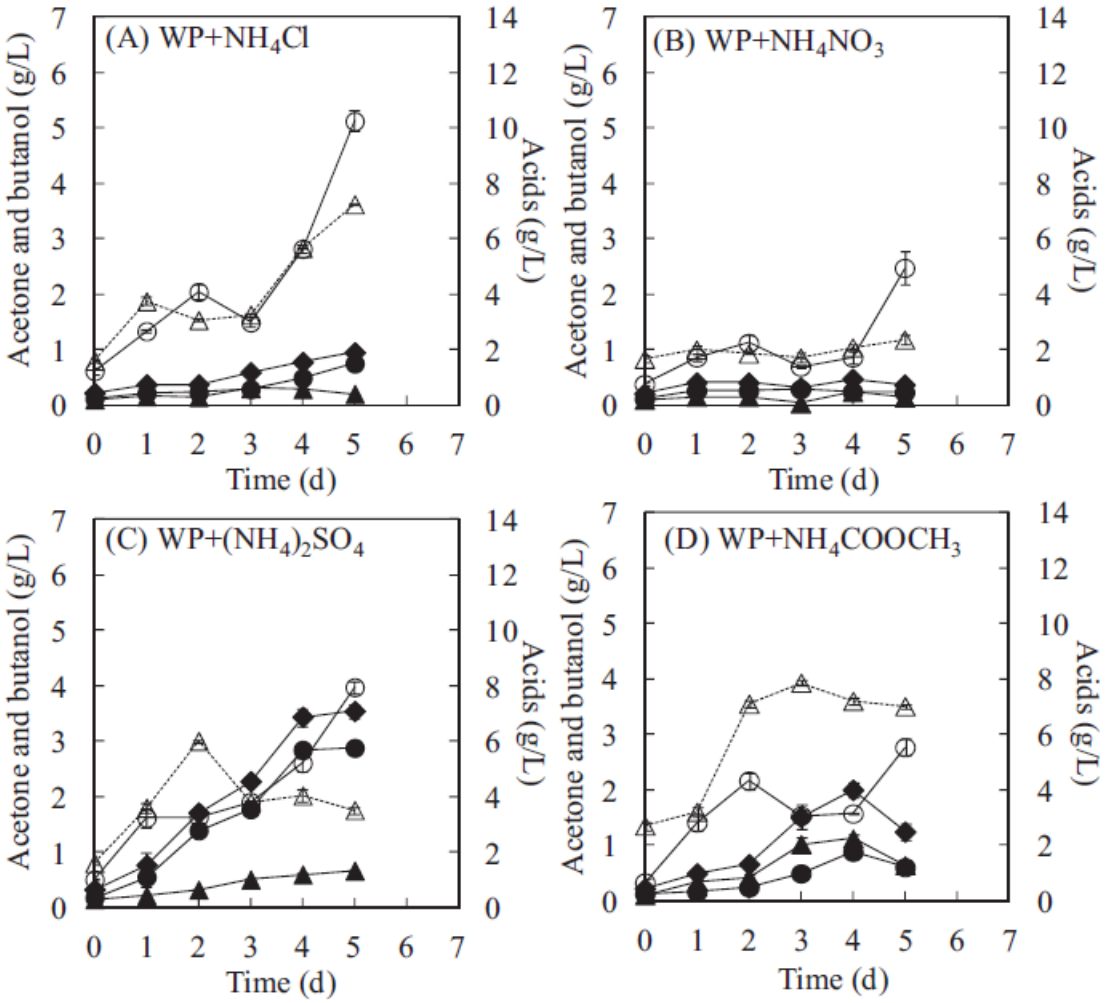

Figure 14. Effect of inorganic nitrogen sources added together with detoxified DCH on formation of products by growing cells of *C. beijerinckii* TISTR 1461. (A) Yeast extract (YE), (B) Whey protein (WP), (C) Isolated soy protein (ISP) and (D) Urea. (▲) Acetone; (●) butanol; (◆) acetone and butanol (AB); (△) acetic acid; (○) butyric acid.

2.6 Effect of C/N ratio

The C/N ratio in the medium has been determined to be one of the important factors that affects the growth and solvent production of *Clostridium*. The cells grow well and more solvents are produced at a lower C/N ratio (Lai and Traxler, 1994). According to the results from the previous section, whey protein and ammonium sulfate were used as organic and inorganic nitrogen sources, respectively, and their concentrations were varied to obtain different C/N ratios. Figure 15 shows the effect of the C/N ratio on the performance in the conversion of detoxified DCH to butanol by *C. beijerinckii* TISTR 1461. The production of acetone and butanol (AB) increased drastically with an increasing nitrogen concentration from 1 to 4 g-N/L with a corresponding C/N ratio decreased from 28 to 7 (Figure 15a-d). However, the AB production significantly decreased with a further increase in nitrogen concentration from 4 to 8 g-N/L (C/N ratio was 3.5, Figure 13d).The highest butanol production of 3.42 g/L was obtained at 4 g-N/L with a butanol yield of 0.28 C-mol butanol/C-mol carbon. It should be noted that more acetic acid was produced than was consumed and this led to a higher final concentration. Therefore, the butanol yield was most likely calculated based on the consumption of only sugars.

Table 16 compared the butanol production from the lignocellulosic biomass in this study with results from other studies using solventogenic clostridia. The lignocellulosic biomass is a potential low cost feedstock for biofuels due to its abundance and sustainability. Cost-effective and easily-degradable feedstocks are required for butanol production. Another strategy would be selecting suitable strains or optimizing the fermentation conditions to improve the efficiency of converting substrate to butanol. Several attempts using lignocellulosic biomass have shown that it is also possible to use hydrolysate from agricultural wastes for butanol production (Jesse *et al*., 2002; Wang *et al*., 2009; Liu *et al*., 2010; Qureshi *et al*., 2010a,b; Wang and Chen, 2011; Lin *et al*., 2011; Ibrahim *et al*., 2012). However, one single crop residue/biomass would not be sufficient to meet the demand for biofuel. With this view, there is a need to explore new and abundant agricultural substrates for butanol production. One of many criteria that could be used for feasible evaluation would be the production yield. The butanol yield in this study was lower than those in several reports (Jesse *et al*., 2002; Wang *et al*., 2009; Wang and Chen, 2011) but it should be

noted that this yield depends on many factors such as Clostridia strain, sugar concentration, nitrogen source and its concentration and also culture condition. Since the decanter cake gave a higher yield of butanol to wheat bran (Liu *et al*., 2010), corn fiber (Qureshi *et al*., 2008), corn straw (Lin *et al*., 2011) and oil palm empty fruit bunches (OPEFB) (Ibrahim *et al*., 2012), it could be suggested that decanter cake could be another lignocellulosic biomass with a high potential for use as feedstock for butanol production.

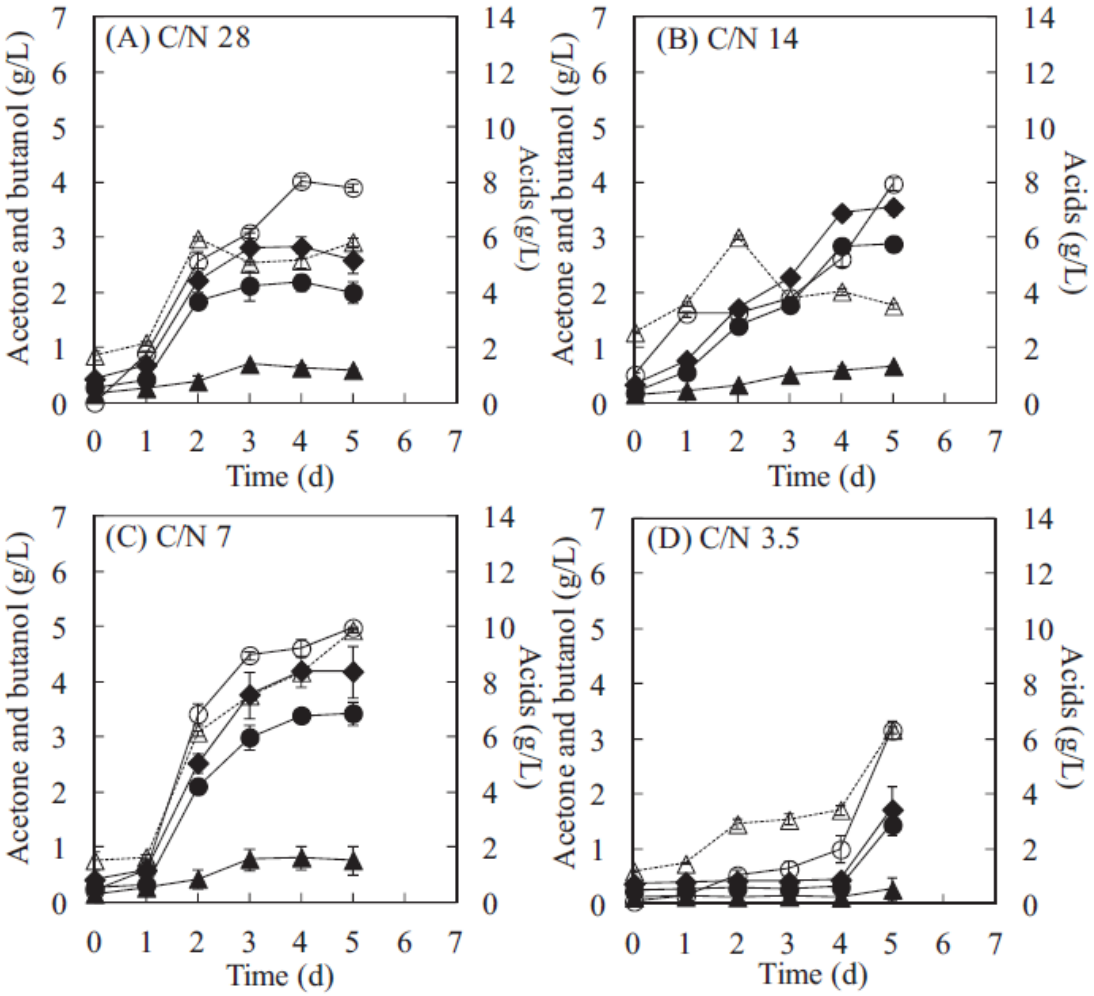

Figure 15. Effect of different C/N ratios on the products formed by growing cells of *C. beijerinckii* TISTR 1461. (a) C/N 28, (b) C/N 14, (c) C/N 7 and (d) C/N 3.5. (▲) Acetone; (●) butanol; (◆) acetone and butanol (AB); (△) acetic acid; (○) butyric acid.

Table 15 Comparison of butanol production from lignocellulosic biomass in this study with result from other studies

Carbon source (sugar concentration)	Nitrogen source (concentration)	Butanol (g/L)	Butanol yield[a]	Microorganism	Reference
Packing peanut hydrolysate (47.7 g/L)	Yeast extract (1 g/L) and ammonium acetate (2.2 g/L)	13.0	0.36	*C. beijerinckii* BA 101	Jesse *et al*. (2002)
DDGS hydrolysate (23.5 g/L)	Yeast extract (1 g/L) and ammonium acetate (2.2 g/L)	10.7	0.34	*C. acetobutylicum* P 260	Wang *et al*. (2009)
Wheat bran hydrolysate (53.1 g/L)	Yeast extract (1 g/L) and ammonium acetate (2.2 g/L)	8.5	0.26	*C. beijerinckii* ATCC 55025	Liu *et al*. (2010)
Corn fiber hydrolysate (46.3 g/L)	Yeast extract (1 g/L) and ammonium acetate (2.2 g/L)	6.4	0.23	*C. beijerinckii* BA 101	Qureshi *et al*. (2008)
Corn stover hydrolysate (53.52 g/L)	ammonium acetate (4.3 g/L)	8.3	0.32	*C. acetobutylicum* ATCC 824	Wang and Chen (2011)
Corn straw hydrolyste (42-44 g/L)	Yeast extract (5 g/L), tryptone (5 g/L), soya peptone (5 g/L)	6.2	0.23	*C. acetobutylicum* CICC 8008	Lin *et al*. (2011)
OPEFB hydrolysate (32.2 g/L)	Yeast extract (6 g/L)	0.7	0.21	*C. butyricum* EB 6	Ibrahim *et al*. (2012)
Decanter cake hydrolysate (20 g/L)	Whey protein (2 g-N/L) and ammonium acetate (2 g-N/L)	3.4	0.28	*C. beijerinckii* TISTR 1461	This work

[a] Butanol yield was calculated based on Cmol butanol/Cmol carbon source. DDGS: distiller's dried grains with solubles, OPEFB: Oil palm empty fruit bunches.

2.7 Response surface methodology for butanol production using the detoxified DCH as a co-substrate with butyric acid

In this study, sugars from detoxified DCH were added as a co-substrate with butyric acid in a nitrogen-free medium for butanol production by *C. beijerinckii* TISTR 1461. The effect of the sugar concentration in combination with butyric acid concentration on butanol production, were investigated using response surface methodology (RSM). The central composite design (CCD) with two variables at three levels leading to a total 13 sets of experiments, were carried out (Table 16). The results obtained were analyzed by ANOVA. The second order regression equation for acetone (Y_1), butanol (Y_2) and AB production (Y_3) as a function of sugar (x_1) and butyric acid (x_2) are given as follows:

$$Acetone\ (g/L) = 9.832 - 1.206x_1 + 0.395x_2 - 0.003 \times x_1x_2 + 0.033x_1^2 - 0.017x_2^2 \quad (2)$$

$$Butanol\ (g/L) = -10.56 + 1.029x_1 + 2.077x_2 - 0.015 \times x_1x_2 - 0.031x_1^2 - 0.098x_2^2 \quad (3)$$

$$AB\ (g/L) = -0.674 - 0.182x_1 + 2.467x_2 - 0.018 \times x_1x_2 + 0.0018x_1^2 - 0.115x_2^2 \quad (4)$$

The models fitted satisfactorily to the experimental data as indicated by their goodness of fit expressed by R^2 and P value. The R^2 values of the models for Y_1, Y_2 and Y_3 were 0.95, 0.92 and 0.98, respectively. This indicated that up to 92–97% of the variations in acetone and butanol production can be explained by this equation. The P values of the model for acetone, butanol and AB production were 0.0002, 0.0012 and 0.0001, respectively. The P value ≤ 0.05 indicated the significance of the coefficients. A regression model was employed to develop response surface plots as shown in Figure 16.

Table 16. Experimental range and levels of the two independent variables used in RSM in terms of coded and actual factors and experimental data for the two-factor with three-level response surface analysis.

Trail	Independent variables		Dependent variables		
	Sugar (g/L)	BA (g/L)	Acetone (g/L)	Butanol (g/L)	AB (g/L)
1	-1(10.00)	0(10.00)	3.51	5.00	8.51
2	1(20.00)	0(10.00)	0.24	5.53	5.77
3	0(15.00)	1(15.00)	0.54	2.95	3.49
4	-1(10.00)	1(15.00)	2.48	4.08	6.56
5	1(20.00)	-1(5.00)	0.25	3.80	4.05
6	1(20.00)	1(15.00)	0.27	2.12	2.39
7	0(15.00)	0(10.00)	0.97	6.94	7.87
8	-1(10.00)	-1(5.00)	2.16	4.25	6.41
9	0(15.00)	-1(5.00)	0.70	4.26	4.96
10	0(15.00)	0(10.00)	0.92	6.67	7.58
11	0(15.00)	0(10.00)	0.88	6.86	7.74
12	0(15.00)	0(10.00)	0.92	6.45	7.37
13	0(15.00)	0(10.00)	0.84	6.79	7.63

Based on response surface plot, the interaction between two variables and their optimum levels can be easily understood and located. From Figure 15a, it was established that the effect of butyric acid concentration on the acetone production was insignificant. The acetone production mainly depended on the sugar concentration and it decreased with increasing sugar concentration. On the other hand, the sugar concentration affected the butanol production less than the butyric acid concentration did (Figure 16b). At a butyric acid concentration of 5 g/L, a sugar concentration in the range of 12–17 g/L was enough for maximizing butanol production up to the maximum level of 4.5 g/L, and AB up to 6.5 g/L. With a higher butyric acid concentration of 10 g/L, the optimal sugar concentration for butanol production was 15 g/L. At this combination of butyric acid and sugar, all acetic and

butyric acids were consumed and more than 60% of the sugar was utilized. This then gave a maximum butanol production of 6.94 g/L with a butanol yield of 0.47 C-mol butanol/C-mol carbon. At sugar concentrations higher than 15 g/L, the butanol production became lower. Typically, the level of sugar concentration that had an adverse effect on butanol production was >100 g/L (Ezeji *et al*., 2003; Ezeji *et al*., 2004a). Therefore, the reason for the lower butanol production was most likely due to the presence of inhibitors at higher levels. When the concentration of butyric acid was increased >10 g/L, the butanol production decreased. This could be due to the inhibitory effect of butyric acid at high concentrations. The maximum AB production of 8.51 g/L (Figure 16c) was obtained at 10 g/L butyric acid and 10 g/L sugar concentration. It should be also noted that a lower sugar concentration favored acetone production.

Al-Shorgani *et al*. (2012) studied the influence of varying glucose/butyric acid ratio on butanol production by C. *saccharoperbutylacetonicum* N 1-4. They reported that the use of 20 g/L glucose in the presence of 10 g/L butyric acid gave the highest butanol production of 12.99 g/L, while Tashiro *et al*. (Tashiro *et al*., 2007) produced only 7.5 g/L butanol from the same combination of glucose and butyric acid. In the study of Baba *et al*. (Baba *et al*., 2011), a continuous high cell density system of *C. saccharoperbutylacetonicum* N1-4 was operated at a dilution rate of 0.85 h^{-1} by feeding a nitrogen-free medium containing glucose and butyric acid (20 and 10 g/L, respectively).Their system gave the highest butanol concentration of 9.4 g/L. In this study, the butanol production from detoxified DCH and butyric acid was lower than that obtained from glucose and butyric acid (Figure 8) and those reported in the literature. This was likely because some extent of inhibitors still remained in the DCH. This study has shown that the sugars derived from decanter cake could be used as a co-substrate for direct conversion of butyric acid to butanol in the nitrogen-free medium.

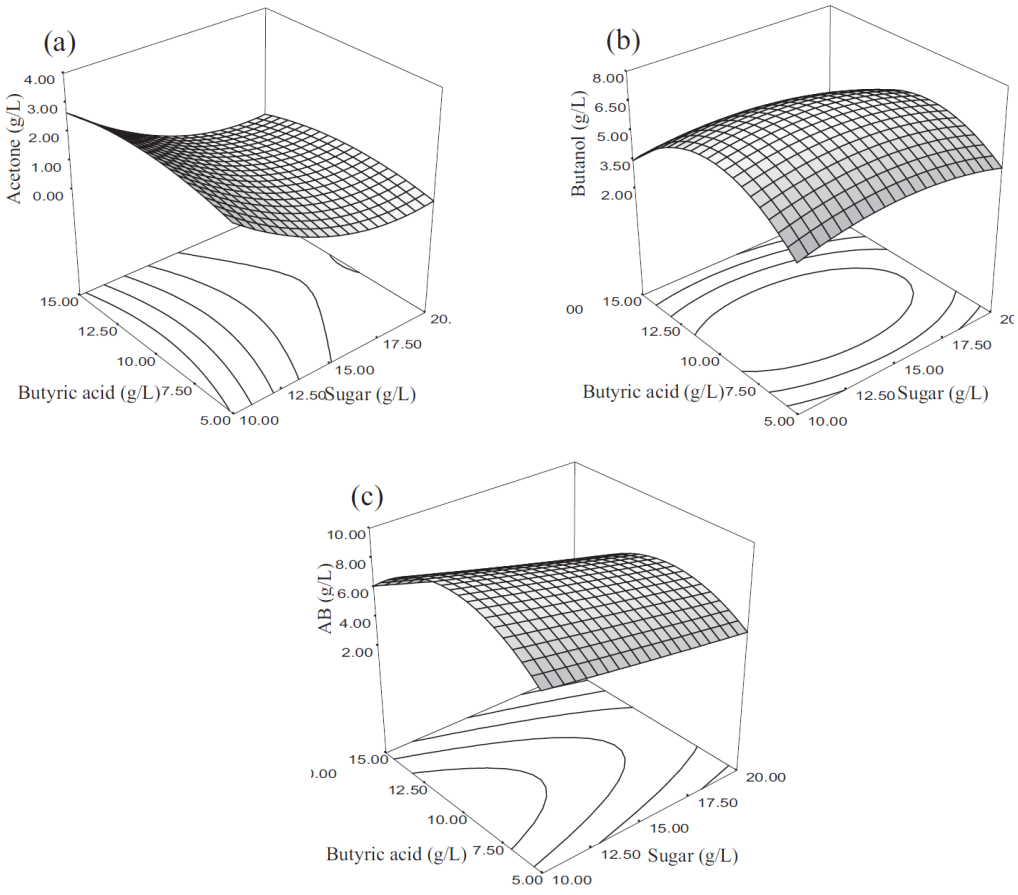

Figure 16. Response surface plots and contour plots obtained from using detoxified DCH as a co-substrate for direct conversion of butyric acid to butanol by *C. beijerinckii* TISTR1461 cultivated in a nitrogen-free medium. (a) Acetone, (b) butanol and (c) acetone and butanol (AB)

2.8 ABE production from DCH in the bioreactor

2.8.1 ABE production from glucose

The ABE production from glucose was performed using *C. beiferinckii* TISTR 1461 in 1L bioreactor, an experiment was performed where the initial pH of the media was 6.26. Time courses of OD, pH, ABE and acids are shown in Figure 17. The OD increased during first 24 h indicating the vigorous growth of the *Clostridium*. The *Clostridium* entered acidogenesis phase and produced acetic and butyric acids during first 18 h leading to a decrease in the pH to 4.99 After 18 h, acid continuously

decreased and the *Clostridium* shifted its metabolic path to produce ABE leading to an increase in the pH up to 7.63. The total sugar rapidly decreased during 24 h of fermentation and depleted at 30 h.

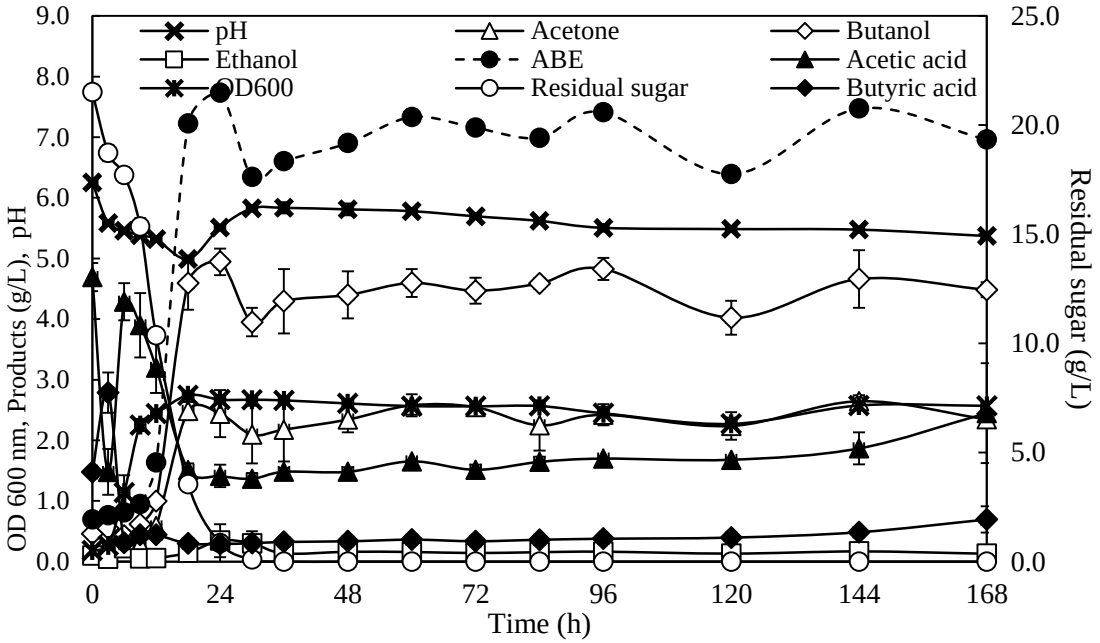

Figure 17. Growth and metabolic activity of *C. beijerinckii* TISTR 1461 in anaerobic bioreactor using nitrogen-rich TYA medium containing glucose as a carbon source.

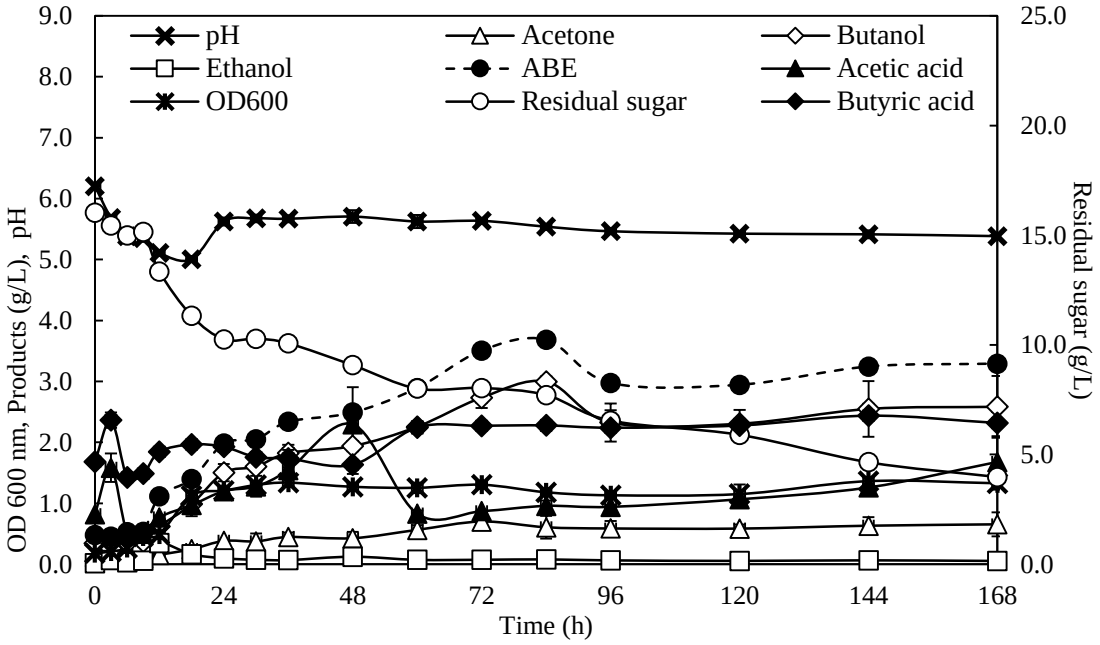

Figure 18. Growth and metabolic activity of *C. beijerinckii* TISTR 1461 in anaerobic bioreactor using nitrogen-free medium.

Figure 18 shows the growth and metabolic activity of *C. beijerinckii* TISTR 1461 in anaerobic bioreactor using nitrogen-free medium. The cells gradually consumed produced mainly acetic acid to the maximum level of about 2 g/L while sugar and butyric acid remained constant until 48 h. As a result from the production of acetic acid, the butyric acid pH slightly dropped during 18 h and increased later. Lee *et al.*, (2008a) reported that the butyric acid have promoted earlier shift to the solventogenic phase. This general trend is probably owed to the fact that the inclusion of butyric acid in the medium aids to stimulate the consumption of glucose associated with cell growth even though the specific growth rates for all bacteria decrease with increasing butyrate concentrations glucose might be utilized for other cell purposes such as maintenance of cell activity and synthesis of enzymes required for solventogenic functions.

In general, it has been reported that butyric acid plays four main interdependent roles in Clostridial fermentation systems: firstly, it is used as metabolic substrate for butanol synthesis by the cells; secondly, it buffers the medium pH preventing it to drop below critical levels that compromise cell stability. Third, it

inhibits cell growth by reducing the specific growth efficiency; and fourth, it appears to enhance the substrate consumption (Lee *et al*., 2008b). So, the cells consumed glucose and produced mainly acetic acid as a primary metabolite. As acetic acid was built up in the system the medium pH dropped rapidly and increased when the cells shifted their metabolic state from acidogenesis to solventogenesis. However, if the pH drops below the critical level then the metabolic switch of the cells is negatively affected and acidogenesis is the dominant phase.

2.8.2 ABE production from detoxified DCH

ABE production in TYA medium containing detoxified DCH as a carbon source was performed in bioreactor. The profile of pH, residual sugar, acid, ABE production and inhibitors are shown in Figure 19. Similar fermentation results were obtained. The maximum concentration with those using glucose (Figure 16) butanol 3.94 was produced from 20 g/L of total sugar in the fermentation using DCH.

The change of furfural and HMF in the culture medium during ABE fermentation by *C. beijerinckii* TISTR 1461 are shown in Figure 18b. The furfural concentration decreased from 0.33 g/L to 0.19 g/L within 168 hours of ABE fermentation. The initial concentration of 0.91 g/L HMF reduced to 0.39 g/L within 168 h of fermentation. The drop in furfural and HMF could be due to their metabolism to furfuryl alcohol and HMF alcohol, respectively (Boopathy *et al*., 1993). This biotransformation appears to use aldehyde as an electron acceptor, simply converting furfural and HMF to furfuryl alcohol and HMF alcohol by $2e^-$ reduction (Belay *et al*., 1997; Boopathy *et al*., 2009). Cofactors (NADH and NADPH) are involved in the detoxification of furans (furfural and HMF) by microorganisms (Miller *et al*., 2009). These cofactors are also involved in butanol synthesis and play important roles during the transition from acidogenesis to solventogenesis (Grupe and Gottschalk, 1992). Conversion of furfural to furfuryl alcohol by yeast (Villa *et al*., 1992; Palmqvist and Hahn-Hägerdal, 2000), bacteria (Boopathy *et al*., 1993; Gutiérrez *et al*., 2002) and archaea (Belay *et al*., 1997; Boopathy, 2009) have been previously reported.

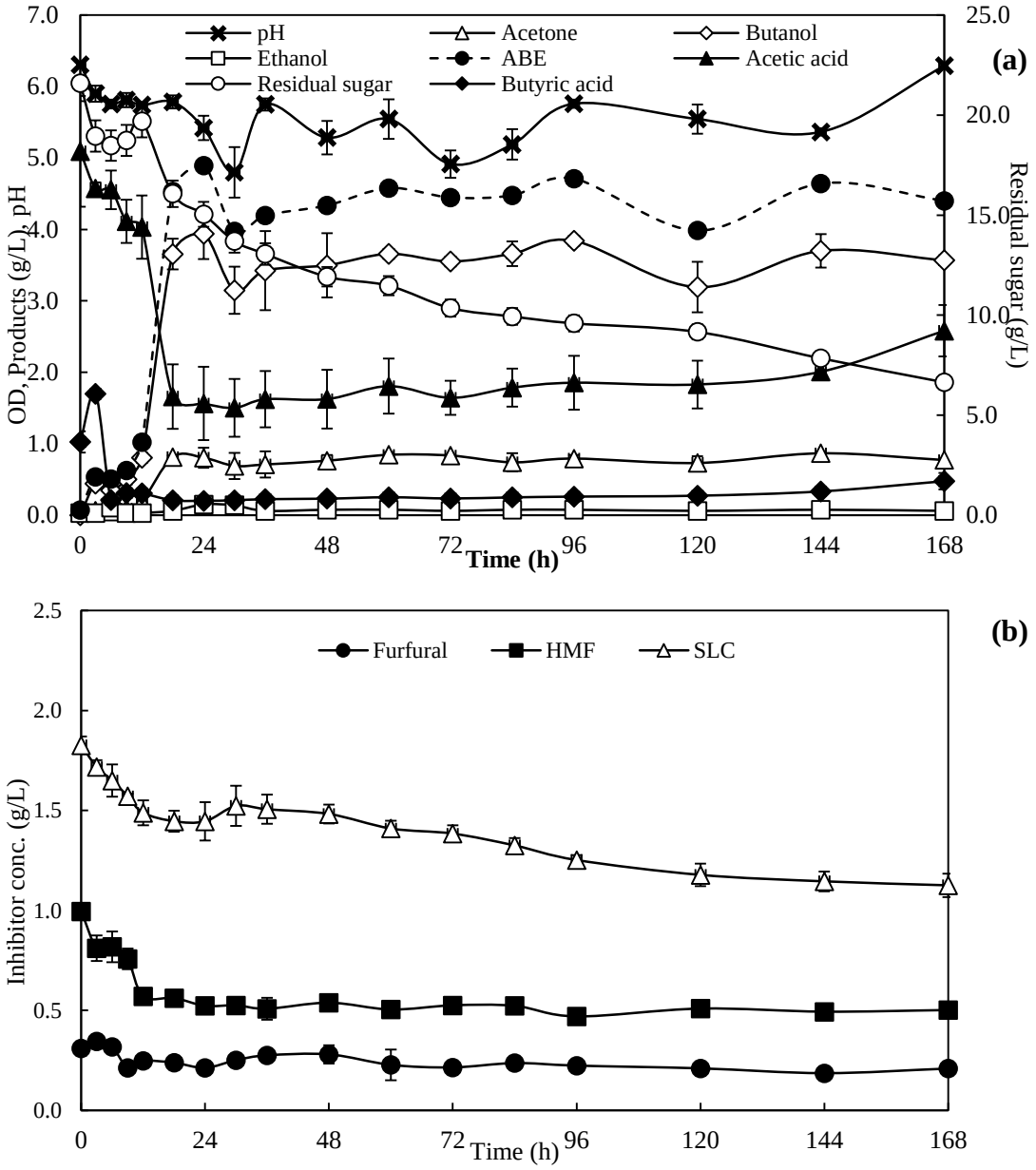

Figure 19. ABE production from detoxified DCH by *C. beijerinckii* TISTR 1461 in modified TYA medium (a) pH, sugar utilization and solvent and acid production; (b) inhibitor reduction profile

2.8.3 ABE production from DCH in solventogenic mode

DCH after detoxification was used for ABE fermentation in solventogenic mode in 1-L bioreactor (Figure 20). The butanol was slightly higher than that in the serum bottle. The butyric acid concentration increased at 60 h due to the metabolism of *Clostridium*. In this experiment, no ethanol was detected in the fermentation broth and low acetone (0.54 g/L at the end of fermentation) was produced. The addition of butyric acid to the culture of *C. beijerinckii* TISTR 1461 increased the concentration of ABE as it was found in the serum bottle (Figure 7b). According to Gottschal and Morris (1981) reported, the stimulation of ABE production was initiated at an earlier stage when an elevated concentration of acids (acetic acid and butyric acid) was initially added to the medium, but increased acid levels do not necessarily improve the final production of butanol.

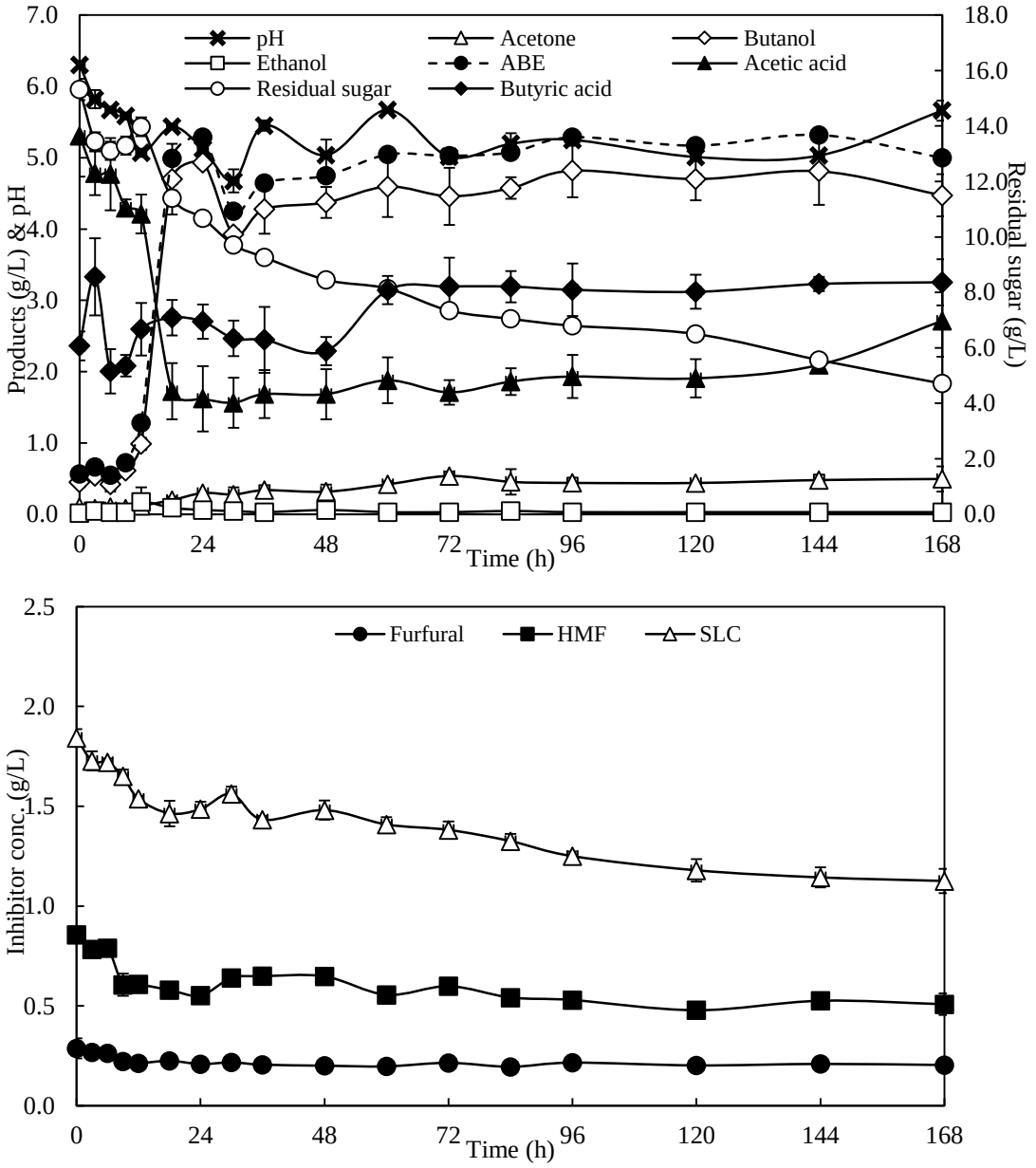

Figure 20 ABE production from DCH in solventogenic mode by *C. beijerinckii* TISTR 1461 in N-free medium (a) pH, sugar utilization and solvent and acid production; (b) inhibitor reduction profile

2.9 ABE fermentation with gas stripping for butanol recovery

To improve sugar conversion and butanol production, gas stripping as an online product recovery technique was employed to relieve butanol inhibition in the ABE fermentation. Gas stripping removes only the volatile solvent, not nutrient, and would not harm cells in the fermentation (Ezeji *et al*., 2004; Xue *et al*., 2012). Figure 20 shows ABE production from glucose with gas stripping for butanol recovery. Gas stripping was turned on at 24 h to continuously remove solvents from the fermenter. With continuous gas stripping at 1.25 L/min, acetone and butanol concentration gradually increased and increased after 30 h (Figure 20). The batch fermentation was continued for 168 h. Over this period, a total of 22.20 g/L glucose was consumed, and 10.52 g/L of butanol, 9.88 g/L of acetone and 0.39 g/L of ethanol were produced. The butanol concentration in the condensate was 2 g/L. No acetone, ethanol and acids were detected in the condensate. These results indicated that gas stripping was more selective in removing butanol than acetone and ethanol, and it did not remove acids, which are not volatile at the fermentative pH of 5.0.

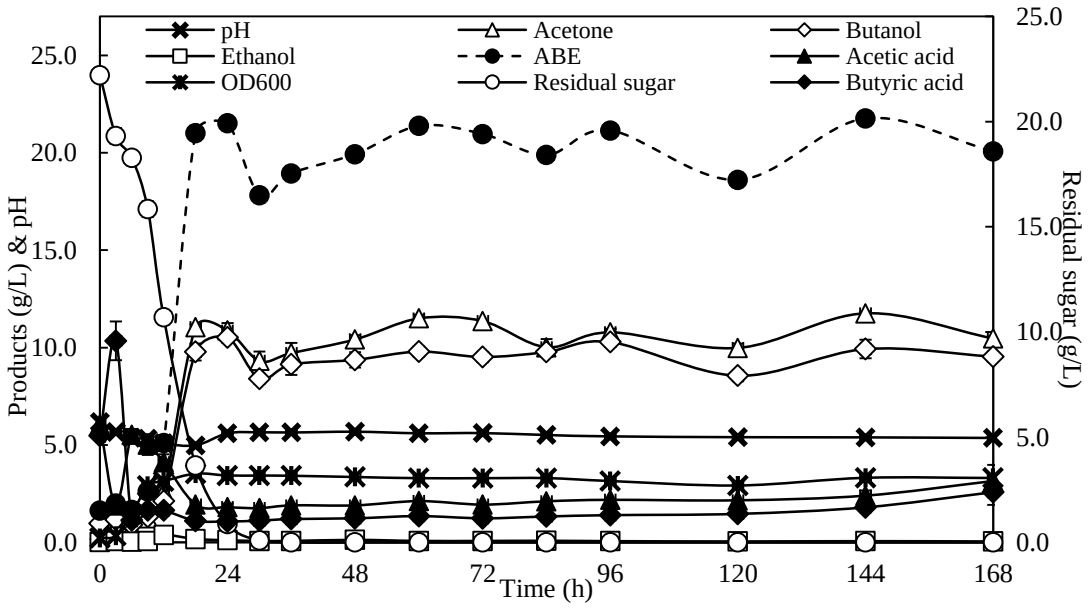

Figure 21. Growth and metabolic activity of *C. beijerinckii* TISTR 1461 in anaerobic bioreactor with integrated with gas stripping.

The finding that no acetic and butyric acid could be detected in the condensate has been reported by several researchers (Ezeji *et al*., 2003; Ezeji *et al*., 2005). They also reported that although the gas stripping did strip out acetone and ethanol in the case of using the model solution containing 0.48 g/L acetone and 0.02 g/L ethanol, in the fermentation on broth the concentrations of acetone and ethanol might be lower than the minimum requirement for stripping. The presence of cells might also reduce the stripping ability for these compounds.

A total 10.54 g/L of ABE was produced of which 6.22 g/L was butanol, when gas stripping was applied. The ABE yield was 0.637 g/g and butanol yield was 0.376 g/g. Compared to the fermentation shown in Figure 17, gas stripping increased the butanol production by 22.34% with the same sugar consumption.

In the fermentation using DCH and gas stripping (Figure 22), *C. beijerinckii* rapidly started producing butanol during 18 h of cultivation and approximately 6.11 g/L of butanol was produced as the most abundant product. The acetic acid in the medium was consumed (4.28 g/L) and butyric acid was detected at high concentration at 3 h (9.58 g/L).

In the fermentation integrated with gas stripping, the final butanol was 6.22 g/L or 1.58 fold of that without gas stripping (3.94 g/L). About 2 g of butanol were removed from the medium by gas stripping. The total ABE productivity was 0.86 g/L/h. A yield of 0.93 ABE produced/g sugar consumed was obtained. The estimated production rate of butanol was 0.34 g/L/ in the exponential growth phase (3-18 h) and 0.15 g/L/h in the stationary phase (24-72h).

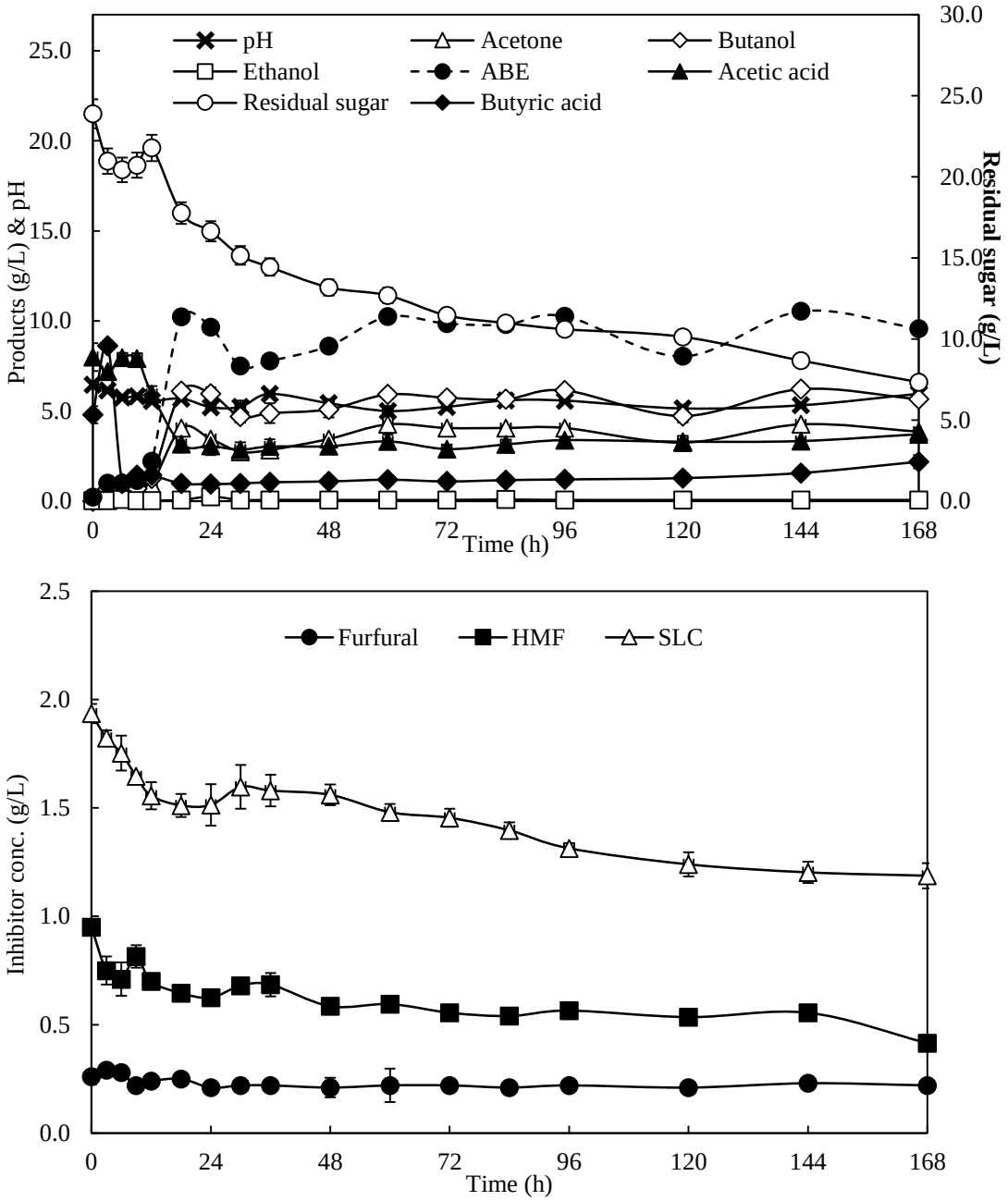

Figure 22. ABE production from detoxified DCH by *C. beijerinckii* TISTR 1461 in modified TYA medium with integrated with gas stripping (a) pH, sugar utilization and solvent and acid production; (b) inhibitor reduction profile

3. ABE production by immobilized cells

Immobilized cell system has been suggested as an effective method for improving butanol production, since it is potential to increase process productivity, ease to separate cell mass from the bulk liquid, reduced risk of contamination, better operational stability and cell viability for several cycles of fermentation and reduce production costs. The most suitable carriers for cell immobilization are entrapment in calcium alginate beads because these techniques are simple, cost effective and non-toxic (Behera *et al*., 2010). There is also a surge in the attempts toward discovery out a renewable and biodegradable carrier which is basically not synthetic, easy to use, inexpensive and available naturally in abundance. The advantages accruable from such biomaterials (sawdust, wood chips/shavings, rice husks, cotton towels, and straw) are reusability, freedom from toxicity problems, mechanical strength for necessary support, long time operation, high fermenting activity and opening of spaces within the matrix for growing cells (Guénette and Duvnjak Z, 1996; Das *et al*., 1993; Shukla *et al*., 1989; Huang *et al*., 2004; Forberg and Haggstrom, 1985)

In this study, several support matrices included oil palm shell (OPS), empty fruit bunch (EFB), palm press fiber (PPF) and oil palm frond (OPF) were evaluated for the immobilization of *C. beijerinckii* TISTR 1461 in ABE fermentation. The optimal size and number of support matrices used for cell immobilization in butanol production were investigated. A repeated-batch operation using immobilized cells was also discussed in this study.

3.1 Sample preparation and morphological analysis

The support materials were prepared into 1-2 mm size (Figure 23) and studied by SEM (Figure 25). The ideal immobilization support material should be non-toxic highly porous and can provide large surface area for cell attachment. All the materials screened fulfill the requirements of an ideal immobilization support material including nontoxic nature, easy obtainability, reusability and high surface area and porous for cell attachment. The adsorption capability and strength of binding are also important factors that determine the selection of a suitable support material. The selected and morphologically examined matrices were further used to establish batch fermentation system for butanol production. The OPF that showed the highest butanol

production is convenient when applying in either repeated-batch or continuous operation mode. In addition to a simple operation and easy availability, also has low price OPF. Therefore, OPF were chosen as the support matrices for cell immobilization in the following experiment.

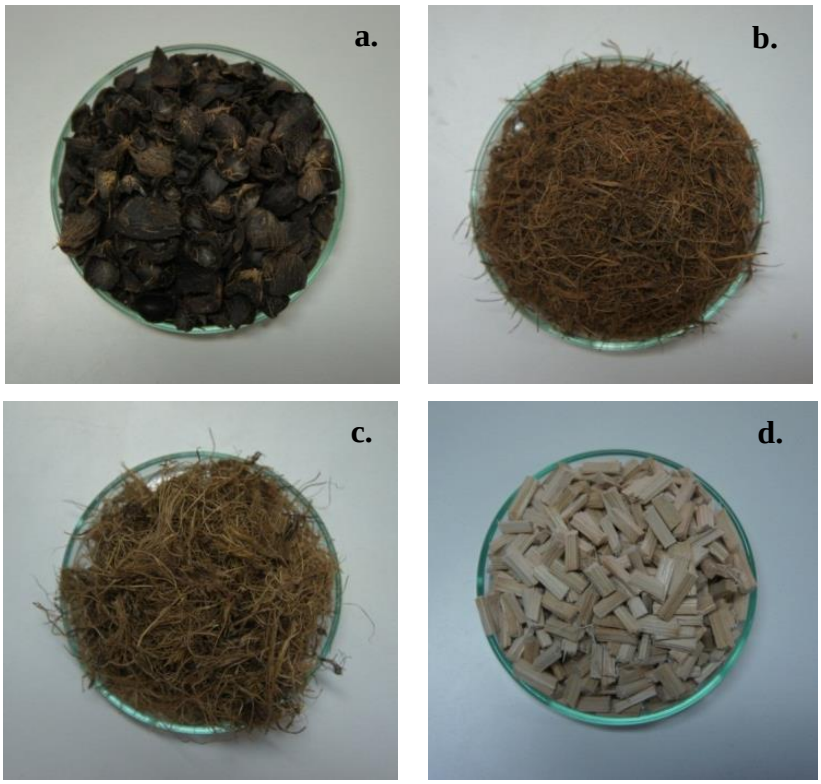

Figure 23 The digital image of immobilization support matrices; (a) oil palm shell (OPS), (b) palm press fibers (PPF), (c) empty fruit bunch (EFB) and (d) oil palm frond (OPF).

The support material surfaces before and after the cell immobilization observed by SEM are shown in Figures 25 and 26, respectively. It was found that OPS showed non-uniform rough surface and PPF and EFB showed smooth surface only OPF showed porous structure with smooth surface (Figure 25e, 25f, 25g and 25h). While the OPS and PPF showed less cells adherence (Figure 25a, 25b, 25c and 25d) due to smooth surface which lead to the detachment of cells. Apart from that, the OPF showed high number of cell immobilization either in adsorbed condition (Figure 25g and h). Many researchers (Huang *et al*., 2004; Survase *et al*., 2011; Tripathi *et al*., 2010) have reported that the clostridium can adsorb and grow on lignocellulosic materials without any additional chemicals. It was clear that the cell were adhered onto the OPF surfaces as shown in Figure 25 (g and h). However, due to technical difficulties, there was no way to measure how many cells were adhered either on the surface or inside the OPF.

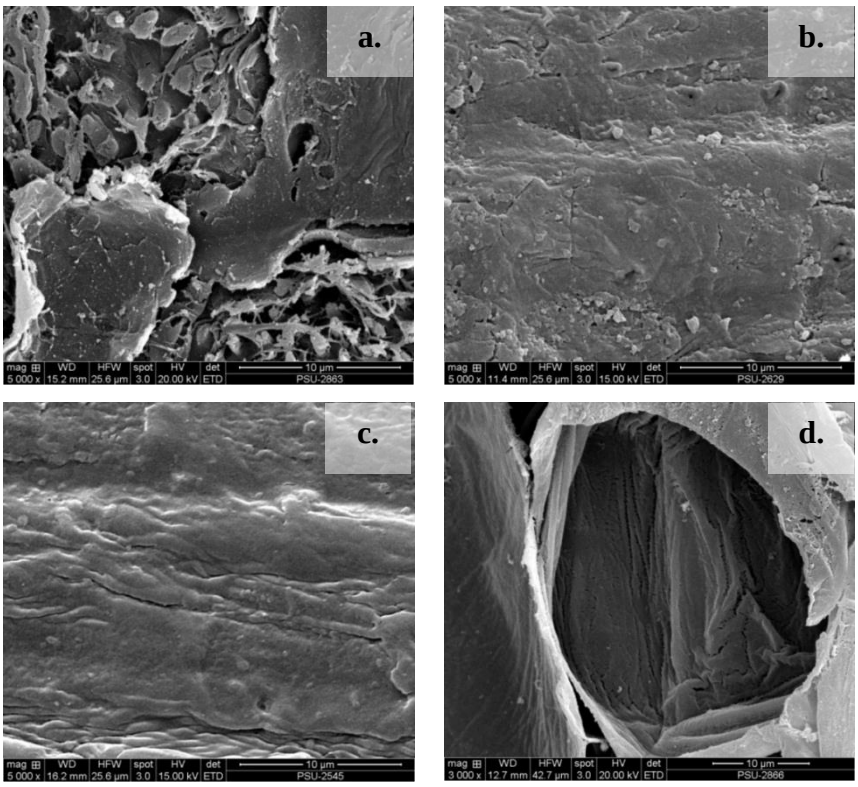

Figure 24. Scanning electron microscopic (SEM) images of different support matrices showing the surface morphology of (a.) oil palm shell (OPS), (b.) palm press fibers (PPF), (c.) empty fruit bunch (EFB) and (d.) oil palm frond (OPF).

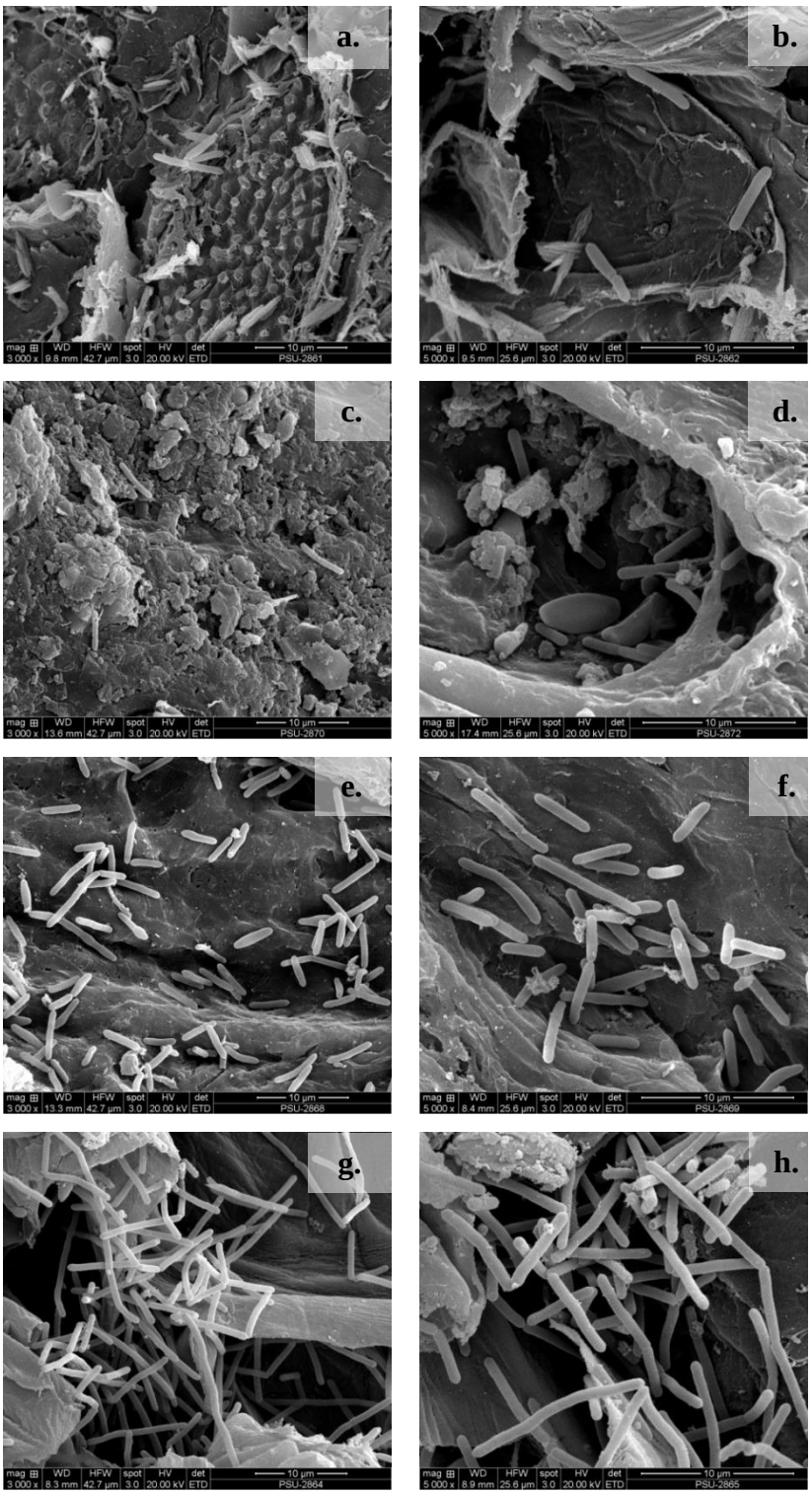

Figure 25. Scanning electron microscopic (SEM) showing that cells were adhered on OPS (a and b), PPF (c and d), EFB (e and f) and OPF (g and h).

3.2 Cells immobilization

The cell immobilization done by adsorption provides a direct contact between nutrients and the immobilized cells minimizing the diffusion problems. This technique involves the transport of cells from the bulk phase, followed by the adhesion and subsequent colonization of the matrix surface. Both electrostatic and hydrophobic interactions are believed to govern the cell-support adhesion, which is the key step in controlling the cell immobilization on the matrix (Kilonzoa *et al*. 2011).

Figure 23 shows the effect of support materials on butanol production and sugar utilization in solventogenic mode. In this study, 15 g/L glucose and 10 g/L butyric acid were used as substrates in the medium. The TYA medium was generally used for growth and proliferation of clostridial cells, while N-free medium is a production medium with high substrate concentration. In this set, the cell population was increased by growing cells in TYA medium and simultaneously immobilizing them on the support matrices for 24 h. Once the cells were immobilized, the TYA medium was replaced with N-free medium for solvent production. The utilization of glucose by clostridial cells was analyzed at various time intervals. It was found that the addition of support materials help in improving the butanol production. OPF was found to be the best support material. The maximum butanol concentration of 9.31 g/L was obtained with OPF followed by empty fruit bunch (EFB), oil palm shell (OPS) and palm pressed fiber (PPF), respectively. Survase *et al*. (2012) reported previously that the coconut fibers and wood pulp are the most promising support matrices for Clostridia. Tripathi *et al*. (2010) screened agarose–alginate cryogel beads and compared with the other potential immobilization matrices such as coconut fibers, brick pieces, and burnt coal. They reported that cryogel beads was effective for active immobilization and can be further studied for continuous production process. Shamsudin *et al*. (2006) investigated ABE production by *Clostridium saccharoperbutylacetonicum* N1-4 using different immobilization materials and concluded that passive immobilization could increase the production by 215.12%. In contrast to synthetic matrices, wood chips, straw, loofa sponge, bagasse, coconut fibers, and other fibrous matrices provide adequate supporting surfaces for cell adsorption due to their high specific surface area, void volume, mechanical strength

and permeability, low pressure drop, less diffusion problems and non-toxicity, biodegradability and durability, and low cost and high availability (Ogbonna *et al*. 2001; Survase *et al*. 2009; Huang *et al*. 2004). The immobilized cell systems are able to maintain high cell concentrations, have improved reaction rates, and are stable at high dilution rates with a little cell washout (Welsh *et al*. 1987).

The rate of glucose consumption in PPF was slowest as compared to other matrices. The low glucose utilization might be because of ineffective cell adherence which occurred on the support matrices that most of the bacterial cells came out when the TYA medium was replaced with N-free medium. It was also possible that the rest of the cells which adhered on the support matrices were dead due to medium shock i.e. from TYA to N-free medium. But in case of OPF, the cells might be safely entrapped inside and there might be neither cell loss nor medium shock. This then encourage the butanol production in the N-free medium.

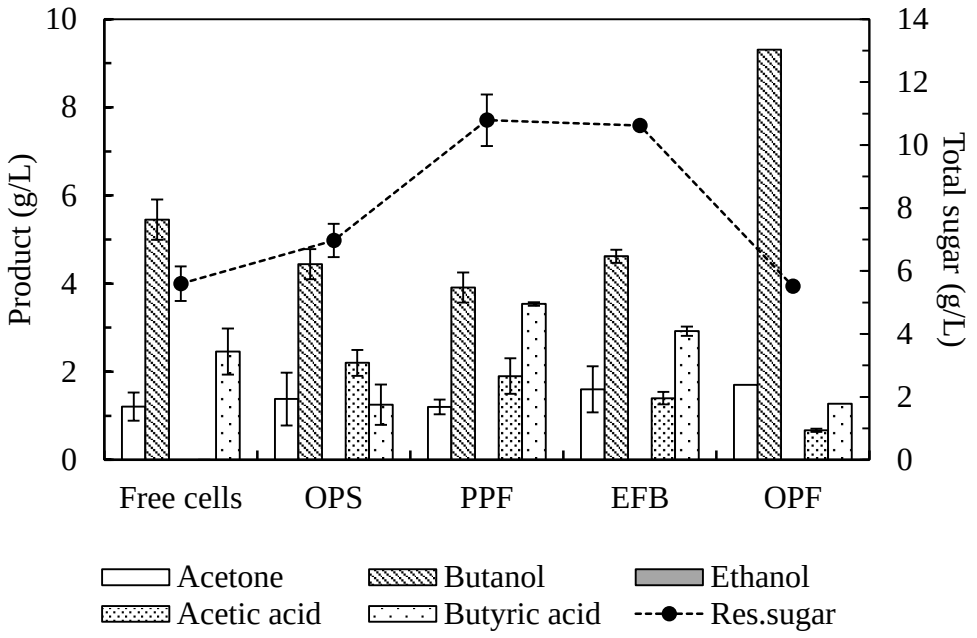

Figure 26. Cell immobilization on various support materials for butanol production by *C. beijerinckii* TISTR 1461. OPS: oil palm shell, PPF: palm pressed fiber, EFB: empty fruit bunch, OPF: oil palm frond and Res. sugar: residual sugar.

3.3 Repeated batch fermentation on different support materials

Repeated batch fermentation is the process that the portion of fermentation is withdrawn at time intervals and the residual part of the broth is used as an inoculum for the next batch. This process aimed to increase the productivity, and it has several advantage compared to a batch fermentation such as no new inocula requirement for each batch and long-term productivity (Anastassiadis and Rehm, 2006).

The butanol production by the four different support materials were compared. Changes of pH, optical density, total sugar and butyric acid of the butanol production from the glucose by *C. beijerinckii* TISTR 1461 immobilized on the 5 g of support material during the repeated batch fermentation (five cycles) are shown in Figures 27 and 28. The fermentation time of each cycle was controlled at 72 h.The old fermentation medium were removed from the serum bottle by syringe, and the immobilized cells on support material was used in the next batch operation. The batch fermentation using the fresh medium with suspended cells as a control. In the 2nd cycle, the butanol concentration decreased significantly likely due to acid crash effect from the 1st cycle. It is generally accepted that acid crash is caused by the fast accumulation of butyric acid during the fermentation. In the previous study of Richter and co-worker (2011), they found that when decreasing the concentration of glucose and butyric acid in single stage fermentation did not circumvent acid crash problem.

In the first cycle of the immobilized *C. beijerinckii* TISTR 1461 on OPS, PPF, EFB and OPF the butanol production were 5.55, 3.91, 4.62 and 9.31 g/L, respectively, which were higher than that using free cell (5.68 g/L). After completion of the first cultivation, all of the liquid was removed and new medium was added. In this way, the immobilized cells could maintain the solventogenesis phase, making the subsequent cycle shorter and increasing the yield. During the sequential cultivation, cell growth was observed in every cycle. A sharp decrease of optical density (OD 600 nm) value was noticed after reaching the maximal solvent production in all cycles. Similar observations were reported earlier and explained as autolysis (Croux *et al*., 1992; Yang *et al*., 2013).

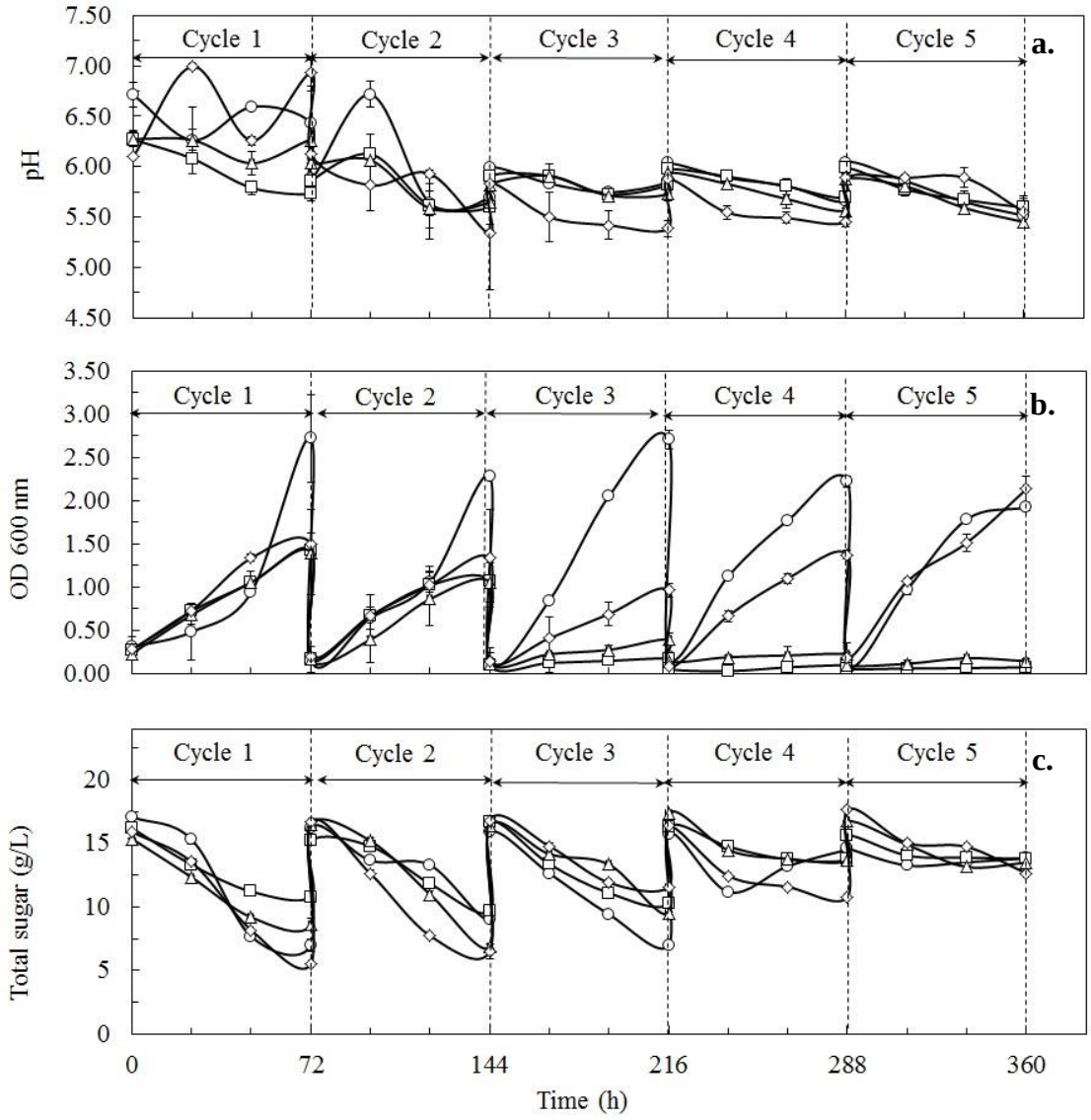

Figure 27. (a) pH, (b) OD 660 nm and (c) sugar profiles in repeated batch fermentation of N-free medium for butanol production by immobilized cells on different materials (OPS, ○; PPF, □; EFB, △; OPF, ◇).

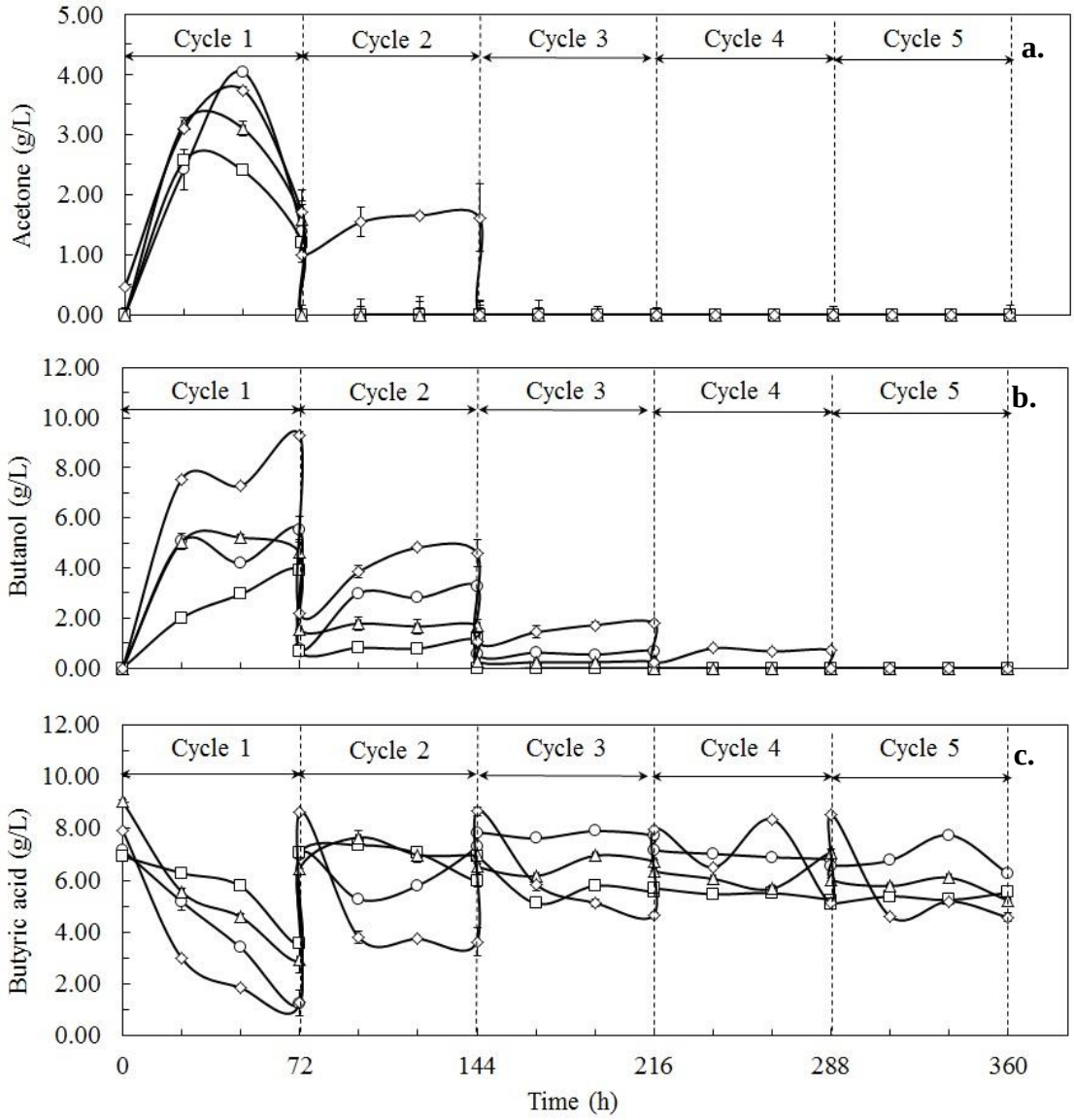

Figure 28. (a) acetone (b) butanol and (c) butyric acid in repeated batch fermentation of N-free medium for butanol production on different immobilization materials (OPS, ○; PPF, □; EFB, △; OPF, ◇).

Autolysin production by the solventogenic clostridia and the effect of butanol on clostridial cells during the solventogenic phase of butanol fermentation is worthy of mention. Barber *et al*. (1979) reported the production of greater concentrations of autolysin toward the end of the exponential growth phase of *C. acetobutylicum* (onset of solventogenesis) and was accompanied by lysis of culture cells and decrease in ABE-producing cells. They also reported the direct linkage of the autolytic degradation in solvent producing cells of *C. acetobutylicum* and the presence of butanol. It was found that the concentrations of butanol were involved in the induction of release of cell-free autolysin during the solventogenic phase (Barber *et al*., 1979; Jones and Woods, 1986).

The first fermentation cycle showed a high production of 9.31 g/L butanol and 11.01 g/L ABE, with glucose consumption over 65.28%. Out of five, this cycle showed the highest value of OD and productivity. The first cycle was followed by a cycle with diminished process efficiency as it was also observed by the lower glucose consumption, lower product, yield and productivity, and accumulation of acids.

When comparing the immobilized cells to the free cells, the differences in the performances under the same condition were observed. Although the fermentation cycles started with the same inoculum size in both immobilized and free cells, the OD value in fermentation using immobilized cells, was lower than that of free cells. However, the production of butanol and total solvent were 1.75-fold and 1.76-fold higher than those of the free cells, considering the three initial sequential batches studied. This indicated that the cells in the support materials may contribute substantially to the ABE production by maintaining its solventogenic stage. In general, it has been observed that immobilized cells perform better than free form cells (when the immobilization method does not offer limitations in mass transfer or cell viability) and potentially offer high tolerance to harsh environment (Huang *et al*., 2004; Chen *et al*., 2013; Efremenko *et al*., 2011)

Qureshi and co-worker (2004) assumed that ‘growing adsorbed’ cells support growth and all newly grown cells did not adsorb (some may be attached firmly, some may be attached loosely, and some may not be attached at all). The medium contained newly grown and desorbed cells (possibly loosely attached). The

exact reason for desorption of loosely attached cells was not known; however, it appeared that chemotaxis plays an important role (Guttierez and Maddox, 1987). During chemotaxis cells tended to migrate away from the toxic chemicals such as butanol. Since butanol was produced in the reactor, loosely attached cells might tend to detach and became free cells. It should be noted that no chemicals were used to adsorb the cells to the matrix, rather it was a natural process whereby cells themselves bind to the support. However, it is known that microbial cultures produce polysaccharides which may help in the adsorption process in addition to charges on the cells and support. The yield of butanol by immobilized cell on OPF in this study (0.13) was comparable to those of other studies using different support materials and other immobilization method (Table 18).

Table 17. Comparison of batch fermentation by immobilized cells on different support materials

Immobilization method	Cultivation time (h)	Initial glucose (g/L)	Parameter				References
			P_{ABE} (g/L)	P_{BtOH} (g/L)	$Y_{butanol}$ (g/g)	Q_{BtOH} (g/l/h)	
pretreated cotton towels	84	60	19.8	12.3	0.205	0.15	Chen *et al*., 2013
pretreated cotton towels	nd	60	-	13.85	0.23	-	Chen *et al*., 2013
wood pulp	110	60	18.88	-[a]	0.37	0.17	Survase *et al*., 2012
coconut fiber	110	60	18.6	-	-	-	Survase *et al*., 2012
coal	141	60	19.87	11.6	0.23	0.08	Tripathi *et al*., 2010
coconut fiber	141	60	20.29	11.6	0.23	0.14	Tripathi *et al*., 2010
clay brick	141	60	24.37	13.7	0.27	0.17	Tripathi *et al*., 2010
OPS	72	20	5.82	5.55	0.45	0.08	This study
PPF	72	20	5.11	3.91	0.55	0.05	This study
EFB	72	20	6.22	4.62	0.66	0.06	This study
OPF	72	20	11.01	9.31	0.76	0.13	This study

[a] = not available

P_{ABE} : *total solvent concentration,* P_{BtOH} : *butanol production,* Y_{BtOH} : yield of solvent, Q_{BtOH} : butanol productivity

3.4 Effect of OPF amount on butanol production

Because the immobilized cells on OPF was the most successful for butanol production and better than the free cells, the effect of amount on butanol fermentation was then further investigated OPF. The results are shown in Figure 28 and 29. Three amounts of OPF for cell immobilization were 3 %, 5 % and 7 %. The repeated-batch fermentation by *C. beijerinckii* TISTR 1461 immobilized on 3 %, 5 % and 7 % were carried out. According to the optimum condition for batch process, the fermentation time of each cycle in the repeated-batch system was kept constant at 72 h. In the first cycle, the maximum butanol production using 3, 5 and 7 % were 7.94 ± 0.23 g/L, 9.31 ± 0.15 g/L and 9.98 ± 0.54 g/L, respectively. In the 1–5 cycles, the residual sugar concentrations using 7 g of OPF were approximately 1.31, 7.14, 8.14, 15.34 and 16.44 g/L (Figure 29c), respectively.

The increased optical density indicated the detachment of the bacterial cells from the OPF in each cycle and the vigorous growth was observed until the 5^{th} cycle. It was possible that some immobilized cells were detached into the broth and others grew and continuously attached in the support materials in each cycle. Therefore, OPF containing active bacterial cells acted as the source of inoculum for butanol production in the next cycle.

Considering the higher production of solvents at the end of the first cycle in comparison to free cells, this implied that the cells in the OPF were more efficient in the uptake and recycling of acids, detoxifying the cellular environment and contributing to additional carbon for an improved solventogenesis (Formanek *et al*., 1997).

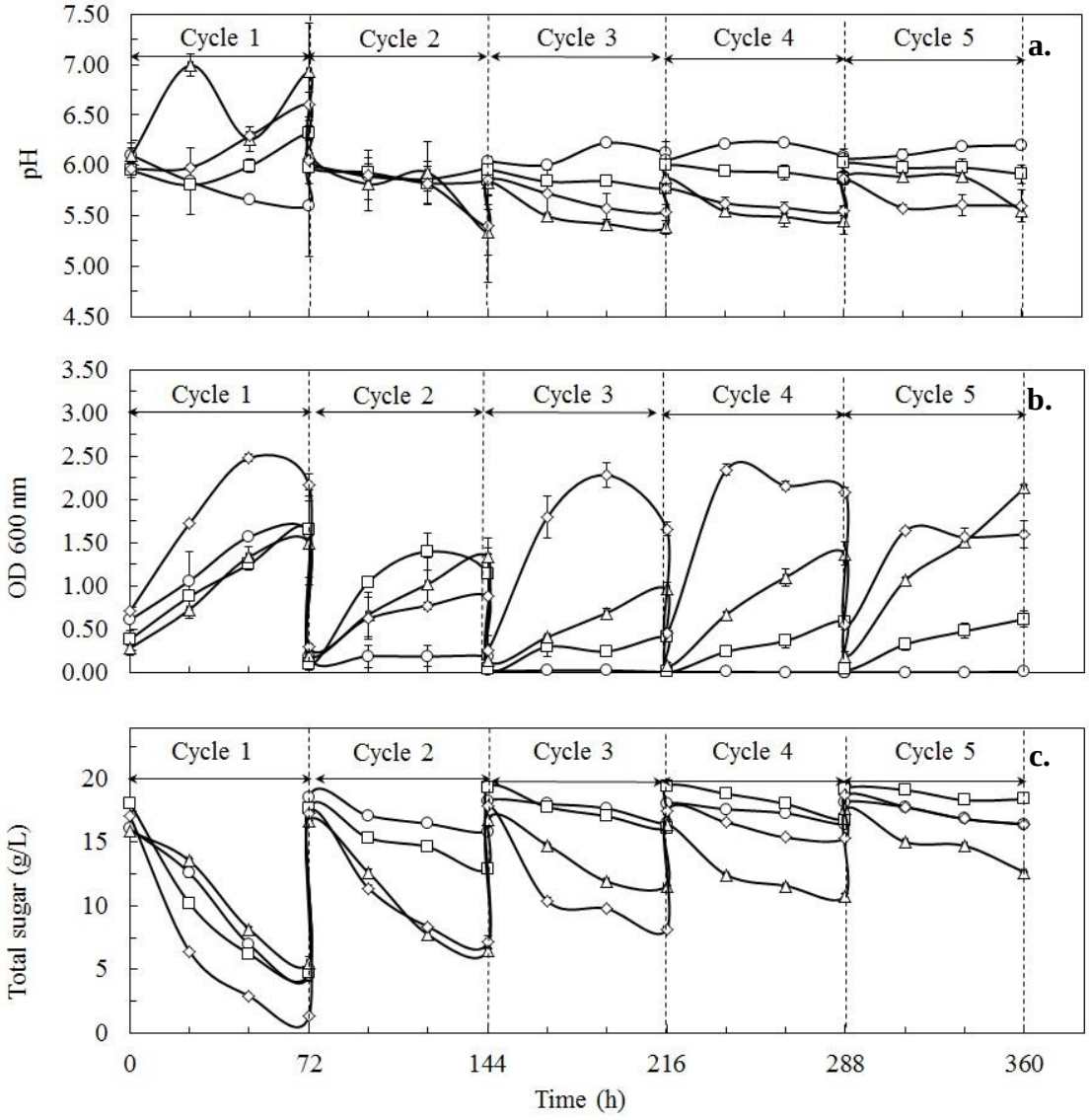

Figure 29 (a) pH, (b) growth and (c) total sugar profile in repeated batch fermentation by immobilizeds cells on OPF (free cells, ○; 3 %, □; 5 %, △; 7 %, ◇)

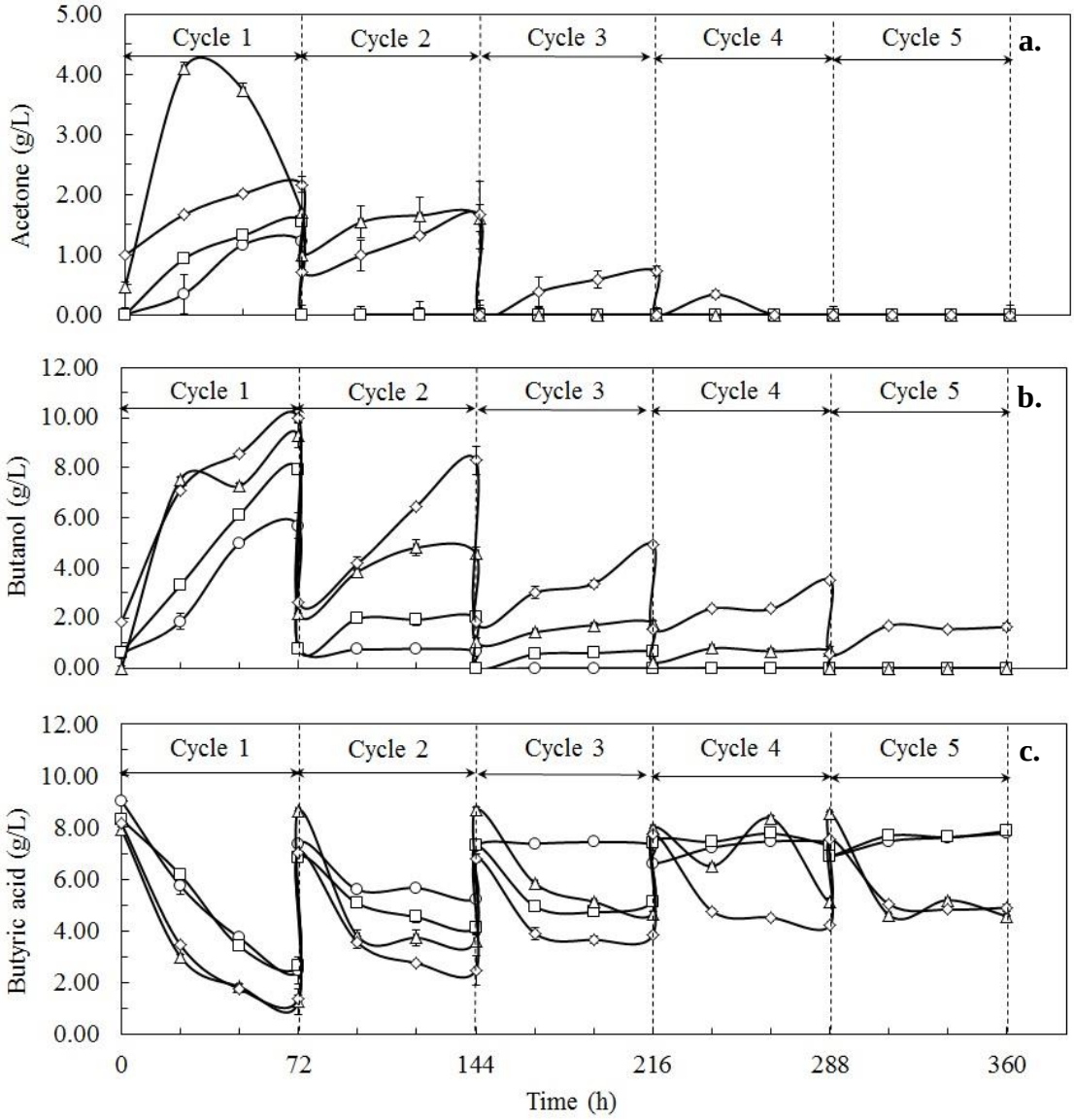

Figure 30. (a) acetone, (b) butanol and (c) butyric acid in profile in repeated batch fermentation by immobilized cells on OPF (free cells, ○; 3 %, □; 5 %, △; 7 %, ◇)

However, fluctuations in the production values were observed in the different cultivation cycles. The fluctuation in production values along the sequential batch was also observed by Chen *et al*. (2013) when using immobilized *C. acetobutylicum* CGMCC5234 on pretreated cotton towels. This suggested that it is a regular aspect in sequential batch that production and yields cyclically increased first and then dropped. The variations in each cycle were probably due to the limitations of a batch system, where different levels of inhibition occur, due to the accumulation of products in the vicinity of the cells and thus resulted in different activities in each cycle.

The reduction in the process efficiency in the second cultivation cycle of the immobilized cells could be explained by the deleterious effect of product concentration in the preceding cycle which showed high butanol production, reaching 9.98 g/L. Those values are equal to or above the concentrations (0.1–0.15 M) that are known to cause 50% inhibition of both cell growth and sugar uptake. It negatively affects the cell membrane fluidity and a number of physicochemical characteristics inside the cells, such as the ATPase activity (Lee *et al*., 2008a; Kumar and Gayen, 2011). The higher concentration of butyric acid in the second cultivation cycle indicated that the solventogensis became inhibited due to an acid crash, where an excess of acid production occurs without a significant switch to solventogenesis (Maddox *et al*., 2000).

Table 19 compares the fermentation performance during each cycle in the repeated batch fermentations. A relatively stable butanol yield of 0.32-0.61 g/g was obtained. However, there was a significant drop in butanol productivity after the first cycle, indicating that the accumulation of butanol would inhibit its biosynthesis in the cells. Both solvent productivity and glucose utilization rate decreased after the second cycle and dropped significantly from the third cycle to the fourth cycle (Figure 28 c and 29 a,b), indicating the decrease in cell viability and productivity. Due to the extended fermentation time, dead cells and non-active cells might coexist with actively solvent-producing cells in the fermenter (Qureshi *et al*., 1988). It was also possible that ABE fermentation might fail due to exhaustion of the nutrients. (Ezeji *et al*., 2003, 2004b). Therefore, nutrient supplementation with yeast extract in the

subsequent feeding cycles might be needed was able to maintain a stable fermentation for an extended period.

Table 18 Results achieved during the repeated-batch fermentation in 7 % OPF for butanol productivity by *C. beijerinckii* TISTR 1461

Cycle (h)	ABE (g/L)	$Y_{(B/C)}$	Q_B (g/L/h)
1 st (72)	9.98 ± 0.54	0.34	0.139
2 nd (144)	8.31 ± 0.36	0.43	0.058
3 rd (216)	4.95 ± 0.06	0.30	0.023
4 th (288)	3.52 ± 0.52	0.61	0.012
5 th (360)	1.65 ± 0.41	0.32	0.005

$Y_{(B/C)}$: yield of butanol to the substrate consumed (C-mol/C-mol), Q_B : butanol productivity

3.5 Two-stage fermentation

Since the results from the reported fermentation indicated that the nutrient limitation would suppress the solventogenesis activity of the cells. The two-stage fermentation in which TYA medium was used in the first stage for reactivating the cells and N-free medium was used in the second stage for butanol production. Measurement of metabolic products throughout the entire operating period of the run showed the acidogenesis and solventogenesis in the first and second stages, respectively (Figure 30 and 31). In the two-stage fermentation, 20 g/L glucose in the first stage and 15 g/L glucose and 10 g/L butyric acid were used in the second stage. The results showed that the cells immobilized on OPF could be reused 3 times for butanol production without losing their viability. The effect of duration time in the first stage was also investigated. It was found that 24 h was the suitable for maximum butanol production (Figure 31b)

Figure 31 also shows the production of gas by the Clostridia. In this study, the butanol production was also slightly higher than that reported by Guo *et al*. (2012). In the acidogenic phase which usually takes place in the exponential growth phase, substrate is converted into acetic acid, butyric acid, H_2 and CO_2 that are only

two components in the evolved gas H_2 and CO_2. H_2 is generated in the electron transport shuttle system by the catalysis of hydrogenase, and CO_2 is generated in pyruvate → acetyl-CoA and acetoacetyl-CoA → acetone routes. Therefore, the accumulated gas corresponds to the production of acid as shown in Figure 30. When the acidogenic phase successfully shifts into the solventogenic phase, acetate and butyrate are re-assimilated and converted into acetyl-CoA and butyryl-CoA, respectively, accompanied with acetoacetyl-CoA converting into acetone. During this period, large amount of CO_2 has to be released while H_2 generation is decreased as butanol synthesis needs a large amount of NADH. Therefore, when the shift from acidogenic phase to solventogenic phase occurred, a reduced ratio of H_2/CO_2 could always be observed (Jones and Woods, 1986). ABE production in two-stage fermentation have been reported either in batch or continuous cultures with either freely suspended or immobilized cells. A comparison of the two-stage batch fermentation using different *Clostridium* species are shown in Table 20. The developed process in this study shows superior yield when compared with the work of Xue *et al*. (2012) and Guo *et al*. (2012).

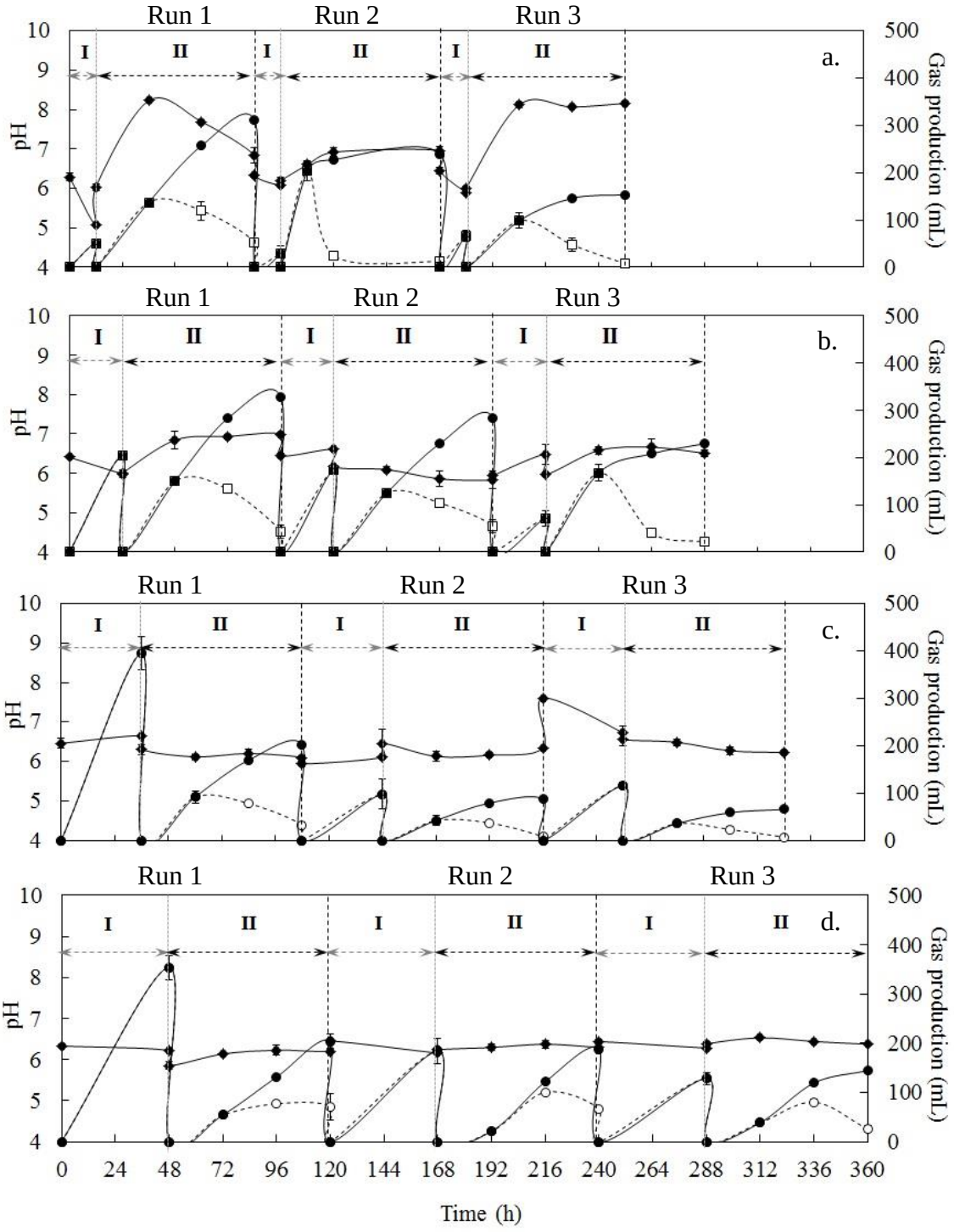

Figure 31. Effect of duration time in the first stage on pH and gas production of stage by immobilized cells on OPF repeated-batch fermentation two stage fermentation of OPF. The time in the first stage was a. 12 h, b. 24 h, c. 36 h, d. 48 h (pH, ◆; daily gas production, ○; cumulative gas production, ●)

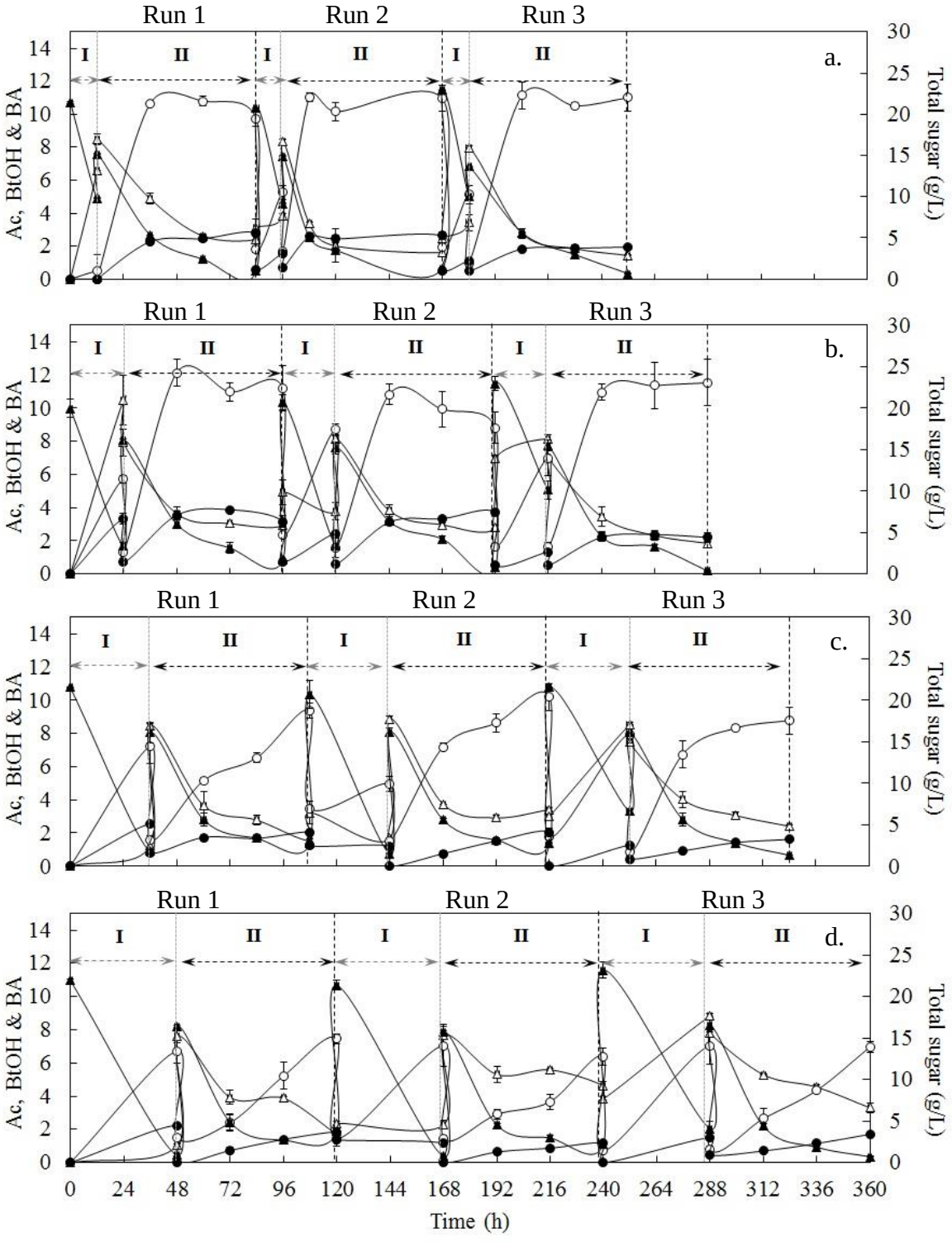

Figure 32. Effect of duration time in the first stage on acetone, butanol, butyric acid and total sugar profile of stage by immobilized cells on OPF repeated-batch fermentation two stage fermentation of OPF. The time in the first stage was a. 12 h, b. 24 h, c. 36 h, d. 48 h (acetone, ●; butanol, ○; butyric acid, △; total sugar, ▲).

Table 19. Solvent production by two-stage fermentation by free and immobilized cells

Two-stage system	Strain	Glucose:BA (g/L)	Initial pH	Parameter				Reference
				P_{ABE} (g/L)	P_{BtOH} (g/L)	Y_{BtOH} (g/g)	Q_{BtOH} (g/l/h)	
Batch								
Immobilized cells with *in situ* product removal	*C. acetobutylicum* JB200	30:0	5.00	25.50	16.20	0.20	0.30	Xue *et al*., 2012
Free cells with pH-control	*C. acetobutylicum* XY16	10:0	4.70	19.60	11.50	0.20	0.40	Guo *et al*., 2012
Immobilized cells on OPF	C. *beijerinckii* TISTR 1461	15:10	6.50	17.47	11.70	0.61	0.16	This study
Continuous								
Free cells	*C. acetobutylicum* ATCC 824	60:0	6.00	-[a]	5.93	0.16	0.40	Lai and Traxler, 1994
Free cells, membrane evaporation	*C. beijerinckii* NRRL B592	60:0	4.70	9.27	9.10	0.25	1.72	Gapes *et al*., 1996
Immobilized cells with, pervaporation	*C. beijerinckii* NRRL B592	60:0	4.70	9.30	-	0.24	0.20	Mutschlechner *et al*., 2000
Free cells with gas stripping	*C. acetobutylicum* B5313	60:0	6.50	20.30	13.60	0.24	0.01	Bankar *et al*., 2013

[a]Not available

P_{ABE} : *total solvent concentration,* P_{BtOH} : *butanol production,* Y_{BtOH} : yield of solvent, Q_{BtOH} : butanol productivity

3.6 Two-stage fermentation of DCH in bioreactor

In the two-stage fermentation of DCH (Figure 33). The concentration of butanol in the first stage were 6.26, 4.45 and 6.59 g/L in 1st, 2nd, and 3rd cycle, respectively. They were slightly lower than those in the fermentation using glucose. When DCH was used as the substrate in the second stage, Solvents volumetric productivity in the second stage was 0.12 g ABE/L/h. This solvent productivity is close to that was achieved with batch fermentation using glucose (0.15 g/L/h). The initial pH (6.5) in the first-stage has a significant influence on the acid formation. Several researchers have reported that a fermentation that produced high acid in the first-stage did not produce high solvents in the second stage, irrespective of the low pH in the second stage, as the cells were not able to switch their metabolic state to solventogenesis (Lai and Traxler, 1994; Godin and Engasser, 1989, 1990; Andersch *et al*., 1983). It is evident that the conditions set in the first reactor determine whether solvent production occurs in the second reactor. Godin and Engasser (1989) concluded that the second stage reactor was dependent on the organic acids (2 g/L) and metabolic state of the cells produced in the first reactor. The high pH in the first stage also resulted in no acetone production. Hüsemann and Papoutsakis (1989) reported that batch or continuous cultures at pH 6 are incapable of producing acetone as the enzymes CoA-transferase and acetoacetate decarboxylase are not present at this pH. Lai and Traxler (1994) also found that there was no acetone produced in a continuous fermentation set at pH 6. However in their two-stage fermentations with the first stage at pH 6, 2.7 g/L acetone was produced.

A comparison of the two-stage fermentation kinetics is shown in Table 21. The process in this study shows superior solvents production when compared with the work of Bahl e*t al*. (1982) and Lai and Traxler (1994). The stable butanol production attained in the repeated-batch fermentation demonstrated the long-term operation stability of the immobilized cells. This implied the good stability and viability of the *C. beijerinckii* TISTR 1461. In addition, cell immobilization in the FBB offered another advantage: gas stripping was more efficient when there were less suspended cells in the broth, which significantly reduced gas stripping selectivity for solvents and could cause severe foaming problem (Ezeji et al., 2004b).

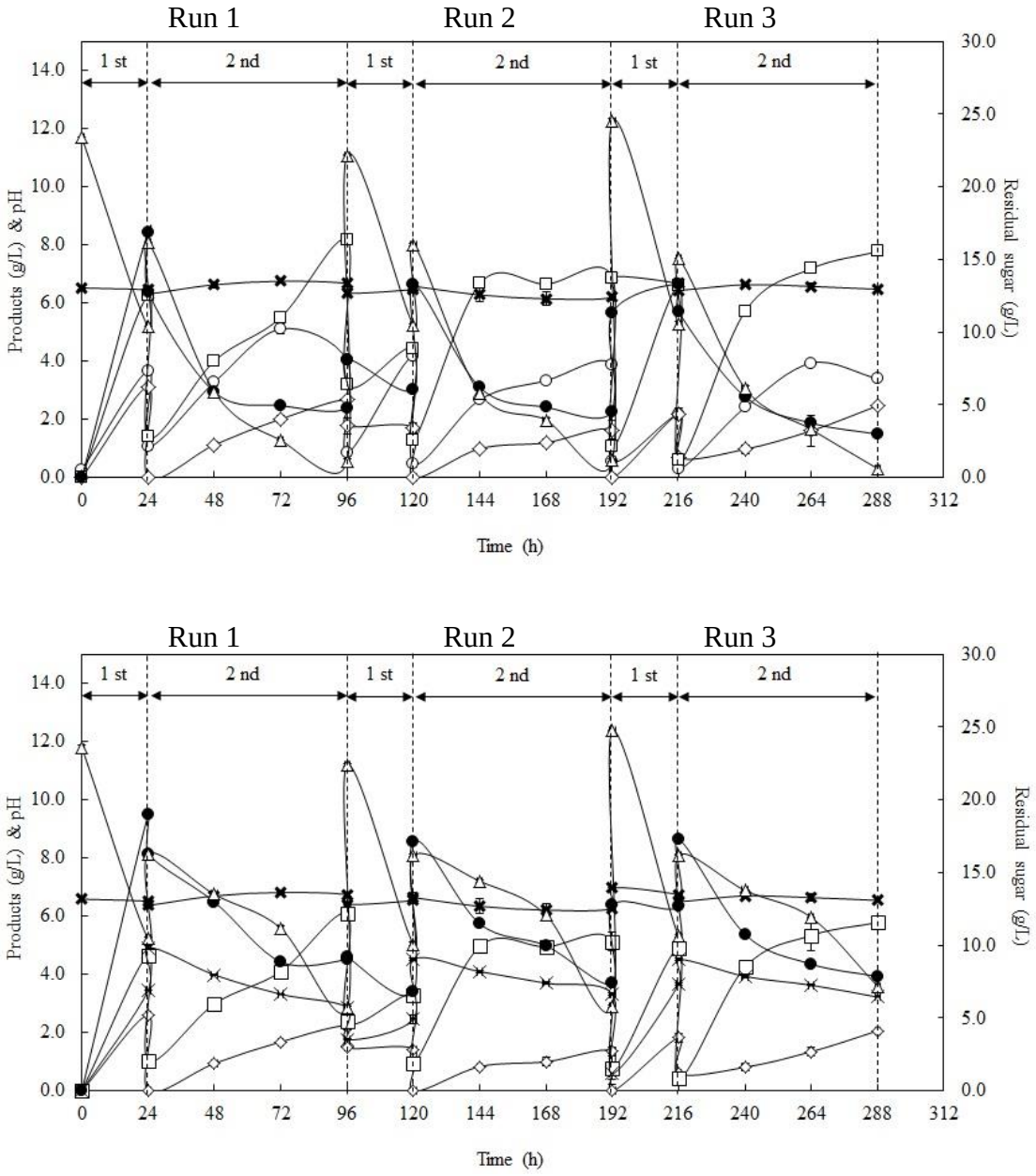

Figure 33 Two-stage fermentation profiles by immobilized *C. beijerinckii* TISTR 1461. (a) glucose and butyric acid (b) DCH and butyric acid without gas stripping (pH, ✖; acetone, ◇; butanol, □; acetic acid, ×; butyric acid, ●; residual sugar, △)

3.7 Two-stage fermentation integrated with gas-stripping

Butanol and ethanol are known to be strongly inhibitory to the organism, and hence their removal is obviously advantageous. The preferential extraction of solvents by the extractant shows a minimum effect on butyric acid and acetic acid (reaction intermediates) extraction, enabling them to be available for further butanol production. Inability to extract these acids was favorable for higher solvent production, since it promotes the solventogenic phase and increases total solvent in the fermentation broth. A two stage fermentation system integrated with gas-stripping was performed. The experiment consisted of three consecutive batch fermentations (Figure 34). Gas stripping preferentially removed butanol over acetone from the fermentation broth that not only alleviated butanol toxicity but also altered the fermentation kinetics. Typical ABE fermentation usually produced butanol and acetone at 2:1 ratio, as seen in the batch fermentation. In contrast, the overall butanol/acetone ratio was significantly higher at 1:1 ratio in two stage fermentation with gas stripping for continuous product removal. Maximum total solvent production of 16.42 g/L (acetone, 8.27 g/L; butanol, 8.15 g/L; and no ethanol) was observed in first stage.

In this study, 3.57 g/L butanol was obtained from detoxified DCH and it was further improved to 5.66 g/L in the gas-stripping integrated fermentation process. To our best knowledge, this study was the first one to demonstrate the feasibility of producing butanol from DCH in ABE fermentation with integrated gas stripping to recover butanol and alleviate butanol toxicity. DCH as an alternative feedstock to the current foodbased substrates should help to improve the process economics of the ABE fermentation. It should be noted that the nutrients and nitrogen source present in the modified TYA medium used in this study can be replaced with low-cost industrial materials such as corn steep liquor. However, further process optimization would be necessary before commercial application. In butanol fermentation with gas stripping, gas bubbles as transporting media have low efficiency in transferring butanol from fermentation broth to the condensate. But since the productivity in butanol fermentation is much higher than that in ethanol fermentation, the gas-stripping rate of butanol could be compatible with the butanol production rate under rational process

design. In addition, gas stripping is the only clean technique without any contamination resource to contact the fermentation broth.

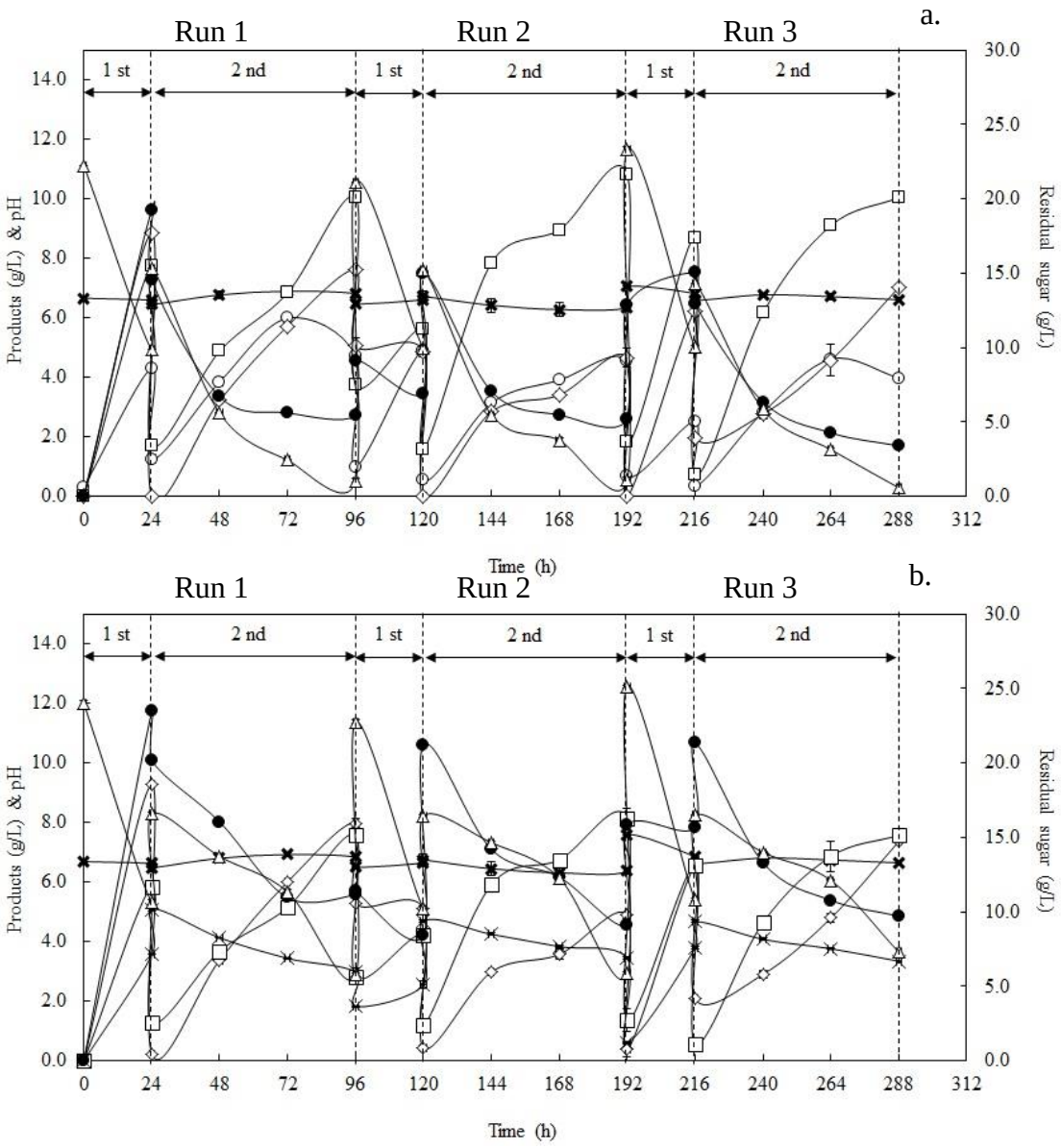

Figure 34. Two-stage fermentation profiles by immobilized *C. beijerinckii* TISTR 1461. (a) glucose and butyric acid (b) DCH and butyric acid with gas stripping (pH, ✖; acetone, ◇; butanol, □; acetic acid, ×; butyric acid, ●; residual sugar, △)

Table 20. A comparison of the two-stage fermentation kinetics

References	Parameter			
	Butanol (g/L)	Total ABE (g/L)	Butanol productivity (g/L/h)	ABE productivity (g/L/h)
Bahl *et al*., 1982[a]	10.5	16.5	0.38	0.54
Frick and Schügerl ,1986[b]	9	15.4	-	1.93
Godin and Engasser, 1988[c]	13	20	-	0.57
Lai and traxler, 1994[d]	5.9	9.1	0.4	0.6
Setlhaku *et al*., 2013[e]	9.5	14.6	0.38	0.58
Setlhaku *et al*., 2013[e]	12	19.5	0.92	1.47
Glucose (without gas stripping)[f]	8.2	11.3	0.16	0.15
Glucose (with gas stripping)[f]	10.8	14.1	0.12	0.21
DCH (without gas stripping)[f]	6.08	8.68	0.08	0.12
DCH (with gas stripping)[f]	8.15	10.2	0.11	0.14

[a] Synthetic medium 60 g/L glucose, phosphate limitation. Stage 1: pH 4.3 and D = 0.125 h^{-1}. Stage 2: pH 4.3 and D = 0.04 h^{-1}

[b] Complex medium, 6 g/L yeast extract, 10 g l−1 tryptone, 60 g /L glucose. pH 4.3 in both stages.

[c] Complex medium, 6 g/L yeast extract, 70 g/L1glucose. Stage 1: pH 4.5 and D = 0.1 h^{-1}. Stage 2: pH 4.5 and D = 0.04 h^{-1}.

[d] Synthetic medium, 60 g /L glucose. Stage 1: pH 6.0 and D = 0.075 h^{-1}. Stage 2: pH 4.5 and D = 0.06 h^{-1}.

[e] Complex medium, 5 g/L yeast extract, 60 g/L glucose. Stage 1: pH 4.5 and D = 0.1 h^{-1}. Stage 2: pH 4.5 and D = 0.04 h^{-1}. D close to zero in fed-batch.

[f] Nitrogen free medium: pH 6.5

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

CONCLUTIONS

This study has shown that the non-growing cells of *Clostridium* spp. could convert butyric acid to butanol by their solventogenic activities in the nitrogen-free medium. The most effective strain for this conversion was *C. beijerinckii* TISTR 1461. It produced the maximum butanol of 12.0 g/L with a high butanol yield of 0.68 C-mol/C-mol and a high butanol ratio of 88 % when using the optimal combination of glucose of 15 g/L and butyric acid of 10 g/L. This strategy is more promising than using growing cells due to its: (a) allowing for direct conversion of butyric acid to butanol without losing carbon flow towards cell growth and led to a higher yield of butanol from carbon used and (b) producing a higher butanol ratio in the ABE products and a higher final concentration of butanol.

This study has also shown that it is possible to produce butanol from decanter cake waste from palm oil mill after hydrolysis by nitric acid and detoxification by activated charcoal. Detoxified decanter cake hydrolysate (DCH) supplemented with whey protein and ammonium sulfate was used as a cost-effective growth medium for butanol production by growing cells. In addition, detoxified DCH without addition of nitrogen sources was used as a co-substrate with butyric acid for direct conversion to butanol. Therefore, decanter cake is another lignocellulosic material that could be used as a low-cost substrate for butanol production.

The immobilization of the cells on oil palm frond (OPF) has been performed. Repeated-batch fermentation using immobilized cells on OPF demonstrated that immobilized cells was an efficient method for long-term butanol production. The operation of two-stage fermentation was shown to be the most effective method for sustainable butanol production by immobilized cells. Table 21 summarized improvement of butanol production and in this study.

Table 21. Summary of improvement of butanol production in this study

Experiments		Butanol (g/L)	Butanol Yield (g/g)	Butanol Productivity (g/L/h)
Part I	- Butanol production from 20 g/L glucose	8.23	0.46	0.114
	- Butanol production by solventgenesis phase in N-free medium containing 15 g/L glucose and 10 g/L butyric acid	12.0	0.68	0.160
Part II	- Butanol production from DCH (total sugar 20 g/L)	3.42	0.28	0.048
	- Butanol production from DCH in solventogenesis phase containing 15 g/L total sugar and 10 g/L butyric acid	6.94	0.47	0.096
Part III	- Butanol production using immobilized cells on OPF in repeated batch fermentation	9.98, 8.31, 4.95, 3.53 and 1.65 in the 1st to 5th cycle	0.34, 0.43, 0.30, 0.61 and 0.32 in the 1st to 5th cycle	0.139, 0.058, 0.023, 0,012 and 0.005 in the 1st to 5th cycle
	- Butanol production using immobilized cells on OPF in two-stage fermentation	6.30, 7.41 and 4.40 in the 1st to 3rd cycle	0.34, 0.39 and 0.22 in the 1st to 3rd cycle	0.047, 0.053 and 0.031 in the 1st to 3rd cycle

SUGGESTIONS

1. The process of using non-growing cells should be further investigated in a continuous system for better understanding in its sustainability.
2. The immobilized cells should be further applied in a continuous and large scale production.
3. The effect of the cell concentration on solvent production should be studied.

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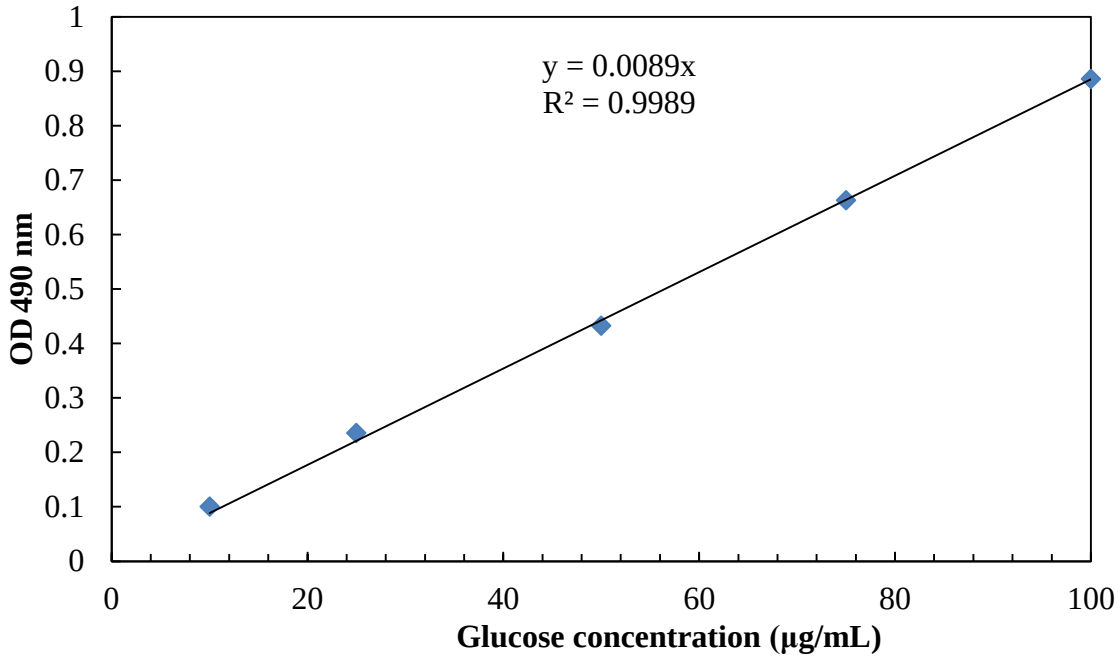
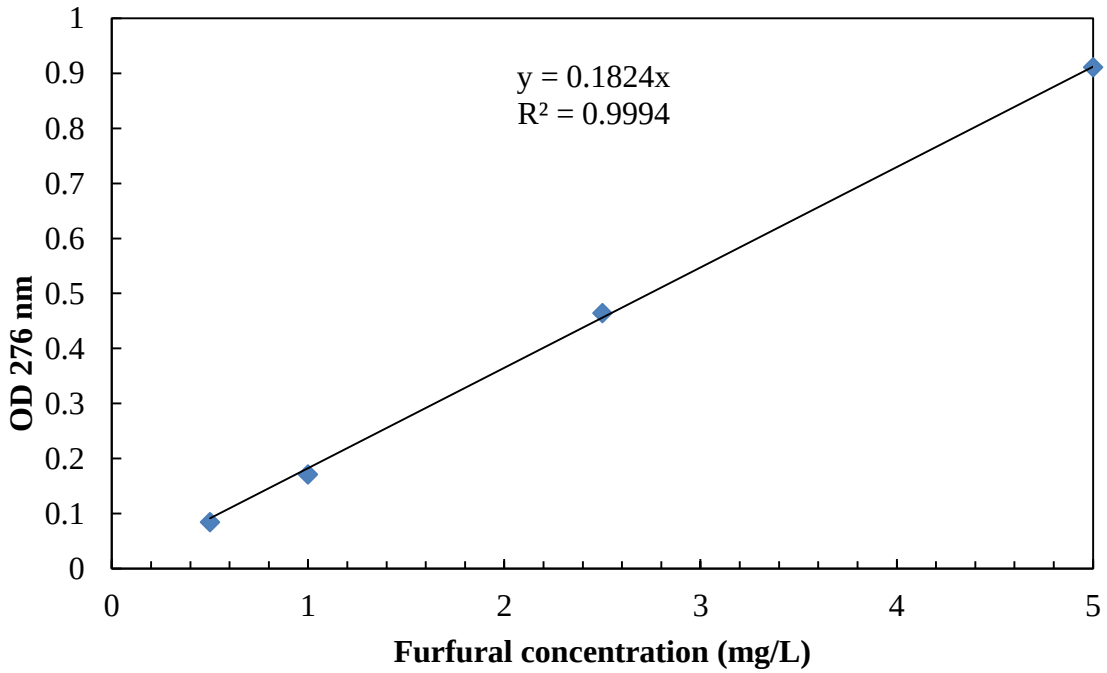
APPENDIX A

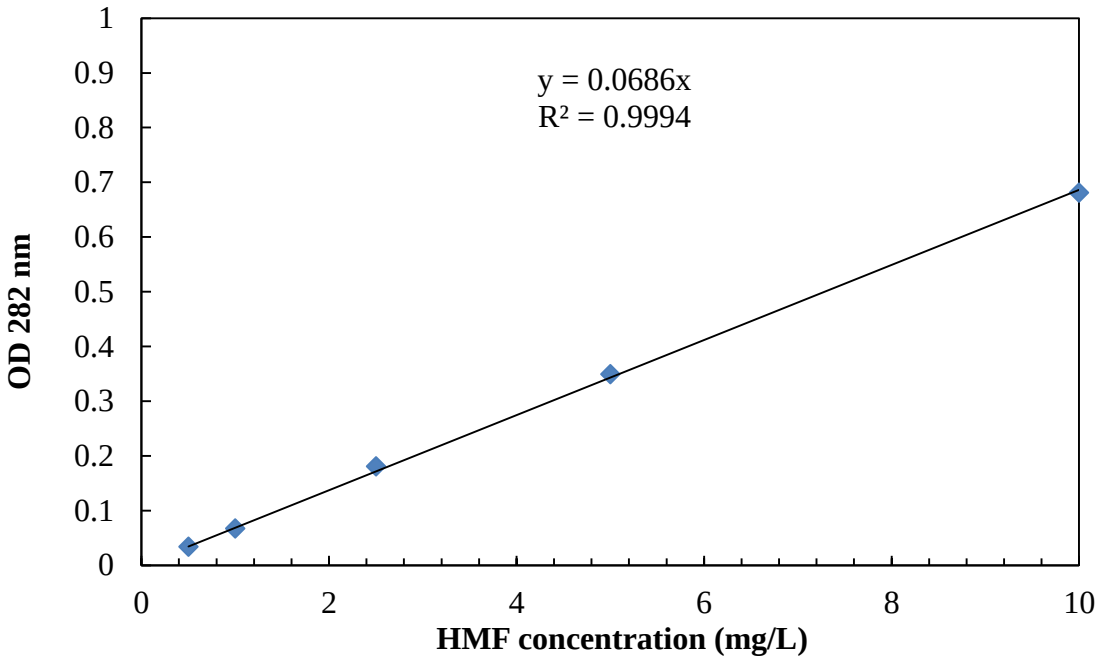
1. Glucose standard curve for total sugar



2. Furfural standard curve



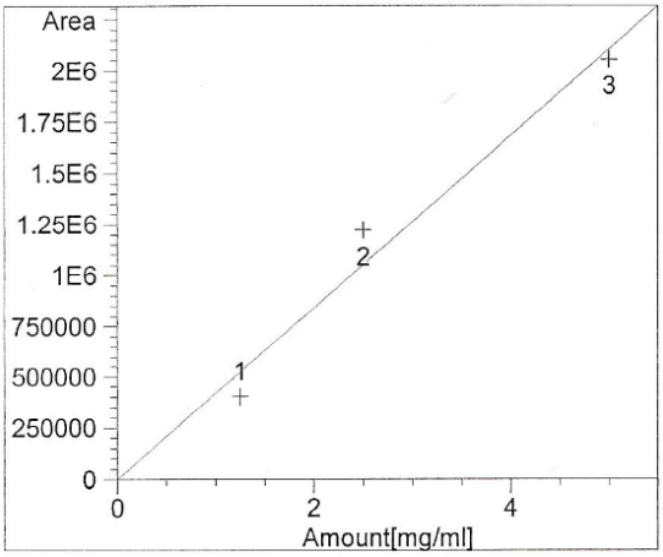
3. 5- Hydroxymethylfurfural (HMF) standard curve



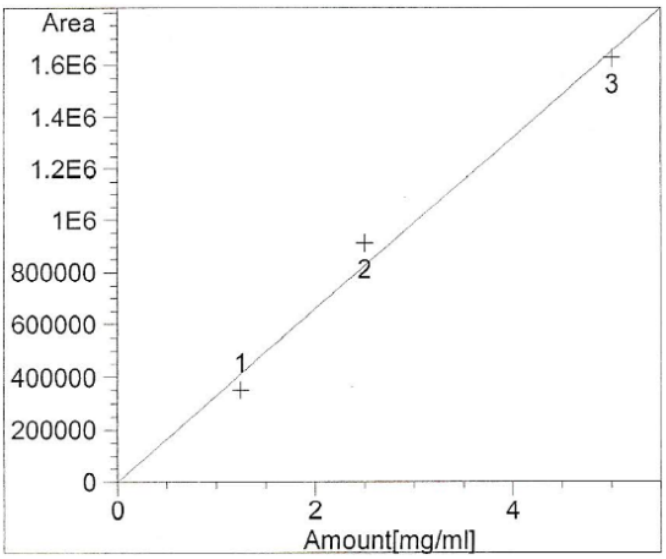
APPENDIX B

1. Standard curve of sugar by HPLC

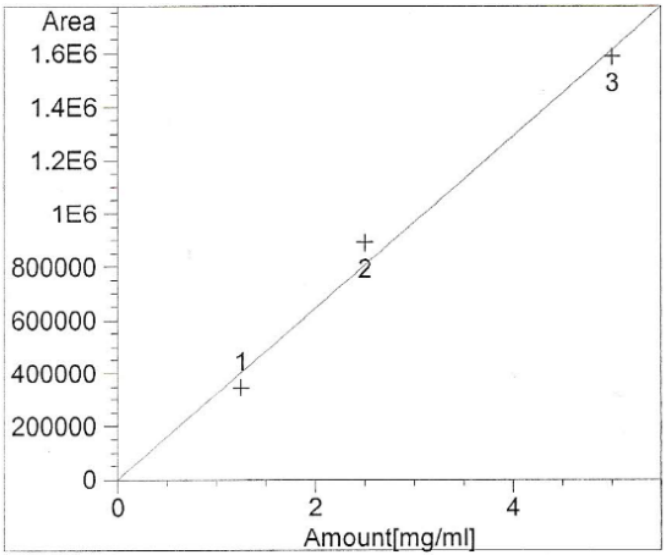
1.1 Standard curve of glucose by HPLC.



1.2 Standard curve of xylose by HPLC.

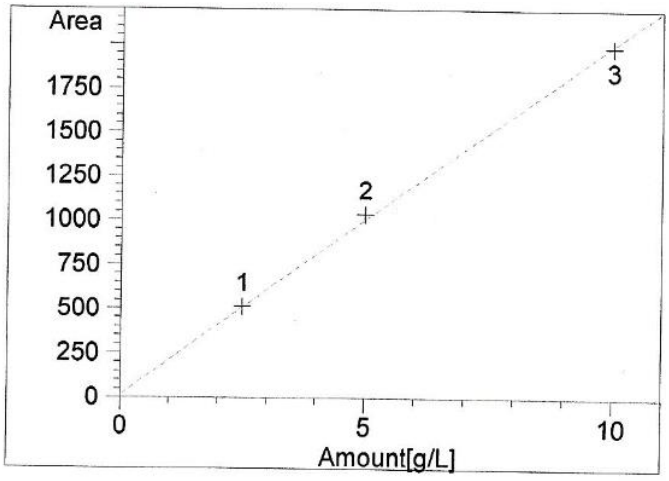


1.3 Standard curve of arabinose by HPLC.

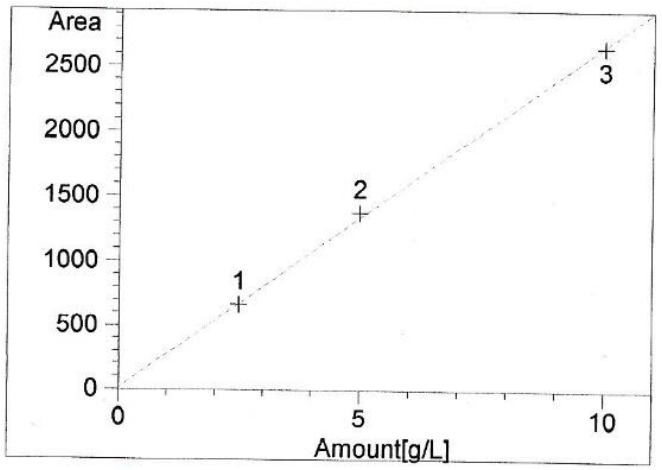


2. Standard curve of ABE and acid.

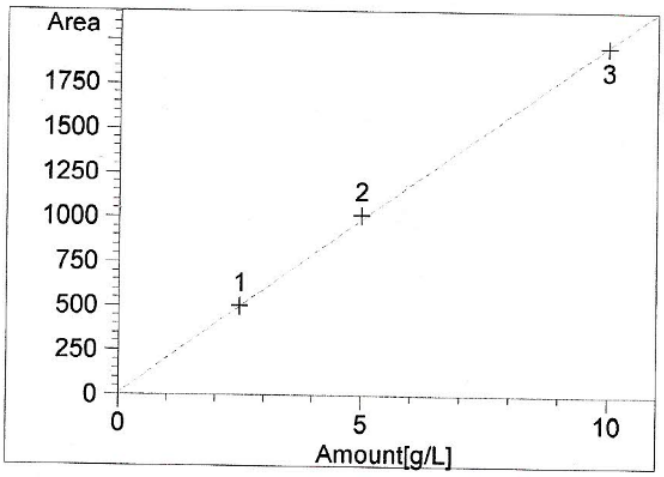
2.1 Standard curve of acetone.



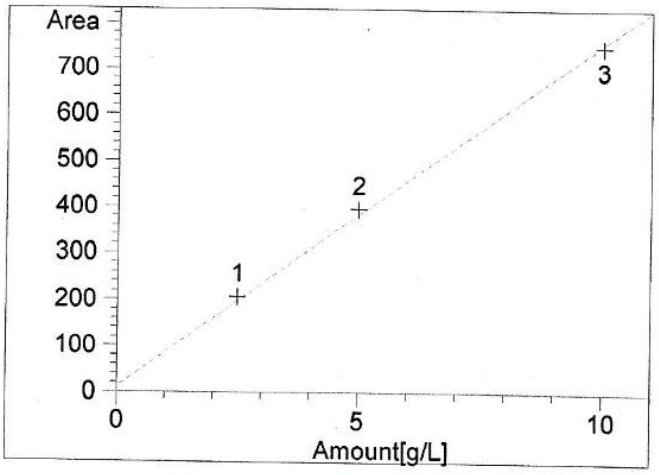
2.2 Standard curve of butanol.



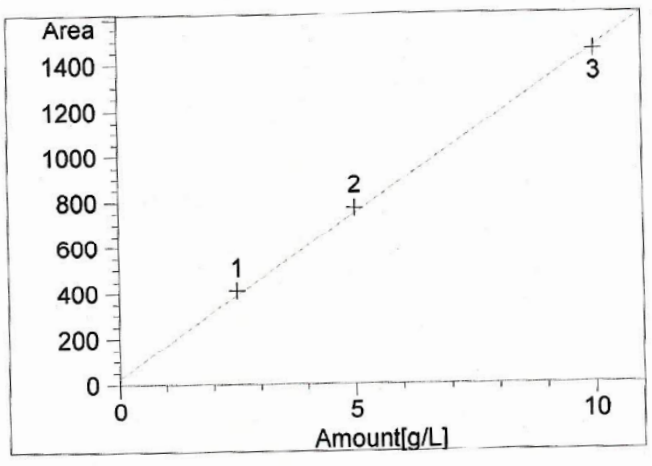
2.3 Standard curve of ethanol.



2.4 Standard curve of acetic acid.



2.5 Standard curve of butyric acid.



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TRF Master Research Grants Co-funding or TRF-MAG Co-funding, The Thailand Research Fund (TRF)

List of Publications and Proceedings

Publication

Loyarkat, S., Cheirsilp, B. and Umsakul, K. 2013. Direct Conversion of Sugars and Organic Acids to Biobutanol by Non-growing Cells of *Clostridium* spp. Incubated in a Nitrogen-Free Medium. Appl. Biochem. Biotechnol. 171(7): 1726-1738.

Loyarkat, S., Cheirsilp, B. and Umsakul, K. 2013. Decanter cake waste as a renewable substrate for biobutanol production by *Clostridium beijerinckii*. Process Biochem. 48(12): 1933-1941.

Loyarkat, S. and Cheirsilp, B. Butanol production by immobilized *Clostridium beijerinckii* TISTR 1461 in repeated-batch fermentation of glucose and butyric acid. (Submitted)

Presentations

Loyarkat, S., Cheirsilp, B. and Umsakul, K. 2012. Optimization of Solventogenic Phase of *Clostridium* spp. in Nitrogen-free Medium for Acetone-Butanol-Ethanol production. The 1st Forum of the Deans of ASEAN Plus Three Graduate Schools (AGRC) on March 2-3, 2012, Chaing-Mai, Thailand (Oral presentation)

Patent

Loyarkat, S. and Cheirsilp, B. Two-phase continuous production of butanol from sugar and organic acids by non-growing immobilized *Clostridium* sp. cells. (In preparartion)