DEVELOPMENT OF LACTIC ACID PRODUCTION PROCESS FROM CASSAVA BY USING LACTIC ACID BACTERIA

Pailin Boontawan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology

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การพัฒนากระบวนการผลิตกรดแล็คติคจากมันสำปะหลังโดยแบคทีเรียกรดแล็คติค

นางไพลิน บุญทาวัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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งานวิจัยนี้ได้ดำเนินการศึกษาการคัดแยกและการระบุชนิดของแบคทีเรียกรดแล็คติค จากกากของเสียจากการหมักมันสำปะหลังในประเทศไทย การศึกษาลักษณะทางฟีโนไทป์และจีโนไทป์ของประชากรแบคทีเรียกรดแล็คติค มีการใช้ลำดับยีน 16S rDNA เชื้อแบคทีเรียสายพันธุ์ที่คัดแยกพร้อมกับทำการระบุชนิดได้ คือ *Pediococcus pentosaceus* ถูกเลือกมาใช้เป็นหัวเชื้อในกระบวนการหมักในขั้นตอนต่อไป เชื้อสายพันธุ์นี้มีความสามารถในการผลิตกรดแล็คติคเป็นผลผลิตเพียงอย่างเดียว ที่มีความบริสุทธิ์เชิงแสงของกรดแล็คติคชนิด L ได้ สำหรับกระบวนการเพื่อที่จะลดต้นทุนแหล่งสารอาหารในกระบวนการหมักกรดแล็คติค เพื่อที่จะพัฒนาสารอาหารที่มีราคาถูกลง จึงมีการใช้แป้งมันสำปะหลังที่ทำการย่อยด้วยเอนไซม์สองชนิด คือ เอนไซม์อะไมเลส และกลูโคอะไมเลส เป็นสารอาหารที่เป็นแหล่งคาร์บอนหลัก ขณะที่ สารสกัดยีสต์ที่ได้จากยีสต์เหลือทิ้งจากโรงงานผลิตเบียร์ใช้เป็นแหล่งไนโตรเจน สำหรับรูปแบบการหมักต่างๆ แบ่งออกเป็น กระบวนการหมักแบบกะ ที่มีและไม่มีการควบคุมค่าความเป็นกรด-ด่าง แบบกึ่งกะที่แบ่งออกเป็น การป้อนอาหารเป็นระยะๆ แบบกึ่งกะที่มีอัตราการป้อนอาหารคงที่ และแบบกึ่งกะที่มีการป้อนอาหารแบบเอกซ์โปเนนเชียล ตามลำดับ ผลการศึกษา พบว่า การหมักแบบกึ่งกะที่มีการป้อนอาหารแบบเอกซ์โปเนนเชียล โดยสามารถผลิตกรดแล็คติคได้ที่ความเข้มข้นสูงสุด 150 กรัมต่อลิตร ระยะเวลาดำเนินการ 72 ชั่วโมง โดยที่กระบวนการหมักดังกล่าวแสดงค่าผลผลิตที่สูงมากกว่า 0.9 (กรัมของผลผลิตต่อกรัมของซับสเตรต) จากการทดลองผลิตผลของกรดแล็คติคกระบวนการหมักแบบกึ่งกะ ที่มีการป้อนอาหารแบบเอกซ์โปเนนเชียล มีค่าสูงกว่าการป้อนอาหารเป็นระยะๆ แต่อย่างไรก็ตาม ค่าผลผลิตของการป้อนอาหารเป็นระยะๆ มีค่ามากกว่า ดังนั้นในการทดลองต่อไป จึงทำการแยกกรดแล็คติคออกจากกระบวนการหมักจึงเลือกการป้อนอาหารเป็นระยะๆ การแยกผลิตภัณฑ์กรด-L-แล็คติค หรือแล็คเตต นั้นมีการใช้เทคนิคอิเลคโตรดิไอออนไนเซชัน ที่มีการศึกษาค่าความเข้มข้นเริ่มต้นของแล็คเตตที่มีผลต่อการเจริญของเซลล์ จากการคำนวณทางคณิตศาสตร์ของการยับยั้งโดยผลิตภัณฑ์สามารถอธิบายได้ว่า แล็คเตตที่ค่าความเข้มข้นประมาณ 80 กรัมต่อลิตร มีผลโดยตรงต่อเซลล์ จึงทำให้เกิดการศึกษาทดลองกระบวนการแยกผลิตภัณฑ์ออกจากระบบเพื่อป้องกันการยับยั้งดังกล่าว ซึ่งระบบจะมีการควบคุมสภาวะต่างๆของระบบอิเลคโตรดิไอออนไนเซชัน และดำเนินการแยกแล็คเตตออกจากน้ำหมักในระหว่างการหมัก เพื่อลดความเข้มข้นของแล็คเตตในถังหมัก ทำให้ค่าอัตราการตายจำเพาะของเซลล์ มีค่าลดลงจาก 0.026 ต่อชั่วโมง เป็น 0.0054 ต่อชั่วโมง ค่าครึ่งชีวิตของเซลล์ดีขึ้น อีกทั้ง ความเข้มข้นสูงสุดของแล็คเตตที่ถูก

แยกออกมา ทำให้ในสารละลายมีค่า 185 กรัมต่อลิตร โดยมีค่าความบริสุทธิ์เชิงแสงของกรดแล็คติคชนิด L มากกว่า 95 เปอร์เซ็นต์ ที่ได้จากการวิเคราะห์ด้วยคอลัมน์ไครัล จากผลการทดลองที่ได้นี้ แสดงให้เห็นว่า *Pediococcus pentosaceus* เป็นแบคทีเรียสายพันธุ์หนึ่งที่สามารถพัฒนาเพื่อนำไปสู่การผลิตกรดแล็คติคทางการค้า พบว่า สามารถผลิต และสามารถนำไปใช้ร่วมกับกระบวนการหมักที่มีประสิทธิภาพสูง เพื่อนำไปสู่การพัฒนาการ ทั้งนี้สรุปได้ว่าแป้งมันสำปะหลังที่ผ่านการย่อยแล้ว เสริมด้วยสารสกัดยีสต์จากโรงงานผลิตเบียร์ สามารถลดต้นทุนของสารอาหารได้ถึง 80 เปอร์เซ็นต์ และอาจใช้เป็นประโยชน์สำหรับกระบวนการผลิตกรดแล็คติคขนาดใหญ่ต่อไปได้

สาขาวิชาจุลชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PAILIN BOONTAWAN : DEVELOPMENT OF LACTIC ACID PRODUCTION PROCESS FROM CASSAVA BY USING LACTIC ACID BACTERIA. THESIS ADVISOR : ASST. PROF. SUNTHORN KANCHANATAWEE, Ph.D. 204 PP.

L-(+)-LACTIC ACID/ LACTIC ACID BACTERIA/ PHENOTYPIC AND GENOTYPIC CHARACTERIZATION/ CASSAVA STARCH/ SPENT BREWER'S YEAST EXTRACT/ FERMENTATION PROCESSES/ ELECTRODEIONIZATION

Isolation and identification of lactic acid bacteria from fermented cassava waste in Thailand were performed in this work. The phenotypic and genotypic characterizations using 16S rDNA gene sequences on lactic acid bacteria population were investigated. The most efficient strain identified was *Pediococcus pentosaceus*, and it was used as the starter culture in subsequent fermentation processes. The strain showed homo-fermentative characteristic with high optically pure L-(+)-lactic acid. In order to develop cheaper nutrient sources, cassava starch was treated with α-amylase and gluco-amylase before being used as the main carbon source whereas spent brewer's yeast extract was used as a supplementary nitrogen source. Different fermentation modes were attempted, including the batches with non-controlled and controlled pH, intermittent fed-batch, constant feed rate fed-batch, and exponential fed-batch, respectively. The experimental result was obtained from exponential fed-batch system with the highest lactic acid concentration of approximately 150 g/L within 72 h of the operating time. The fermentation performance also exhibited a high production yields ($g_{product}/g_{substrate}$) of more than 0.90. The lactic acid productivity of exponential feeding showed a high value; however, the production yield of intermittent fed-batch was higher. As a result, this

feeding strategy was chosen for *in situ* lactic acid recovery process. Extractive fermentation of L-(+)-lactic acid (lactate) was carried out using the electrodeionization (EDI) technique. The effect of initial lactate concentrations on microbial growth was initially investigated. A mathematical simulation of the product inhibition was successfully illustrated. It was found that the critical lactate concentration at which the cell growth severely hampered was approximately 80 g/L. Various operating conditions were investigated to assess the EDI performance. The system was subsequently applied for *in situ* removal of lactate from the fermentation broth. Specific deactivation constant (k_d) was substantially reduced from 0.026 h^{-1} to 0.0054 h^{-1} resulting in much improved half life of the biocatalyst. The highest lactate concentration in the receiving solution was obtained at 185 g/L. More than 95% optical purity of L-(+)-lactic acid was obtained using a chiral HPLC column. This finding revealed that *P. pentosaceus* was one of the most promising strain for commercial L-(+)-lactic acid production. In conclusion, hydrolyzed cassava starch supplemented with spent brewer's yeast extract can be effectively used to reduce the nutrients cost by 80%, and it might provide substrate for a large scale lactic acid production.

School of Microbiology

Academic Year 2010

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LIST OF ABBRVIATIONS

ATP	=	Adenosine triphosphate
cm^2	=	Centrimeter squre
cfu	=	Colony forming unit
°C	=	Degree celcius
MRS	=	De Man Rogosa and Shapes
DNA	=	Deoxyribonucleic acid
DCW	=	Dry cell weight
et al.	=	el alia (and others)
g	=	Gram
g/mol	=	Gram per molar
g/s	=	Gram per second
HPLC	=	High performance liquid chromatography
h	=	Hour
L	=	Liter
μm	=	Micrometer
mL	=	Milliliter
mm	=	Millimeter
min	=	Minute
nm	=	Nanometer
N	=	Normality
OD	=	Optical density

LIST OF ABBREVATIONS (Continued)

ppm	=	Part per million
%	=	Percentage
%(w/v)	=	Percentage weight by volume
h^{-1}	=	Per hour
rpm	=	Revolution per minute
rDNA	=	Ribosomal deoxyribonucleic acid

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Low cost medium for lactic acid fermentation have been developed for economic lactic acid fermentation. Agricultural products such as corn, potato, and wheat containing substantial amount of starch have been preferred as raw materials for lactic acid production. However, it is expected that cassava which can be supplied constantly at lower costs and riches in carbohydrate could be used as a suitable renewable resource in substitution of other agricultural products. In lactic acid fermentation, lactic acid bacteria have complex nutritional requirements especially carbon source and nitrogen source. Yeast extract was generally reported as the most employed nitrogen source, but it is an expensive substance. Therefore, it is very attractive to develop a more economical method for lactic acid production using a cheaper nitrogen source. Spent brewer's yeast extract is a nutritious waste product of the alcoholic beverage and, therefore, could be considered as a feasible, inexpensive alternative nitrogen source for lactic acid fermentation. This research work focuses on the development of an efficient and economical process for the lactic acid production. This involves the screening of potential lactic acid bacteria, media formulation with a view to replace expensive ingredients; such as, yeast extract and peptone, by renewable lower cost materials, development of fermentation processes, and finally *in situ* lactic acid recovery using electrodeionization (EDI) technique.

1.2 Research objectives

1.2.1 To isolate and identify potential lactic acid bacteria with high volumetric productivity and product yield of lactic acid from various sources.

1.2.2 To optimize fermentation conditions and suitable substrate for lactic acid production.

1.2.3 To study lactic acid production by using cassava as carbon source and spent brewer's yeast extract extract as nitrogen source in batch culture.

1.2.4 To optimize lactic acid production in batch and fed-batch processes.

1.2.5 To recover and concentrate lactic acid from fermentation broth using electrodeionization (EDI) technique.

1.3 Research hypothesis

Isolated lactic acid bacteria could produce lactic acid as its major product, with a high yield, and high growth rate. Cassava starch and its products could be used for lactic acid production by lactic acid bacteria at the optimum production conditions. Lactic acid could be recovered from fermentation broth, and concentrate with high yield by using EDI technique.

1.4 Scope and limitation of the study

This work involves investigation of lactic acid production starting from isolation of potential lactic acid bacteria (LAB), media formulation, fermentation processes, and *in situ* recovery of lactic acid using EDI technique. The ultimate objective of the whole thesis is to improve value of the agricultural product for the efficient production of lactic acid by fermentation processes. Lactic acid fermentation was performed using cassava

starch as the main substrate supplemented with nitrogen source such as spent brewer's yeast extract extract. Effect of carbon sources and nitrogen sources was investigated in order to obtain optimum media formulation. In addition, fermentations were carried out in laboratory bioreactors either in batch or in fed-batch system. Moreover, lactic acid recovery process was investigated using EDI technique. Mass transfer characteristic was studied in both abiotic and biotic system. Finally, combination of lactic acid fermentation and *in situ* product removal via EDI technique was performed. Its performances in terms of product yield and volumetric productivity were investigated, and compared with conventional fermentation methods.

1.5 Expected results

1.5.1 High lactic acid yield from fermentation processes under the optimum condition will be obtained.

1.5.2 Microorganism can utilize cassava starch and spent brewer's yeast extract as carbon source and nitrogen source for lactic acid production, respectively.

1.5.3 Successful continuous recovery of lactic acid from fermentation broth using EDI technique will be realized.

CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid

Lactic acid was firstly discovered in sour milk in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component. In 1789, Lavoisier named this milk component (acide lactique), which became the possible origin of the current terminology for lactic acid. However, it was in 1857 when Louis Pasteur discovered that it was not a milk component, but a fermentation metabolite generated by certain microorganisms (Wee *et al.*, 2006).

Lactic acid (IUPAC systematic name: 2-hydroxypropanoic acid), also known as milk acid, is a chemical compound that plays a role in several biochemical processes. It is a carboxylic acid with a chemical formula of $C_3H_6O_3$. It has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid (AHA). In solution, it can lose a proton from the acidic group, producing the lactate ion ($CH_3CH(OH)COO^-$). Lactic acid is soluble in water and water miscible organic solvents but insoluble in other organic solvents (Narayanan *et al.*, 2004). Lactic acid exists as two optical isomers. One is known as L-(+)-lactic acid or (*S*)-lactic acid, and its mirror image is D-(-)-lactic acid or (*R*)-lactic acid. L-(+)-lactic acid is the biologically important isomer. Figure 2.1 illustrates the two optical isomers of lactic acid, and some physiochemical properties of lactic acid are shown in Table 2.1.

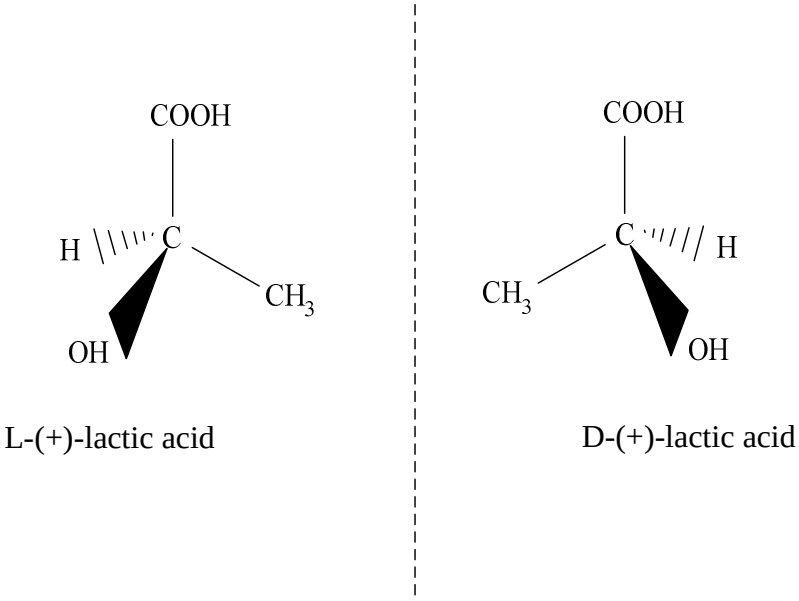


Figure 2.1 Chemical structure of L-(+)-lactic acid and D-(-)-lactic acid.

Table 2.1 Physiochemical properties of lactic acid.

	Properties	**Value**
	Empirical formula	$C_3H_6O_3$
	Chemical name	2-hydroxypropanoic acid
	Dissolution constant (k_a)	1.37×10^{-4}
	Molecular weight	90.08 g/mol
	Normal boiling point, °C	122 °C at 14 mmHg
	Melting temperature, °C	L: 53 °C
		D: 53 °C
		D/L: 16.8 °C
	Density, d_4^{20}, g/ml	1.1

Source: (Datta *et al.*, 1995).

2.1.1 Application of lactic acid in polymer industry

Recently, a great interest in biodegradable lactide polymers has accelerated researches on the production of pure L(+)- or D(-)-lactic acid as bulk raw material, and efforts have been made to enhance the productivity and economy of the L(+)- or D(-)-lactic acid production processes (Champomier-Vergs *et al.*, 2002). The optically pure lactic acid can be polymerized into a high molecular mass poly-lactic acid (PLA) through the serial reactions of polymerization, depolymerization, and ring opening polymerization (Vijayakumar *et al.*, 2008). Two molecules of lactic acid can be dehydrated to yield lactide, a cyclic lactone. A variety of catalysts can polymerize lactide to produce either heterotactic or syndiotactic polylactide, biodegradable polyesters with valuable (*inter alia*) medical properties, which are currently attracting much attention.

These polymers are transparent and their degradation can be controlled by adjusting the composition, and molecular weight. Their physical properties approach those of petroleum derived plastics. Lactic acid esters like ethyl/butyl lactate can be used as green solvents. They are high boiling, non-toxic, and biodegradable components. Poly L-lactic acid with low degree of polymerization can help in controlled release or degradable mulch films for large-scale agricultural applications (Gupta *et al.*, 2007). Moreover, polymers of lactic acids are biodegradable thermoplastics. The resultant polymer, poly-lactic acid (PLA), has numerous uses in a wide range of applications, such as protective clothing, food packaging, mulch film, trash bags, and short-life trays, respectively.

2.1.2 Other applications

Lactic acid is used in the food industry for several aspects. Lactic acid has a long history of uses for fermentation and preservation of human foodstuffs (Davison *et al.*, 1995). Lactic acid can also be found in various processed foods, usually either as a pH

adjusting ingredient, or as a preservative (either as antioxidant or for control of pathogenic microorganisms). It has many pharmaceutical and cosmetic applications including formulations in tropical ointments, lotions, anti-acne solutions, humectants, parenteral solutions, and dialysis applications, for anti carries agent. In addition, technical grade lactic acid is used as an acidulant in vegetable and leather tanning industries. Lactic acid is being used in many small scale applications like pH adjustment in hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyl resin modifier, solder flux, lithographic and textile printing developers, adhesive formulations, electroplating and electro-polishing baths, and detergent builders, respectively (Vijayakumar *et al.*, 2008).

Biological production of lactic acid has attracted a great deal of interest due to its potential uses in many industries, and the production of polylactide polymer. Although the biological production of lactic acid has some advantages over chemical synthesis, it still requires the cheaper substrates for industrial feasibility (Lim *et al.*, 2008).

2.2 Lactic acid production

2.2.1 Lactic acid production by chemical reaction

Lactic acid can be manufactured by either chemical synthesis or fermentation. The commercial process for chemical synthesis is based on lactonitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered, and purified by distillation. It is then hydrolyzed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and lactic acid. Lactic acid is then esterified with methanol to produce methyl lactate before being purified by the means of distillation, and is hydrolyzed by water under acid catalyst to produce lactic

acid and methanol. The chemical synthesis method produces a racemic mixture DL-lactic acid. This process is represented by the following reactions (Narayanan *et al.*, 2004).

(a) Addition of Hydrogen Cyanide

$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \underset{\text{Hydrogen cyanide}}{HCN} \xrightarrow{\text{Catalyst}} \underset{\text{Lactonitrile}}{CH_3CHOHCN}$$

(b) Hydrolysis by H_2SO_4

$$\underset{\text{Lactonitrile}}{CH_3CHOHCN} + H_2O + \tfrac{1}{2}H_2SO_4 \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Ammonium salt}}{\tfrac{1}{2}(NH_4)_2SO_4}$$

(c) Esterification

$$\underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH} \longrightarrow \underset{\text{Methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O$$

(d) Hydrolysis by H_2O

$$\underset{\text{Methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH}$$

2.2.2 Lactic acid production by fermentation processes

On the other hand, an optically pure L(+)- or D(–)-lactic acid can be obtained by microbial fermentation when the appropriate microorganism is selected. The optical purity of lactic acid is crucial to the physical properties of poly(lactic acid) (Antonio *et al.*, 2000), and an optically pure L(+)- or D(–)-lactic acid, rather than racemic DL-lactic acid. Lactic acid can be polymerized to produce a high crystalline PLA that is suitable for commercial uses. As a result, the biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative way to prevent environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources (Han and Pan, 2009).

2.2.3 Microorganisms for the lactic acid production

Microorganisms with a capability to produce lactic acid can be divided into two groups namely bacteria and fungi (Litchfield, 1996). Most investigations of lactic acid production were carried out with lactic acid bacteria, and filamentous fungi. For example, *Rhizopus* species utilize glucose aerobically to produce lactic acid (Zhou *et al.*, 1999). Fungal fermentation has some advantages in which *Rhizopus oryzae* requires not only a simple medium and produces L(+)-lactic acid, but it also requires vigorous aeration because *Rhizopus oryzae* is an obligate aerobe (Tay and Yang, 2002). *Rhizopus* species such as *R. oryzae* and *R. arrhizus* have amylolytic enzyme activity, which enables them to convert starch directly to L(+)-lactic acid (Yin *et al.*, 1997). However, fungal fermentation showed low production rate, below 3.0 g/L/h, is probably due to the low reaction rate caused by mass transfer limitation of substrate. The lower product yield from fungal fermentation is partially attributed to the formation of by-products, such as fumaric acid and ethanol (Wee *et al.*, 2006). Although there have been persistent attempts to produce lactic acid through fungal fermentation, LAB have been commonly used for the production of lactic acid due to the aforementioned disadvantages of fungal fermentation. Moreover, lactic acid bacteria were performed the desirable activities which they promote (Vijayakumar *et al.*, 2008).

2.3 Lactic acid bacteria

The lactic acid bacteria are a group of Gram positive bacteria. Other main characteristics include non-respiring, non-spore forming, cocci or rods, and produce lactic acid as the major end product from the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, all fermented milks, cassava (to produce *gari* and *fufu*), and most fermented vegetables. Historically, bacteria from the genera *Lactobacillus, Leuconostoc,*

Pediococcus and *Streptococcus* are the main species involved. Lactic acid bacteria were recently reviewed by Wessels and his colleagues (Wessels *et al.*, 2004). They lack the ability to synthesize cytochromes and porphyrins (components of respiratory chains), and therefore cannot generate ATP by creation of a proton gradient. The lactic can only obtain ATP by fermentation, usually of sugars. Phosphotransferase system (PTS) is a distinct method used by bacteria for sugar uptake where the source of energy is from phosphoenolpyruvate (PEP). Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles ATP per glucose consumed. Since they do not use oxygen in their energy production, lactic acid bacteria happily grow under anaerobic conditions, but they can also grow in the presence of oxygen. They are protected from oxygen byproduct (e.g. H_2O_2) because they have peroxidases. Furthermore, they convert carbohydrate to lactic acid plus carbon dioxide and other organic acids without the need for oxygen that described as microaerophilic as they do not utilize oxygen. Some of the families are homofermentative which produce only lactic acid with small amounts of alcohol.

The taxonomy of lactic acid bacteria has been based on the gram reaction and the production of lactic acid from various fermentable carbohydrates (Salminen *et al.*, 1998). These organisms are heterotrophic and generally have complex nutritional requirements because they lack many biosynthetic capabilities. Most species have multiple requirements for amino acids and vitamins. Because of these, lactic acid bacteria are generally abundant only in communities where these requirements can be provided. They are often associated with animal oral cavities and intestines (e.g. *Enterococcus faecalis*), plant leaves (*Lactobacillus, Leuconostoc*) as well as decaying plant or animal matter such as rotting vegetables, fecal matter, compost, etc (Yun *et al.*, 2003). Their growth lowers both carbohydrate content of the foods that they ferment, and pH due to lactic acid production. It

is this acidification process which is one of the most desirable side-effects of their growth. The fermentation (and growth of the bacteria) is self-limiting due to the sensitivity of lactic acid bacteria to such acidic pH (Kostinek *et al.*, 2005).

2.3.1 Natural habitat of lactic acid bacteria

LAB can be isolated from various natural sources. For example, lactococci can be found in milk and milk products. Previous studies have shown their probiotic activity such as the ability to inhibit the growth of other bacteria (Charalampopoulos *et al.*, 2003). Diversity and density of lactic acid isolated from Algerian raw goats' milk in arid zones were studied by determination of morphological, cultural, physiological and biochemical characteristics. Two hundred and six lactic acid bacterial strains were isolated, 115 of them belonging to lactic acid cocci and others to the genus *Lactobacillus*. The representative species of the total cocci were *Lactococcus* sp. (76.16%), *Streptococcus thermophilus* (14.78%) and *Leuconostoc* sp. (8.6%), respectively. The dominating species is *Lactococcus lactis* subsp. *lactis*. Lactobacilli species found in local raw goats' milk and their proportion were: *Lb. curvatus* (25.25%), *Lb. helviticus* (10.98%), *Lb. plantarum* (9.89%), *Lb. reuteri* (9.89%), *Lb. casei* (7.69%), *Lb. brevis* (5.49%), *Lb. bulgaricus* (5.49%), *Lb. paracasei* (4.39%) and *Lb. acidophilus* (2.19%) (Badis *et al.*, 2004). It has also been reported that some lactic acid bacteria isolated from the gastrointestinal tract of fish can act as probiotic (Olympia *et al.*, 1995).

The variation of the ecological parameters acting on the microbial association such as the nature of cereal, temperature, size of inoculum, and length of propagation intervals leads in each case to a characteristic species association. Cereals are suitable fermentable substrates for the growth of potentially probiotic microorganisms. Previous studies showed that four potentially probiotic strains (*Lactobacillus fermentum, L. reuteri, L. acidophilus*

and *L. plantarum*) were cultured in malt, barley and wheat media. All strains attained high cell populations (8.1-10.1 $\log_{10}$ cfu/g). The malt medium supported the growth of all strains more than barley and wheat media due to its chemical composition, while *L. plantarum* and *L. fermentum* appeared to be less fastidious and more resistant to acidic conditions than *L. acidophilus* and *L. reuteri* (Talamond *et al.*, 2002). Another study showed that the natural sour cassava starch fermentation was mainly due to the action of lactic acid bacteria. Fermentation temperature and duration as well as the composition of the microflora influenced the expansion properties of the final cassava sour starch. However, some LAB strains (such as *Lactococcus lactis*, *Streptococcus* sp., *Enterococcus saccharolyticu*, *Lactobacillus plantarum*, and *Leuconostoc mesenteroides*) involved in the natural sour cassava starch fermentation were isolated, identified and characterized using classical microbiological techniques (Ampe *et al.*, 2001).

2.3.1.1 Lactic acid bacteria in food

Lactic acid bacteria (LAB) are very important in the food and dairy industries because lactic acid and other organic acids produced by these bacteria act as natural preservatives as well as flavor enhancers. LAB find increasing acceptance as probiotic which aid in stimulating immune responses, preventing infection by enteropathogenic bacteria, and treating and preventing diarrhea. Fermented foods constitute a substantial part of the diet in many African countries are considered as an important means of preserving and introducing variety into the diet, which often consists of staple foods such as milk, cassava, fish and cereals. For example, Ben saalga is a traditional Burkinabè gruel obtained by cooking a diluted fermented paste of pearl millet (*Pennisetum glaucum*). This fermented food is widely accepted and consumed by the population, particularly by young children. The processing of pearl millet into ben saalga comprises the following successive main steps: soaking the grains (first fermentation), grinding and

filtration of humid flour, decanting (second fermentation) and cooking (Sanni *et al.*, 2002). The production of this fermented food is still largely a traditional art associated with poor hygiene, inconsistent quality presentation and short shelf life. The preparation of this indigenous food generally depends on a spontaneous or chance inoculation by naturally occurring lactic acid bacteria (LAB) and the use of starter cultures is still at very early development stages. LAB play an essential role in the majority of food fermentations, and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, and vegetable and bakery products. One of the most important contributions of these microorganisms is the extended shelf life of the fermented product by comparison to that of the raw substrate. Growth of spoilage and pathogenic bacteria in these foods is inhibited due to competition for nutrients and the presence of starter-derived inhibitors such as lactic acid, hydrogen peroxide and bacteriocins. Bacteriocins are heterogeneous group of antibacterial proteins that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties (Lee, 1997). Currently, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by LAB as biopreservatives as well as the application of bacteriocinogenic LAB in starter cultures. According to the generally poor sanitary conditions of ben saalga and other traditional fermented foods, the use of selected bacteriocinogenic LAB with antimicrobial activity against the most frequent foodborne pathogenic bacteria could be an affordable way to improve the safety of these fermented foods. For examples, a total of 14,020 lactic acid bacteria (LAB) are isolated from *Nham* and two traditional Indonesian fermented foods "*Tapai*" (fermented tapioca), and "*Tempoyak*" (fermented durian flesh). Chilli puree and fresh goat's milk are used as sources

for the isolation of lactic acid bacteria (LAB), and the total amount of 126 isolates are obtained (Visessanguan *et al.*, 2006).

2.3.1.2 Lactic acid bacteria in agricultural products

The major genera found in the microflora of fermented or sour cassava-starch were *Streptococcus*, *Bacillus*, *Lactobacillus* and *Saccharomyces* with amylase activity (Lacerda *et al.*, 2005). Lactic acid bacteria predominated whereas the presence of moulds was not significant. Traditional fermentation of cassava is dominated by a lactic acid bacteria (LAB) population. A total of 139 predominant strains isolated from fermenting cassava were identified using phenotypic tests and genotypic methods. Moreover, fermented tapioca was used as sources for the isolation of lactic acid bacteria (LAB). A total of 126 isolates were obtained. In addition, by sequential screening for catalase activity and Gram-staining, 55 were determined to be LAB, and out of which 16 were established to be homofermentative.

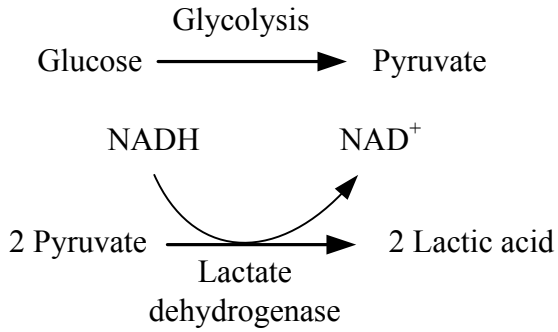
Moreover, Thailand is the world's largest exporter of tapioca starch and starch derivatives with annual production of over 2 million tons of starch. Development of lactic acid production using cassava as the main substrate is very attractive because it is a cheap source, contains high starch content with low quantity of impurities, and also abundant in Nakhon Ratchasima province (Tonukari, 2004).

2.3.2 Characterization of lactic acid bacteria

The lactic acid bacteria belong to two main groups, the homofermentors and the heterofermentors. The pathways of lactic acid production differ for both types. Homofermentative produces mainly lactic acid whilst heterofermentative produces lactic acid plus appreciable amount of ethanol, acetate, and carbon dioxide, respectively.

The lactic acid bacteria are also divided into three groups based on fermentation patterns:

1. Homofermentative produces more than 85% lactic acid from glucose (Salminen *et al*., 1998). One mole of glucose can be converted to two moles of lactic acid, generating a net of 2 ATPs per mole of glucose metabolized.



2. Heterofermentative produces only 50% lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide, one mole of glucose converted to one mole of lactic acid, one mole of ethanol, and one mole of CO_2 (Salminen *et al*., 2004). One mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolized. Because of the low energy yields, lactic acid bacteria often grow more slowly than microbes capable of respiration, and produce smaller colonies of 2-3 mm.

$$\text{Acetaldehyde} + \text{NADH} + H^+ \longrightarrow \text{Ethanol} + NAD^+$$

$$C_6H_{12}O_6 \longrightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2$$

Glucose — Lactic acid — Ethanol — Carbon dioxide

3. Less known heterofermentative species which produce DL-lactic acid, acetic acid and carbon dioxide.

The ability of lactobacilli to convert lactose to lactic acid is employed in the successful treatment of lactose intolerance. People suffering from this condition cannot metabolize lactose due to lack or dysfunction of the essential enzyme systems. Lactic acid,

by lowering the pH of the intestinal environment to 4 to 5, inhibits the growth of putrefactive organisms and *E. coli*, which require a higher optimum pH of 6 to 7. They are differentiated from other organisms by their ability to ferment hexoses to lactic acid. The lactic acid bacteria have limited biosynthetic ability, requiring preformed amino acids, B vitamins, purines, pyrimidines, and typically a sugar as energy source. A rich medium is usually employed when cultivating LAB. These multiple requirements restrict their habitats to areas where the required compounds are abundant (animals, plants, and other multicellular organisms). LAB can grow at temperatures from 5-45 °C, and not surprisingly are tolerant to acidic conditions. Most strains are able to grow at pH 6.

There are two main hexose fermentation pathways that are used to classify LAB genera. Under conditions of excess glucose and limited oxygen, homolactic LAB catobolize one mole of glucose in the Embden-Meyerhof-Parnas (EMP) pathway (Figure 2.2) to yield two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles ATP per glucose consumed. Representative homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus* and group I lactobacilli.

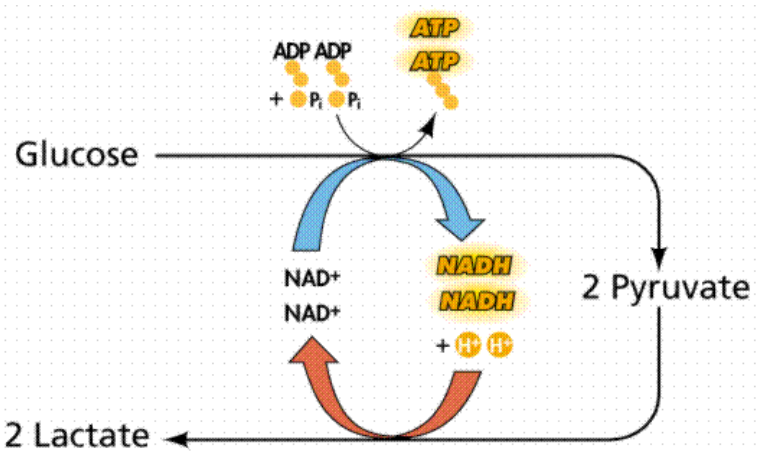


Figure 2.2 Embden-Meyerhof-Parnas (EMP) pathway (Casey and Ingleew, 1986).

Heterofermentative LAB utilizes the pentose phosphoketolase pathway. One mole Glucose-6-phosphate is initially dehydrogenated into 6-phosphogluconate, and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP is further metabolized into lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. Theoretically, the end products (including ATP) are produced in equimolar quantities from the catabolism of one mole glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and group III lactobacilli.

2.3.3 Enzymes for lactic acid fermentation

Lactic acid is produced in the form of L(+) or D(-) lactic acid or as its racemic mixture. Organisms that form the L(+) form or D(-) form have two lactate dehydrogenases (LDH), which differ in their stereospecifity. Some *Lactobacilli* produce L(+) form, which on accumulation induces a racemase, which converts it into D(-) lactic acid until equilibrium is obtained. When this organism is grown in continuous culture, a shift in pH from acidic to alkaline causes it to catabolize sugar in a heterofermentation mode by the phosphoketolase split pathway. This implies that lactate dehydrogenases in lactic acid bacteria are under the control of not only allosteric affects, but also gene expressions (Narayanan *et al.*, 2004).

2.3.4 Catabolic pathways in lactic acid bacteria

Lactic acid bacteria ferment sugars resulting in homo-, hetero-, and mixed acid fermentation. The two major pathways for better assimilation of glucose and xylose in lactic acid are the Embden-Mayerhof-Parnas (EMP) pathway and the pentose phosphoketolase (PK) pathway (Vijayakumar *et al.*, 2008) shown in Figure 2.3.

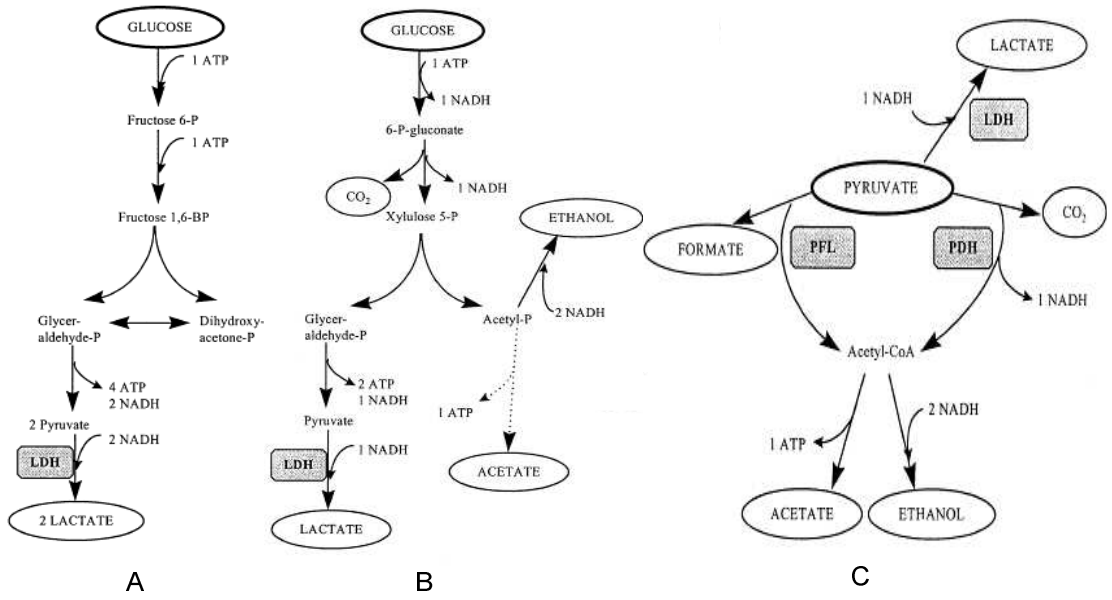


Figure 2.3 Catabolic pathways in LAB. Homofermentation (A), heterofermentation (B) and mixed acid fermentation (C). P = 5 phosphate, BP = 5 bisphosphate, LDH = 5 lactate dehydrogenase, PFL = 5 pyruvate formate lyase, and PDH = 5 pyruvate dehydrogenase (Hofvendahl and Hahn-Hagerdal, 2000).

Glycolytic pathway of excess sugar and limited oxygen are reported an in-depth account of the biochemical pathways for the both homo- and hetero-fermentative (Wessels *et al.*, 2004). Homofermentation gives only lactic acid as the end product of glucose metabolism, and the Embden-Meyerhof-Parnas pathway is used (Thomas *et al.*, 1979). In heterofermentation equimolar amounts of lactic acid, carbon dioxide and ethanol or acetate are formed from glucose via the phosphoketolase pathway (Axelsson, 2004). The ratio of ethanol and acetate formed is dependent on the redox potential of the system (Kandler, 1983). Reduction potential is a measure of the tendency of a chemical species to acquire electrons and thereby be reduced. This pathway is used by facultative heterofermentors, such as *Lb. casei*, for the fermentation of pentoses, and for the fermentation of hexoses and pentoses. Mixed acids are formed by homofermentors such as lactococci during glucose

limitation (Fordyce *et al.*, 1984), and during growth on other sugars or at increased pH and decreased temperature. Ethanol, acetate and formate are formed in addition to lactic acid. The homofermentative pathway is used, but the difference is in the metabolism of pyruvate in which lactic acid is also metabolized into formate and acetyl-CoA by pyruvate formate lyase (PFL). In the presence of oxygen PFL is inactivated, and an alternative pathway of pyruvate metabolism becomes active via pyruvate dehydrogenase (PDH), resulting in the production of carbon dioxide, acetyl-CoA and NADH (Hofvendahl and Hagerdal, 2000).

2.4 Isolation and screening of lactic acid bacteria

The seemingly simplistic metabolism of lactic acid bacteria has been exploited throughout the history of agriculture. Domestication of lactic acid bacterial strains passed down through various sources. Lactic acid bacteria are also critical for the production of sourdough, and numerous indigenous food fermentations. They are indigenous to food-related habitats, including plant (fruits, vegetables, and cereal grains) and milk environments. In addition, they are naturally associated with the mucosal surfaces of animals, e.g., small intestine, colon, and vagina. The isolates are obtained from plant, dairy, and animal habitats, implying wide distribution and specialized adaptation to these diverse environments. Identification and classification of bacteria is a hard task since the beginning of microbiology. It could be worthwhile to begin providing some basic definitions taken from the edition of Bergey's Manual of Systematic Bacteriology (1994). Classification is the arranging of new isolate belongs to one of taxonomic groups (taxa) on the basis of similarities or relationships. Nomenclature is the assignment of names to the taxonomic groups according rules (Morelli, 1997).

Identification of lactic acid bacteria may be achieved using classical microbiological methods that are relatively simple to perform, but they often lack discriminatory power, and

reproducibility at species level (Ehrmann and Vogel, 2005). For reliable species identification, both phenotypic and genotypic methods are used to obtain unambiguous identifications (Vandamme *et al*., 1996). The term LAB is not limited to a strictly defined taxonomic group of microorganisms, but it comprises a wide range of phylogenetically related genera of Gram positive bacteria with several biochemical, and ecological features in common (Hofvendahl and Hahn-Hagerdal, 1997).

2.4.1 Physiology of lactic acid bacteria

Lactic acid bacteria comprise a wide range of genera including a considerable number of species. It belongs to the clostridia branch of the gram positive bacteria. Lactic acid bacteria belong to the Gram-positive phylum with a low G+C (guanine plus cytosine) content (<50%) (Schleifer and Ludwing, 1995). In PCR experiments, the GC-content of primers are used to predict their annealing temperature to the template DNA. DNA with high GC-content is more stable than DNA with low GC-content which indicates a higher melting temperature.

Nevertheless, the latter are also considered as lactic acid bacteria, because of similar physiological and biochemical properties and the sharing of some common ecological niches such as the gastro-intestinal tract (GIT) (Klein *et al.*, 1998). Important physiological features for taxonomic considerations are cell wall composition, cellular fatty acids, other characteristics of the cell, carbohydrate fermentation patterns, resistance to different NaCl concentrations, growth on different nutrient media, growth at defined temperatures, and resistance against antibiotics (Klein *et al.*, 1998). Some of physiological characteristics are interesting for their function as probiotics, a pre-condition of which is survival in the gastro-intestinal tract. This is based on their resistance to low pH and /or bile and their temperature growth ranges (Fuller, 1989).

Moreover, lactic acid bacteria unable to synthesize ATP by cellular respiration, but lactic acid produced from energy-conserving fermentation of sugars (Hofvendahl and Hagerdal, 2000). They have high acid tolerance and survive pH 5.0. This gives them a competitive advantage over other bacteria. On the other hands, the optimal temperature for growth varies between from 20 to 45 °C (Dicks *et al.*, 1995). Most of them are considered GRAS (generally regarded as safe), but some strains of e.g. streptococci are pathogenic. The most important species with *Bifidobacterium* seemed to be possible to differentiate based on their biochemical reactions, namely unique fructose-6-phosphate phosphoketolase pathway (Klein *et al.*, 1998). However, the classifications of lactic acid bacteria were focused on intense taxonomic study with an increasing urgency for a polyphasic approach involving both phenotypic and phylogenetic characterization of bacteria.

2.4.2 Phenotypic characterization

In some carbohydrate fermentation patterns, such as those of lactose, maltose, trehalose, and D-xylose, and could not be identified to the species level on the basis of phenotypic characterization. Different phenotypic methods are used to identify lactic acid bacteria important for fermentation technology. The primary objective of the microbiological analysis (e.g. control of food quality, food preservation, efficiency of starter cultures and the monitoring of particular species/strains) were performed, the taxonomic level of the microbial discrimination depends upon the sensitivity of the technique used and may range from genus (or species) to strain level (subtyping) (Guarneri *et al.*, 2001). In general, more than one method is usually needed to obtain both identification and typing of unknown isolates. However, these methods are not sufficient to characterize sub-species and strains in a genus. Thus, new methods have been developed depending on genotypical features and effectively used for the definition of the bacteria.

Recently, detection and identification of microorganisms were performed mainly through biochemical and phenotypic methods. The mentioned methods are labor intensive, time consuming and do not always give unequivocal results. In response to this situation, more robust genetic methods based upon molecular biology have been recently developed for the identification and subtyping of bacteria. Molecular techniques provide outstanding tools for the typing, taxonomy and evolution of bacteria involved in processes (Germond *et al.*, 2003).

2.4.3 Genotypic characterization

The genetic interrelationships of members of the lactic acid bacteria have been studied extensively in 16S rDNA sequence, and DNA-DNA hybridization experiments. Therefore, a method that is universally suitable for the lactic acid bacteria with a high resolving power both on the species and intraspecies level would be a highly valuable tool. In this regard, PCR based genomic fingerprinting techniques are believed to have the most potential, and are easy to perform (Adiguzel and Atasever, 2009). Previous studied examines nine other lactic acid bacteria genomes representing the phylogenetic and functional diversity of lactic acid-producing microorganisms. Phylogenetic analyses, comparison of genomic content across the group, and reconstruction of ancestral gene sets reveal a combination of gene loss and gain during the co-evolution of lactic acid bacteria. Among PCR-based techniques, it is widely recognised that RAPD-PCR could be a rapid and reliable method for infra- and inter-specific differentiation of most food-associated bacterial species. Several studies have reported success in using RAPD-PCR for differentiation of lactic acid baceteria strains (Baruzzi *et al.*, 2000) (Olive and Bean, 1999). Although variability of RAPD fingerprints have been observed, reproducibility could be achieved under carefully controlled conditions (Guarneri *et al.*, 2001).

Methods to understand the link between structural diversity and functioning of complex ecosystem need to be developed so that the questions of how diversity influences functions can be addressed (Amann *et al.*, 1995). Among the methods designed to gain access to the physiology and genetics of microorganisms, metagenomics, the genomic analysis of a population of microorganisms, has emerged as a powerful centerpiece. It could be useful to identify which organisms are present in ecosystem, what they do, and how their genetic information can be beneficial to mankind (Randazzo *et al.*, 2009).

2.5 Nutrient requirement for lactic acid fermentation

All lactic acid bacteria require a source of nutrients for metabolism. The fermentative bacteria require carbohydrates, either simple sugar such as glucose and fructose or complex carbohydrates such as starch or cellulose. The energy requirements of lactic acid bacteria are very high. Limiting amount of available substrate can stop their growth. It is necessary to supplement the fermentation media with sufficient nutrients for rapid lactic acid production. If small amounts of other nutrients were supplemented to the process, then the efficiencies of lactic acid fermentation would be improved significantly.

2.5.1 Carbon sources

The biotechnological production of lactic acid from cheap raw materials is necessary. This is because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for lactic acid production should have the following characteristics: cheap, low levels of contaminants, rapid production rate, high yield, little amount of by-product formation, ability to be fermented with some pre-treatment, and year-round availability (Vickroy, 1985). However, this is still economically unfavourable because the refined carbohydrates are too expensive

that they eventually result in higher production costs. Therefore, there are attempts to select cheap raw materials for the economical lactic acid production. Cheap raw materials, such as wheat starch, cellulosic materials, whey, and molasses, have been used for lactic acid production. Among these, starch and cellulosic materials are currently receiving a great deal of attention, because they are cheap, abundant, and renewable. Consequently, inexpensive starch, starch derivatives or starch containing waste materials as substrate would offer great advantages when combine with minimal preprocessing and supplementation (Wee, 2006).

Alternative carbon sources

Cassava is grown for its enlarged starch-filled roots, which contains nearly the maximum theoretical concentration of starch on a dry weight basis among food crops. Fresh roots contain about 30% starch and very little protein. Tapioca (*Manihot esculenta* Crantz) is known by various names in different regions of the world. It is called “tapioca” or “cassava”, and “tapioca” is the name given to the starch. The chemical compositions of cassava grown in Thailand are shown in Table 2.2. Cassava starch, a dominant source of starch in Thailand, possesses a strong film, clear paste, good water holding property, and stable viscosity. Unlike other starch sources, such as corn, rice and wheat, tapioca roots contain high starch content and a very low quantity of impurities. Thai tapioca starch industry has over fifty years experience resulting in highly developed processing technology being used by most Thai manufacturers (Tonukari, 2004). The most important characteristics of native Thai tapioca starch is white in color, absence in unpleasant odor, possesses high ratio of amylopectin to amylose (80:20), and provides a high peak viscosity which is very useful in many applications. Amylopectin is a soluble and highly branched polymer of glucose found in plants. It is one of the two components of starch, the other being amylose. Glucose units are linked in a linear way with α-(1→4) glycosidic bonds.

Branching takes place with α- (1→6) bonds occurring every 24 to 30 glucose units. In contrast, amylose contains very few α-(1→6) bonds, which causes it to be hydrolyzed more slowly but have higher density. These characteristics can be further improved by modification (Sriroth *et al.*, 1999).

Table 2.2 Chemical composition of starch sources**.**

Chemical composition (% dry basis)	**Starch sources**			
	Cassava root	**Wheat (whole grains)**	**Potato (whole grains)**	**Maize (whole grains)**
Moisture	70.0	68.3	75.8	75.0
Starch	24.0	26.2	19.9	19.0
Fiber	2.0	2.0	1.1	2.7
Protein	1.0	1.2	1.8	3.2
Other substances	3.0	2.3	1.4	0.1

Source: (Tonukari, 2004; Parigi-Bini and Chiericato, 1976).

2.5.2 Nitrogen sources

Biotechnological production of lactic acid on either a glucose or a lactose based medium requires supplementation, for example, yeast extract (Hujanen and Linko, 1994). Supplementation with yeast extract had a significant effect on lactic acid concentration, volumetric productivity, and substrate conversion. The most common and effective supplement therefore seems to be 10 g/l of yeast extract (Aeschimann and von Stockar, 1990). When a yeast cell is inactivated, a natural digestion process called “autolysis” starts. During this process the yeast's own enzymes breakdown proteins and other parts of the cell. This causes the release of peptides, amino acids, vitamins and other yeast cell components which, once the insoluble components have been removed, is called “Yeast Extract”.

Yeast Extract is rich in nitrogen, vitamins and other nitrogenous growth factor stimulating compounds, and therefore is used as an ingredient in media for the cultivation of microorganisms. Moreover, all published reports have shown that lactic acid production increases with the concentration of the supplement (especially yeast extract) (Lund *et al.*, 1992). The typical composition of yeast extract is 8 -12% of total nitrogen content (organic and inorganic compound), 50-75% of protein content, 3-5.2% of fermentable nitrogen content, 4-13% of total carbohydrate content, and very little of lipid content, respectively. In microbial fermentations, the cost of the fermentation medium can account for almost 30% of the total cost (Rivas *et al*., 2004). Most studies reported on lactic acid production by lactic acid bacteria were performed in media containing expensive nutrients such as yeast extract and peptone (Mercier *et al.*, 1992). In this context, the search for alternative, low-cost media for lactic acid fermentation has an obvious economic interest. However, the development of an alternative nitrogen source is a prerequisite for the economical production of lactic acid, because yeast extract is a relatively expensive nitrogen source for industrials use. Therefore, it is essential to develop a more economical method for lactic acid fermentation, using materials as a cheaper nitrogen source.

Alternative nitrogen sources

Nutritional requirements of LAB are fastidious, culture media have to be supplemented with various peptide sources and growth factors (Amrane, 2000). According to literature, yeast extract is the best source of growth factors for LAB among the different nitrogen sources tested (Barrette *et al.*, 2001). Various amounts of B vitamins have been studied to replace yeast extract. Yeast extract exhibited clearly the most significant effect on lactic acid production of LAB, particularly in the beginning of growth, with lactic acid concentration increasing linearly with the increase of yeast extract level (Hujanen and Linko, 1996).

Basically dead yeasts are consider, this waste fraction represents a potential source of nutrients, particularly after being subjected to autolysis. Autolysis is the hydrolysis of cellular components by hydrolytic yeast enzymes (lipase, protease, etc.) (Vakhlu and Kour, 2006). The main events that occur during this process are breakdown of cell membranes, release of hydrolytic enzymes, liberation of intracellular constituents, and hydrolysis of intracellular biopolymers into products of a low molecular weight (Guilloux-Benatier and Chassagne, 2003). Literature studies have considered the use of yeast autolysates as nutrients in wheat fermentations and alcoholic production by recombinant *Escherichia coli* (Jones and Ingledew, 1994). Autolysates of brewery yeast biomass have been used for growth of *Lactobacillus plantarum* in whey or *Bacillus thuringiensis kurstaki* (Saksinchai *et al.*, 2001). Spent brewer's yeast extract is a by-product of alcoholic fermentation. Thailand has several large breweries, and there is an abundant waste yeast products generated from the industrial. Brewer's yeast is currently used as an ingredient for animal feed. If brewer' yeast extract can be efficiently use as a raw material for the production of value-added products with commercial importance, then a considerable reduction in fermentation costs would be possible. The disposal of excess brewer's yeast wastes can be detrimental to environmental quality, leading to research into brewer's yeast wastes utilization. In order to compensate the costs for evaporation and powdering of brewer's yeast extract as an equivalent but a cheaper nitrogen source for complete replacement of yeast extract in the medium.

2.6 Lactic acid fermentation processes

Fermentation is defined as an energy yielding process whereby organic molecules serve as both electron donors and electron acceptors. The molecule being metabolized does not have all its potential energy extracted from it. Hence, lactic acid bacteria are widely

used as a low cost method for food preservation by fermentation and generally no or little heat is required during the fermentation. In fermentation, pyruvic acid molecules are turned into waste product and a little bit of energy (only two ATP molecules per molecule of glucose is produced by the homofermentative pathway through the Embden-Meyerhof pathway. Yeast extract clearly exhibits the most significant effect on lactic acid production, especially at the beginning of growth, with the lactic acid concentration increasing nearly linearly with an increase in yeast extract level. The importance of yeast extract at the early stages of fermentation was probably caused by the amino acids and vitamins that are crucial for growth. However, if the medium contained a sufficient quantity of yeast extract from brewing as a source of nitrogen and growth factors, the amount of commercial yeast extract could be reduced or even replaced without a decrease in lactic acid production.

2.6.1 Batch fermentation

Lactic acid production in batch fermentation is widely studies in batch fermentation by lactic acid bacteria (Yun and Ryu, 2001). The species of *Lactobacillus* used to ferment sugar depends mainly upon the type of carbohydrate being employed. Strains of *L. delbruecki* find favor when production is based on maize sugar; the inoculum is grown up in batches and these are added directly to the fermentor containing several thousand gallons of medium. For whey fermentation, strains of *L. bulgaricus* are necessary because this species is able to utilize the milk sugar (lactose). The fermentation proceeded for up to six days, and the concentration of sugar in the medium dropped to around 0.2 per cent (Brock and Madigan, 1991). An amylolytic lactic acid producing *L. amylovorus* produced 36 g/L of lactic acid in mixed cultures with *L. casei* at 37 °C in 48 h, when initial barley flour concentration was 180 g/L woth a yield of 20% (Javanainen and Linko, 1995). Lactic acid production was investigated for batch of *Enterococcus faecalis* RKY1, using wood hydrolyzate and corn steep liquor. When wood hydrolyzate (equivalent to 50 g/L glucose)

supplemented with 15-60 g/L corn steep liquor was used as a raw material for fermentation, up to 48.6 g/L of lactic acid was produced with volumetric productivities ranging between 0.8 and 1.4 g/L/h with a yield of 97.2% (Wee *et al.*, 2006). Literature reviews of lactic acid production from various materials shown in Table 2.3.

Table 2.3 Lactic acid fermentation from agricultural resources by batch fermentation.

Raw materials	**Organisms**	**Lactic acid (g/L)**	**Productivity (g/L/h)**
Waste paper	*Rhizopus oryzae* NRRL 395	49.1	1.8
Molasses	*L. delbrueckii* NCIMB 8130	90.0	3.8
Corn starch	*L. amylophilus* GV6	76.2	0.8
Wheat hydrolyzate	*L. lactis* ATCC 19435	106.0	3.3
Acorn starch hydrolyzate	*L. rhamnosus* HG 09	57.6	1.6

Source: (Wee *et al*., 2006).

Moreover, an amylolytic lactic acid bacterium was capable to produce 18.3 g/L of lactic acid from starch with a product yield of 61% (based on total carbohydrates consumed) (Zhand and Cheryan, 1991). However, the major disadvantage of batch fermentation is that lactic acid concentration and productivity decrease due to inhibition of high substrate concentration. This phenomenon is the typical property of batch fermentation. On the other hand, as the time goes by the concentration of lactic acid are increased but it can inhibit cell growth and product formation.

2.6.2 Fed-batch fermentation

Lactic acid production by *Lactobacillus casei* fermentation using different fed-batch feeding strategies was studied. According to the experimental results, exponential fed-batch culture is an effective method for the fermentation of L-lactic acid. In exponential feeding glucose solution (850 g/L), the maximum lactic acid concentration was obtained at 210 g/L. L-lactic acid yield, the maximal dry cell weight and productivity were up to 90.3%, 4.30 g/L, and 2.14 g/L/h, respectively. Comparing with the traditional batch culture, the exponential feeding glucose and yeast extract culture showed 56.5% improvement in L-lactic acid production, 68.6% improvement in dry cell weight and 59.7% improvement in productivity, respectively (Ding and Tan, 2006). Batch, fed-batch, and continuous fermentations are the most frequently used methods for lactic acid production. Higher lactic acid concentrations may be obtained in batch and fed-batch fermentation than in continuous fermentation.

2.7 Factors affecting on lactic acid fermentation

However, there are still several researches that need to be addressed in order to produce lactic acid within the targeted cost, development of high performance lactic acid producing microorganisms and lowering the cost of the raw material. Many factors affected in lactic acid fermentation have been investigated. The optimization of fermentation processes requires profound knowledge of the factors determining microbial metabolism, and the influence of process parameters.

2.7.1 Effect of temperature

Temperature and pH are the key environmental parameters that affect the fermentation process (Yuwono and Kokugan, 2008). Low temperature has been reported to

positively influence the outgrowth of contaminating microorganism, thereby influencing the performance of the lactic acid production were investigated (Neysens and Vuyst, 2005). The temperature giving the highest productivity lower than the temperature resulting in highest lactic acid mass concentration and yield (Hujanen and Linko, 1996).

For *Lactobacillus amylophilus*, which is known to grow at 15 °C but not at 45 °C, the optimal temperatures were 25 °C and 35 °C for maximum productivity and yield, respectively (Yumoto and Ikeda, 1995). Some previous reported investigated the cultivation temperature on the solid-state fermentation of lactic acid production by controlling the growth temperatures at 22, 30, 35, and 40 °C. The results from measuring the residual starch and reducing sugar in 4 h and 8 h indicated that there was increased in starch hydrolysis and reducing sugar accumulation as the temperature increased from 22-30 °C, and a further increase from 30-40 °C resulted in a slight improvement for the saccharification in both *Rhizopus oryzae* 2062 and *Rhizopus oryzae* 36017 cultures. Therefore, the lactic acid production and biomass growth were affected by the temperature (Huang *et al.*, 2005).

2.7.2 Effect of pH

The fermentation pH is either set at the beginning and then left to decrease due to acid production or it is controlled by an addition of alkaline solutions. The optimal pH for lactic acid production varies between 5.0 and 7.0. A pH below 5.7 was optimal for *Lactobacillus* strains, which are known to tolerate lower pH than *lactococci*. The previous studies investigated the influence of culture pH on lactic acid fermentation from molasses where lactic acid fermentations were performed on a jar fermentor at 38 °C and pH 5.0-9.0. Although the optimum pH for cell growth of *Enterococcus faecalis RKY1* was seen to be 8.0, the lactic acid fermentation at pH 7.0 was completed faster than that at pH 8.0. The cell growth at pH 5.0 almost ceased after 10 h of fermentation, the highest lactic acid mass

concentration was obtained at pH 7.0 with a comparable yield with pH 6.0 (Wee *et al.*, 2004). Moreover, some reported showed the effect of various initial pH on the lactic acid production of the immobilized *Lactobacillus delbrueckii* during the batch fermentation of liquid pineapple waste. At initial pH 6.5, cell started to utilize glucose earlier and at a faster rate than at other initial pH. Maximum lactic acid concentration was attained at initial pH 6.5. Further increase in initial pH beyond 6.5 does not improve the lactic acid production (Idris and Suzana, 2006). It is possible that the higher initial pH brought too much stress on the microorganism metabolic abilities (Vijayakumar *et al.*, 2008).

2.7.3 Effect of incubation period

Previous reported represented that an increase in lactose utilization and subsequent lactic acid production was found incubation time up to 36 h and thereafter no improvement in both the functions was observed (Panesar *et al.*, 2010). This could be attributed to the growth of the culture reached to the stationary phase and as a consequence of metabolism, microorganisms continuously change the characteristics of the medium and the environment. The incubation period of 48 h has been generally used for lactic acid production using different lactobacilli cultures (Gandhi *et al.*, 2000). In addition, the different optimal conditions reported by various workers for maximum lactic acid production could be explained by the differences in the nature of the strains and medium composition used in their studies.

2.7.4 Effect of agitation

Different lactic acid bacterial strains differed in their requirement for growth conditions. The consequence of agitation speed on lactic acid fermentation efficiency was carried out. For the strain *Lactobacillus rhamnosus*, the maximum lactic acid concentrations

could be achieved when fermentation was carried out at pH 6, temperature of 40°C and agitation speed of 150 rpm, which was in accordance with a previous report (Hofvendahl and Hagerdal, 2000) the optimal condition for lactic acid is pH 5.0-6.8, temperature 30-45°C with continuously agitating at 100-200 rpm (Timbuntam *et al.*, 2008).

2.8 Inhibition of lactic acid fermentation

The major problems associated with lactic acid production are substrate inhibition, end-product (lactic acid) inhibition, and by-product formation, respectively. There are different strategies to check the end-product inhibition for example neutralization of lactic acid with suitable alkali, but there are fewer attempts to account for the substrate inhibition (Porro *et al.*, 1999). End product inhibition occurs due to rise in pH of extracellular medium during lactic acid production that results in disturbance of NADH/NAD^+ ratio. It has been evidenced that conversion of pyruvate to lactic acid requires high cytosolic NADH and a high NADH/NAD^+ ratio increases *ldh* activity (Garrigues *et al*., 1997).

2.8.1 Substrate inhibition

At high concentrations, some substrates also inhibit the enzyme activity. Uncompetitive inhibition is substrate inhibition which occurs at high substrate concentrations. It happens when two molecules of substrate can bind to the enzyme, and thus block activity. Some paper showed that high initial lactose concentration of 100 g/L in cheese whey reduced both the specific growth rate and substrate utilization rate due to the substrate inhibition phenomenon (Tango and Ghaly, 1999).

2.8.2 End-product inhibition

For microorganisms, limitation of growth and acid production by the end-product is well known. Lactic acid production processes traditionally suffer from end-product inhibition. The inhibition mechanism of lactic acid is probably related to the solubility of the undissociated lactic acid within the cytoplasmic membrane and the insolubility of dissociated lactate, which causes acidification of cytoplasm and failure of proton motive forces (Wee *et al.*, 2006). It eventually influences the transmembrane pH gradient and decreases the amount of energy available for cell growth (Goncalves *et al.*, 1997). The inhibition of growth in *Lactobacillus acidophilus* were investigated, by acidification of cytoplasm via the acid produced, below an organism-specific threshold pH level of 4.4 (Kashket, 1987). However, the mechanism for lactic acid inhibition of growth is not fully understood. The accepted mechanism of inhibition by weak organic acids is related to the solubility of the non-dissociated form within the cytoplasm membrane and the insolubility of the ionised acid form (Gatje *et al.*, 1991). This causes acidification of the cytoplasm and the collapse of the motive force, resulting in inhibition of nutrient transport (Bender and Marquis, 1987). In addition, effect of inhibition by acetic acid on anaerobic bacteria is related to the accumulation of metabolic intermediates of terminal reactions (McDonald *et al.*, 1990). It suggested that the capacity of lactobacilli to tolerate lower pH values than other lactic acid bacteria, such as *Streptococcus* and *Leuconostocos*, originates from their more basic cytoplasmic pH values, allowing higher concentrations of non-dissociated acids to be tolerated. In addition, it could also be related to the higher activity of the ATPase responsible for the extrusion of H^+, or related to the more efficient excretion of lactic acid by lactobacilli (Gatje *et al.*, 1991). The lactic acid is excreted in *Lactobacillus helveticus* as transported lactate ions and by simple diffusion (of the non-dissociated form of lactic acid). This could be due to the decrease of intracellular pH, which reduces ATPase

activity, since it has been shown the ATPases isolated from different lactic acid bacteria have different pH optima, ranging from 5.5 to 7.5 depending on their origin (Sturr and Marquis, 1992).

Therefore, to alleviate the inhibitory effect of lactic acid during the fermentation, it must be removed selectively *in situ* from the fermentation broth. Recently, various attempts have been carried out to remove the lactic acid simultaneously as it is formed. There are studied the reactive extraction of lactic acid from the fermented broth. They indicated that *in situ* extraction was possible with the use of di-*n*-octylamine and with adjustment of the fermentation broth to a pH 5.0 by ammonia. In their study, the system of electrodialysis fermentation with a level meter was the most efficient system and a higher yield could be obtained if the glucose concentration in the broth could be controlled to remain at a lower level (Hano *et al.*, 1993).

2.9 Extractive fermentation

The fermentation medium contains lactic acid or its salt or the mixture of both. A class of advantageous processing approaches involves removal of lactic acid from the fermentation broth or other mixture, while leaving the lactate (at alkali condition) behind in the fermentation broth. The separation can, in some instance, occur within the fermentor or it can be conducted on solution material removed from the fermentor. As lactic acid is formed and accumulated, pH of the fermentation broth goes on lowering, that affects the productivity of microorganism. At the highest concentration, biomass and lactic acid concentration diminished, indicating that higher concentrations also inhibits the process by product inhibition (Lund *et al.*, 1992). The influence of lactate concentration on the specific growth rate, and it has been observed that this inhibition was reduced when the culture broth was neutralized by ammonia instead of sodium hydroxide, but; this phenomenon is

not well explained (Belfares *et al.*, 1993). However, conventional method in controlling of pH is carried out by addition of lime and thus converting lactic acid to calcium lactate to avoid this problem. Downstream processing of this broth includes precipitation and acidification of calcium lactate to lactic acid. Alternatively, lactic acid can be continuously removed from the fermentor by adsorption, extraction or membrane separation.

Extraction and purification of lactic acid from fermented medium is a difficult operation. It is impossible to crystallize the acid from an aqueous solution because of its low melting point (53 °C) and distillation of concentrated solutions under reduced pressure often leads to some loss of lactic acid (Gupta *et al*., 2007). The several processes involving salt formation have been used to extract acid. Salt with secondary and tertiary alklyamines are sufficiently stable to be extracted from aqueous solutions using organic solvents. The solvent is then evaporated off and salt decomposed to give free lactic acid. Moreover, the method for extracting lactic acid is by esterification. This involves preparing an ester of lactic acid, distilling this to remove impurities and recovering lactic acid and alcohol after hydrolysis. Esterification is used to prepare high grade lactic acid for use in manufacture of bioplastics.

2.10 Purification techniques of lactic acid

A number of approaches can be used for separation of lactate salt from fermented medium including extraction by organic solvents, ion-exchange separation, adsorption, vacuum distillation, and membrane separations. Each of these exhibits some advantages and disadvantages that are also reported with fermentation processes. The choice of the separation process should be based on the efficient and economically usage of these extractants. Technological advancements in the major process components-fermentation, primary and secondary purification, polymerization, chemical conversion of lactic acid and

its derivatives would enable low cost large volume and environment friendly production of lactic acid. Recent advancements in membrane based separation and purification would enable lactic acid production without producing salt or gypsum by products. In recently issued patents, an osmotolerant strain of lactic acid bacteria and a configuration of desalting electrodialysis, water splitting electrodialysis and ion exchange purification, a concentrated lactic acid product containing less than 0.1% proteinaceous components can be produced by a carbohydrate fermentation (Narayanan *et al*., 2004).

2.10.1 Adsorption

Recovery of carboxylic acids from fermentation broths presents a challenging separation problem, because of the dilute, complex nature of fermentation broths. Important characteristics of extractants and solid sorbents are a high capacity for the acid, a high selectivity for the acid as opposed to water and substrate (e.g., glucose), regenerability, and, depending upon the process configuration, the biocompatibility with microorganisms. Many fermentation processess, such as that for lactic acid production, are subject to end-product inhibition. If a solid sorbent can be used *in situ* or in an external recycle loop, higher overall yields can be achieved (Frieling, 1999). However, elution steps are the most difficult and problematic part as a large volume of eluent is required with adjusting of the pH.

2.10.2 Solvent extraction

Solvent extraction has been used for the purification of carboxylic acid such as lactic acid and succinic acid. But these solvents *in-situ* are toxic as they rupture the cell membrane causing the metabolite to leak out. Long chain alcohols such as 1-octanol and 1-decanol were found to be less toxic than other diluents. In addition, they reduce the toxicity

of solvents on the cells. Bio-compatibility and price of the solvent are the major concern when using solvent extraction processes (Hong *et al*., 2001).

2.10.3 Membrane separation processes

As mentioned earlier, lactic acid production processes traditionally suffer from end-product inhibition. An undissociated lactic acid passes through the bacterial membrane and dissociates inside the cell. It eventually influences the transmembrane pH gradient and decreases the amount of energy available for cell growth (Gonçalves *et al.*, 1997). In parallel with fermentation kinetic studies, the use of membranes to extract fermentation products from the aqueous phase was introduced many years ago in order to improve the traditional production processes (Doig *et al.,* 1998). In many particular systems, membrane separations are the most effective techniques. Membrane separation has been widely used for purification concentration of fluid mixtures. Theory of membrane transport is concerned with chemical nature of the membrane, physical structure and physio-chemical

properties of the mixture to be separation. The main interest will be focused on the absorption of organic compounds into the membrane surface and the diffusion across the membrane matrix (Li *et al.,* 2000). However, the fermentation step is needed for an economically favorable production. So, this work will be emphasized on membrane separation technique coupling with lactic acid fermentation.

2.10.3.1 Electrodialysis (ED) technique

Electrodialysis (ED) is usually considered as a desalination process, but there is much wider scope for this technique because ED is, in many cases, a powerful separation method when charged compounds have to be separated from a solution.

For instance, ED can be used to recover free lactic acid from fermentation broth. Electrodialysis of the permeate allows good lactic acid separation and concentration, up to 13% w/v of lactic acid. The acid can further be concentrated by evaporation. The fermentation step is needed for an economically favorable production (Lund *et al.*, 1992). Electrodialysis fermentation (EDF) is promising because it can remove produced lactic acid continuously from the fermentation, and maintain the pH of the broth. In this study, an EDF method with the conventional fermentation method was compared with EDF with continuous medium feed in order to increase the productivity of lactic acid. However, the acid can further be concentrated by evaporation which increase the cost of the process (Habova *et al.*, 2004).

2.10.3.2 Electrodeionization (EDI) technique

Using present technology, the volumetric productivity of lactic acid fermentation is known to be rather small as accumulation of lactic acid inhibits the process by product inhibition. Unfortunately, owing to its physicochemical properties,

lactic acid can not be continuously extracted from the fermentation medium by simple operation, which means that a large proportion of the cost of lactic acid production is associated with recovery and purification operations. The pH of the fermentation broth decrease and the fermentation is inhibited when lactic acid is produced. Therefore, the recovery of lactic acid from the fermentation broth is very important for increasing its productivity. Electrodeionization (EDI) utilizes chemical-free regeneration. EDI is a continuous process where ion exchange resins are being continuously regenerated by the DC electric field. Compared with deionization process of water, there is no breakthrough of ions as happens in conventional ion exchange operations, therefore the quality of the water

remains at a constant high level of purity. The electric field also provides a bacteriostatic environment inside of the EDI cell, inhibiting the growth of bacterial and other organisms.

EDI has significantly lower operating costs than conventional ion exchange processes. Only a relatively small amount of electric power is needed to provide high purity water (Widiasa *et al*., 2004). The lack of acid and caustic regeneration means less operator attention and lower labour costs. Capital costs can also expect to be lower, especially because no chemical storage, pumping and neutralization equipment is required. Moreover, EDI has a significantly smaller footprint than conventional ion exchange processes. In an EDI device, the space within the ion depleting compartments (and in some cases in the ion concentrating compartments) is filled with electrically active media such as ion exchange resin. The ion exchange resin enhances the transport of ions and can also participate as a substrate for electrochemical reactions, such as splitting of water into hydrogen (H^+) and hydroxyl (OH^-) ions. Different media configurations are possible, such as intimately mixed anion and cation exchange resins (mixed bed or MB) or separate sections of ion exchange resin, each section substantially comprised of resins of the same polarity e.g., either anion or cation resin (layered bed or LB and single bed or SB). Figure 2.4 shows the electrodeionization process using a combination of ion selective principle and materials as ion exchange resins sandwiched between two electrodes (anode (+) and cathode (-)) under a DC voltage potential. Literature reviews on separation of lactic acid from fermentation broth using EDI technique is scarce. Only one related work can be obtained for recovery of citric acid production (Wenten, 2004). Hence, it is very interesting to investigate EDI technique for *in situ* recovery of L(+)-lactic acid from fermentation broth.

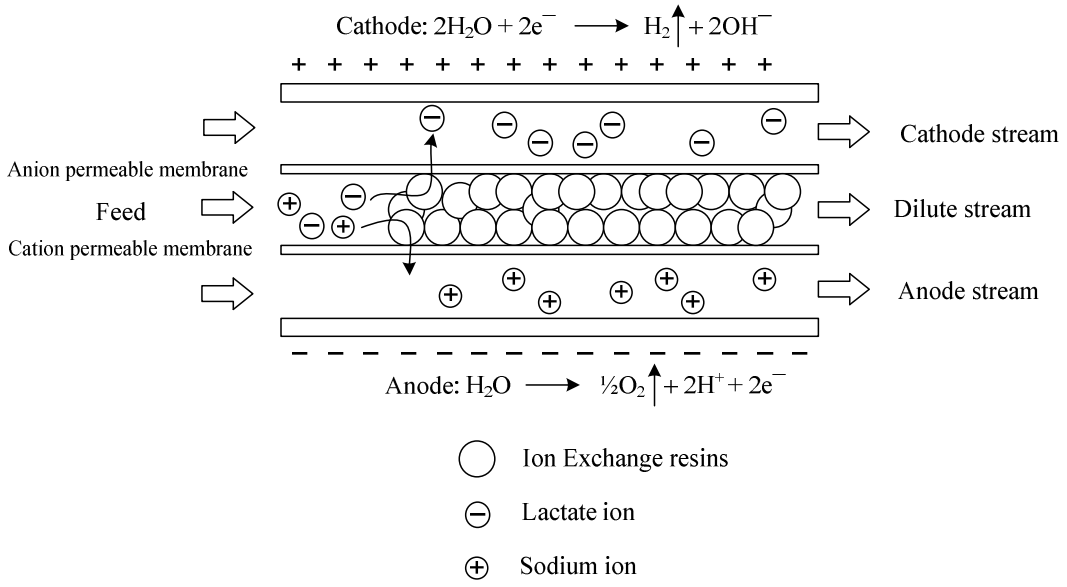


Figure 2.4 Schematic diagram of electrodeionization (EDI) process for recovery of lactic acid from fermentation broth.

2.11 *In situ* product removal system

Biocatalytic processes for the manufacture of small, highly functionalized molecules frequently have limited productivity. A common reason for this is the presence of the reaction products that can cause inhibitory or toxic effects (making poor use of the enzyme), or promote unfavourable equilibria (giving low conversions). In each case, the product needs to be removed as soon as it is formed in order to overcome these constraints and hence increase the productivity of the biocatalytic process. Therefore, *in situ* product removal and the process research are required for its implementation (Lye and Woodley, 1999).

The productivity of biocatalytic processes is frequently limited by the need to operate the reaction under conditions unsuited to the biocatalyst (microorganism). Indeed, this differentiates biological from chemical catalysis, in that the optimal environment for the

biological catalyst has been carefully evolved for operation under natural physiological conditions, whereas chemical catalysts are designed for a specific conversion at user-defined conditions. Inevitably, the need for high process productivities outside the environment for which the biocatalyst was evolved leads to compromises over bioreactor design and operation. In several cases, however, compromises are not sufficient and it is necessary to maintain one environment around the biocatalyst while the bulk of the reactor operates under different conditions. This philosophy of compartmentalization is, in principle, an effective strategy to overcome the problem of low productivities. It has already been successfully applied to cases where the substrate concentrations need to be different for the reactor and the biocatalyst. A further need is for compartmentalization of the product owing to inhibitory (reversible loss of catalytic activity), or toxic (irreversible loss of catalytic activity) effects on the biocatalyst to product degradation and/or to unfavorable reaction equilibrium.

In situ product removal methods address these limitations by selectively removing the product from the vicinity of the biocatalyst as soon as it is formed and can also provide further benefits for the subsequent downstream processing. *in situ* product removal methods can increase the productivity or yield of a given biocatalytic reaction by any of the following means: (1) overcoming inhibitory or toxic effects; (2) shifting unfavourable reaction equilibria; (3) minimizing product losses owing to degradation or uncontrolled release; and (4) reducing the total number of downstream-processing steps (Freeman *et al.*, 1993). Despite these advantages, however, the application of *in situ* product removal to the production of small molecules has been slow except in a limited number of well-known cases. The primary examples are of low-value, high-volume products, such as the removal of organic acids and solvents from fermentation processes. In conclusion, the potential of the technology can be fully exploited (Lye and Woodley, 1999).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemical agents and nutrients

All chemicals used for chemical reaction were analytical grades, and some chromatographic grades for chemical analysis by HPLC, and were purchased from Fluka, United Kingdom; Himedia, India; and Merck, Germany. Lactic acid (p.a. purity) was obtained from Sigma-Aldrich (Montreal, Que., Canada), and was used as standard for HPLC analysis. Yeast extract, MRS medium, beef extract and peptone were obtained from Difco (Difco laboratories, Detroit, MI, USA).

3.2 Screening and identification of selected isolates of lactic acid bacteria

3.2.1 Isolation methods

Lactic acid bacteria (LAB) in this study were isolated from several different sources. Lactic acid bacteria were isolated from samples of starchy raw materials such as two types of cassava starch, three types of cassava dregs, one types of rice vermicelli, seven types of bran, and three fermented cassava waste of cassava starch processing factories in Nakhon Ratchasima, Thailand. Each of these (10 g) was separately blended with 100 ml 0.85% NaCl solution; 10 ml of this blended sample was subsequently added to 100 ml MRS broth in a 250 ml Erlenmeyer flask. The flasks were shaken in an incubator (New Brunswick Scientific, USA) at 200 rpm, 30 °C for 24-48 h (Mohd Adnan and Tan, 2007). The aliquot of the culture from each of the flasks was diluted serially. The samples

were also serially diluted with 0.85% NaCl solution, and 100 μL of each dilution were isolated by spread-plating onto MRS agar plates. A duplicate set of culture plates were incubated for 24-48 h at 30 °C in under anaerobic condition using the Gas Pack system (Merck Anaerocult type C) (Booysen *et al.*, 2002; Muyanja *et al.*, 2003). The samples were serially diluted in physiologic saline up to 10^{-6} and appropriate dilutions were spread on MRS agar plates. From each dilution, 0.1 mL volumes of isolated strains were pour-plated in duplicate on MRS agar, and they were cultivated at temperature 30 °C for 24 hours under anaerobic condition. The colonies were randomly (different colony types) picked from plates with 30-300 colonies. Several representative strains displaying the general characteristics of lactic acid bacteria were chosen from each plate for further studies. Each of the isolates were repeatedly streaked in order to purify the isolates, which were maintained on MRS agar slants for immediate use and in 15% glycerol for storage at -20 °C. All isolates of LAB were selected to identify the lactic acid yield in MRS medium by HPLC analysis.

3.2.2 Morphological characterization

For the primarily selection, the isolates which had the highest of %yield were selected for this experiments. Besides the isolates which had highest %yield in group of heterolactic bacteria, were also selected. Cell morphology was observed by Gram's staining under bright field microscopy. Gram-positive bacteria were stained dark blue or violet by crystal violet and safranin-O. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet. Catalase activity was initially tested by placing a drop of 3% hydrogen peroxide solution on the cells(Guyot *et al.*, 1998). Immediate formation of bubbles indicated the presence of catalase in the cells. For gas production determination, bacterial were

added in MRS test tubes containing the Duram tube (Nomura *et al.*, 2006) to determine whether the isolate strains produces carbon dioxide during fermentation (CO_2 from glucose or gluconate). The identification procedures were determined according to the criteria established in Bergey's Manual of Determinative Bacteriology. An isolate was deemed to be a homo-fermentative lactic acid producer if no gas was produced (no CO_2 from glucose or gluconate). Plates were incubated at 30 °C for 24-48 h (Holt *et al.*, 1994). All tests were performed in duplicate.

3.2.3 Biochemical characterization

The previous procedure was repeated in order to purify the isolates, and to be able to select the high ability in term of growth and good characterization. According to these tests, the isolates were selected as potential lactic acid bacteria. Biochemical characterization of isolated strains was carried out using API 50 CHL tested kit following the manufacturer's instructions (BioMerieux, Marcy-l'Etolite, France). The API 50 CHL system was used to determine the biochemical results of carbohydrates or sugar utilization of the lactic acid bacteria. Reproducibility was verified by repeated analysis using cultures grown on MRS agar and sub-cultured in liquid MRS medium prior to inoculation. The cultures were inoculated in 10 ml MRS broth for overnight. Culture was harvested by centrifugation at 12,000 g for 1 min, then the mixture solutions were washed twice with sterile physiological saline (0.85% sodium chloride) and pellets or cells were suspended in API 50 CHL medium (API systems, BioMérieux). Using sterile Pasteur pipettes, homogenized suspensions of the cells in the medium, with subsequent vortex mixing, were transferred into each of the 50 wells on the API 50 CH strips of 49 different compounds (and one control). This was done for all isolates and type strains. All wells were overlaid with sterile mineral oil (Merck, Germany) to affect anaerobiosis. Strips were moistened and covered as recommended by the manufacturer and incubated at 30 ^{o}C. Changes in color

from violet to yellow were monitored after 24 h and 48 h. Then the APILAB Plus software version 5.0 from BioMérieux and Analytab Product's computer database were used for comparison of carbohydrate assimilation and/or fermentation patterns and bacterial identification (Muyanja *et al.*, 2003).

The selected isolates of lactic acid bacteria was confirmed for their cell morphology using scanning electron microscopy (SEM) at the electron microscopy laboratory of the Instrument Buildings of the Center for Scientific and Technological Equipment, Suranaree University of Technology.

3.2.4 Phenotypic and genotypic characterization of lactic acid bacterial strains

The strains which showed the high performance in lactic acid production were selected for phenotypic and genotypic characterization. Cell morphology and cell characterization were investigated. Growth ability of the isolates was performed at different NaCl concentration at 1.5, 2.5, 5, and 7.5 % (w/v), temperature between 15, 30, 45, and 50 °C, respectively. The initial pH ranging from 3.0, 4.0, 5.0, 6.0 and glucose fermentation patterns were investigated. All tests were done in duplicate.

The isolate which could produce high lactic acid yield and able to grow in wide range of environmental condition was considered to the best strain for further studies. Application of molecular genetic techniques to identify lactic acid bacteria has resulted in significant changes in their taxonomic classification. These techniques have been considered as an appropriate technique for the differentiation and characterization of lactic acid bacteria. DNA extractions according to the methods of Pitcher *et al*. (1989) were carried out. Reference strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), LTH (Universität Hohenheim, Germany) and ATCC (American Type Culture Collection).

3.2.4.1 DNA extraction

Isolated strains were collected from MRS agar were grown to middle log phase in MRS broth (to an optical density of 1.4 at a wavelength of 600 nm), and harvested using centrifugation technique (8000×g, 5min, 4 °C). The cell pellet was rinsed twice with sterile saline solution (0.85%, w/v, NaCl), and was suspended in 50 μl sterile distilled water before the cells were boiled in a hot water bath (95 °C) for 15 min. The DNA of isolates collected from MRS plate was extracted with phenol-chloroform, as described by Dellaglio *et al*., 1973. DNA extracts were frozen at -20 °C.

3.2.4.2 PCR amplification of 16S rDNA

DNA templates for PCR amplification were prepared by previous method as described earlier (Marmur, 1961). DNA coding for 16S rDNA regions was amplified by means of PCR with *Taq (Thermus aquaticus)* polymerase, as described by Kawasaki (Kawasaki *et al.*, 1993), Yamada (Yamada *et al.*, 2000), and Katsura (Katsura *et al.*, 2001). A PCR product for sequencing 16S rDNA regions was prepared by using the following two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3'), positions 9-27 on 16S rDNA by the *E.coli* numbering systems; (Brosius *et al.*, 1981) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'), position 1509-1492 on 16S rDNA by the *E.coli* numbering system; (Brosius *et al.*, 1981). One hundred μl of a reaction mixture contained 15-20 ng of template DNA, 2.0 μmole each of the two primers, 2.5 units of *Taq* polymerase , 2.0 mM $MgCl_2$, 0.2 mM dNTP and 10 μl of 10x *Taq* buffer, pH 8.8, containing $(NH_4)_2SO_4$, which was comprised of 750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$ and 0.1% Tween 20. The PCR amplification was programmed to carry out an initial denaturation step at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 2 min, followed by a final amplification step at 72 °C for 3 min (Brosius *et al.*,

1981). The PCR product was analyzed by 0.8% (w/v) agarose gel electrophoresis and purified product. After purification PCR product was stored at -20 °C for further step.

3.2.4.3 Direct sequencing of 16S rDNA

Direct sequencing of the single-banded and purified PCR products (ca. 1500 bases on 16R rDNA by the *E. Coli* numbering system; (Brosius *et al.*, 1981) was carried out. The primers 20F, 520R (5'-GTA TTA CCG CGG CTG-3'), position 536-519), for partial sequencing and additional 1500R, 520F (5'-CAG CAG CCG CGG TAA TAC-3', position 519-536), 920F (5'- AAA CTC AAA TGA ATT GAC GG-3', position 907-926) and 920R (5'-CCG TCA ATT CAT TTG AGT TT-3'), positions 926-907) for full length sequencing were used for sequencing of 16S rDNA. Ten µl of a sequencing reaction mixture contained 5-20 ng of template DNAs, 2.0 µl of BigDye™ terminator ready reaction mixture, 5-20 ng of DNA template, 1.6 pmole of sequencing primer, 1.5 µl of 5x BigDye™ sequencing buffer and deionized water. The PCR reactions were carried out as follows: an initial denaturation step at 96 °C for 30 sec, 25 cycles of denaturation at 96 °C for 10 sec, annealing at 50 °C for 5 sec and elongation at 60 °C for 4 min. Eighty µl of freshly prepared ethanol/acetate solution was added to the sequencing reaction mixture in 1.5 min microcentrifuge tube and mixed well with a brief vortex. The mixture was left to stand at room temperature for 15 min and centrifuged at the maximum speed of 14,500 g for 20 min at room temperature. The ethanol solution was immediately removed carefully from the tube with an aspirator equipped with a fine tip. The resulting DNA pellets were washed by adding 250 µl of 70% ethanol to the tube, and vortexed briefly. The precipitated DNA was collected by centrifugation for 5 min at the maximum speed. The remaining ethanol was carefully removed from the tube with an aspirator equipped with a fine tip. The DNA obtained was dried in a heat box at 90 °C for 1 min, and the dried DNA was stored at either

4 °C or -20 °C. The DNA pellets were suspended in 20 µl of a terminator sequencing reagent, mixed on a vortex and spun down. The double stranded DNA was completely separated by heating at 95 °C for 2 min, and immediately placed on ice, until ready to load on instrument. The DNA sequencing was performed by the analyzer and using the standard of the database for identification of the nearest sequences. The sequences were then compared to those in GenBank (National Center for Biotechnology Information; www. ncbi.nig.gov). For phylogenetic tree analyzed, multiple alignments were generated using Multiple Alignment, which was based on Fast Fourier Transform (MAFFT).

3.3 Medium formulation of lactic acid fermentation by lactic acid bacteria

Since a potential lactic acid bacterium was isolated from fermented cassava waste, it was expected that the strain possessed high amylolytic activity. The first step to examine its fermentation performance was to test its ability to hydrolyze cassava starch. In order to accomplish the experiment, 50 µL of sample (culture) was put into a hole on starch agar plates containing 2.0% agar and 1% starch at pH 7.0. The plates were incubated at 30 °C for 24 h. Diameters of the clear zone around the hole were measured with a present of iodine solution as shown in Figure 3.1. Amylase activity in this research was the value of the diameter of the clear zone surrounding the hole (X) minus the diameter of the hole (Y) as present in Figure 3.2 (Laloknam *et al*., 2009).

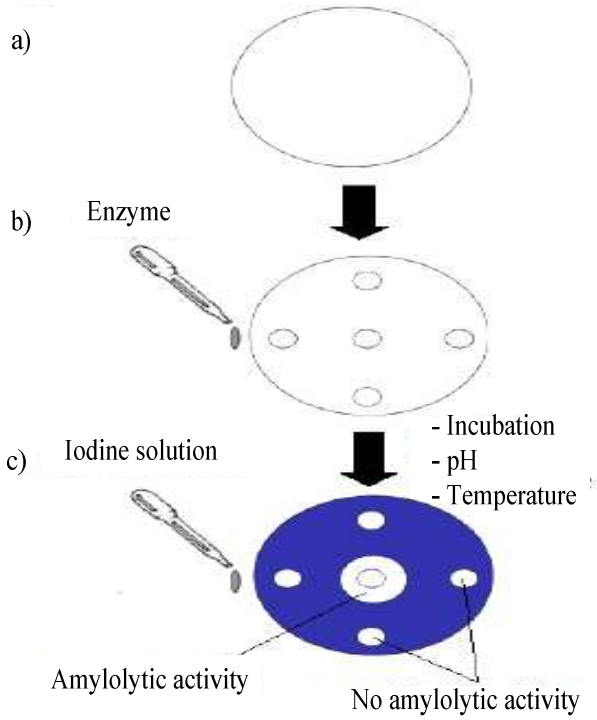


Figure 3.1 Schematic representation for detection of amylolytic activity: a) Starch agar, b) hole in starch agar, and c) amylolytic activity after iodine solution was presented (Laloknam *et al*., 2009).

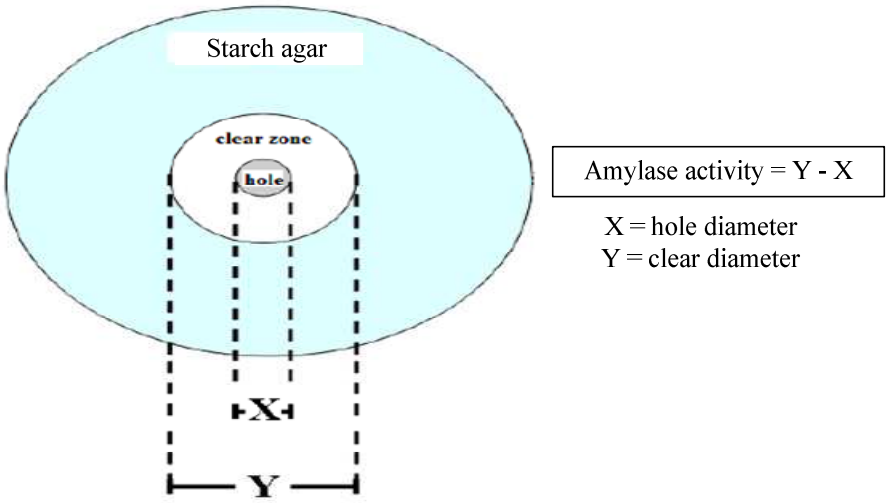


Figure 3.2 A conventional method for calculation of amylase activity (Laloknam *et al*., 2009).

3.3.1 Amylase activity assay

In order to correctly assay the α-amylase activity, 0.1 ml of appropriately diluted enzyme solution was incubated with 0.8 ml of a solution containing cassava starch in 0.1 mol/L of citrate-phosphate buffer (Petrov *et al.*, 2008). The reaction was stopped by the addition of 0.1 ml of 1 mol of H_2SO_4. After incubation, residual starch contents after different lengths of time were determined colorimetrically at a wavelength of 620 nm. The iodine complexing ability of starch according the method of Giraud *et al*., 1993 by adding 0.1 ml of the reaction mixture to 2.4 ml of an iodine solution containing (g/L in distilled water): KI, 30; I_2, 3, diluted to 4%. One enzyme unit was defined as the amount of enzyme hydrolyzing 10 mg starch in 30 min.

3.3.2 Cassava starch hydrolysis

Prior to fermentation, cassava starch was processed with two distinct steps by enzymatic hydrolysis. The liquefaction by α-amylase (Sigma-Aldrich, USA) was firstly used with the concentration of 0.5 Unit/mg starch at 55 °C for 24 h, then enzyme was added into the cassava starch. Stability of enzyme activity should be checked before use in the process. The α-amylase breaks down long-chain starch, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and limit dextrin from amylopectin. Secondly, the saccharification step for liberation of glucose molecules was carried out using gluco-amylase (Oriental Yeast Co. Ltd. Tokyo, Japan) with the working concentration of 0.5 Unit/mg starch at 40 °C for 24 h. For the preparation of hydrolyzed cassava starch, 100 g of cassava starch was produced equivalent to 40 g of glucose. When the starch hydrolysis was completed, the slurry was cooled to room temperature (Zhengdong *et al*., 2009).

3.3.3 Effect of carbon sources on lactic acid fermentation

The optimum conditions for growth and fermentation were investigated by using the selected lactic acid bacterium. To investigate the requirements for complex carbon sources, the experiments were performed either omitting one of the organic carbon sources from the modified MRS medium. Lactic acid bacteria which express high stereo-selectivity, and high yields of lactic acid production were selected. The selected lactic acid bacteria (10% inoculums size) were inoculated into the modified MRS medium. Effects of glucose concentrations were investigated. The initial glucose concentrations varied between 20-200 g/L in batch cultures. In addition, the effects of different concentrations of cassava starch (1-10%), hydrolyzed cassava starch (1-10%), glucose syrup, and different sugars were investigated. For cultivation, the following modified MRS medium has been used: 20 g/L carbon sources, 15 g/L nitrogen sources, 5 g/L sodium acetate, 2 g/L triammonium citrate, 2 g/L $Na_2HPO_4.2H_2O$, 0.1 g/L $MgSO_4.7H_2O$, 0.05 g/L $MnSO_4.4H_2O$, and 1 ml Tween 80, pH at 6.0 (Ding *et al*., 1997). All tests were performed in six parallel 1 Litre bioreactors (working volume 700 mL) of Multifors® benchtop bioreactor system, INFORS-HT, Switzerland. The fermentation was carried out within the period of 0-72 h. All experiments were performed at 30 °C and agitation speed of 200 rpm. Sampling was done every 3 h interval for the first 12 hours, and then every 6 hour interval for further analysis. The optimized condition and medium were further used in process development.

3.3.4 Effect of nitrogen sources on lactic acid fermentation

To investigate the requirements for organic nitrogen sources, the experiments were performed either omitting one of the organic nitrogen sources from the modified MRS medium (20 g/L of carbon source). The highly effective lactic acid bacterium which possesses desirable characteristics such as homo-fermentative ability,

good fermentation performance was selected. The different nitrogen sources were investigated such as peptone, whey, corn steep liquor, yeast cell, and spent brewer's yeast extract, respectively. The effect of different concentrations of nitrogen sources (5-20 g/L) were also investigated following modified MRS. Spent brewer's yeast extract was prepared according to a previous work (Saksinchai *et al*., 2001). However, it was reported that spent brewer's yeast extract lacks of growth factors especially iron. Therefore, 2 mg/L of ferrous sulfate ($FeSO_4$) was added. As a result, a cost-effective fermentation medium using spent brewer's yeast extract was developed. Spent brewer's yeast extract showed its high content of protein, vitamins, minerals, and also growth factors which necessary for the growth of lactic acid bacteria. Commercial yeast extract costs 55 Dollars per kilogram (Bi *et al*., 2011), and the production cost for our spent brewer's yeast extract was approximately 15 Euros per kilogram. Compared with commercial MRS medium, the calculated production cost for this newly developed fermentation medium was 80% cheaper. All tests were performed six parallel 1 Litre bioreactors (working volume 700 mL) of Multifors® benchtop bioreactor system, INFORS-HT, Switzerland, and sampling was performed for further analysis. The optimized condition and medium will be further used in process development. All experiments were carried out in duplicate.

3.3.5 Preparation of spent brewer's yeast extract

Spent brewer's yeast was collected from local breweries. The solid content of 20% (w/v) were resuspended using distilled water and then transferred to a glass bottle. The slurry was autolyzed for 24 h at 50 °C with stirring. The autolyzate was then heated to 85 °C and kept at this temperature for 15 min to inactivate residual enzyme activity. It was centrifuged at 6,000 rpm for 15 min. The clear supernatant was poured off and concentrated

by evaporation technique. The concentrate was subsequently spray-dried. The spent brewer's yeast extract powder was stored at 4^{o}C for further use (Saksinchai *et al.*, 2001).

3.3.6 Effect of others factor on lactic acid fermentation

The culture was inoculated into the medium to determine the optimum condition for lactic acid fermentation processes. The main aim of this study was to investigate the effects on the amelioration of lactic acid production. The fermentation experiments were conducted. The experimental studies were investigated as followed:

(a) The temperature giving the highest productivity was in some cases lower than the temperature resulting in highest LA concentration and yield, whereas in others the same temperature gave the best results in all categories (Hofvendahl and Hagerdal, 2000). The effects of temperature between 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, or 55 °C were investigated for lactic acid fermentation (Busairi, 2002). All of determinations were analyzed and the optimum temperature was selected for further study.

(b) Some enzymes have ionic groups on their active site, and these ionic groups must be in the correct form (acid or base) to function. Variation in the pH of the medium results in changes in the ionic form of the active site. Therefore, the activity of the enzymes were significantly affected the reaction rate for cell growth and lactic acid production. The effects of initial pH (4.0, 5.0, 6.0, 7.0, or 8.0) were investigated for lactic acid fermentation (Yuwono and Kokugan, 2008). The optimum pH was selected for further study.

(c) Batch experiments were determined the effect of agitation rate on lactic acid production in order to obtained the optimum stirrer speed for improving the lactic acid productivity at the initial operation in batch fermentation mode. Agitation speeds were varied at 100, 200, or 300 rpm. The optimum agitation speed was selected for further study.

(d) To evaluate the end product inhibition, the lactic acid solution was individual added in the medium to make up the desired lactic acid concentration ranging from 0 to 90 g/L for investigates of cell growth in lactic acid fermentation.

All experiments were carried out in six parallel 1 Litre bioreactors (working volume 700 mL) of Multifors® benchtop bioreactor system, INFORS-HT, Switzerland.

3.4 Lactic acid fermentation processes

3.4.1 Batch fermentation

All fermentations were performed in a 2 L or 10 L bioreactor (Sartorius, Germany) containing initial 1.2 L of fermentation medium. The operating temperature of 30 °C was set for all experiments with the agitation speed of 200 rpm in order to ensure the homogeneity of the system. The inoculum size of 10% (v/v) was prepared in MRS medium at 30 °C for 12 h using an incubator (New Brunswick Scientific, USA) at 200 rpm agitation. The medium formulation which yields the best performance was chosen for batch fermentation in bioreactor. All experiments were performed at optimal growth temperature at 30 °C, pH 6.0 without aeration. The pH was monitored by a pH sensor (Mettler Toledo, Switzerland) and automatically maintained by the addition of 5M NH_4OH. Fermentation ended when either glucose was completely consumed or no net change in its concentration for more than 6 h. Samples were withdrawn aseptically at regular intervals for further analysis.

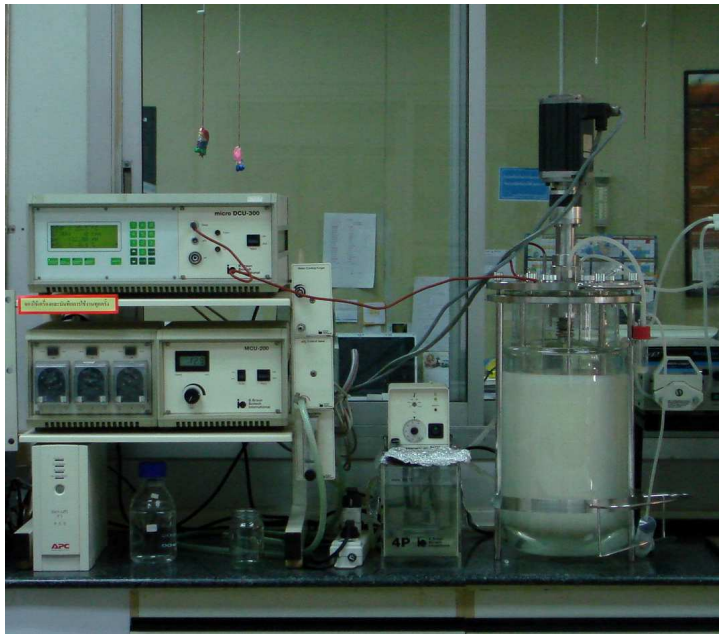


Figure 3.3 Equipment for fermentation processes of lactic acid production.

3.4.2 Fed-batch fermentation

The suitable conditions (section 3.4.1) were used for lactic acid production in fermenter. In fed-batch modes, the bioreactor was initially operated as batch cultures with a working volume of 1.2 L at 30 °C, for 17 h prior to adding the feeding solution of hydrolyzed cassava starch (5 times concentration of medium equivalent to 200 g/L of glucose) plus 15 g/L of spent brewer's yeast extract. The culture volume can also be maintained practically constant by feeding the concentrated medium solution. The feeding solution was pumped into the bioreactor using a variable speed peristaltic pump (Cole Parmer, USA). Intermittent feeding was carried out by the addition of feeding solution to keep the concentration of glucose in an appropriate range (5-10 g/L) (Wu *et al.*, 2009). In constant feed rate, the feeding solution was supplied at the flow rates between 0.01-0.3 g/s, respectively.

For exponential fed-batch fermentation, the substrate mass feeding rates were manipulated by using Equation (1) which is derived with the assumption of a constant biomass yield and maintenance coefficient throughout the experiment.

$$F_s = \left(\frac{\mu}{Y_{x/s}} + m \right) V_0 X_0 e^{\mu t} \quad (1)$$

Where V_0 is the initial volume, X_0 is the initial cell concentration before fed-batch, μ is the specific growth rate, $Y_{X/S}$ the biomass yield on substrate, m represent maintenance coefficient, t is the fed-batch time, respectively (Korz *et al*., 1995). The substrate feeding began at the end of batch mode by using a computer controlled peristaltic pump.

3.5 Analysis of fermentation broth

During fermentation, a 10 mL samples were withdrawn under aseptic conditions. After centrifugation at 12,000 rpm for 10 min, the supernatant was collected for further analysis.

3.5.1 Reducing sugar

Reducing sugars present in the broth were estimated by using 3,5-dinitrosalicylic acid reagent (DNS method) (Miller, 1959). Reducing sugar present in the broth was estimated. This method tests for the presence of free carbonyl group (reducing sugars). This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions. The 0.5 mL of supernatant was taken and mixed with 0.5 mL of 3,5-dinitrosalicylic acid- reagent in the 16 mL test tube. The development of color was

conducted by boiling the reaction tube for 5 min. The concentration of reducing sugar was calculated against standard glucose concentration of 0.2-1.0 μmol/mL.

3.5.2 Growth and biomass measurement

Growth and biomass was measured with spectrophotometer (Hitachi, Japan) by optical density at a wavelength of 600 nm wavelength against the medium as blank and gravimetrically, respectively. The gravimetric cell mass concentration measurement began by using a manifold filtration unit. For the determination of dry weight, ten millilitres of cell suspensions were centrifuged at 12,000 rpm for 10 min, washed twice with deionized water, and dried at 105 °C until a constant weight was achieved (24 h). The cell density was then converted to dry weight (g/L) using an appropriate calibration curve. Dry weight of biomass was calculated from a calibration curve between O.D. and dry cell weight (1 OD_{600} unit=0.34 g dry weight/L). Cell cultures were appropriately diluted in sterile medium to obtain OD_{600} of less than 0.4, and calibration curves between OD_{600} and dry cell weight were established for each of the strains. Specific growth rate (μ) was calculated as (1/X) x (dX/dt). Growth and lactic acid yield ($Y_{X/S}$ and $Y_{P/S}$) were calculated as the slope of the linear regressions of either biomass or lactic acid versus residual substrate (as total sugars) during then exponential growth phase.

The percentages of substrate conversion were calculated using the following expression:

$$GC = \frac{100 \times (S_0 - S)}{S_0} \qquad (2)$$

S_0 = initial glucose concentration (g/L)

S = final glucose concentration (g/L), to time when maximum of lactic acid concentration.

3.5.3 Lactic acid analysis

During fermentation, a 10-ml sample was collected under aseptic condition. The result is expressed as lactic acid and the yield (%) was calculated as the grams of acid that were produced from 100 g of reducing sugars consumed. At specific intervals of time, the vials were removed and the fermented broth was centrifuged at 12,000 rpm for 10 min and the supernatant was analyzed by HPLC. The cultured medium was filtered through 0.45 μm membrane filter. Organic acids were analyzed by HPLC (Merck–Hitachi). Five microliters of the sample was injected into the HPLC system equipped with an Aminex HPX-87 H column (Bio-Rad Co., USA) and RI detector. The column temperature was maintained at 65 °C. The mobile phase was 10 mM H_2SO_4 at flow rate of 0.6 mL/min (Thang and Novalin, 2008).

The titratable acidity is expressed as % lactic acid (CH3CHOHCOOH, molecular weight = 90) and is determined by titration with 0.1 N NaOH using phenolphthalein as indicator according to the AOAC (1975) method. The titratable acidity (as lactic acid) was calculated using the relationship (reproducibility ± 0.01% of lactic acid):

$$\%\ \text{titratable acidity} = \frac{mL \times N \times 90 \times 100}{V \times 1000} \tag{3}$$

Where; mL = volume of 0.1 N NaOH used; N = normality of 0.1 N NaOH; V=Volume of sample used.

The result is expressed as lactic acid and the yield (%) was calculated as the gradient of the curve obtained by plotting product concentration against substrate concentration.

$$Y_{P/S} = \frac{g_{lactic}}{g_{substrate}} \tag{4}$$

3.5.4 Determination of lactic acid isomers

Analysis of D(-) and L(+) lactic acid concentrations were measured enzymatically based on colorimetric determination by using the Test-Combination of Boehringer (Mannheim, Germany) by measuring the absorbance at a wavelength of 340 nm. In this method, known amounts of production medium were taken during fermentation and centrifuged at 12,000×g for 10 min. The supernatant was used directly for determination of lactic acid concentration. The assays are specific for either D-lactic acid or L-lactic acid. The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.107 mg/L of sample solution at the maximum sample volume of 1.50 mL (or to 1.60 mg/L with a sample volume of 1.0 mL). The absorbance of the solutions (A1) was read against blank at 340 nm after approximately 3 min and the reactions were initiated by addition of 0.02 mL of (D-LDH)/(L-LDH) suspensions and then the second absorbance of the solutions (A2) were read at the end of the reaction (approximately 20 min). In situations where the reaction did not stop after 20 min, the absorbances were read at 5 min intervals until the absorbance either remained the same, or increased constantly over 5 min. The D-(-) and L-(+)-lactic acid were also determined by enzyme test kit according to the manufacture's instruction.

Enantioseparation process was carried out by an introduction of neutralized and purified lactic acid samples into a HPLC (Agilent technologies, USA) equipped with a chiral column (Sumichiral OA-6100, S.A.S Corp., Japan), diode array detector (DAD). The column was set at room temperature. The mobile phase consisted of 2 mmol/L $CuSO_4$ in H_2O/acetonitrile solution (98:2) with the flow rate of 1 mL/min.

3.6 EDI experimental set-up

Experimental set-up for separation of lactic acid from solution using EDI technique was shown in Figure 3.4. All experiments were carried out at room temperature, around 25-30 °C. The body of the EDI cell was constructed from polycarbonate. Cation and anion exchange membranes were Neosepta BP-1, and AM-1 (Tokuyama, Japan). The effective surface area of each membrane was 90 cm^2. All diluted compartments were filled with mixed-bed ion-exchange resins (purolite strong acid cation-exchange, C-100E and strong base anion resins, Amperlite IRA-400): 50%/50% or 40 g. Platinum and stainless steel were used for anode and cathode, respectively. The internal spacer for each compartment was 8 mm. The EDI process involved two independent streams: feed solution stream and concentrated solution stream. The concentrated stream was split and pumped through both concentrated compartments and electrode compartments as concentrate and electrodes rinse, respectively. The EDI system was operated continuously. The flow rate of the feed solution was operated at 4 L/h. The influences of various operating conditions on electrical resistance and ions transfer rate were assessed. After the experimental run, samples were taken for analysis of lactic acid by either titration with NaOH solution (0.01 N) and HPLC measurement.

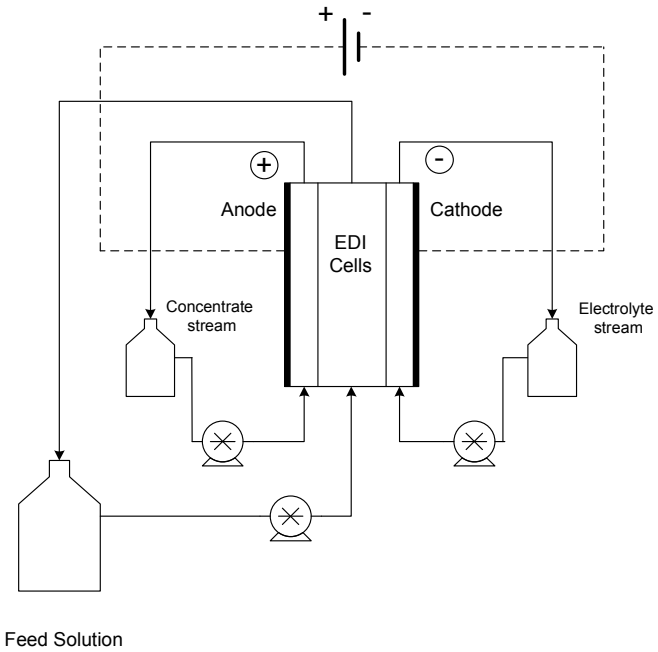


Figure 3.4 Experimental set-up for separation of L(+)-lactic acid from fermentation broth using EDI technique.

3.6.1 Adsorption characterization

In the study of equilibrium, 5 g of dry resin and 100 mL of either aqueous lactic acid solutions (the concentration between 0-90 g/L) were contacted in 250 mL flask at 30 °C. The initial pH was fixed at 6.0. Temperature and pH were the same as in equilibrium studies. After contacting phases with magnetic stirring until the equilibrium was reached, samples of the supernatants were withdrawn and analyzed for lactic acid by HPLC.

3.6.2 EDI technique characterization

A DC programmable power supply was in-house constructed, and was used to produce direct electric current. It could supply voltage, and current density in the range of 0-70 Volts, and 0-45 mA/cm^2, respectively. An adjustable power supply was specially constructed, and was used to produce direct current.

3.7 Membrane bioreactor set-up for *in situ* lactic acid removal by EDI technique in fed-batch fermentation

The regenerated culture from agar slant in MRS medium was transferred to the bioreactor. The inoculum sizes were a volume equivalent to 10% of the reactor working volume. The fermentation medium was sterilized at 121 °C for 15 min. In the fermentation processes, the culture pH was automatically controlled at desired values (6.0) by the addition of 5M NH_4OH. The bioreactor was operated under anaerobic condition, agitation at 200 rpm, and temperature at 30 °C. Impeller speed was monitored, and regulated using an external electric control unit. In order to avoid cell damage due to direct contact with the electrodes, a submerged microfiltration (ceramic membrane size 0.2 μm) membrane module was internally installed to process only the clarified broth. It was used for extracted the bacterial cells of the fermentation broth. The microfiltration permeate was re-circulated across the feed side and concentrated side by a peristaltic pump, and then was directly fed into the EDI stage. Back washing was carried out periodically when filtration rate was too slow. Fed-batch fermentation of lactic acid coupling with EDI experimental set up was operated as shown in Figure 3.5.

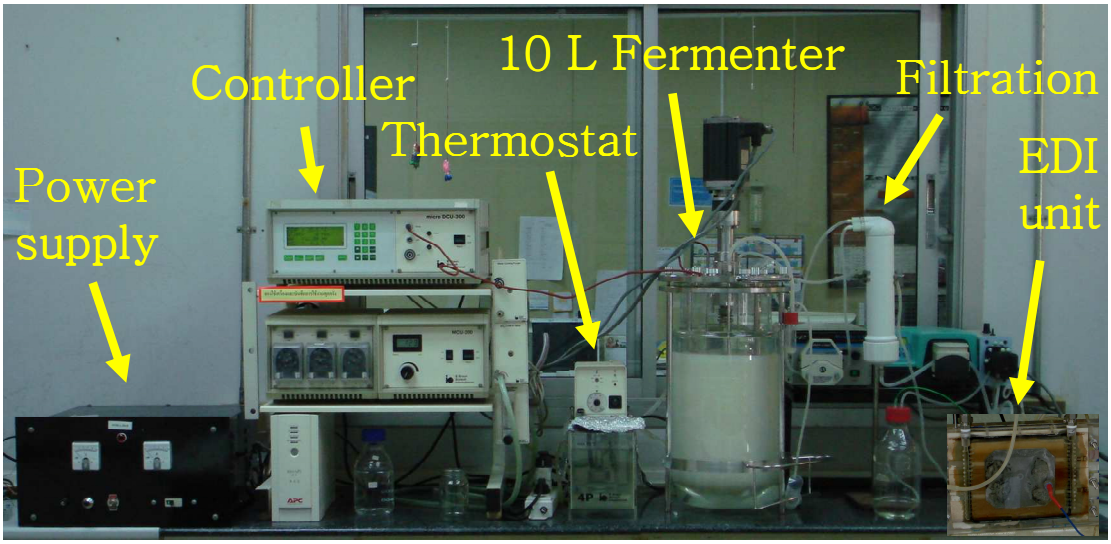


Figure 3.5 Membrane bioreactor set up for fed-batch fermentation of lactic acid by EDI technique.

However, the influence of lactate concentration to fermentation performance by the biocatalyst has been studied intensively. The coupling of a biological reaction with a membrane in only one unit is a very interesting configuration for the reaction where the continuous elimination of metabolites is necessary to maintain a good productivity.

The recovery of lactate (R) from the feed stream was calculated using the following equation:

$$R = \frac{C_{C,f}V_{C,f} - C_{C,i}V_{C,i}}{C_{F,i}V_{F,i} - C_{F,f}V_{F,f}} \times 100\% \qquad (5)$$

Where, $C_{C,f}$ is the final lactate concentration of the concentrate solution, $V_{C,f}$ is the final volume of concentrate solution, $C_{C,i}$ is the initial lactate concentration of concentrate solution, $V_{C,i}$ is the initial volume of concentrate solution, $C_{F,i}$ is the initial lactate concentration of feed solution, $V_{F,i}$ is the initial volume of feed solution, $C_{F,f}$ is the final lactate concentration of feed solution, and $V_{F,f}$ is the final volume of feed solution, respectively.

3.8 Viable cell measurement by fluorescence microscopy

Measurement of cell viability was accomplished using fluorescence microscopy method. Propidium iodide (PI; Sigma) was used as a fluorescent probe that binds to DNA, but cannot cross an intact cytoplasmic membrane (Amanullah *et al.*, 2003). In addition, 4′-6-diamidino-2-phenylindole (DAPI) was an AT-selective DNA stain which caused a 20-fold enhancement in fluorescence when it combined with DNA. In order to avoid any clumping of cells, the cell were centrifuged and re-suspended to 25% of the original broth volume using phosphate buffer solution (pH 7.2). The working concentrations of DAPI and PI in the buffer solution were 1 mg/mL. The stained samples were then fixed into glass slides and examined under a microscope. PI fluorescence was measured at 630 nm, whereas DAPI was measured at 525 nm.

The number of viable cells (C_X) were compared with the number of viable cell at the beginning of the run (C_X^0), and the ratios at time (t) were subsequently used to calculate the deactivation constant (k_d) as follows;

$$\frac{C_X}{C_X^0} = e^{-k_d t} \tag{6}$$

Take natural logarithmic on both sides of the equation gives

$$\ln \frac{C_X}{C_X^0} = -k_d t \tag{7}$$

A plot of $\ln C_X/C_X^0$ against time (hours) results in a straight line, and the deactivation constant (k_d) can be directly estimated from the slope. In addition, the half-life or the period of time it takes for a substance undergoing decay to decrease by half of the biocatalyst can then be calculated as;

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{8}$$

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Screening and isolation of lactic acid bacteria (LAB) for lactic acid production

4.1.1 Isolation of LAB

The aim of this section was to isolate, and to carry out taxonomic determination of the potential strain for bioprocess development of lactic acid production. MRS agar was employed as a selective media for isolation of lactic acid bacteria. Approximately 20 colonies were randomly picked from plates containing 20 to 200 lactic acid bacteria. Plates were examined by eye, and the different colony types were individually picked. They were propagated twice and streaked on MRS broth to obtain the pure cultures. Experimental results revealed that fifty bacterial isolates were obtained. All of them showed Gram-positive and catalase-negative characteristic. A total of fifty isolates of LAB were isolated from the different sources. Initially, the isolated strains were named according to their origin. For example; twenty-three isolates from fermented food were named as N(n), five isolates from bran were named as N(n)B(n), and twenty-two isolates from fermented cassava waste of cassava starch processing factories in Nakhon Ratchasima, Thailand were defined as MB(n), respectively.

4.1.2 Selection of LAB for lactic acid production

All isolates of LAB were used to identify the lactic acid yield in MRS medium at pH 6.0. The concentrations of lactic acid were determined by using HPLC. The lactic acid yield can be calculated as % yield following the Equation (9):

$$\%\ \text{Yield} = \frac{\text{Grams of lactic acid produced}}{\text{Grams of total sugar consumed}} \times 100 \qquad (9)$$

Experimental results for product yield were shown in Table 4.1.

Table 4.1 The lactic acid yield by all isolates of LAB.

No.	Isolate code number	% Yield of lactic acid production
1	N10	26.56
2	N11	30.45
3	N12	90.78
4	N13	42.34
5	N14	40.23
6	N15	31.08
7	N16	20.81
8	N17	12.54
9	N18	15.89
10	N19	32.14
11	N20	49.98
12	N21	20.34

Table 4.1 (Continued).

No.	Isolate code number	% Yield of lactic acid production
13	N22	30.87
14	N23	2.98
15	N24	40.65
16	N25	5.47
17	N26	10.89
18	N27	3.54
19	N28	10.98
20	N29	32.34
21	N30	23.32
22	N31	20.19
23	N32	18.43
24	N1B1	80.52
25	N2B2	42.34
26	N2B3	29.87
27	N2B4	60.89
28	N3B2	13.11
30	MB12	19.43
31	MB20	78.43
32	MB21	80.42

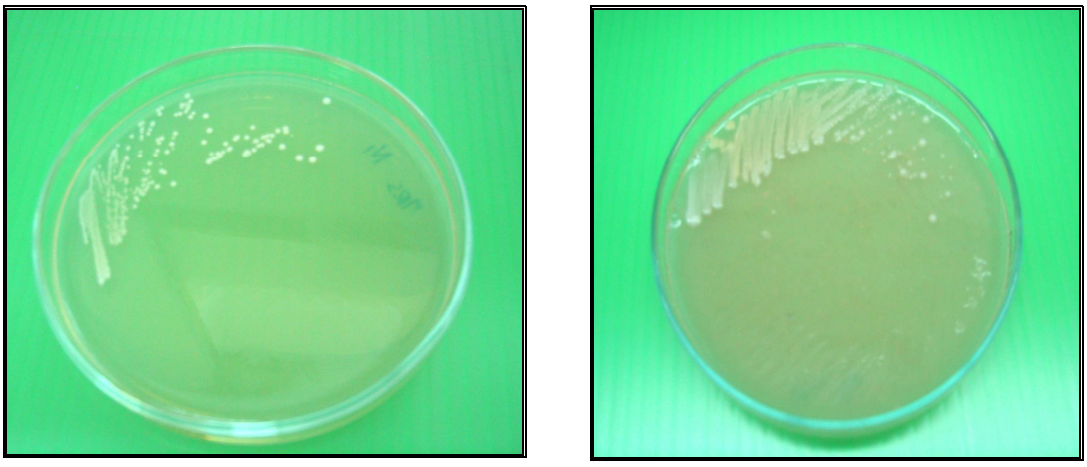
Table 4.1 (Continued).

No.	Isolate code number	% Yield of lactic acid production
29	MB10	80.98
33	MB30	76.48
34	MB40	85.17
35	MB50	79.89
36	MB51	34.56
37	MB52	90.13
38	MB61	75.45
39	MB63	13.54
40	MB64	31.09
41	MB65	46.78
42	MB72	6.67
43	MB73	2.32
44	MB74	31.09
45	MB75	46.78
46	MB76	50.15
47	MB77	54.65
48	MB78	21.76
49	MB79	17.43
50	MB80	5.76

From Table 4.1, it could be seen that 10 isolates could produce high yields of lactic acid (more than 75%) whereas other 40 isolates may be classified as heterofermentative LAB because of their low yield of lactic acid production. As a result, 10 isolates with the high of product yield were selected for subsequent characterization experiments. However, they could be readily distinguished by microscopic examination. The determinations of the strains were further investigated according to their morphological, cultural, physiological, and biochemical characteristics by the procedures described in the Bergey's Manual of Systematic Bateriology (Holt *et al*., 1994).

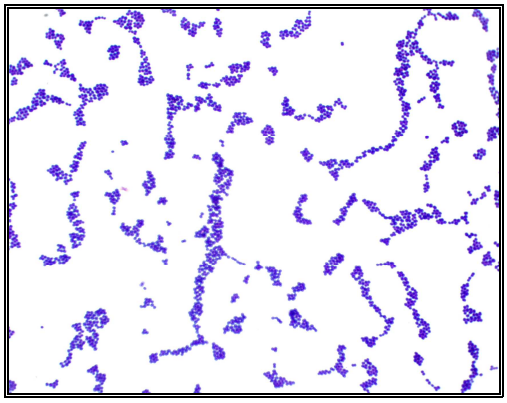
4.1.3 Morphological and phenotypic characterization of LAB

In this section, the 10 isolates were further tested for morphological and phenotypic characterization. The single colonies of sample isolates grown on MRS medium agar were illustrated in Figure 4.1. Microscopic observation showed typical morphological characterization of the example isolated strains appeared as short rods, long, thin and cocci shaped, etc. Gram's stains of the isolates were shown in Figure 4.2.

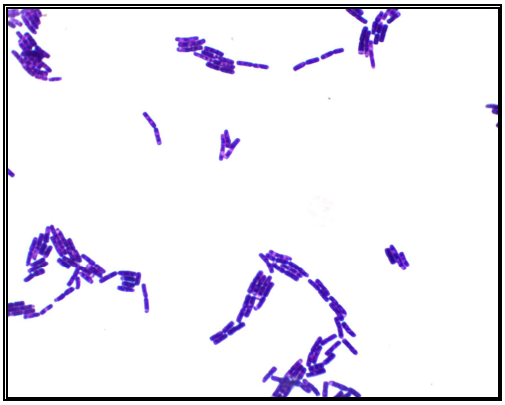


(A) (B)

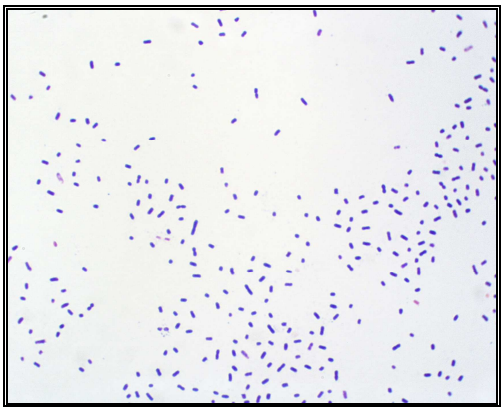
Figure 4.1 Samples of lactic acid bacteria colonies grown on MRS medium agar.



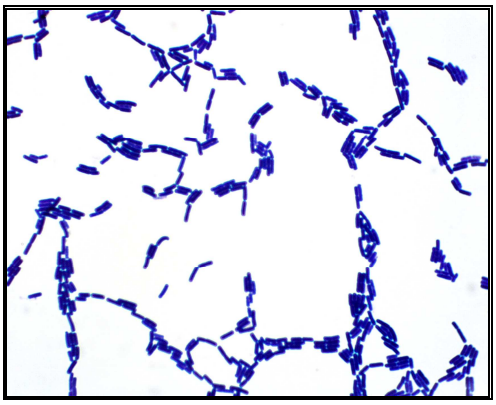
MB50



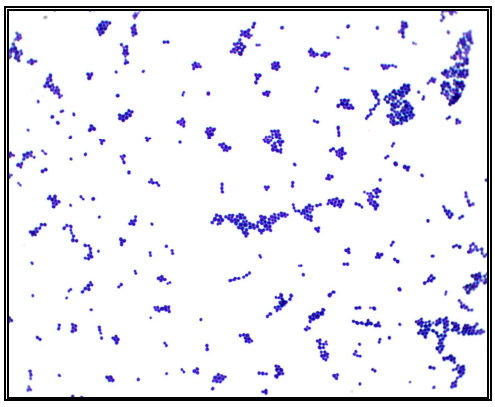
MB30



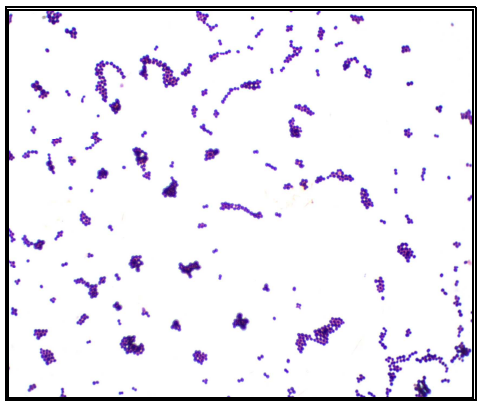
MB10



MB20

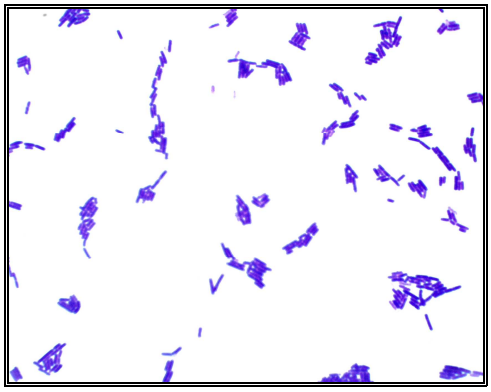


MB2B1

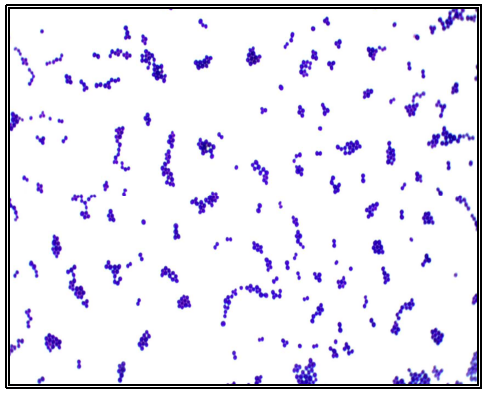


MB61

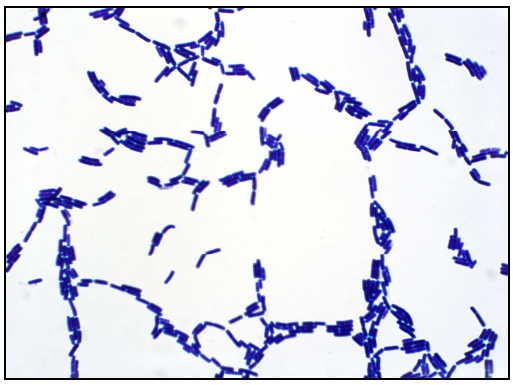
Figure 4.2 Photomicrograph of the isolates under bright field microscopy (×1000).



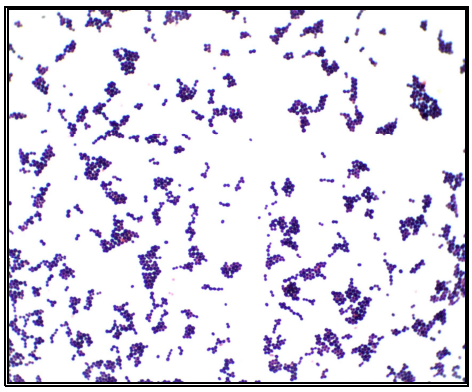
MB40



N12



N1B1



MB52

Figure 4.2 (Continued).

The morphological and physiological (catalase test and CO_2 production) of the 10 isolated strains were investigated. This diversity of species is relative and dependent primarily on the nature of the material isolated and the different criteria used for each study. According to Table 4.2, morphological and physiological characteristics of the 10 isolates strains giving details on cell morphology (rod, cocci, and ovoid shape) that were subdivided into two groups: cocci (46%), and rods (54%). Furthermore, microbial growth capacity, and gas production (homo- or hetero-fermentative) were investigated. For the bacterial growth in MRS medium, growth caused turbidity of the medium. The growth after inoculation, profuse growth, good growth, and poor growth were presented as 50 % (+++), 40 % (++),

and 10 % (+), respectively. Bacterial growth depends on culture medium with its ability to produce a high concentration of biomass. The variation of different nutrients in the medium are needed for lactic acid bacterial strains. The group of complex nutrients, i.e., skimmed milk, yeast extract, and peptone were used to satisfy the complex demands of the bacteria. A complex medium, especially, MRS medium is usually employed and supported the growth when cultivating of LAB.

These findings showed that lactic acid bacteria isolated in this study were grown well and some strains showed good performance of homo-lactic acid fermentative, and suitable to use as starter culture in the subsequent fermentation processes. For further consideration, isolates which produced gas were discarded because they deemed to be hetero-fermentative. The MB 40 was also chosen because of its possessed high % lactic acid yield and high growth, although it produced gas and belonged to hetero-fermentative group. In conclusion, four isolates namely MB52, MB40, N12, and N1B1, respectively, were selected according to their high growth rate, and were further investigated for morphological and phenotypic characterizations.

Table 4.2 Cell morphology and growth performance of lactic acid bacterial strains.

Strain code	Cell shape	Color	Form	Elevation	Margin	Microbial growth (turbidity)	Gas production
N12	Cocci	White	Circular	Flat	Entire	+++	-
N1B1	Rod	White	Circular	Raised	Entire	+++	-
MB50	Cocci	White	Punciform	Flat	Entire	++	-
MB40	Rod	White	Circular	Raised	Undulate	+++	+
MB30	Rod	White	Irregular	Flat	Undulate	++	-
MB10	Rod	White	Circular	Flat	Entire	++	-
MB20	Rod	White	Punciform	Flat	Undulate	++	-
MB2B1	Cocci	White	Circular	Raised	Entire	+	+
MB52	Cocci	White	Circular	Flat	Entire	+++	-
MB61	Cocci	White	Circular	Flat	Undulate	++	-

Legend: profuse growth (+++), good growth (++), moderate to poor growth or a positive reaction (+), no growth or no reaction (-).

Table 4.3 showed general characteristics of the four isolated strains. Most strains possessed cocci or short rods shape with their cell size ranging from 0.5-1.5 μm. The strain MB52 had a special characteristic of tetrad cell organization. Morphology revealed white, circular, and smooth colonies for MB52, and N12 whereas MB40 and N1B1 colonies were white, circular, and convex, respectively. For physiological characterization, most strains grew at 30-45 °C except MB52 and N1B1 which tolerated the temperature up to 50 °C. The ability to grow at high temperature is a desirable trait as this could translate to increased rate of growth and lactic acid production. A high fermentation temperature could reduce contamination by other microorganisms (Mohd Adnan and Tan, 2007). Furthermore, MB52

was the most tolerant to high NaCl concentration compared to the other three isolates. It grew in the concentration for of salt up to 7.5% whilst the rest could grow in up to 5% salt concentration. Bacteria adapt to hyper-osmolarity by accumulation, synthesis and transport of compatible solutes to restore turgor. It was well documented that osmo-protectants could play additional positive roles, and beneficial effects have been demonstrated on membrane integrity, protein folding and stability, thermo-protection (Baliarda *et al.*, 2003).

Table 4.3 Morphological, and physiological characterization of the isolated strains (Codes MB52, MB40, N12, and N1B1).

Characteristics	**Strain code**			
	MB52	**MB40**	**N12**	**N1B1**
Cell morphology	Cocci in tetrads	Short rods	Cocci	Short rods
Cell diameter size	0.6-1.0 mm	0.5-0.8 mm	0.5-1.0 mm	0.5-1.5 μm
Gram stain reaction	+	+	+	+
Spores formation	-	-	-	-
Colony morphology	White, circular, smooth	White, Circular, convex	White, Circular, smooth	White, Circular, convex
Catalase activity	-	-	-	-
Fermentation types	Homo	Hetero	Homo	Homo
Glucose fermentation	+	+	+	+
Growth temperatures:				
15 °C	+	-	+	+
30 °C	+	+	+	+
50 °C	+	-	-	-

Table 4.3 (Continued).

Characteristics	Strain code			
	MB52	**MB40**	**N12**	**N1B1**
Growth (pH):				
3	-	-	-	-
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
NaCl concentration (%w/v):				
1.5	+	+	+	+
2.5	+	+	+	+
5.0	+	+	+	+
7.5	+	-	-	-

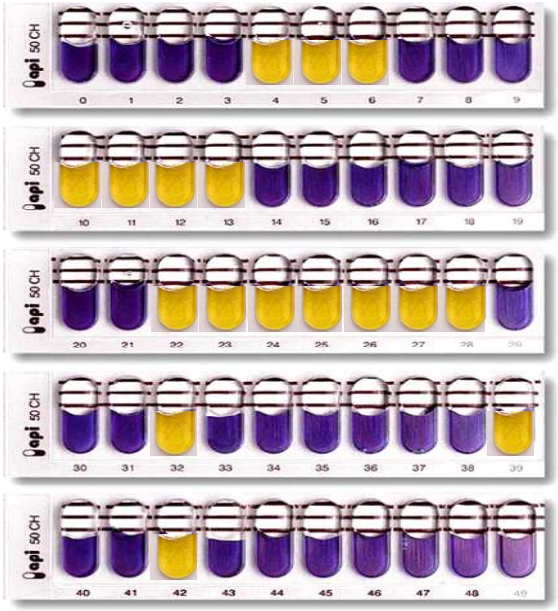
Legend: positive (+), negative (-).

From these studies, accumulation of osmo-protectants or compatible solutes had been considered to be the potential lactic acid bacteria. Similarly, a higher tolerance to lactic acid was a desirable trait for an industrial strain of LAB as it could produce more lactic acid in the fermentation broth without adversely affecting itself. The strain MB52 could be protected to be able to grow at higher NaCl concentration compared with the other isolates. During fermentation process, lactic acid was being produced by the cells whereas alkali was pumped into the broth to prevent excessive reduction in pH. The free acid would be converted to its salt form of lactate which would increase the osmotic pressure on the cells. Therefore, an LAB strain with high osmo-tolerance would be desirable for industrial application. These rapid screening processes resulted in domination of MB52 in a wide range of tested conditions.

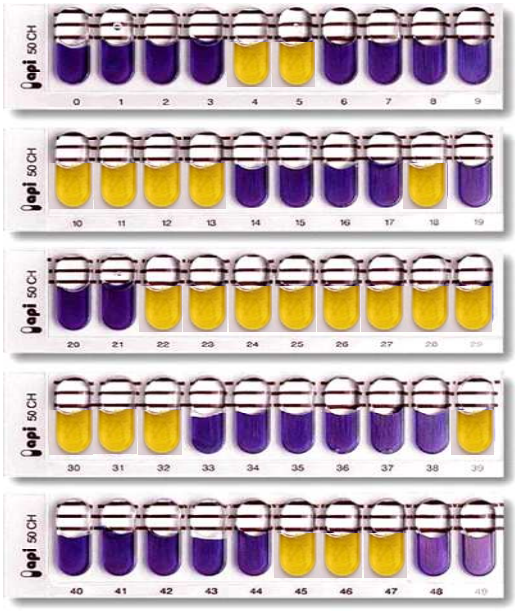
Homo-fermentative lactic acid bacteria grow substantially faster than other bacteria present in the same ecological niche. This results in very rapid domination of this type of bacteria in a wide range of environments. The higher growth rate of lactic acid bacteria is a result of their simple primary metabolism, and their ability in adaptation to rich environments.

4.1.4 Biochemical properties of LAB strains

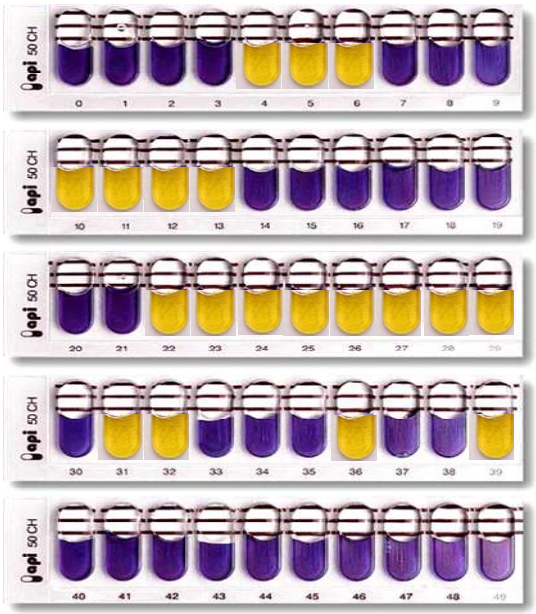
From the previous section, nutritional requirements of the selected lactic acid bacteria had been determined, and the commonly physiological reactions had been carried out: for example; Gram staining, the catalase test, and spore formation, etc. It was now necessary to identify the strains according to their biochemical properties. In this work, all of the four LAB strains of the culture collection were investigated. For identification of the lactic acid bacteria, API 50 CH test kit (bioMerieux™) was used. The API 50 CHL test kit is a standard system associated with the study of the assimilation-fermentation of 49 different compounds (plus one control). When the tube and the cupules were completely filled, then these were incubated the strips at the optimum temperature at 30 °C for 24 to 48 hours (shown in Figure 4.3). Lactic acid bacteria could live in the absence as well as in the presence of the atmospheric oxygen indicating that they are facultative anaerobes (Jensen and Seeley, 1953). Base on the result, 4 strains of LAB isolated were well grown and were able to utilize several sugars. During incubation, sugars fermentation resulted in the pH reduction of the corresponding media. This resulted in a change of color in the strips.



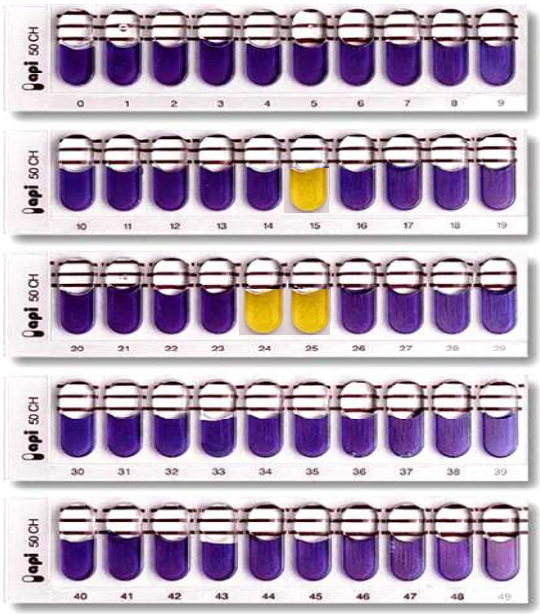
(A)



(B)



(C)



(D)

Figure 4.3 The interpretation of each test of the biochemical profile after incubation: MB52 (A), MB40 (B), N12 (C), and N1B1 (D).

The first tube, which did not contain any active ingredient, was used as a negative control. Interpretation of each test as positive (+), negative (-), and doubtful (?) was tabulated as shown in Table 4.4. The experimental results of biochemical profile were identified using API's computer database for comparison of assimilation and/or fermentation patterns. Moreover, the result from this procedure were also used for other purpose, for example, epidemiological grouping into types of the interested lactic acid bacteria, taxonomical analysis of a group of lactic acid bacteria, and classification of an unknown bacterial population into homogeneous groups.

Table 4.4 Biochemical characterization of the tested strains by API 50 CHL test kit.

Cultures / **Sugars**	**MB52**	**MB40**	**N12**	**N1B1**
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-arabinose	-	-	-	-
L-arabinose	+	+	+	-
D-ribose	+	+	+	-
D-xylose	+	-	+	-
L-xylose	-	-	-	-
D-adonitol	-	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	-
D-galactose	+	+	+	-
D-glucose	+	+	+	-
D-fructose	+	+	+	-

Table 4.4 (Continued).

Cultures / Sugars	MB52	MB40	N12	N1B1
D-mannose	+	+	+	-
L-rhamnose	+	-	-	+
Dulcitol	-	-	-	-
Inositol	-	-	-	-
D-mannitol	-	+	-	-
D-sorbitol	-	-	-	-
Methyl-αD-Mannopyranoside	-	-	-	-
Methyl-αD-Glucopyranoside	-	-	-	-
N-acetylglucosamine	+	+	+	-
Amygdalin	+	+	+	-
Arbutin	+	+	+	+
Esculin	+	+	+	+
Salicin	+	+	+	-
D-cellobiose	+	+	+	-
D-maltose	+	+	+	-
D-lactose (bovine origin)	+	+	+	-
D-melibiose	+	+	-	-
D-saccharose (sucrose)	+	+	+	-
D-trehalose	+	+	+	-
Inulin	+	-	-	-

Table 4.4 (Continued).

Cultures / Sugars	MB52	MB40	N12	N1B1
D-melezitose	-	-	-	-
D-raffinose	-	-	-	-
Amidon (starch)	+	-	+	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
Gentiobiose	+	+	+	-
D-turanose	-	-	-	-
D-lyxose	-	-	-	-
D-tagatose	+	-	-	-
D-fucose	-	-	-	-
D-fucose	-	-	-	-
D-arabitol	-	+	-	-
L-arabitol	-	+	-	-
potassium gluconate	-	+	-	-
potassium 2-ketogluconate	-	-	-	-
potassium 5-ketogluconate	-	-	-	-

Legend: positive (+), negative (-).

Table 4.5 detailed the identification results of the 4 isolated strains. From this phenotypic affiliation, it was observed that three of the four isolates had metabolic traits identity of more than 99.6% whilst one isolate possessed identity of 98.00%. As a result, the

isolates MB52, MB40, N12, and N1B1 were classified as *P. pentosaceus, L. brevis, L.lactis*, and *L. delbrueckii*, respectively. The agreements of API 50 CHL data by mathematic analysis (T-test) were also determined with the values ranging from 0.83-0.99.

Table 4.5 Identification results of the 4 isolated strains by API 50 CHL test kit.

Samples codes	**Significant strains**	**Performance of identification**	**% identity**	**T-test**
MB52	*P. pentosaceus*	Excellent identification	99.9	0.99
MB40	*L. brevis*	Excellent identification	99.9	0.83
N12	*L. lactis*	Very good identification	99.6	0.95
N1B1	*L. delbrueckii*	Very good identification	98	0.93

Among the strains tested, *P. pentosaceus* showed a good agreement with the objectives of this work especially its high growth and good tolerant for osmotic pressure. As a result, this strain was suitable for further bioprocess development. The distribution of the lactic acid bacteria species are related to molecular studies. In addition, phenotypic identification is based on physiological, biochemical and chemotaxonomic methods (Montel *et al.*, 1991). These methods are sufficient for a preliminary characterization but not for unequivocal identification purposes. Several molecular techniques have been applied for LAB identification which permits accurate and fast identification. Previously, it is difficult to identify closely related type strains because of some similarities in phenotypic characteristics. The use of molecular approach for classification and identification of microorganism is now a regular technique which provides a powerful tool for determining

the phylogenetic and evolutionary relationships of microorganisms. Therefore, the significant LAB characterization has been demonstrated in the next section.

4.1.5 Genotypic characterization

Despite the wide application of LAB , they are currently differentiated by the determination of DNA-DNA similarity, their G+C content and, with more difficulty, by physiological characterization (Tanasupawat *et al.,* 1993). On the other hands, biochemical identification is not accurate for determining the genotypic differences of microorganisms. The molecular technique (genotypic characterization) has been applied in this experiment. Moreover, these methods are fast, practical, easy to perform, inexpensive, and do not need a high level of technical skill. A preliminary estimation of taxonomic distribution was used as criteria in our selection of code strains MB52, MB40, N12, and N1B1 for further investigations. We carried out the identification by using selective PCR amplification and subsequent sequencing of the 16S rDNA genes directly from the isolated LAB. The four strains were identified on the basis of their 16S rDNA homologies with entries in the GenBank-EMBL databases. Thus, its application as an additional taxonomic criterion for distinguishing these bacteria from other members of the family was indicated by genotypic characterization.

DNA extraction and gel electrophoresis were determined under comparison with standard sequence which was needed to get the best results. DNA were extract, and used as template for PCR amplification. PCR products were detected for the partial nucleotide sequences. In addition, using 16S rDNA gene sequences to infer organismal phylogeny has been criticized, as it assumes that one molecule reflects organismal evolutionary history. The results of % similarity and strain homology of four isolates were shown in Table 4.6.

The results were interpreted in relation to the currently accepted, belong to a species, for which the name *Pediococcus pentosaceus, Lactobacillus delbrueckii*, *Lactococcus lactis*, and *Lactobacillus brevis*.

Table 4.6 The similarity of 16S rDNA sequence of four isolates compared with strains from database.

Isolates	**Compared with strains from database**		
	Strains homology	**Accession No.**	**%Similarity**
MB52	*Pediococcus pentosaceus* strain NS01 16S ribosomal RNA gene, partial sequence	GU358408	100%
MB40	*Lactobacillus brevis* 16S ribosomal RNA gene, partial sequence	HQ702478	99%
N1B1	*Lactobacillus delbrueckii* strain SDa4-56 16S ribosomal RNA gene, partial sequence	HQ720141	98%
N12	*Lactococcus lactis* strain IMAU40136 16S ribosomal RNA gene, partial sequence	FJ749404	99%

For the biochemical tested when these strains were grow in rich carbohydrate or substrate medium, they were known to exhibit a homolactic acid fermentation pattern. It appears that, under aerobic or anaerobic conditions, hexose degradation was typically of the homolactic type. *P. pentosaceus* could also produce lactic acid with a high yield if the appropriate carbohydrates were used as carbon source. Moreover, *P. pentosaceus* could utilize a flavoprotein enzyme system for the transport of electrons to oxygen, resulting in hydrogen peroxide production (Dobrogosz and Stone, 1962). The significant of oxidative metabolism in organisms such as the lactic acid bacteria did not contain cytochromes. It

was showed the properly and predominant LAB for further experimental studies in lactic acid fermentation.

Figure 4.4 showed the nucleotide pattern of isolated strain by the genotypic technique. The total congruence of the phylogenetic trees allows taxonomic assignments to be made with much more conviction than if based on DNA sequence. The partial sequence of 16S rDNA of the isolate MB52 (Figure 4.4) was obtained from the sequence analysis, and compared with other sequence from GenBank database. When examined by BLAST similarity analysis, the 16S rDNA sequence from isolates MB52 was produced closely related 100% with *Pediococcus pentosaceus* (shown in Figure 4.5).

```
  1 GTACTGATTG AGATTTTAAC ACGAAGTGAG TGGCGAACGG GTGAGTAACA
 51 CGTGGGTAAC CTGCCCAGAA GTAGGGGATA ACACCTGGAA ACAGATGCTA
101 ATACCGTATA ACAGAGAAAA CCGCATGGTT TTCTTTTAAA AGATGGCTCT
151 GCTATCACTT CTGGATGGAC CCGCGGCGTA TTAGCTAGTT GGTGAGGTAA
201 AGGCTCACCA AGGCAGTGAT ACGTAGCCGA CCTGAGAGGG TAATCGGCCA
251 CATTGGGACT GAGACACGGC CCAGACTCCT ACGGGAGGCA GCAGTAGGGA
301 ATCTTCCACA ATGGACGCAA GTCTGATGGA GCAACGCCGC GTGAGTGAAG
```

Figure 4.4 Relevant parts of the nucleotide sequences (350 bp) from the isolated strain (MB52) which present homology with the nucleotide region of partial 16S rDNA.

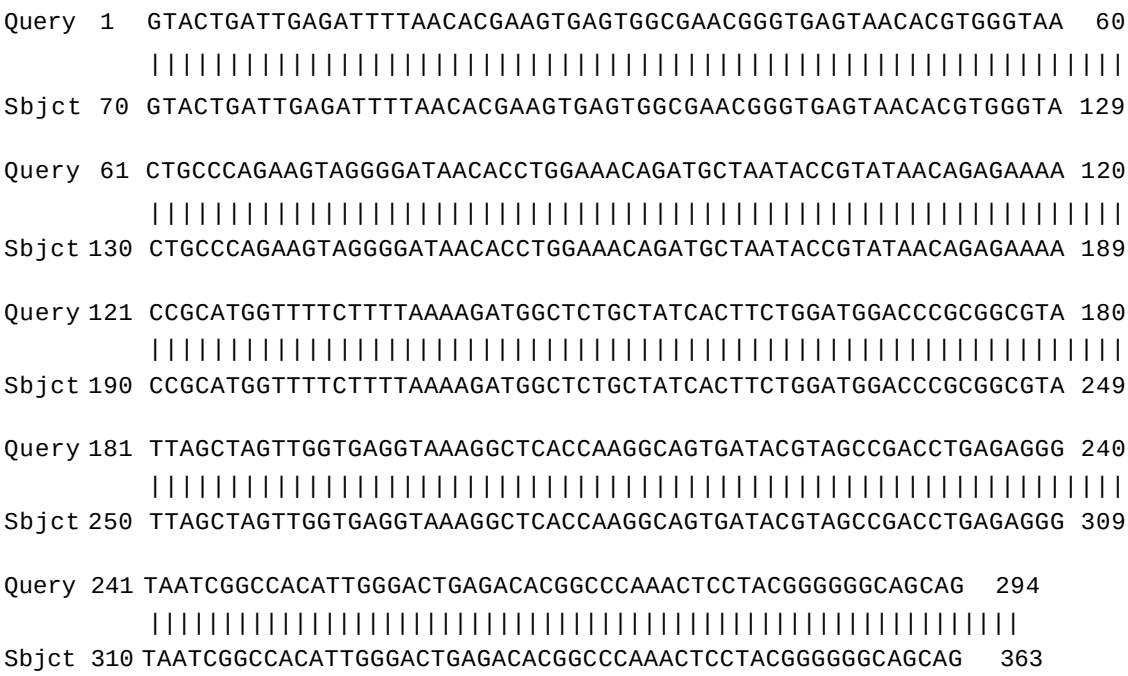


Figure 4.5 Nucleotide sequence alignment between the isolates MB52 (upper line) and *Pediococcus pentosaceus* NS01 16S (GU358408; below line) which were performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://ncbi.nlm.nih.gov/BLAST).

The phylogenetic construction was carried out from a library data which was a collection of database entries (as shown in Figure 4.6). In particular, the isolated strain was identified, even though it was placed in a branch of phylogenetic tree (indicated with a brighter line) which was reliably judged by the software. The closest relative of the isolated strain was *P. pentosaceus*. As a result, phylogenetic tree construction confirmed that this strain belonged to *P. pentosaceus*. Of the remaining strains, a group was identified by PCR, and were allocated in the correct library unit to increase its identification ability. From the results of phylogenetic tree, biochemical analysis of the strains were related, and had significant clearly in particularly specification.

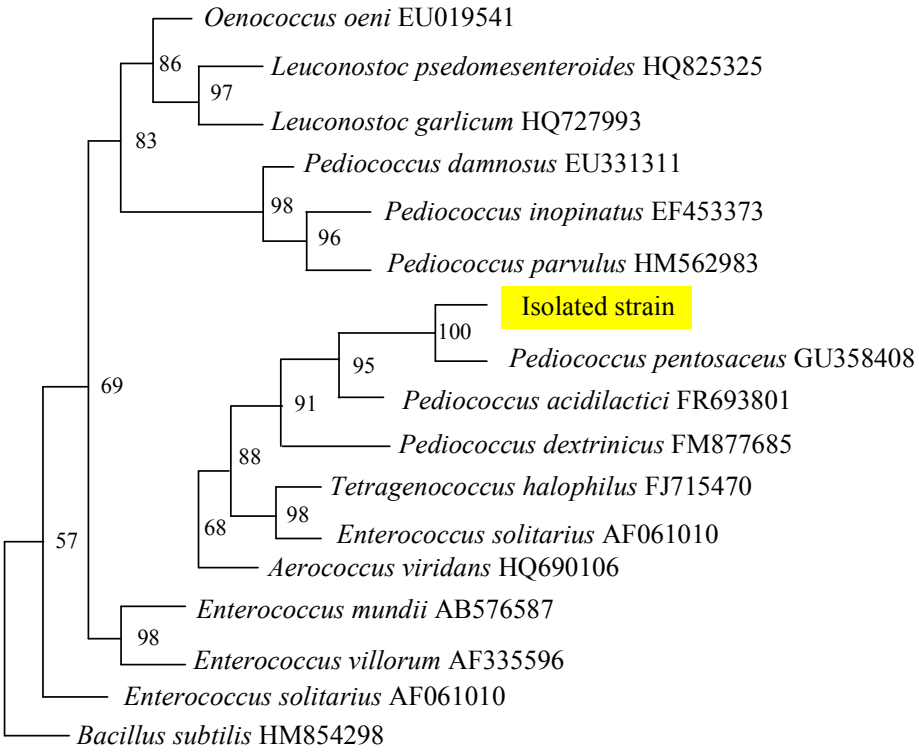


Figure 4.6 Construction of phylogenetic tree obtained with the isolated strain (MB52) based on 16S rDNA.

In conclusion, a more rapid, and accurate method for genotype determination is the molecular biological approach of identifying bacteria based on the DNA sequence of the gene that codes for 16S rDNA (Barney *et al.*, 2001). Phylogenetic analyses, comparison of genomic content across the group, and reconstruction of ancestral gene sets reveal a combination of gene loss and gain during the co-evolution of lactic acid bacteria.

The high resolution of scanning electron microscopy makes it an ideal technique for studies of the cell surface. The scanning electron-microscopy (SEM) was also investigated using 10,000× magnifications as shown in Figure 4.7. The bacterial cells *(P. pentosaceus)* clearly showed tetrad characteristic with the size of approximately 1 μm in diameter.

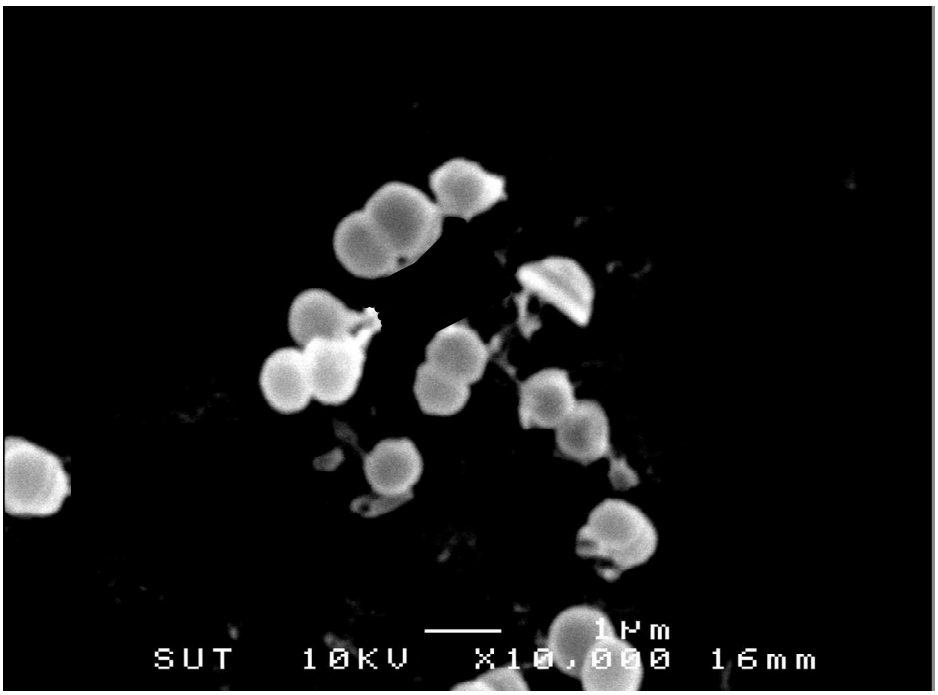


Figure 4.7 SEM micrograph of the isolated lactic acid bacterial strain *(P. pentosaceus).*

4.2 Medium formulation of lactic acid bacteria in fermentation processes

4.2.1 Investigation of amylolytic activity

The amylase activity of *P. pentosaceus* was investigated in this section. The result of amylolytic activity characterization was shown in Figure 4.8. During hydrolysis reaction, the starch molecules in the agar plate were broken down due to amylolytic activity and produce a clear zone surrounding its colony. Clear zone diameter of the sample was measured, and the amylolytic activity was roughly estimated using the method previously described. It was obviously seen that *P. pentosaceus* was able to produce amylolytic enzyme which produce a clear zone with a diameter of approximately 5.0 mm.

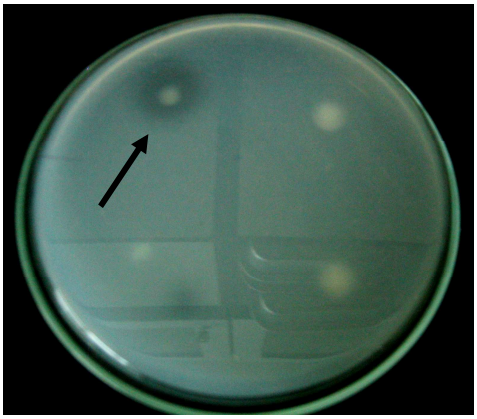


Figure 4.8 Formation of clear zone resulting from hydrolysis of the starch agar.

The sample was then further characterized for its amylase activity. The extracellular enzymatic activities of the culture were determined by taking the sample from the beginning of the stationary phase (Petrov *et al.*, 2008). During the incubation, the bacteria secreted amylolytic enzymes into the fermentation broth in order to hydrolyze the large molecule of starch into smaller molecules of fermentable sugars prior to taking back into the cell. The time course of hydrolysis reaction was illustrated in Figure 4.9. During hydrolysis reaction, the concentration of starch solution constantly reduced with time. Amylolytic activity gradually increased to 0.3 U/mL within 30 h before reached its plateau throughout the experiment. However, a small reduction of starch concentration was observed at only 0.36 g/mL from 60 h of operation. As a consequence, it was be able to conclude that low amylolytic activity was obtained when only *P. pentosaceus* was employed as the sole biocatalyst.

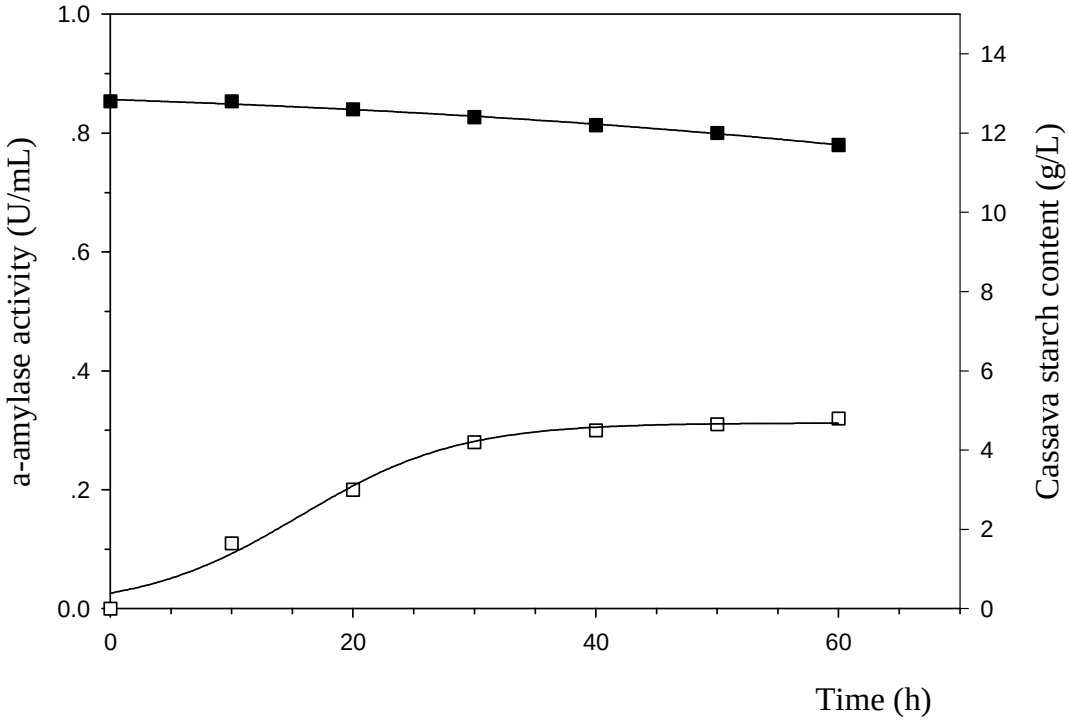


Figure 4.9 Time course of amylase activity and starch hydrolysis: initial concentration 20 g/L of cassava starch, 30 °C, pH 6.0, agitation speed of 200 rpm, amylase activity (open square), and starch content (closed square).

In addition, the strain possessed poor productivity for amylase activity which would result in low volumetric productivity. Although this homofermentative lactic acid is highly productive, its direct application in starch fermentations is restrained by the absence of high amylolytic properties. On the other hand, the most usual method for overcoming this particular problem is the preliminary treatment of the starchy material with α-amylase or amyloglucosidase mixture. External supplementation of the enzyme is very necessary, otherwise the future industrial application would not be possible. Therefore, subsequent lactic acid fermentation experiments by *P. pentosaceus* were carried out with the help of enzymatic hydrolysis of cassava starch. In the next experiment, bioprocess developments were carried out, and the effect of various carbon sources, nitrogen source, and operating factors on lactic acid fermentation performance were investigated.

4.2.2 Effect of glucose concentration

The improvement of lactic acid production has been studied under the control of various operating factors and media components. The fermentative production of lactic acid is interesting due to the prospect of using readily available, and cheap raw agricultural materials. Experiments concerning the influences of various carbon sources were investigated with the objective of establishing an inexpensive fermentation medium. *Pediococcus pentosaceus* was locally isolated from cassava waste. This strain showed its high yield, and volumetric productivity. It was generally known that glucose was a readily metabolizable carbon source. However, an insight into substrate utilization performance was necessary since high concentration of glucose could lead to high osmotic pressure of the cells resulting in substrate inhibition (Hofvendahl and Hagn-Hagerdal 2000). Initial glucose concentrations were varied between 20-200 g/L in batch cultures (Goncalves *et al*., 1991). Specific growth rates were measured during the exponential phase at each glucose concentration by plotting the natural logarithm of cell concentrations against time (data not shown). The kinetic of substrate inhibition could be expressed by an extended Monod model as followed (Luong 1987),

$$\mu = \mu_{max} \frac{C_S}{K_S + C_S} \left[1 - \left(\frac{C_S}{C_S^*} \right)^n \right] \qquad (10)$$

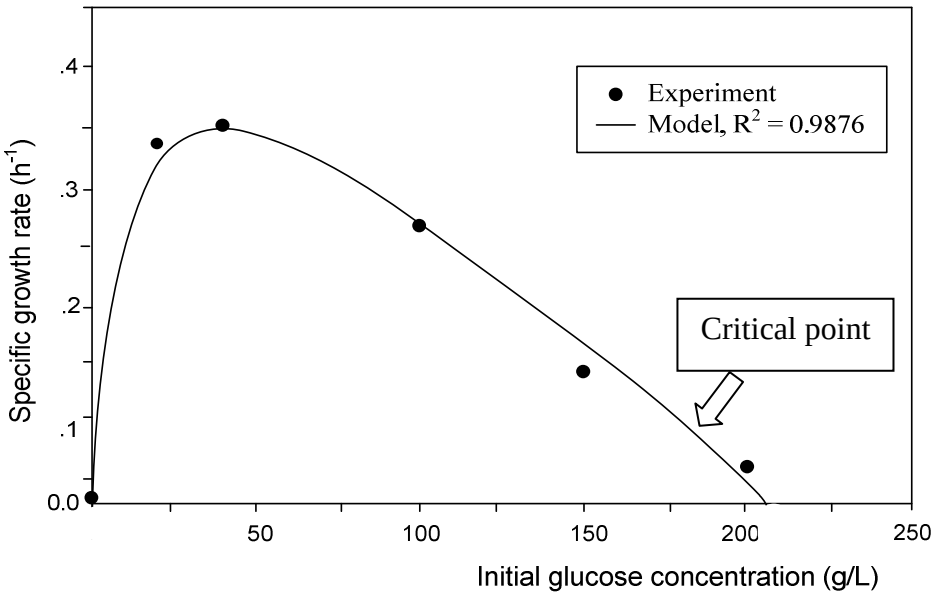
Whereas μ_{max} is the maximum specific growth rate (h^{-1}), K_S is the substrate saturation constant (g/L), C_S is the initial glucose concentration (g/L), C^*_S is the critical glucose concentration in which the cell cannot grow (g/L), and n is the power constant, respectively. In Figure 4.10 (A), the effect of initial glucose concentration on specific growth rate was presented. The calculated value rapidly increased at low glucose concentration until reached its maximum point at 0.35 h^{-1}. However, specific growth rate decreased after initial glucose concentration exceeded 35 g/L before reduced to its lowest

value of 0.05 h^{-1} at 200 g/L of glucose concentration. The critical concentration of glucose (C^*_S) which was calculated from the model was approximately 210 g/L. This experimental result revealed that the strain could tolerate high osmotic pressure, and still grew at relatively high glucose concentration. In addition, *P. pentosaceus* was also reported to tolerate high salinity of fermentation media up to 1.25 M of NaCl (Baliarda *et al*., 2003). Figure 4.10 (B) showed the experimental lag time observed for different initial glucose concentrations. Experimental results showed that the lag time (T) increased with increasing initial glucose concentration. The relationship between lag time and initial glucose concentration could be established by using a modified Belehradek function (Eq. 11).

$$T = \kappa + \lambda C_S^{\gamma} \quad (11)$$

Where κ, λ, and γ are the empirical constants of the equation. The kinetic parameters of the above equation were solved using SigmaPlot with the values of 0.44, 0.35, and 0.54, respectively (Lin *et al*., 2008). The experimental results and model were in good agreement with R^2 of 0.9929.

These results indicated that substrate inhibition taken place at high levels of glucose. The specific growth rate was a key control parameter in the industrial production of lactic acid. The condition of the inoculum had a strong influence on the duration of the lag phase. In general, the length of the lag phase will be proportional to the age of the inoculum. A lag phase may also occur if the inoculum is transferred from one set of growth conditions to another. As a result, it was suggested that investigation of glucose concentration is necessary for cost/time saving and improving lactic acid fermentation. Optimization of biomass productivity required that the specific growth rate and biomass yield were as high as possible. In conclusion, the experimental data for the threshold of glucose concentration was in good agreement with the critical concentration. This was clearly due to substrate inhibition, a traditional property of batch fermentations.



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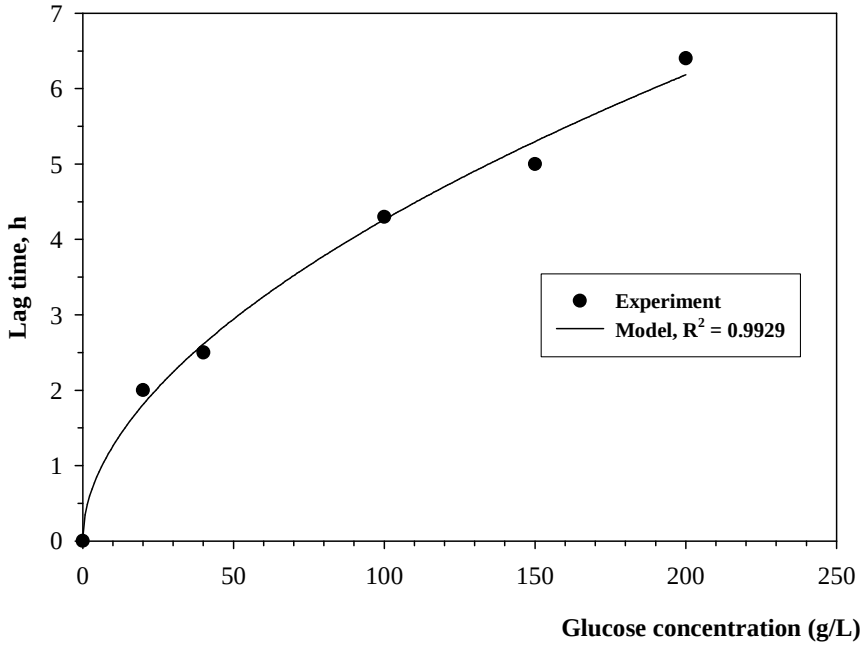


(B)

Figure 4.10 Specific growth rate (A), and lag time (B) as a function of glucose concentration. Initial glucose concentrations between 20-200 g/L were investigated on batch fermentation at 30 °C, initial pH 6.0, and agitation speed at 200 rpm.

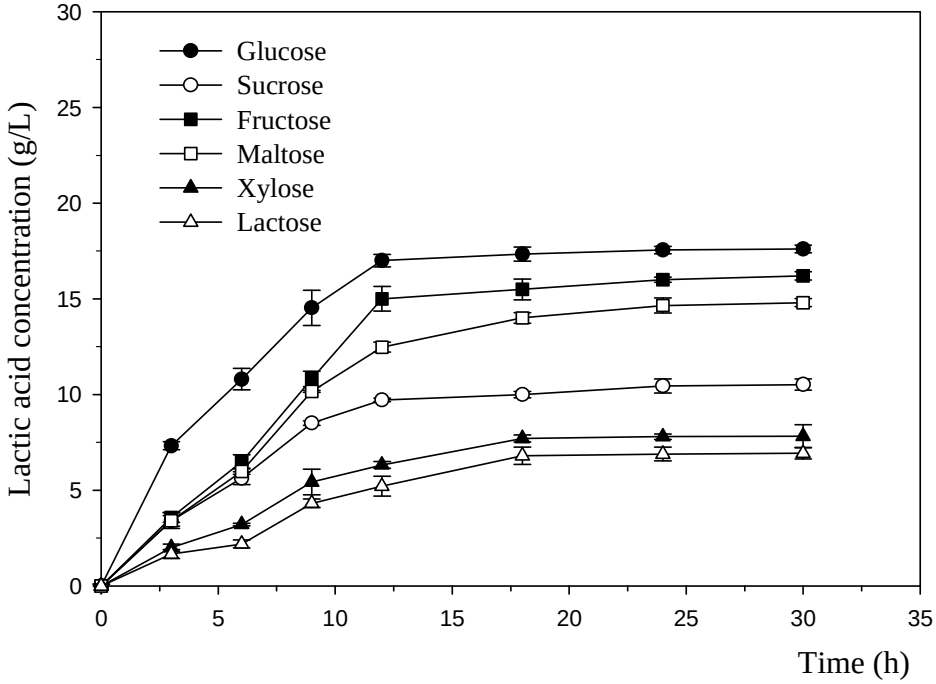
4.2.3 Influence of various carbon sources on lactic acid production

The lactic acid bacteria for industrial process have to metabolize the carbohydrates into optically pure lactic acid through homofermentative pathway and have to require a simple medium composition. Moreover, *P. pentosaceus* could also produce lactic acid with a high yield if the appropriate carbohydrates are used as carbon sources. The homofermentative characteristics of carbohydrate metabolism of *P. pentosaceus* were identified. In this section, lactic acid fermentation by using various carbohydrates as the main carbon source were investigated in modified MRS broth containing 20 g/L of the following carbon sources: lactose, sucrose, maltose, and fructose, respectively (Yun *et al*., 2003). In this work, the several carbon sources were tested in order to get the suitable carbon source for lactic acid production, and it was also possible to use agricultural product in the local area, especially, cassava starch, and its product.

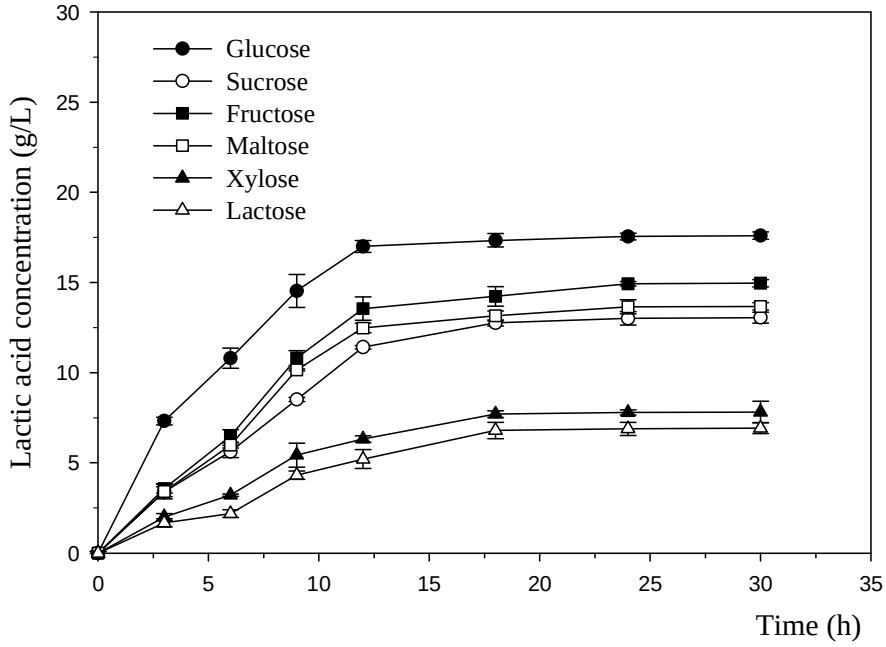
However, it was interesting to see how this strain grew on different kinds of monosaccharide including the large molecule of cassava starch. Nowadays, research effort was focused on investigation for effective nutritional resources including novel progressive fermentation techniques. These enable the achievement of both high substrate conversion, and high production yields. Lactic acid productions had been studied under the control of various operating factors and media components with the objective to achieve high product concentration and low fermentation cost (Ding and Tan, 2006). Figure 4.11 showed the fermentation performances of various sugars in terms of cell concentration, and lactic acid production, respectively. The maximum cell growth and the highest lactic acid concentration were obtained after 24 h of the fermentation time. These experiments were operated with non controlled pH: therefore; pH of fermentation broth rapidly decreased due to accumulation of lactic acid. The fermentation was almost ceased after 24 h when pH reached a value of around 3.0 (data not shown). In fact, there were several reports

demonstrating that lactic acid fermentations could be operated, or only work at all, under conditions where the pH exceeded the p*K*a of the lactic acid produced (p*K*a = 3.86). This was because lactic acid in an un-dissociated form severely inhibited cell growth (Wasewar, 2005).

On the other hands, the maximum cell concentrations of glucose, sucrose, fructose and maltose were obtained at 2.92, 2.32, and 2.88 g/L whereas the highest lactic acid concentrations were 17.60, 13.01, and 14.94 g/L, respectively. When considered the volumetric productivity, the corresponding values were calculated as 1.61, 0.89, 1.19, and 1.11 g/L/h, respectively. As a result, the suitable carbon source for *P. pentosaceus* was in the order of glucose > fructose > sucrose > maltose, respectively.



(A)



(B)

Figure 4.11 Time course of cell growth (A) and lactic acid production (B) as the function of different carbon sources by *P. pentosaceus*.

4.2.4 Effect of cassava starch and hydrolyzed cassava starch on fermentation performance

In this work, the development of lactic acid production process using cassava as the main carbon source was investigated. This procedure was the main objective of this work since cassava was relatively cheap (approximately 0.15 Dollars/kg or five bath/kg (Sriroth *et al*., 2006)) compared with commercial glucose. In addition, it contained high starch content with low quantity of impurities, and also readily available in Nakhon Ratchasima province. The effect of initial concentration of cassava starch and hydrolyzed cassava starch (HCS) on lactic acid production was investigated by an addition of cassava starch into the fermentation medium at initial concentrations of 1, 2, 4, and 10%, respectively. For HCS experiments, pretreatment of starch by using enzymatic process (amylase and gluco-amylase enzyme) was carried out. There are many sugars released from hydrolysis step including glucose, fructose, and especially maltose. These sugars were already investigated in the previous work. The results in Table 4.7 showed that *P. pentosaceus* could utilize all types of carbon source. Glucose was the most favorable carbon source with the maximum specific growth rate of 0.34 h^{-1}, and maximum lactic acid concentration 17.30 g/L which resulted in the production yield of nearly 90%. Fructose, maltose, and sucrose also exhibited their good performance in term of specific growth rate and lactic acid production with the value between 0.19-0.22 h^{-1}, and 13.01-14.94 g/L, respectively. However, as the refined sugars are expensive and it is not economical attractive for industrial production. The experimental results for all initial cassava starch concentrations revealed that cassava starch possesses low specific growth rates, and product concentrations. The low specific growth rates indicated that there was low activity of amylolytic enzyme resulting in slow break down of cassava starch molecule. Generally, lactic acid bacteria are deficient in amylolytic characters especially for the highly branched starch. Cassava starch contains percentage of amylo-pectin of approximately 80%,

therefore, it is necessary to hydrolyze the cassava starch prior to beginning the fermentation for their utilization. Traditionally, the gelatinized cassava starch can be treated with amylase (liquefaction) and gluco-amylase (saccharification) to get the hydrolyzed product. The pre-treatment with α-amylase and gluco-amylase resulted in hydrolysis of cassava starch into glucose including low molecular weight oligosaccharides in which *P. pentosaceus* could directly utilize. As a result, the fermentation performance of lactic acid fermentation using hydrolyzed cassava starch was greatly enhanced. Specific growth rate increased in comparison with the corresponding starch medium. Hydrolyzed cassava starch showed its high potential to be used as the representative carbon source; although, the final acid concentration was lower than the others (refined sugars, especially glucose and fructose). This was probably due to the lower amount of available reducing sugar in the hydrolyzed cassava starch. However, these results showed that hydrolyzed cassava starch possessed comparable fermentation performance with other carbohydrates. Glucose is the most efficient carbon source; however, it is expensive compared to hydrolyzed cassava starch (approximately 0.50 dollars/kg or 15 Baths/kg).

These experimental results confirmed that pre-treatment of cassava starch generally increases the lactic acid yield, fermentation efficiency, and lactic acid productivities. These observed results showed the potential for further improvement of lactic acid production in order to obtain higher fermentation performance approximately 40%.

Table 4.7 Comparison of lactic acid concentrations and specific growth rates obtained from fermentation by *P. pentosaceus*.

Carbon sources	Specific growth rate (h^{-1})	Maximum lactic acid concentration (g/L)
Glucose ±	0.29 ± 0.05	17.30 ± 0.23
Sucrose ±	0.19 ± 0.07	13.01 ± 0.34
Fructose ±	0.22 ± 0.10	14.94 ± 0.41
Maltose ±	0.21 ± 0.09	13.05 ± 0.50
Xylose	0.17 ± 0.11	7.82 ± 0.41
Lactose	0.13 ± 0.05	6.93 ± 0.25
1% of starch ±	0.09 ± 0.02	5.12 ± 0.28
2% of starch ±	0.11 ± 0.04	8.43 ± 0.19
4% of starch ±	0.14 ± 0.03	11.16 ± 0.19
10% of starch ±	0.15 ± 0.02	19.12 ± 0.28
1% of hydrolyzed starch ±	0.11 ± 0.05	9.06 ± 0.33
2% of hydrolyzed starch ±	0.14 ± 0.01	11.25 ± 0.39
4% of hydrolyzed starch±	0.17 ± 0.05	13.80 ± 0.21
10% of hydrolyzed starch±	0.20 ± 0.10	30.84 ± 0.17

: The initial concentration of sugars was 20 g/L (row 1 to 6). Mean values of two replications of analysis ± standard deviation with significant difference of $p \leq 0.05$.

This study clearly demonstrated that lactic acid production was affected by the carbon source used in the fermentation processes. The cassava starch was particularly an alternative substrate to the sugars used in this experiment. However, it was necessary to

hydrolyzed cassava starch into fermentable sugars through enzymatic treatment. These data obtained could surely be used for improvement of lactic acid production by using different feeding strategies.

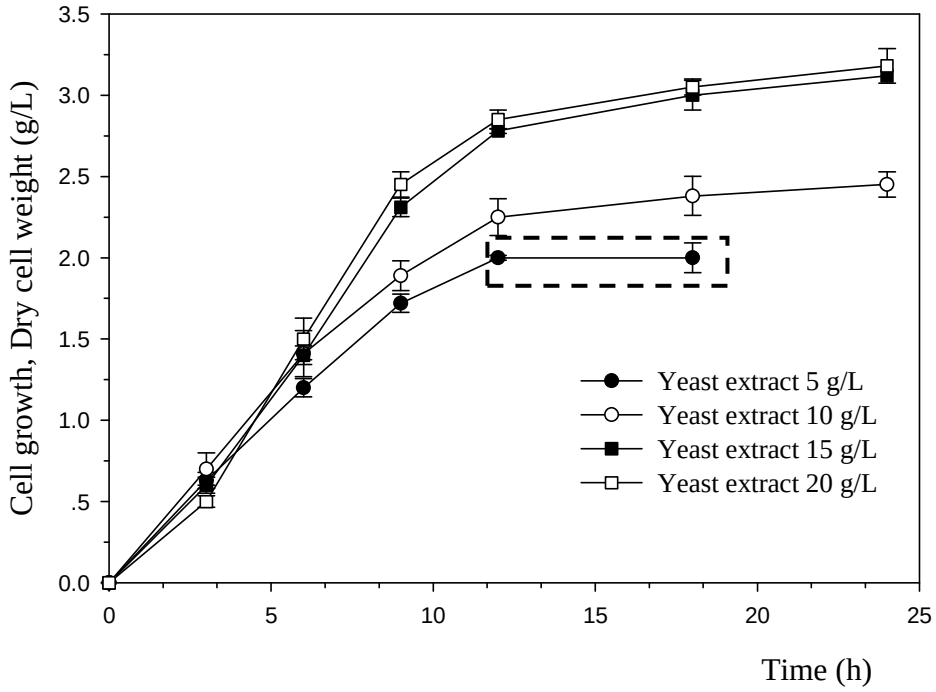
4.2.5 Influence of yeast extract concentration

In order to increase lactic acid production, nutritional depletion was the main concern for carbon and nitrogen sources. The effect of nitrogen source was examined in this work. A recent work had clearly demonstrated that peptidic deficiency was the main factor affecting growth (Amrane and Prigent, 1998). Many nitrogen sources have been used for lactic acid fermentation including corn steep liquor (Cornelius *et al*., 1996), malt sprout extract, casein hydrolysates (Hujanen and Linko, 1996), yeast extract, and also spent brewer's yeast extract as the main objective for this study. Initially, the influence of yeast extract concentrations on lactic acid fermentation from modified MRS medium were investigated by supplemented with the concentration between 5-20 g/L, lactic acid fermentations were performed using 250 mL shake flasks at 30 °C with pH 6.0.

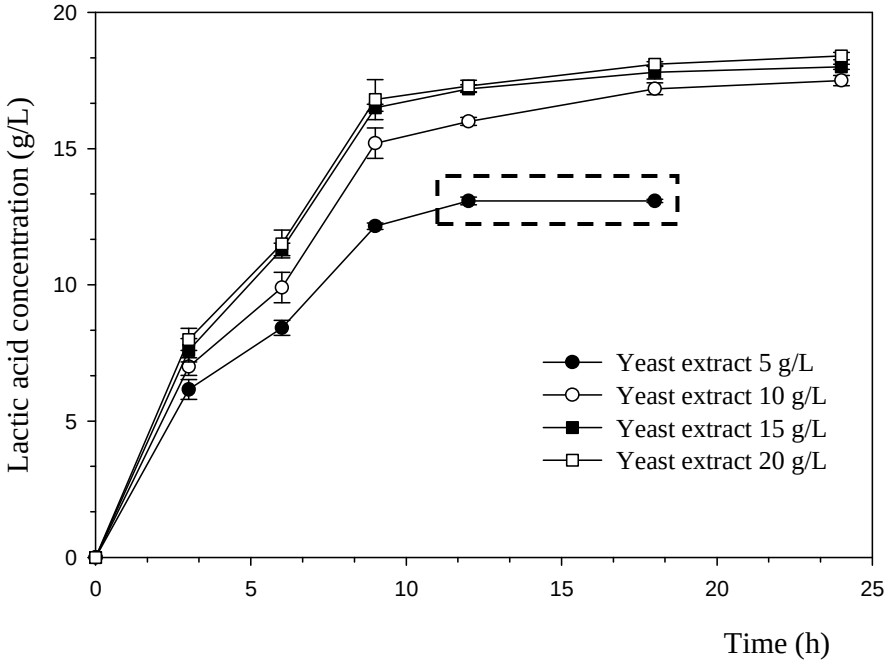
Figure 4.12 showed the time course of lactic acid production and cell growth during fermentation at various concentrations of yeast extract. At 5 g/L of yeast extract, lactic acid fermentation was not completed, and the cell growth was almost stopped after 12 h because the nutrient was depleted. On the other hand, the maximum lactic acid productivity was obtained at 15 g/L whereas dry cell weight (3.18 g/L) was obtained at 20 g/L of yeast extract. From these finding, the increase of yeast extract concentration between 15 and 20 g/L had very little effect on increasing the lactic acid production.

Cessation of growth was due to nutritional limitations, deficiency in peptide sources (amino acids) or in growth factor. These results implied that the lactic acid fermentations should be severely affected by yeast extract concentrations added to the medium. According

to the results, the yeast extract concentration at 15 g/L was recommended for the further experiments.



(A)



(B)

Figure 4.12 Time course of cell growth (A) and lactic acid production (B) in batch lactic acid fermentation from modified MRS medium supplemented with different concentration of yeast extract.

4.2.6 Effect of various nitrogen sources on lactic acid production

In order to develop a economical fermentation, cheap and effective nitrogen source is also necessary to reduce the fermentation cost. Supplementation of nitrogen source into the fermentation medium is important in order to maintain a high yield and productivity. The possibility of using peptone, yeast cell (YC), corn steep liquor, and spent brewer's yeast extract was considered as the sole nitrogen source. These substances could improve nutritional quality of the fermentation medium, because they contain growth promoting compounds in addition to organic nitrogen, and some carbonaceous compounds. The main advantage of this study was substantial lowering the fermentation cost of the media formulation compared to expensive commercial yeast extract. The choice of nitrogen supplementation mainly depended on the local availability. In Nakhon Ratchasima province, there are several large factories producing motor fuel grade ethanol (MFGE) which also use cassava starch as the main substrate. Nearly 2 million liters of MFGE are being produced everyday which ensure the availability of nitrogen source in this region. In this work, the effects of different nitrogen sources having the same elemental nitrogen level (15 g/L) were compared for fermentation performances, and the experimental results were shown in Table 4.8. During the experiment, hydrolysis of organic nitrogen source results in liberation of amino acids which enhanced the lactic acid production. In comparison with typical nutrients such as commercial yeast extract and peptone, spent brewer's yeast extract was a comparable supplementing nitrogen source with high conversion yield and volumetric productivity. The effect of yeast cell supplemented in modified MRS medium was investigated. Yeast cell was not often used as the sole source of nitrogen supplementation because it lacked of several essential minerals. As a result, the utilization rate of glucose and formation of lactic acid were significantly lowered than those observed with the fully supplemented medium.

When supplemented with yeast cell, the maximum lactic acid concentration was obtained at only 10.39 g/L. On the other hands, corn steep liquor was also investigated as another cheap nitrogen source resulting in the maximum acid concentration and dry cell weight of 12.12, and 2.35 g/L, respectively. The result of spent brewer's yeast extract (medium 4) showed a high concentration of lactic acid which relatively closed to the commercial yeast extract supplementation (13.40 g/L, and 17.30 g/L, respectively). This result revealed that spent brewer's yeast extract could be effectively used as nitrogen source to replace an expensive commercial yeast extract. The yeast cell was autolyzed resulting in liberation of water soluble portions such as purine and pyrimidine bases, as well as vitamins B, respectively. Moreover, it also contained nutritious substances such as amino acids, peptides, vitamins, and several organic acids including pyruvic and glyceric acid (Jiang *et al*., 2010). Normally, commercial yeast extract possesses 18 essential elements such as Al, Ba, Cd, Co, Cr, Cu, Fe, Ga, Mg, Mn, Mo, Ni, Pb, Sn, Sr, Ti, V, and Zn, respectively. The information was of general interest and of particular importance when the ash of yeast extract was demonstrated to be essential for growth. On the other hand, the physical of spent brewer's yeast extract were determined showed that appeared the light yellow color, total nitrogen 17.0%, amino nitrogen 3.0%, solid 65.0%, NaCl 4.0%, and residue 10.0%, pH approximately 6.5-7.5. However, it was already reported that spent brewer's yeast extracts lack of iron element (Saksinchai *et al*., 2001). Iron was, therefore, added in spent brewer's yeast extract to fulfill this nutrition requirement for the lactic acid fermentation (medium 5). The experimental result confirmed the importance of iron as the final lactic acid concentration increased from 13.40 to 15.55 g/L. Therefore, this high concentration of lactic acid was attributed to the addition of nitrogen source with essential trace elements. The control medium without nitrogen source (medium 7) resulted in 5.15 g/L of product concentration whereas medium without both carbon and nitrogen sources yielded lactic acid concentration of only 0.94 g/L. Volumetric productivity, and maximum dry cell weight

were also increased to 95% and 91%, respectively when compared between MRS medium, and the medium without carbon and nitrogen source. This reflected the complex nutrient demands of this fastidious lactic acid bacterium due to its limited biosynthesis capacity.

Table 4.8 Media formulation with different nitrogen sources (the concentration of 15 g/L) for lactic acid fermentation by *P. pentosaceus* based on modified MRS medium.

Medium formula	Nutrient supplementation	Lactic acid concentration (g/L)	Cell concentration (g/L)	Substrate conversion (%)
1	Commercial yeast extract	17.30 ± 0.15	2.90 ± 0.25	95.90 ± 0.04
2	Peptone	16.21 ± 0.23	2.80 ± 0.17	93.70 ± 0.12
3	Yeast cell	10.39 ± 0.19	2.14 ± 0.09	75.10 ± 0.19
4	Spent brewer's yeast extract	13.40 ± 0.07	2.76 ± 0.32	89.90 ± 0.20
5	Spent brewer's yeast extract + 0.2% ferrous sulfate	15.55 ± 0.14	2.79 ± 0.25	91.00 ± 0.11
6	Corn steep liquor	12.12 ± 0.08	2.35 ± 0.45	82.70 ± 0.08
7	Except nitrogen source	5.15 ± 0.10	1.48 ± 0.26	42.19 ± 0.14
8	Except carbon source and nitrogen source	0.94 ± 0.11	0.25 ± 0.19	-

In conclusion, the supplementation of nitrogen sources with trace elements resulted in beneficial increase in lactic acid production, substrate utilization, and cell growth. The medium composition had been investigated for many aspects, especially, fermentation performance. The addition of nutrients and higher nutrient mass concentrations generally had a positive effect on the lactic acid production. Media formulation studies demonstrated a significant effect on growth of the bacterial cells. Therefore, this could be concluded that the pronounced difference in lactic acid production in MRS supplemented with spent brewer's yeast extract as nitrogen source could possibly be due to some other growth-promoting factors present in these nutrients. Furthermore, this cheap and readily available spent brewer's yeast extract could significantly reduce the fermentation cost, and make the production process more profitable.

4.3 Influence of factors on lactic acid fermentation

4.3.1 Effect of various temperature on lactic acid production

This work examined the optimum environment condition for microbial growth, and lactic acid production. Temperature adaptations of bacterial growth under different temperature regimes were studied. Better understanding of the temperature effects on lactic acid fermentation will facilitate improvement of the production process. The temperature were monitored and controlled during the fermentation process at elevated temperatures of 30, 37, 40, 45, 50, and 55 °C with fermentation time 30 h as shown in Figure 4.13. The results showed that optimum temperature and time for the lactic acid production was 30 °C for 24 h on the basis of lactic acid content. Temperature is the most important factor on nutrient utilization and cell viability. Higher temperature stimulated the rapid growth of lactic acid bacteria resulted in a rapid decline in pH, and consequently

suppressed the growth of *P. pentosaceus*. Although *P. pentosaceus* could grow at 50 °C, the optimum temperature for its growth were observed between 30-37 °C.

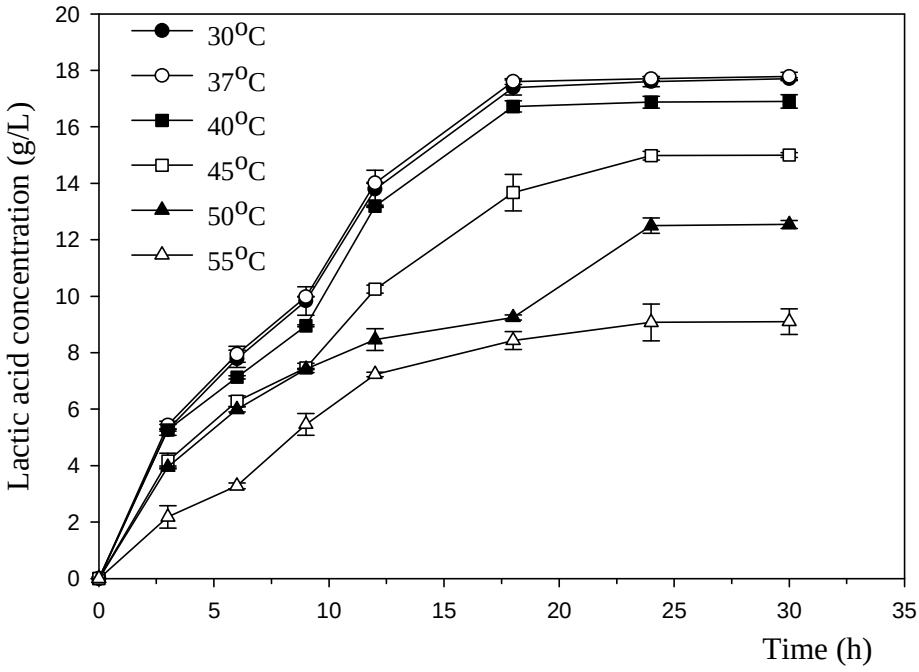
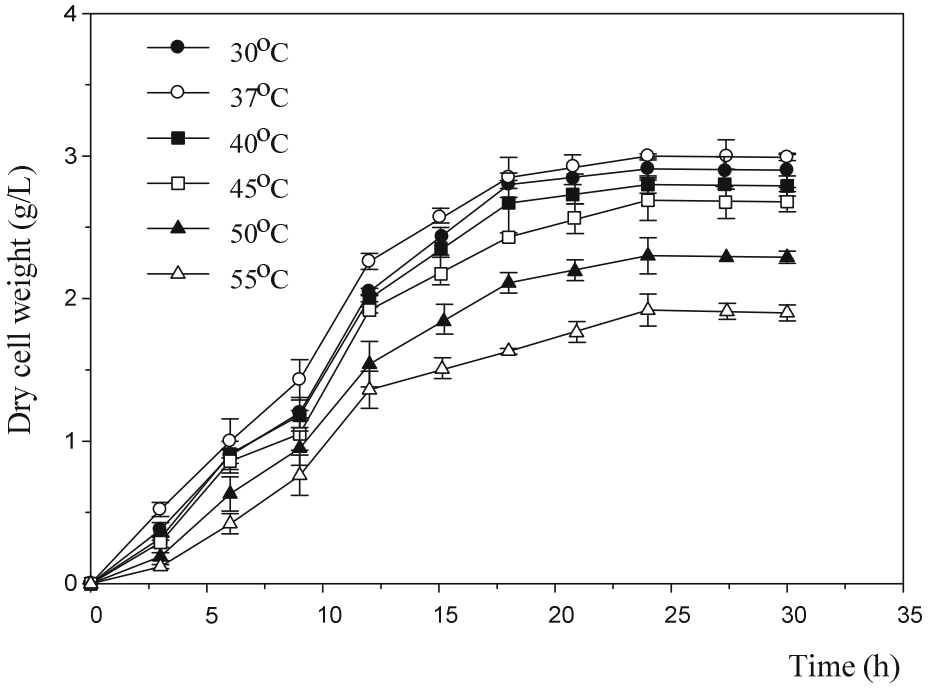


Figure 4.13 The influence of temperature on lactic acid fermentation on modified MRS medium (20 g/L of glucose) by *P. pentosaceus*.

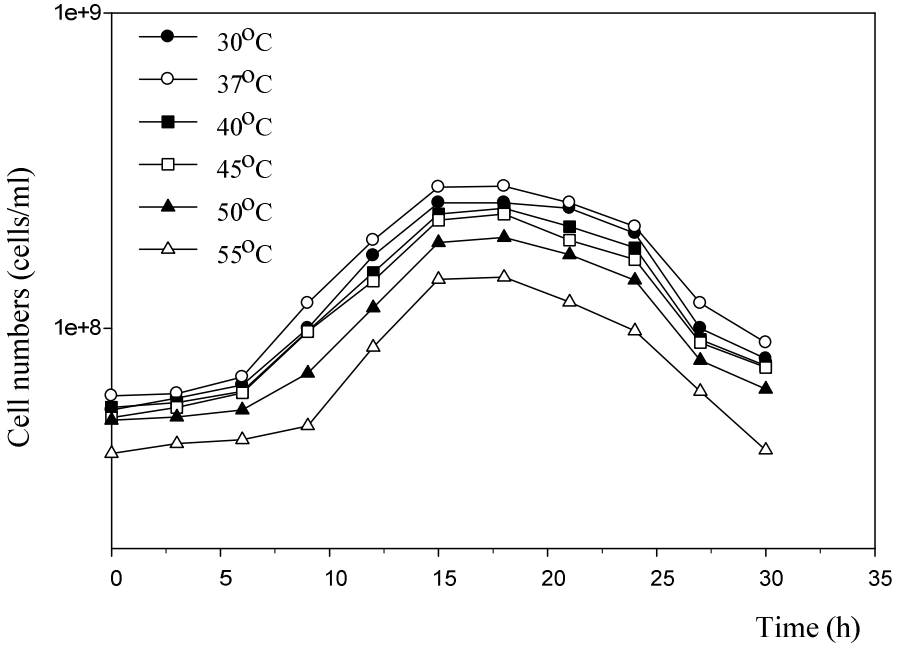
In industrial fermentation processes, the operating temperature of the fermenter is often raised to optimum level to increase microbial activity which depends on the characteristics of the microorganism used as well as on the environmental conditions. Cell changes during fermentation at different temperatures were shown in Figure 4.14 (A). The cell count showed a similar trend. The results indicated that temperature had a pronounced influence on the growth of these microorganisms during lactic acid fermentation.

Moreover, the temperature affects the rate of biochemical reactions, the activity of extracellular enzymes, the generation time, and the activity of the microorganisms involved. Some literatures reported that the rate of reaction for microorganisms increases with

increasing temperature until a limiting maximum temperature is reached (55 °C), after which the growth rate decreases very rapidly (Peleg, 1996). However, when the temperature of the medium is above or below that required for optimum growth, the microbial activity is substantially reduced and the organisms may eventually die. In Figure 4.14 (B), it was showed that the cell growth could modeled with five different phases: lag phase ($\mu \approx 0$, duration time 0-6 h), exponential or log phase ($\mu \approx \mu_{max}$, duration time after 6-12 h), declining growth ($\mu < \mu_{max}$, duration time after 12-16 h), stationary phase ($\mu = 0$, duration time 16-20 h), and death phase ($\mu < 0$, duration time after 20 h). Declining growth normally occurred in cultures when either a specific requirement for cell division was limiting or something else was inhibiting reproduction. In this phase of growth, biomass was often very high and there was no further cell growth.

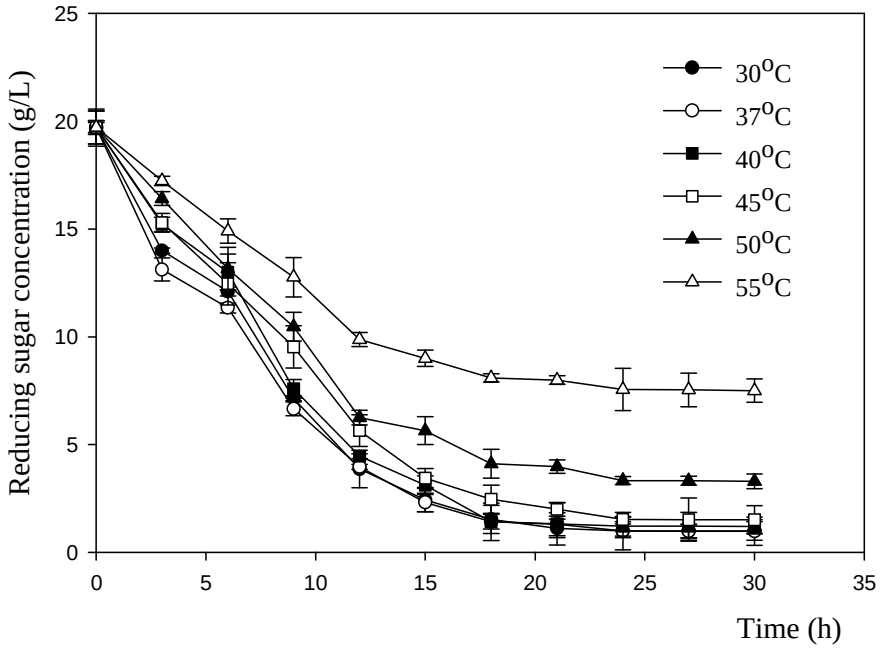


(A)



(B)

Figure 4.14 Effect of temperature on growth or dry cell weight (A), and viable cell count (B), and substrate utilization (C) during batch fermentation.



(C)

Figure 4.14 (Continued).

The fermentation conditions of various temperature were studied, the pH value of the fermentation broth decreased from 6.0 to values less than 4.5 as fermentation proceeded reaching conditions unfavorable for cell growth. Cell population was a measure of the increase in biomass over time and it was determined from the exponential phase. Growth rate was one important way of expressing the relative ecological success of a species or strain in adapting to its natural environment. The duration of exponential phase in cultures depended upon the size of the inoculums, the growth rate and the capacity of the medium and culturing conditions to support cell growth. Biomass estimated need to be plotted over time, and logistical constraints. Cell count and dry cell weight (DCW) are common units of biomass determination. Similarly, at temperature exceeded 50 °C, the cell could not grow. These confirmed that the lactic acid product yield and the cell mass were affected by the temperature for *P. pentosaceus*. Moreover, cell number and lactic acid production in the

fermentation broth depended on the prevailing conditions of temperature. For this reason the control of temperature were necessary during lactic acid fermentation. At 30 °C and 37 °C, the yield and productivity of lactic acid were also similar. The production yield and volumetric productivity were calculated with the time at the exponential phase of the cell growth.

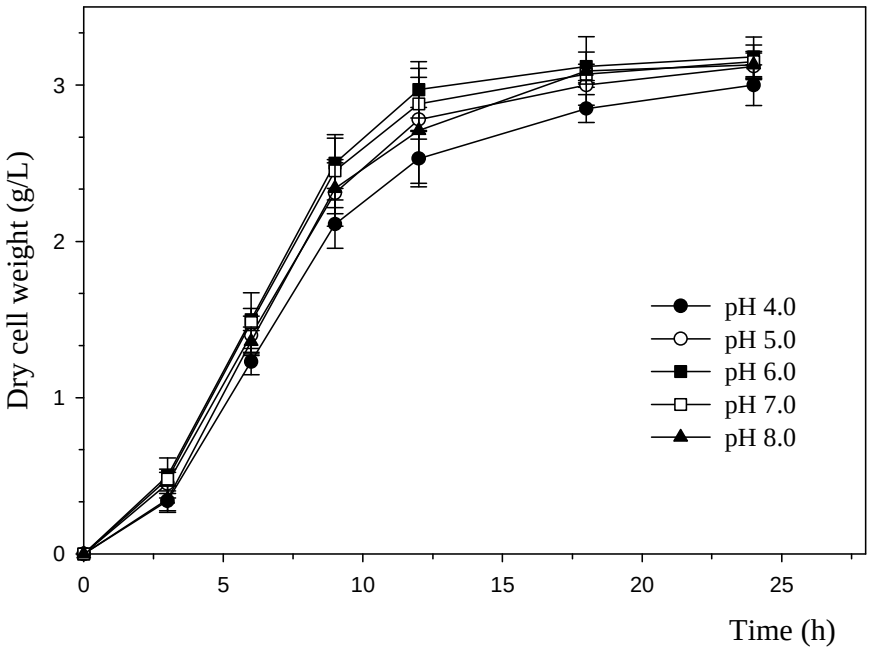
Table 4.9 Effect of initial culture temperature on lactic acid production performance.

Temperature (°C)	**$Y_{X/S}$**	**$Y_{P/S}$**	**Volumetric productivity (g/L/h)**
30	0.15	0.84	1.09
37	0.15	0.84	1.10
40	0.14	0.83	0.96
45	0.14	0.82	0.76
50	0.13	0.79	0.63
55	0.10	0.77	0.47

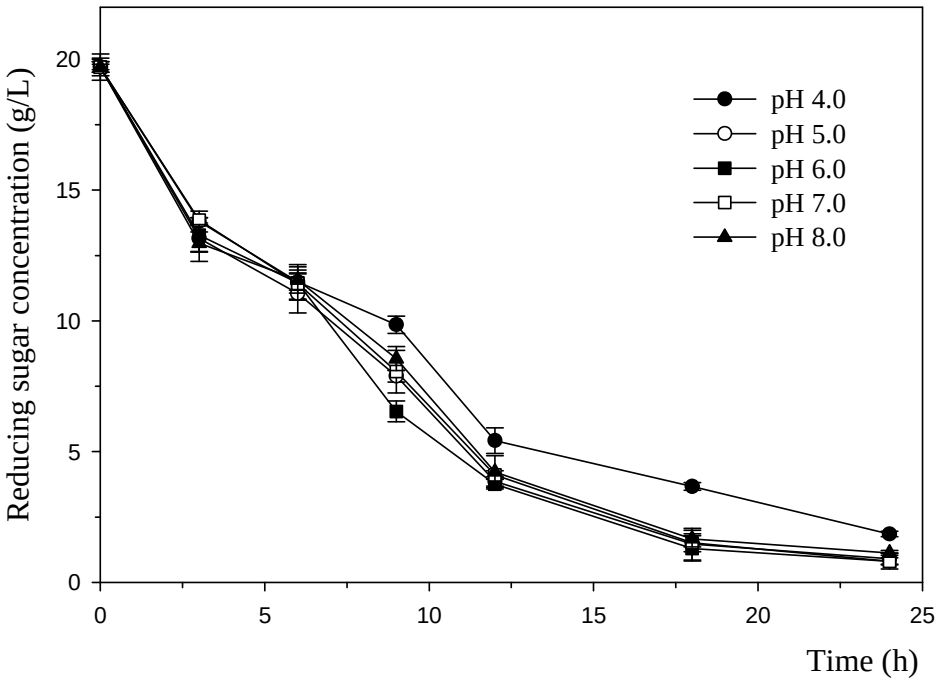
Temperatures were affected cell growth and lactic acid production in fermentation processes. It was, therefore, important that the fermentation temperature must be maintained as stable as possible since bacteria grow optimally within a narrow temperature range. In general, subsequent experiments were conducted at the temperature of 30 °C in order to reduce the cost of electricity of the production process. For this reason, the controls of temperatures were necessary during lactic acid fermentation for lactic acid production.

4.3.2 Effect of various initial pH on lactic acid production

In this section, the effects of different pH levels on the production in batch fermentation were investigated. The effect of pH on cell growth, and substrate metabolism were performed. The initial pH of the medium was maintained at 4.0, 5.0, 6.0, 7.0, or 8.0, respectively, with 5M NH_4OH after the pH dropped to these points due to the production of lactic acid. Time course of cell growth during fermentations at various pH of the cultures were shown in Figure 4.15(A), substrate utilization (Figure 4.15(B)), and lactic acid production (Figure 4.15(C)). Fermentation ended after 18 h of fermentation, and it was observed that either glucose was completely depleted or no change in glucose concentration to lactic acid and the cell growth was almost ceased. In addition, Table 4.10 summarized the fermentation parameters of experiments at various culture pHs, including lactic acid production, lactic acid concentrations, lactic acid yields, and productivities, respectively. Lactic acid yields based on total sugar consumed were ranged between 89.47 and 91.53% at pH 4.0-8.0, and the highest yield of 91.53% was obtained at pH 6.0, and lactic acid productivity reached its highest value of 1.40 g/L/h at pH 6.0.

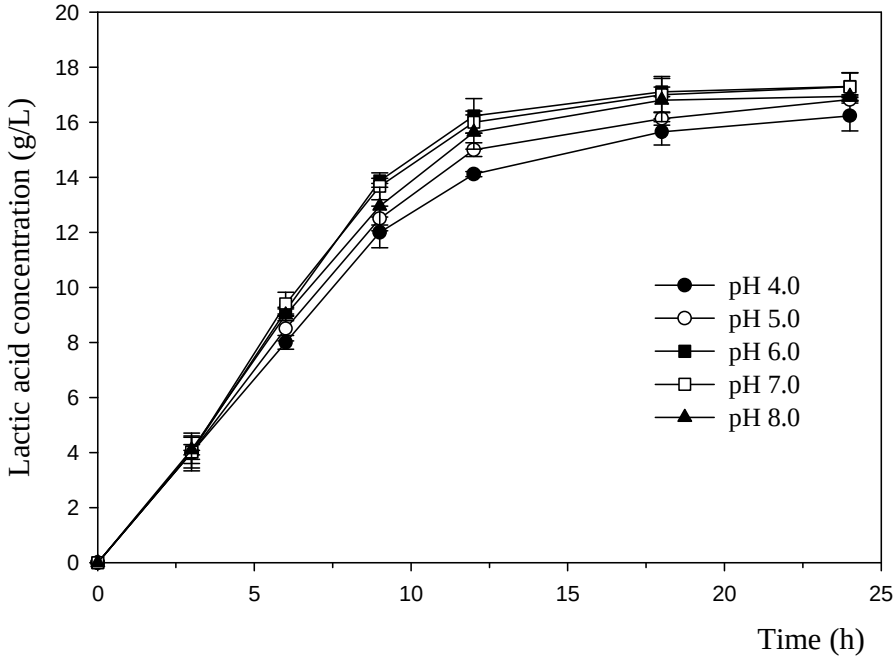


(A)



(B)

Figure 4.15 Time course of cell growth (A), substrate utilization (B), and lactic acid production (C) in batch lactic acid fermentation (modified MRS medium) at different culture pH.



(C)

Figure 4.15 (Continued).

Table 4.10 Effect of culture pH on lactic acid production performance in batch culture of *P. pentosaceus.*

Culture pH	**Lactic acid (g/L)**	**$Y_{P/S}$**	**Maximum DCW (g/L)**	**Volumetric Productivity (g/L/h)**
4.0	16.23	0.91	3.0	1.28
5.0	16.28	0.89	3.12	1.36
6.0	17.3	0.92	3.18	1.40
7.0	17.28	0.91	3.15	1.40
8.0	16.94	0.91	3.13	1.31

Lactic acid yield (%) = (lactic acid produced (g)/total sugar consumed (g)) ×100.

In addition, the maximum specific growth rate (μ_{max}) has been plotted as a function of pH as shown in Figure 4.16. This was in accordance since the μ_{max} was obtained early in the fermentation where the lactic acid concentration was very low. Some literatures reported that low levels of biomass and lactic acid concentration were synthesized at pH values lower than 5.5 (Bai *et al.*, 2004). This effect could resulted from partial loss of the enzymatic activity involved in the biosynthesis of biomass and lactic acid. The experimental results showed that the high level of biomass and lactic acid were obtained when pH was maintained in the pH range 5.0-7.0 with the optimum pH 6.0. The results showed that at each pH value, the reciprocal of the productivity of lactic acid was linearly correlated to the reciprocal of the specific growth rate. Moreover, for pH values lower than 5.0 the limitation of lactic acid production must be different from that obtained at higher pH values: the glucose consumption was much lower, and it took longer time to attain the maximum values of lactic acid concentration. The maximum cell mass obtained for the different pH values did not present significant variations (Goncalves *et al.*, 1997). As a result, it was indicated that the optimum pH for cell growth of *P. pentosaceus* was seemed to be 6.0; lactic acid fermentation at pH 6.0 was completed faster than other pH. It could be concluded that the microorganism, especially, lactic acid bacteria, did not alter its metabolic pathway in the pH range of 5.0-7.0. Furthermore, increase in initial pH beyond 6.5 did not improve the lactic acid production. It was possible that the higher initial pH brought too much stress on the microorganism metabolic abilities (Vijayakumar *et al.*, 2008). Therefore, the optimum pH for lactic acid production was estimated to be 6.0, because the lactic acid productivity should be considered as one of the critical factors for economical lactic acid fermentation. From this finding, *P. pentosaceus* seems to be suitable LAB for further study on lactic acid fermentation under the condition of pH 6.0 and temperature 30 °C.

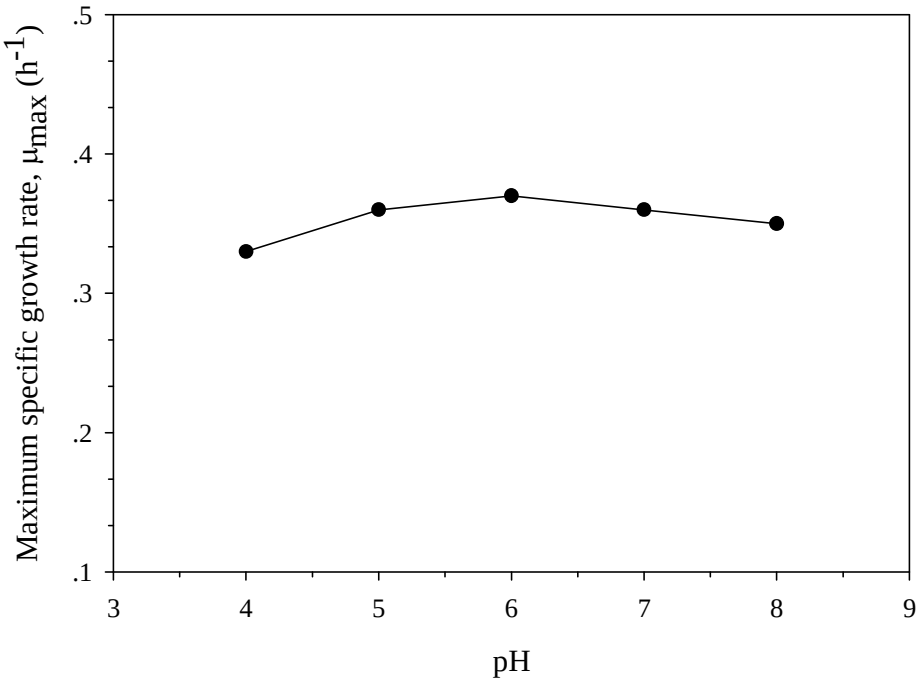


Figure 4.16 Effect of pH on maximum specific growth rate of *P. pentosaceus* on lactic acid fermentation.

4.3.3 Effect of various agitation speed on lactic acid production

Agitation is important for adequate mixing, mass transfer and heat transfer, respectively. It assists not only the mass transfer between the different phases presented in the culture, but also maintains homogeneous chemical and physical conditions in the culture by means of mixing. However, at high agitation speed the biomass concentration may decrease because of cell damaged by the impeller and shear force, while at low agitation speed the liquid or fermentation broth could not homogeneous well and the substrate could not completely utilized. On the other hand, high agitation speed could disrupted the cell by shear forces promoted cell loss (Senthuran *et al.*, 1999). The properties of fermentation broth were affected by power requirement and effectiveness of mixing. Moreover, mixing is

a physical operation which aims to promote homogeneity eliminating gradients of concentration.

The effects of agitation speed on lactic acid production were studied. The influence of cultivation factors on the lactic acid fermentations was observed typically for cell growth, substrate and product concentrations, respectively. Experimental data on lactic acid production, substrate utilization, and cell growth were obtained at different agitation as shown in Figure 4.17. Among the three agitation speeds (100, 200, and 300 rpm), it showed that 100 rpm of the speed significantly affected the fermentation performance. It was clearly due to non-homogeneous condition (lacks uniformity in medium composition) occurred in the fermentation system.

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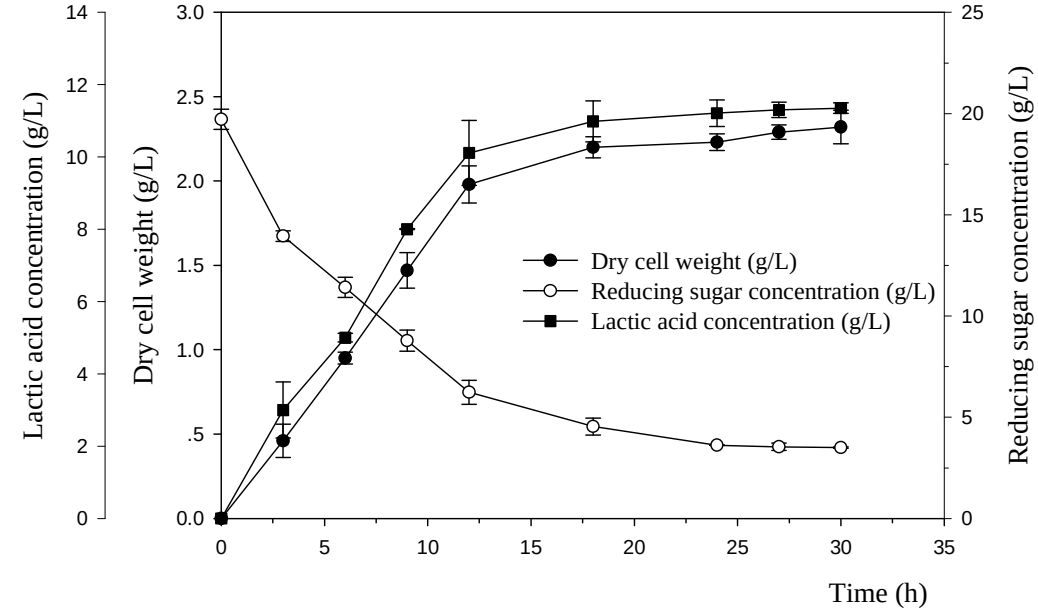
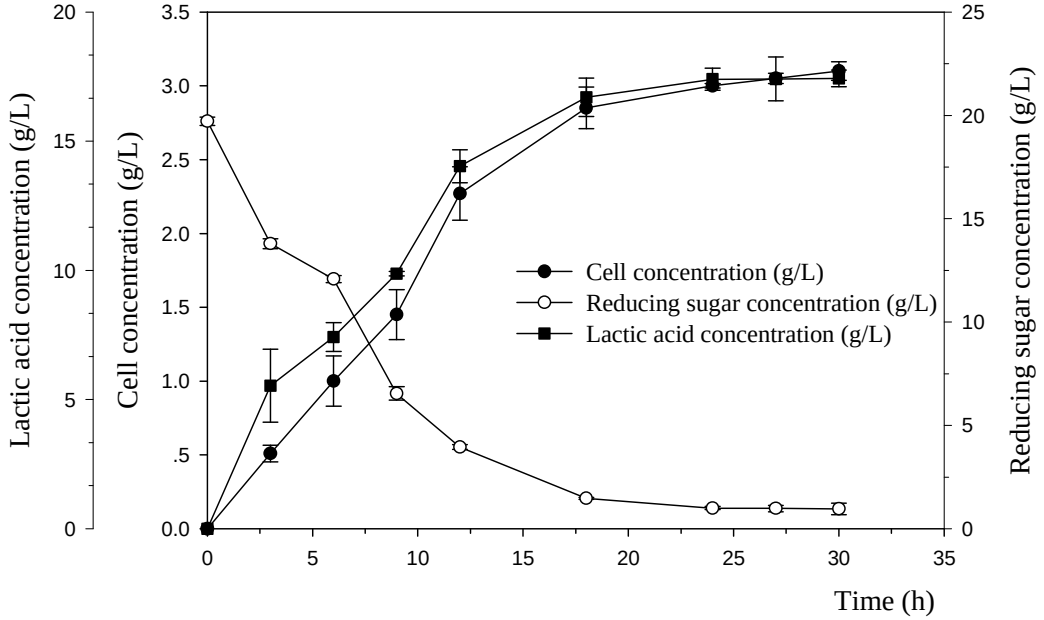
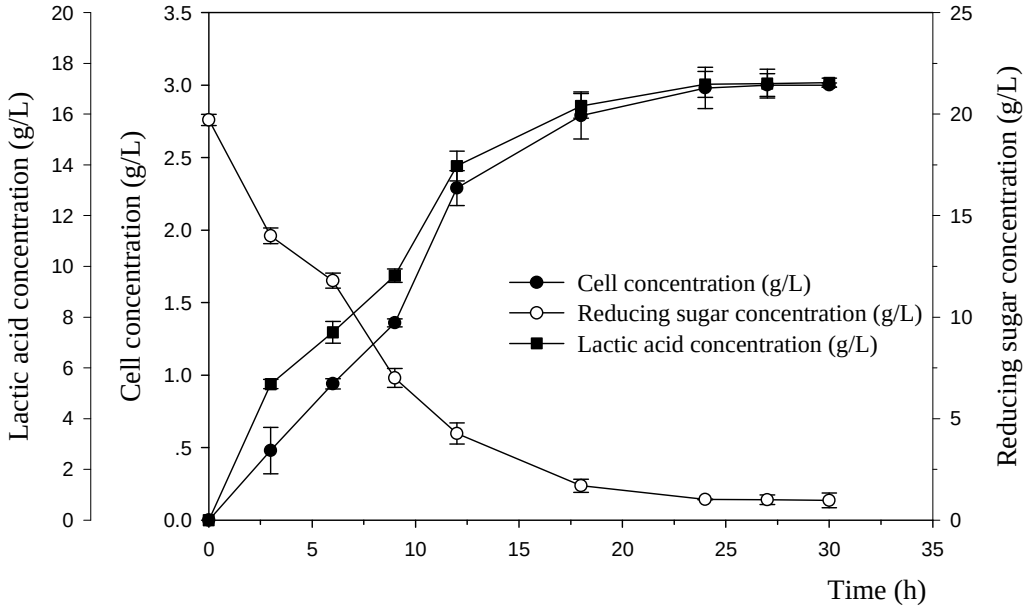


(A)



(B)

Figure 4.17 Effect of agitation speed on the cell growth, reducing sugar concentration and lactic acid production by *P. pentosaceus*: 100 rpm (A), 200 rpm (B), and 300 rpm (C).



(C)

Figure 4.17 (Continued).

Table 4.11 summarized the comparisons of different agitation speed on fermentation performance. Yield of biomass ($Y_{X/S}$) and volumetric productivity were 0.13 (0.96 g/L/h), 0.15 (0.97 g/L/h), and 0.15 (0.97 g/L/h) at the speed of 100, 200, and 300 rpm, respectively. As a result, the latter two agitation speed produced lactic acid of approximately 18 g/L, and there was no significantly different. Some previous paper reported that agitation rate at 200 rpm enhanced fluid-to-particle mass transfer. However, the medium might affect the rheological properties; optimum mixing was required in order to just promote the contact between the bacteria and liquid media. Mixing was an important operation in fermentation which was the main objective to achieve homogeneity of the system. Without agitation, the bacteria will sediment at the bottom and could not fully utilize the carbon source. In addition, the morphology of the microorganism could strongly influence the product formation, since it affected broth rheology and consequently the mass and heat transfer capabilities of the fermentation broth. In this section, the optimum conditions for enhanced

lactic acid production were obtained. Therefore, the agitation speed of 200 rpm was chosen for the subsequent experiment.

Table 4.11 Result of lactic acid productivity at different agitation speeds of fermentation.

Agitation speeds	**$Y_{X/S}$**	**$Y_{P/S}$**	**Volumetric productivity (g/L/h)**
100 rpm	0.13	0.72	0.96
200 rpm	0.15	0.85	0.97
300 rpm	0.15	0.84	0.97

4.4 Lactic acid fermentation methods

4.4.1 Lactic acid fermentation in batch process with non-controlled pH

In a bioprocess development, two main streams are involved including upstream and downstream processes. This work focused on production and separation of lactic acid from fermentation broth. In addition, the use of homo-fermentative with high stereo-selectivity would facilitate further downstream processes. *P. pentosaceus* was successfully isolated and characterized in our laboratory. This strain possesses all desired characteristics of the above mentioned, and was used in all fermentation processes of this work. Firstly, L-(+)-lactic acid fermentation in batch process was investigated. In this work, batch fermentation was studied without pH control, and a 10% (v/v) inoculum grown in MRS broth for 24 h was aseptically inoculated into the fermenter. Experiment was initially

carried out with hydrolysis of 100 g/L cassava starch with α-amylase and gluco-amylase resulted in equivalent to 40 g/L of reducing sugar. The conversion of starch to sugar consumed energy during liquefaction and saccharification (enzymatic hydrolysis), but significantly increased the efficiency of fermentation process. The hydrolyzed starch solution was subsequently supplemented with spent brewer's yeast extract plus ferrous sulphate ($FeSO_4$, 2 mg/L) (Saksinchai *et al.*, 2001) prior to autoclaving. The time course of pH, cell concentration, reducing sugar concentration, and lactic acid concentration during fermentation were shown in Figure 4.18. Glucose concentration rapidly decreased during the first 16 h following by a much slower decreasing rate. Utilization of glucose nearly ceased after 24 h of operation with the remaining concentration of less than 10 g/L. The reason for this high yield was probably the bacteria possessed amylolytic activity which can digest oligosaccharides. The pH of medium also decreased rapidly until reached its final value of 3.90 at the end of the fermentation. It was observed that *P. pentosaceus* possessed a relatively short lag phase followed by exponential phase. The growth entered its stationary phase at 20 h where cell concentration reached plateau of approximately 2.5 g/L until the end of the fermentation process. The maximum lactic acid concentration also rapidly increased since the beginning before reached its plateau of approximately 32 g/L, and was stably maintained throughout the entire stationary phase of fermentation. However, the amount of lactic acid produced at stationary phase (27-34 h) was slightly increased even there were some glucose remained in the medium. This was probably due to the death of the bacteria at lower pH.

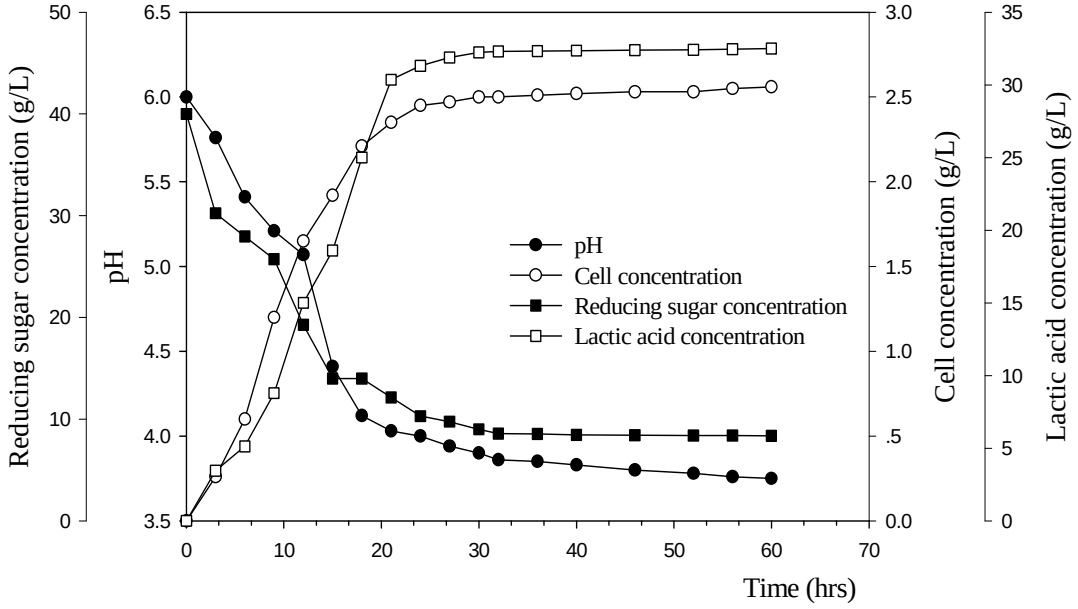


Figure 4.18 Time course of lactic acid production in batch fermentation by *P. pentosaceus* without controlled pH: carbon source as hydrolyzed cassava starch (equivalent 40 g of glucose) and nitrogen source as spent brewer's yeast extract. Experiment was carried out at 30 °C, initial pH 6.0, and agitation speed 200 rpm.

4.4.2 Lactic acid fermentation in batch processes with controlled pH

To overcome product inhibition effect, the pH was regulated during fermentation at its optimal value at which the lactic acid concentration was below the inhibitory threshold. The conventional process for lactic acid production is batch process which normally results in low productivity and high operating costs. In addition, product inhibition occurs as a consequence of failure in proton motive force across the cytoplasm membrane (Goncalves *et al.*, 1997). This problem could be alleviated by controlled pH of the fermentation broth. The dynamics of controlled pH in lactic acid fermentation were illustrated in Figure 4.19. The cells started to enter the exponential growth phase after 3 h of operation, whereas the lag phase was observed at 4 h. Glucose concentration rapidly

decreased at the first 20 h before gradually decreased until the end of fermentation. Lactic acid was mainly produced during the exponential growth phase, and the volumetric productivity reduced when the growth declined. Experimental data showed the highest lactic acid concentration of approximately 50 g/L resulting in 35.4% improvement in lactic acid production compared with the batch process with non-controlled pH. In addition, glucose utilization was also improved with the remaining glucose of lower than 5 g/L.

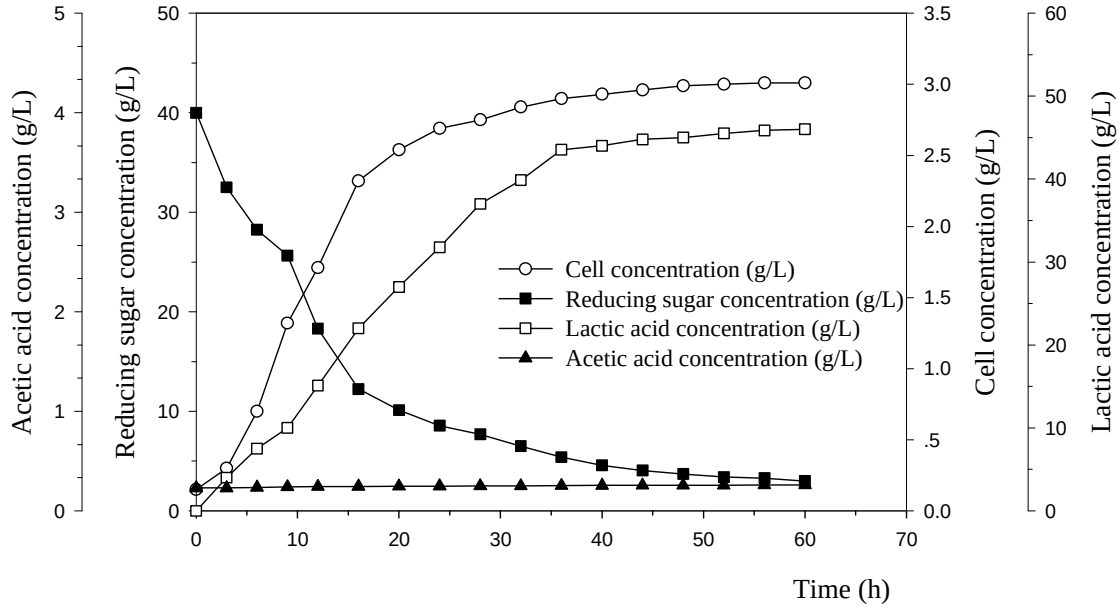


Figure 4.19 Time course of lactic acid production in batch fermentation by *P. pentosaceus* with controlled pH: carbon source contains 100 g of hydrolyzed starch (equivalent to 40 g of glucose) and nitrogen source as spent brewer's yeast extract (15 g/L) supplemented with 2 mg/L of $FeSO_4$ at 30 °C, pH was controlled at 6.0, and agitation speed at 200 rpm.

Although controlled pH fermentation yielded better performance, cell growth ceases and volumetric productivity declined due to substrate limitation at the end of fermentation. This was well known characteristic of batch process. In addition, high substrate concentration could lead to a prolonged lag time, and reduce the specific growth rate.

Therefore, it was very interesting to further investigate on fed-batch process. Substrate feeding strategies may be varied including constant feeding, intermittent feeding, and exponential feeding, respectively. The main objective was not only reduce substrate inhibition effects, but also maximize the volumetric productivity of lactic acid.

During substrate limitation in the fermentation, however, homofermentative could be switched to heterofermentative. Ethanol, acetic acid and formic acid could be formed by difference in the metabolism of pyruvate where lactic acid was also metabolized into formic acid and acetyl-CoA. Alternative pathway of pyruvate metabolism became active via pyruvate dehydrogenase, resulted in the production of carbon dioxide, acetic acid, acetyl-CoA, and NADH (Hofvendahl and Hagerdal, 2000). Therefore, fermentation processes should be operated at sufficient substrate concentration. The substrate should be continuously fed in the fermenter in order to alleviate the substrate limitation. Fed-batch fermentation were attempted in the next section.

4.4.3 Lactic acid fermentation in fed-batch processes

Fed-batch process is a batch process fed continuously or sequentially with substrate (Lee *et al.*, 1999; Roukas and Kotzekidou, 1998). Fed-batch processes were introduced in order to avoid substrate inhibition resulting in high osmotic pressure at high sugar concentrations (Ozmihci and Kargi, 2007). Fed-batch fermentation is advantageous in cases where high substrate concentration is toxic to the culture. These processes resulted in improved sugar utilization and lactic acid production. Fed-batch operation offer special advantages over batch and continuous operations by eliminating substrate inhibition as a result of slow feeding of highly concentrated substrate solution. Fermentation is started with a relatively low substrate concentration (to reduce substrate inhibition) and a low volume. As the substrate is consumed, it is replaced by addition of a concentrated substrate solution

at a low rate while keeping the substrate concentration in the reactor below the toxic level (Qureshi and Blaschek, 2001).

Fed-batch fermentation was carried out in three distinct modes. In the first mode, the *P. pentosaceus* cells were initially grown in batch mode by using a 2 L bioreactor with a working volume of 1.2 L. At the late exponential phase of batch fermentation, highly concentrated hydrolyzed cassava starch solution (200 g/L) supplemented with 15 g/L spent brewer's yeast extract and 2 mg/L of ferrous sulfate was continuously fed into the bioreactor. The fermentation performances of different fed-batch mode were shown in Table 4.12. The different fed-batch feeding strategies were attempted in this work including constant feed rate, intermittent fed-batch and exponential fed-batch, respectively. In comparison, batch with non-controlled pH gave the poorest results in term of lactic acid concentration, biomass, and volumetric productivity. This disadvantage of low volumetric productivity could be avoided, and the lactic acid concentration could be greatly enhanced by the application of low level of initial glucose and repeated feeding during fermentation.

For intermittent fed-batch, the highest lactic acid concentration for constant feed rate fed-batch was obtained at 95.40 g/L with production yield of 91.25%, and cell concentration of 2.51 g/L, respectively. For constant feed rate experiment, different feed rates were preliminarily investigated at the rates between 0.01-0.3 g/s (data not shown). The best result was obtained with the feed rate of 0.1 g/s, and this value was chosen for comparison with other fed-batch methods. Compared to intermittent feeding, constant feed rate fed-batch yielded a better result with the increased lactic acid concentration of 13.3% to 110.10 g/L. Volumetric productivity was also slightly increased to 2.62 g/L/h or 4.1% compared with intermittent feed rate operation.

Table 4.12 Comparison of different fermentation methods on lactic acid production.

Parameter	Batch with non-controlled pH	Batch with controlled pH	Intermittent fed-batch	Constant feed rate fed-batch	Exponential fed-batch
Lactic acid (g/L)	32.30 ± 0.14	49.00 ± 0.05	95.40 ± 0.03	110.10 ± 0.12	155.46 ± 0.21
Productivity (g/L/h)	1.20 ± 0.23	1.56 ± 0.17	2.51 ± 0.07	2.62 ± 0.15	2.80 ± 0.18
Yield (%)	83.13 ± 0.09	87.78 ± 0.15	91.25 ± 0.11	90.12 ± 0.10	90.01 ± 0.06
Dry cell weight (g/L)	2.97 ± 0.02	3.06 ± 0.05	3.57 ± 0.07	3.79 ± 0.09	3.74 ± 0.02
Maximum specific growth rate (μ_{max})	0.37 ± 0.05	0.39 ± 0.03	0.41 ± 0.10	0.40 ± 0.07	0.46 ± 0.03
Final sugar concentration (g/L)	10.19 ± 0.11	5.21 ± 0.07	12.11 ± 0.03	25.87 ± 0.17	5.02 ± 0.09
Initial broth volume (L)	1.20	1.20	1.20	1.20	1.20
Final broth volume (L)	1.32	1.35	1.90	1.97	2.07

: Constant feed rate in fed-batch at 0.1 g/s

: The values given in the Table are the average of duplicate experiments.

Although both fed-batch modes enhanced the lactic acid production over batch fermentation, these processes were still subject to substrate inhibition effect. The level of glucose concentration in the medium remained relatively high during fermentation process. This was because the addition of glucose was not at appropriate value, and glucose utilization rate was not at its maximum.

In order to minimize the effect of substrate inhibition, another fed-batch technique was attempted by following cells growth. Exponential fed-batch was developed using pre-determined feeding strategy according to a previous work (Korz *et al*., 1995). According to Equation 1, the specific growth rate was set at 0.08 h^{-1} which the optimum value was concerning the changing in volume of the system. The typical value of biomass yield on substrate ($Y_{X/S}$), and maintenance coefficient (m) were 0.11 g/g, and 0.04 h^{-1}, respectively which were taken from a previous work (Zhang and Lovitt, 2006). Figure 4.20 showed the time course of exponential fed-batch fermentation. Experimental results showed a significant improvement of lactic acid concentration compared with the other fed-batch modes where the highest product concentration was obtained at more than 150 g/L. This addressed the appropriate addition of carbon source to the medium, and the cells were able to efficiently utilize the substrate. Substrate inhibition was also minimized as the glucose concentration remained lower than 20 g/L throughout the operation. The maximum cell concentration of 3.74 g/L was obtained. However, it was recommended that the fermentation should be stopped after 40 h of operation for the economic point of view. This was because volumetric productivity significantly reduced after lactic acid concentration reached 130 g/L, and residual glucose started to rise. In addition, hydrolyzed starch solution was fed at exponential rate, and prolonged fermentation time could result in much higher operating cost.

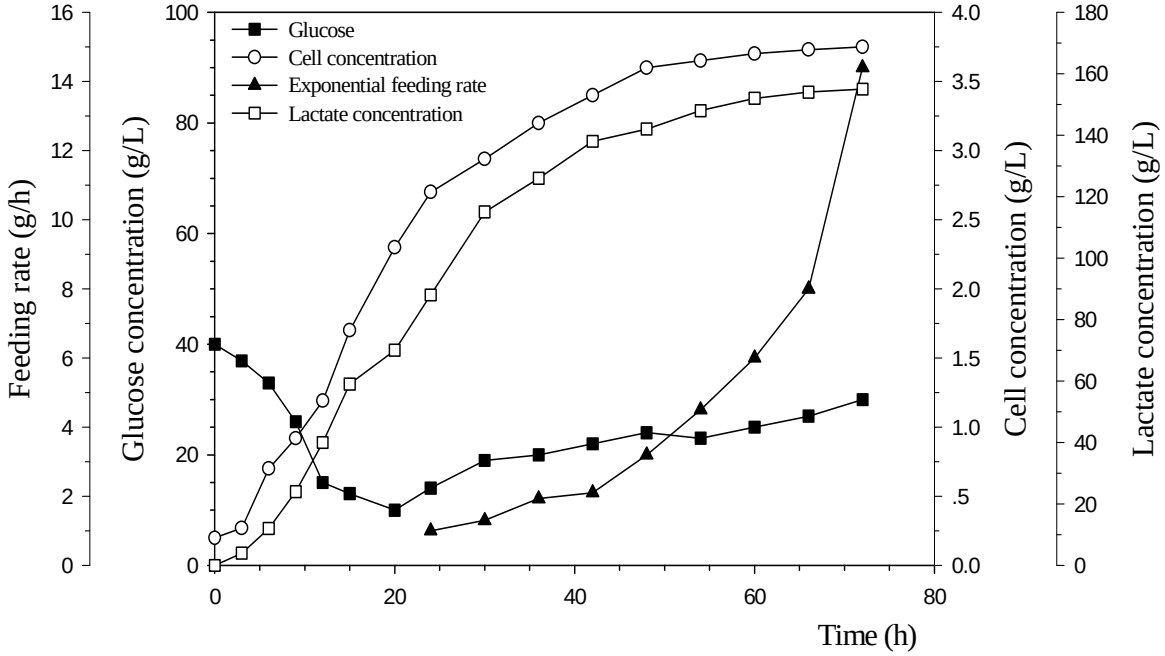


Figure 4.20 Lactic acid production by exponential fed-batch fermentation by *P. pentosaceus* with controlled pH (pH 6.0), temperature at 30 °C, agitation speed at 200 rpm. The feeding solution contains 200 g/L hydrolyzed starch supplemented with 15 g/L spent brewer's yeast extract and 2 mg/L ferrous sulfate.

In conclusion, fed-batch fermentation could greatly enhance lactic acid production, and had advantages over batch process. This process permitted effective control of residual glucose concentration levels (Papagianni *et al*., 2007). Cells proliferate and glucose utilization was maximized. Although substrate inhibition was minimized, the cell mass was maintained at rather constant level, and volumetric productivity reduced at the end of fermentation. This was probably due to inhibition of the cells by lactic acid especially at the concentration higher than 80 g/L. Number of works had already been addressed inhibitory effects of lactic acid to the cells (Wee *et al*., 2006). Since lactic acid was a growth-associated product, it was therefore interesting to compare the productivity and cell viability at different modes of operation including batch with non-controlled pH,

batch with controlled pH, and fed-batch fermentation. However, lactate accumulated in the fermentation broth led to the product inhibition at the end of the fermentation. Application of *in situ* lactate removal was highly encouraged in order to increase production yield and volumetric productivity. In addition, separation of the lactate product facilitated subsequent purification steps because many impurities would be significantly removed.

4.5 Extractive fermentation of lactic acid

Numbers of works have been reported for the end-product inhibition especially non-dissociated form of the lactic acid. Therefore, it is necessary to alleviate toxicity effect to the cell in order to increase yield and volumetric productivity. The main advantage of the fed-batch system is that inhibition and catabolite repression are prevented by feeding of the substrate. If the substrate has an inhibitory effect, intermittent addition improves the productivity of the fermentation by maintaining a low substrate concentration. The parameters of the intermittent fed-batch process are very close to exponential fed-batch process. On the other hand, the disadvantage of using merely exponential fed-batch is that it is difficult to control and requires complicated equipments. Therefore, intermittent feeding strategy was attempted with the main objective of increasing the final product concentration and downstream processes. The method suggested in this study, intermittent fed-batch process could be applied to separation of lactic acid by *in situ* product removal concept.

However, the influence of lactate concentration to fermentation performance by the biocatalyst has to be studied intensively. The coupling of a biological reaction with a membrane in only one unit is a very interesting configuration for the reaction where the continuous elimination of metabolites is necessary to maintain a good productivity.

4.5.1 Product inhibition effect

In order to assess the inhibition kinetic of *P. pentosaceus*, the strain was cultivated on various initial lactate concentrations. The effect of substrate inhibition was already investigated in the early part of this work. Experiments were carried out by using initial lactate concentrations between 0-90 g/L (Lin *et al*., 2004). The specific growth rates as a function of initial lactate concentrations were shown in Figure 4.21. The results confirmed that lactate played an important role on fermentation performance even at low initial lactate concentration. The maximum specific growth rate (μ_{max}) was observed at 0.44 (h^{-1}). The specific growth rate constantly decreased with the increase of initial lactate concentration. At 50 g/L, specific growth rate reduced to approximately 50% and the value rapidly decreased to zero when the concentration reached 90 g/L. Critical lactate concentration (P_{crit}) refers to the concentration at which the cell growth was severely hampered. In this work, it was observed that almost no cell growth occurred at lactic acid concentration higher than 80 g/L. Therefore, this concentration was set as P_{crit} in the Equation (10). The mathematical modeling showed a good agreement with the experimental result. The substrate saturation constant (k_m) of 0.23 g/L was adopted from a previous work (Lee and Dobrogosz, 1965). In addition, the correlation was best fitted with the *n* value of 0.55. This model could be used to predict the inhibitory effect in a wide range of lactic acid concentrations. This is due to the fact that the undisscociated form of lactic acid is easier to be transported across the cell membrane into the cells, resulting in intracellular acidification. It is more reasonable to use the total lactic acid concentration as the model parameter in a pH controlled system that in non-pH controlled system (Lin *et al*., 2004).

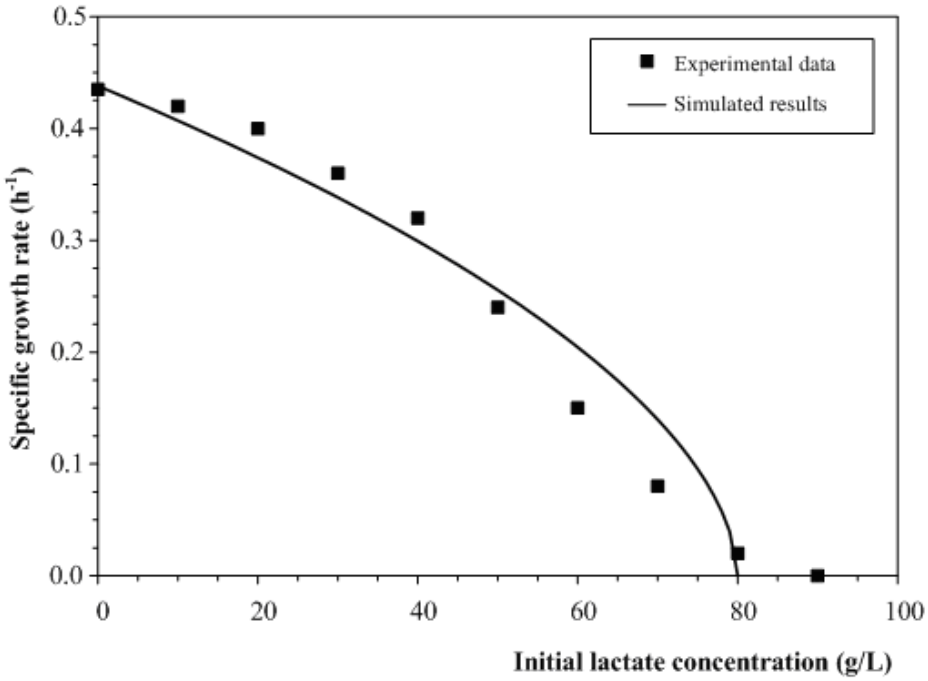


Figure 4.21 Variation of the specific growth rates as a function of the initial lactate concentrations.

4.6 Adsorption characterization

It is generally known that fermentation broth contains impurities which need to be removed during downstream processes. The first operation starts with removal of insoluble particles such as bacterial cells. Recovery of lactic acid from fermentation is traditionally achieved by direct sequestration using adsorption method. Adsorption occurs when components of the liquid are attached on the surface of solid particles due to a binding force. In this case, lactate ions can be attached to anion ion exchange resins as a result of charge difference. When equilibrium was reached, sample in the flask were withdrawn and the capacities of the resin (denoted q, g lactic acid/g dry resin) were calculated by the following equation (Moldes *et al*., 2003):

$$q = \frac{[LAC_0 - LAC_E]V}{w} \quad (12)$$

Where LAC_0 and LAC_E are the initial and equilibrium concentrations of lactic acid (g/L), V is the volume of solution and *w* is the amount of dry resin employed (g).

In addition, the Langmuir's equation is expressed as (Moldes *et al.*, 2003):

$$q = \frac{q_m.[K.LAC_E]}{[K.LAC_E]+1} \quad (13)$$

Where q_m is the maximum resin capacity (expressed as g lactic acid/g dry resin), and the value of K is expressed as the equilibrium constant or Langmuir equilibrium constant. However, the value of q_m cannot be directly obtained by using the graph of Equation 13. As a result, a Lineweaver-Burk regression method can then be used to estimat the value of q_m and K by rearranging the Equation 13.

The arrangement yields:

$$\frac{1}{q} = \frac{1}{q_m} + \frac{1}{q_m K.LAC_E} \quad (14)$$

A plot of 1/q versus 1/ LAC_E yields a slope of 1/ q_m.K whilst the Y intercept is equal 1/ q_m.

The suitability of a given ion exchange resin for lactic acid recovery depends on its physicochemical features (including capacity, and sorption kinetics) (Cao *et al.*, 2002). Amberlite IRA-400 resin has proper pore size and high adsorption capacity for recovery of lactic acid and it can adsorb lactic acid in wide pH range. Application of Amperlite IRA 400 resin were observed in this experiment. The sorption of ion exchange resin could be ascribed to two different phenomena, adsorption and ionic exchange, which could happen simultaneously (Moldes *et al.*, 2003). The component of synthetic feed solution on lactic acid absorption were investigated by plotting the equilibrium lactic acid concentration (LAC_E), and the capacity of the resin or adsorption isotherm (as shown in Figure 4.22).

The sorption capacity of a strong base resin could be increased by acidification to switch the equilibrium towards the molecular form of the acid. Kinetic of lactic acid absorption were carried out to assess the kinetic pattern of lactic acid recovery, necessary for reaching equilibrium. On the other hand, different feed concentration resulted in the different in adsorption capacity. In conclusion, the overall process was made up of sequential stages, including: (1) solute mass transfer through the liquid film surrounding the resin particles; (2) intraparticle diffusion of solute and (3) solute adsorption. Figure 4.22 showed experimental results of adsorption isotherm indicating that the absorption of lactate on IRA 400 resins was Langmuir type.

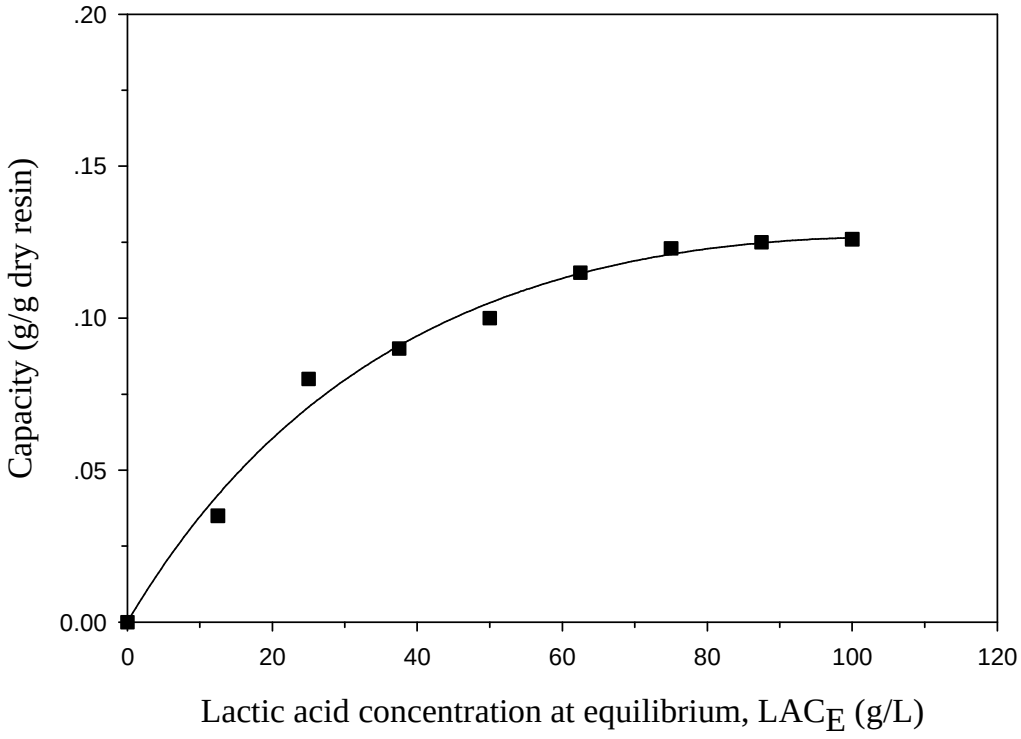


Figure 4.22 Absorption isotherm of lactate on the resin.

In addition, experimental data obtained from absorption experiments were subsequently employed to estimate the value of q_m and K. A Lineweaver-Burk regression were shown in Figure 4.23. The linear regression indicated the $1/q_m$ value of 0.16 with

the R^2 of 0.9634 and the K value of 0.0010 could then be directly calculated from the slope of the graph.

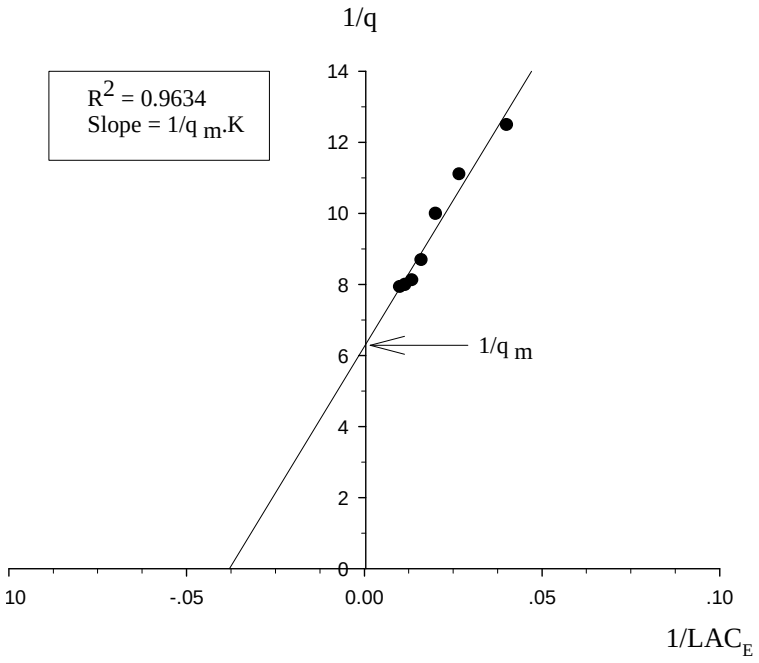


Figure 4.23 A linear regression of a Lineweaver-Burk plot from equation 14.

Absorption has advantages over other purification techniques. The equipment for this process is less expensive, and easier to maintain. However, the desorption or elution is required as the last step to recover the absorbed product. The disadvantages is that expensive solid adsorbents are more easily fouled by the dirtier feed stream and require more expensive regeneration steps and more frequent replacement. In addition, a large amount of eluent is produced causing some environmental problems. Therefore, a highly effective desorption method is required.

4.7 EDI experimental set-up for lactic acid separation

Normally, the fermentation broths contain some purities especially free amino acids and organic acids. Due to the diluted product concentration, more energy consumption will be needed for concentration step to obtain high purity lactic acid.

Moreover, the highest cost of the process for lactic acid production by fermentation processes corresponds to the separation steps that are necessary to achieve the quality requirements of purified lactic acid. Adsorption with anion exchange resins is the conventional technique for recovery of lactic acid from fermentation broth. However, the desorption or elution step is often associated with a large amount of eluent used (NaCl solution) which cause environment problems, and is often associated with significant product lose. In order to reduce purification costs, numerous studies on lactic acid separation have been conducted using EDI technique as it is based on continuous desorption of the lactate ion under direct electrical field. In addition, the overall benefit of this technique coupling with membrane bioreactor was extremely positive. It was a one stage integrated process, with *in situ* pH control, integral product (lactic acid) removal in a concentrated form, and overall enhancement of yield. The approach taken in this study was potentially applicable in a wide range of bio-catalytic and fermentation processes. The trials with model solutions were focused on the determination of the parameters for the EDI experiments and on the investigation of the time course under different conditions.

4.7.1 EDI technique characterization

Nowadays, most industrial producers of lactic acid still employ the precipitation process for the purification of lactic acid, which leads to the generation of crude calcium sulphate, a by-product which is normally dumped to the environment as waste. In addition, the number of purification steps required and their individual step yields determine the overall yield of the process. Currently, the overall yield of typical purification processes composed of about 8-10 unit operations is in the range of 50-80%, a yield that can diminish minor process achievements in fermentation processes (Walter, 1998).

The employment of new purification technologies and integrated process configurations therefore presents exciting possibilities to reduce the number of purification steps, and production costs, respectively. Apart from treatment with alkali solutions, various techniques have been proposed to continuously remove the lactic acid as it is formed, and these range from stripping with organic solvent to the use of electrodialysis systems (Yabannavar and Wang, 1991). An in-house electrodeionization technique was introduced in this study. The proposed theoretical model involved the diffusive transfer from the flowing solution to the ion-exchange resin beads combined with the electrolytic transfer of ions along the chain of ion-exchange beads (Klein *et al.*, 1998). This study quantified the relationships between applied current and mass transfer characteristic to evaluate the performance of the electrodeionization system. Figure 4.24 showed the typical experimental set-up of electrodeionization experiment used in this work.

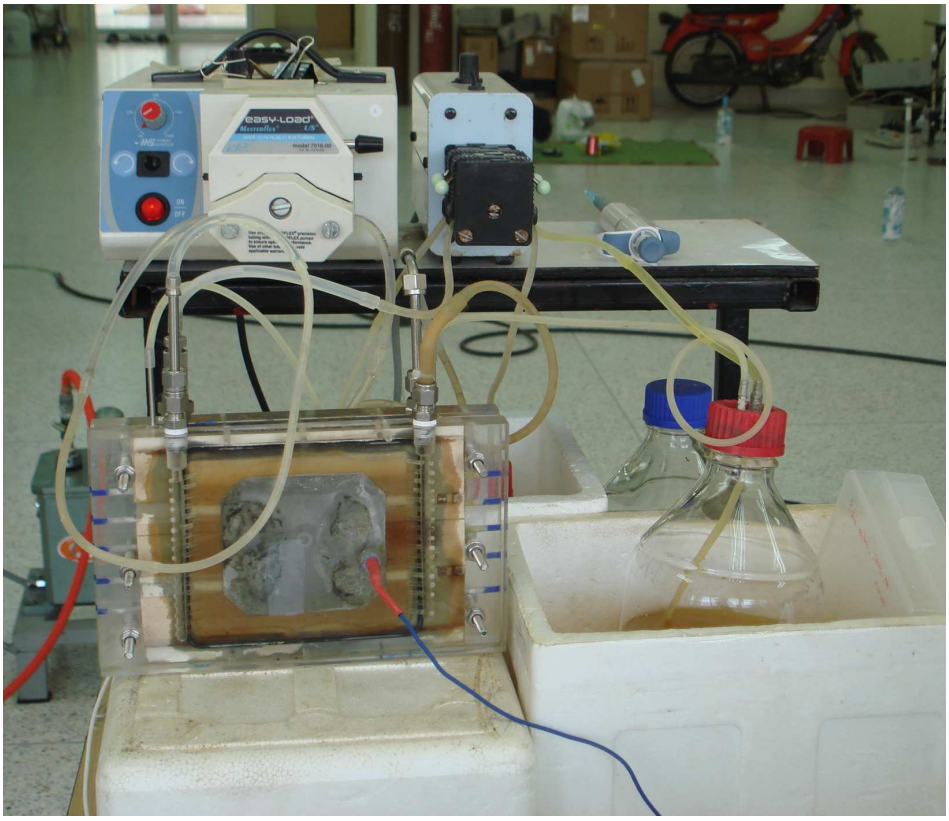


Figure 4.24 Batch experimental set up of in-house EDI system.

The use of EDI in bioseparation is challenging, and the objective of this work is to study the behavior of an EDI system under different operating conditions. Firstly, the EDI process was investigated in abiotic system for the mathematical modeling of lactic acid separation. Several current densities were initially assessed in this experiment. Long-term operation and preliminary technical analysis of the EDI system were also investigated.

4.7.1.1 Batch electrodeionization of synthetic solutions

The most important characteristic of the EDI system is the continuous desorption of charged molecules from mixed-bed ion exchange through ion exchange membranes under direct electric current. Due to the diluted concentration of the feed stream, more energy is required to obtain higher lactate concentration. Figure 4.25 shows a typical behavior of EDI technique for separation of lactate ions from synthetic solution. The initial lactate concentration in the feed stream was 90 g/L, and the operating condition of current density was initially set at 45 mA/cm^2. From the experimental result, it was observed that concentration of lactate in the feed stream constantly decreased with time, whereas lactate concentration in the concentrate stream rapidly increased. However, this operating mode of constant current was switched to constant voltage at 15 Volts (Spiegel *et al*., 1999) when the electrical resistant of the system significantly increased. At low concentration, the amount of lactate transferring across the ion-exchange membrane was faster than film layers surrounding ion-exchange resins and the membrane-aqueous interface. This phenomenon resulted in the reduction of current efficiency which was the ratio of lactate transferred to the total charge supplied. For this case, the operating mode was switched when the lactate concentration decreased to approximately 10 g/L resulting in a sudden increase in voltage. Although concentration of lactate ion in the feed stream played a major role in determination of the current efficiency, quantification of the limiting

current density was not carried out. In fact, changing time for the operating mode was able to carry out by an observation of the sudden increase in voltage. The proposed theoretical model involved the diffusive transfer from the flowing solution to the ion-exchange resin beads combined with the electrolytic transfer of ions along the chain of ion-exchange beads (Monzie *et al.*, 2005).

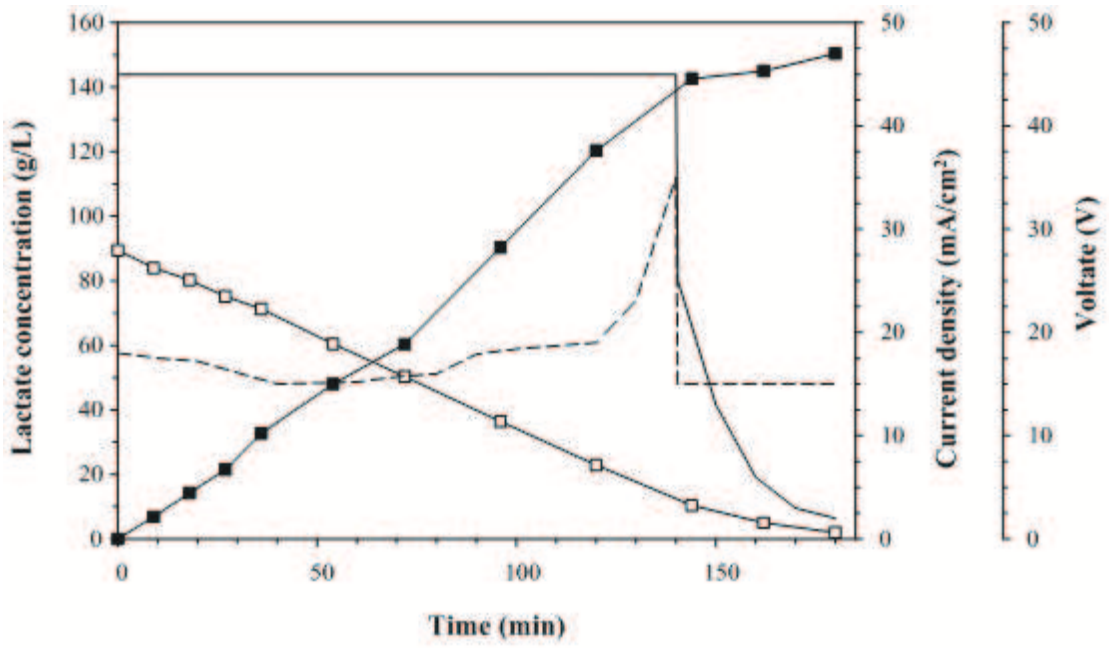


Figure 4.25 The time course of lactate concentration in the feed (⊣□⊢) and concentrate solution (⊣■⊢) during the batch EDI experiment. Current density (——) was initially set at 45 mA/cm^2 and was switched to constant voltage mode when the voltage (- - -) suddenly increased.

Figure 4.26 showed the effect of initial concentrations, and current densities on mass flux of lactate ions across the membrane. The experiments were carried out at initial lactate concentrations ranging from 10 to 125 g/L. Experimental results showed that initial lactate concentration in the feed side was a very important operating factor. At 10 g/L lactate concentration, the mass flux was completely independent on the applied

current density. For higher concentrations, mass flux showed a linear relationship with the current density especially at concentration higher than 40 g/L. This behavior implied that all of electrical power was used to drive lactate ions across the membrane. Current efficiency in this range was therefore close to 100%. When current density was higher than 20 mA/cm^2, however, the lactate fluxes slowly increased, and any increase in current density was not accompanied by a further proportional increase of lactate flux especially at 40 g/L lactate concentration. In conclusion, the current efficiency dramatically decreased when the lactate concentration decreased. Because power consumption depended on current density, this implied that *in situ* removal of lactate from fermentation broth should be carried out at high lactate concentration. In order to avoid product inhibition and low current efficiency, the lactate concentration in the feed must be controlled at the optimum level. The highest performance of current density applied in this study was obtained at 45 mA/cm^2. However, when the current density exceeded limiting current density, concentration polarization could be increased, resulting in decrease of the current efficiency. Separation of lactic acid from the feed solution depends not only on electrical driving force, but also on concentration difference. It was obviously seen that higher concentration difference resulted in higher lactate flux across the membrane.

However, the fermentation in batch reactors was in general, a low productivity process due to the toxic effect of products on micro-organisms (Luedeking and Piret, 1959). The continuous separation of products by this technique would enhance the process productivity. To overcome the inhibitory effects of lactic acid, either the lactic acid must be continuously removed from the fermentation vessel, or it must be neutralised with alkali during the fermentation to convert lactic acid to its less inhibitory dissociated form (Madzingaidzo *et al.*, 2002).

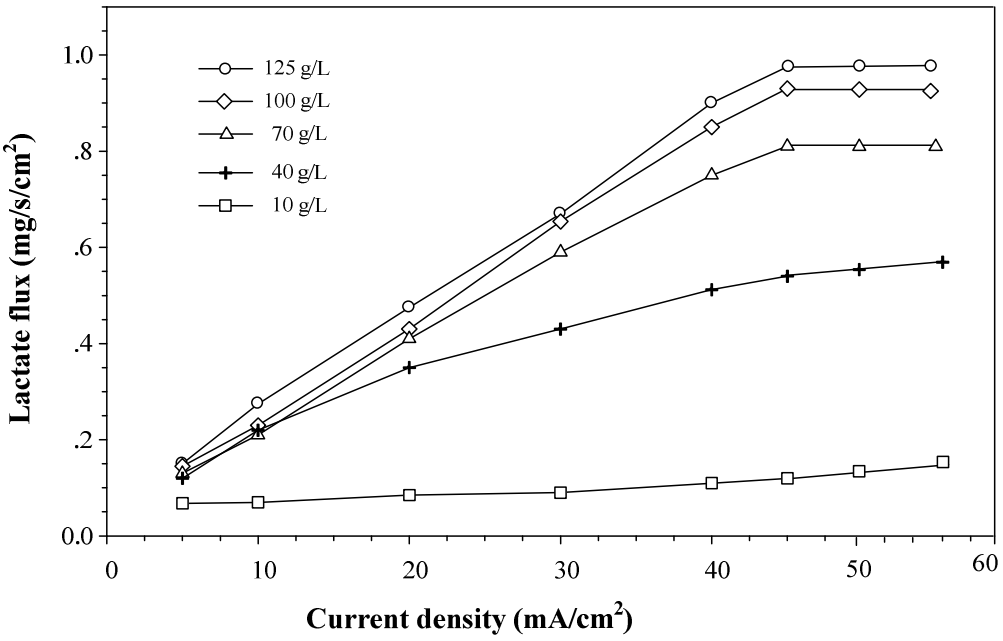


Figure 4.26 The effect of current density on lactate fluxes at different initial lactate concentrations.

4.7.1.2 Batch electrodeionization of clarified broth

Unlike synthetic solutions, application of the electrodeionization process for fermentation broth is complicated. At the end of fermentation process, the broth typically contains bacterial cells, lactic acid product, residual reducing sugar, nitrogenous compounds, and some other impurities. All of these materials need to be completely removed during purification steps in order to produce pure lactic acid. Membrane separation processes are very often applied for recovery of lactic acid from dilute solutions or from fermentation broth (Lazarova and Peeva, 1994; Reisinger and Marr, 1993; Mok *et al.*, 2007). The EDI process was investigated for several factors for example, initial lactic acid concentration, initial pH, amount of current electricity, etc. The disadvantages of the conventional fermentation process are a low reaction rate, an elaborate product recovery, a large amount of byproducts and thereby negative impact on the environment. Moreover, this is most likely due to overcoming product

inhibition in the fermentation process as the lactic acid. There are other possibilities for lactic acid recovery, but solvent extraction, direct distillation, adsorption and other relatively simple methods have certain limitations, which obstruct their wider use (Lee *et al.*, 1998). ED and EDI is one of very promising and perspective methods provided by the rapid development of the membrane processes (Heriban *et al.*, 1993). It has been widely applied and it represents one of the most important membrane processes for environmentally clean technology in biochemical industries (Strathmann, 1992). This approach of combining the advantages of EDI technique could be used.

During the experiment, voltage was kept constant at 15 V, 2.0 Ampere and the process was terminated when the electrical current decreased. Figure 4.27 showed the experimental results of lactic acid separation from fermentation broth in batch fermentation process from hydrolyzed cassava starch as the main substrate. The concentrations of lactic acid were analyzed in the 2 compartments (feed solution, and receiving solution or concentration side). The concentration of the lactic acid in the concentrate solution increased after 4 h by natural diffusion passed through ion exchange membrane. From these finding, lactic acid concentration in the feed stream and product stream changed linearly with time. Moreover, previous reports indicated that the transport rate decreased in the fermentation broth with lower initial lactic acid concentrations (Hobovfi *et al.*, 2004).

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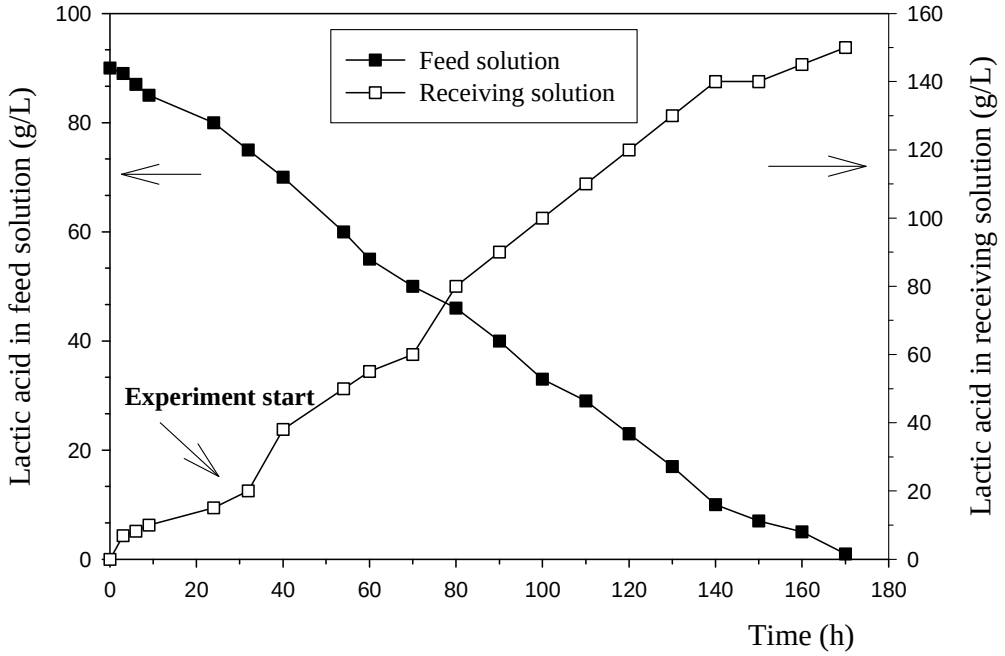


Figure 4.27 The lactic acid concentration in the two phases of feed solution and receiving solution in the EDI system.

Table 4.13 summarized the operating results during batch concentration of lactate ions from clarified fermentation broth. During the course of the EDI experiments, the concentrate and feed volumes changed due to water passage through the membranes simultaneously with the lactate ions by electro-osmosis. This phenomenon resulted in a decrease of the feed volume with an increase of the concentrate volume. Unfortunately, organic nitrogen assays revealed that there was some migration of organic nitrogen compounds from the feed solution into the concentrate solution (Choi *et al.*, 2002). This phenomenon has been reported especially migration of amino acids during electrodialysis of lactic acid from fermentation broth (Wang *et al.*, 2010). Based on Kjeldhal nitrogen assays, organic nitrogen 0.07 g/L in the concentrate solution was obtained from 0.62 g/L in the feed solution. These impurities inevitably influence the final purity, and usually resulted in yellowish color of the purified lactic product. The degree of the concentration

(the ratio of the final concentration in the concentrate stream to the initial feed concentration) could be influenced by the increase of the ratio of the initial feed volume to the initial concentrate volume. During the operation, the volume of the concentrated solution increased. This could be due to the water that passes through the membranes together with the lactate ions by electro-osmosis effect. Water transport index, the ratio of increasing volume to the mass of lactate transported across the membrane, was calculated at 2.09 mL/g which was slightly lower than the previous report (Lee *et al.*, 1998).

Table 4.13 Changes in some parameters during lactic acid separation process by EDI system from fermentation broth.

Factors	Concentrate solution	Feed solution
Before EDI		
Volume (L)	0.3	2
Lactate concentration (g/L)	11.5	40
Glucose concentration (g/L)	0	10
Organic nitrogen (g/L)	0	0.62
After EDI		
Volume (L)	0.44	1.85
Lactate concentration (g/L)	167.5	1.2
Glucose concentration (g/L)	0.15	11
Organic Nitrogen (g/L)	0.07	0.65

In conclusion, the resin in EDI system was more advantaged for the lactic acid separation from fermentation broth. The influence of glucose which was commonly present in the fermentation broth on the EDI experimental run was also studied, and the

experimental results showed a small migration of glucose across the ion exchange membranes. It was clearly seen that glucose could pass through the membrane, although it has no net charge. The possible reason was due to natural diffusion as a result of concentration difference.

4.8 Operation of EDI technique for lactic acid separation from fermentation processes

In order to maximize fermentation performance and to facilitate further downstream processes, an *in situ* product removal technique should be applied in combination with the fed-batch fermentation. By feeding the reactor at an optimum rate, substrate concentration could be kept below inhibitory levels, while the suitable separation technique could be applied simultaneously to remove the product from fermentation broth. Thus, application of these two engineering techniques solved two toxicity problems (substrate and product inhibition) (Qureshi and Blaschek, 2001).

4.8.1 EDI technique for *in situ* lactate removal in batch fermentation process

The overall benefit from the bioreactor was extremely positive in that it was one stage integrated process. Separation of the target product could be obtained in a concentrated form, and could result in an increase of the overall production yield. The EDI process was operated at constant voltage mode (15 Volts) because current efficiency significantly reduced at low lactate concentration region. Figure 4.28 showed the time course of glucose, cell, and lactate concentrations in both feed and concentrate solutions during batch fermentation with EDI process. Glucose concentration was rapidly

decreased for the first 15 h, and the consumption rate was gradually decreased until glucose was completely consumed at 27 h. After a short lag phase of approximately 4 h, cell concentration was rapidly increased before it reached the value of 2.72 g/L at the beginning of stationary phase. The highest cell concentration was obtained at 2.84 g/L. On the other hand, lactic acid was produced and its concentration started to rise even at 3 h of fermentation time. Subsequently, the power supply was switched on, and lactate ions started to migrate into the concentrate solution. Experimental results also revealed constant lactate concentrations below 20 g/L indicating that there was no mass transfer limitation of the voltage used. After 21 h, mass flux of lactate exceeded production rate resulting in decreasing of the lactate concentration until lactate was completely removed. Considering the concentrate solution, lactate concentration linearly increased with time since the DC current was applied. The highest concentration of lactate was obtained at 80 g/L whilst the volumetric productivity was calculated at approximately 3.64 g/L/h. In addition, the recovery ratio of nearly 100% was obtained at the end of fermentation process. In conclusion, this batch fermentation coupling with EDI system showed a high possibility to increase lactate production by subsequent addition of carbon source.

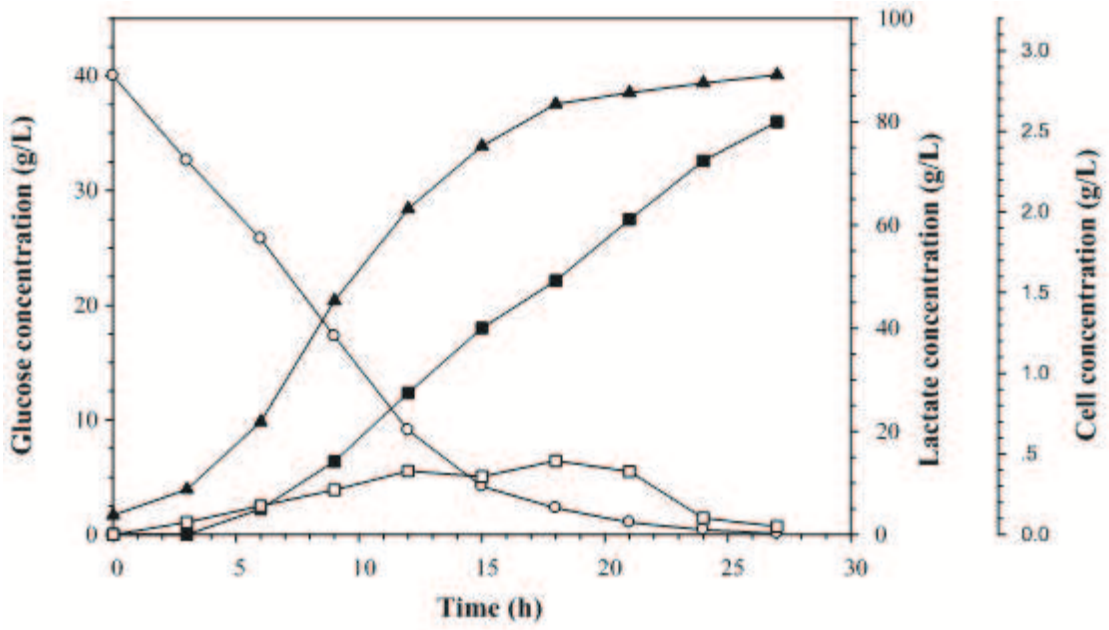


Figure 4.28 The time course of glucose (—○—), cell (—▲—), and lactate concentration in the feed side (—□—), and concentrate side (—■—) during batch electrodeionization experiment. Initial volume of concentration solution was 400 mL, applied voltage was 15 Volts.

4.8.2 Intermittent fed-batch fermentation with and without EDI technique

From the previous section (4.4.3), the lactic acid productivity of exponential feeding showed a high value; however, the production yield of intermittent feeding was higher. As a result, the latter feeding strategy was chosen in this experiment. This section compared fermentation performances between intermittent fed-batch and intermittent fed-batch coupled with EDI technique. Figure 4.29(A) showed time course of intermittent fed-batch fermentation with initial glucose concentration set at 40 g/L. Experimental results revealed that glucose was rapidly consumed within the first 28 h before the first addition of the feeding solution. During this fed-batch fermentation, glucose consumption rate was slightly lower than the previous fermentation, and the

average value was calculated at approximately 2.50 g/L/h. Moreover, fermentation performance was significantly reduced after the second addition of the feeding solution. The fermentation stopped after 78 h because glucose was poorly utilized. The conversion yields for each cycle were estimated as 91.12%, 85.37%, and 70.83%, respectively. On the other hand, average volumetric productivity was calculated at 1.12 g/L/h, and the highest lactate concentration was obtained at 87.24 g/L. This concentration was in accordance with the previous inhibition kinetic study where the value of P_{crit} was observed at 80 g/L. The accepted mechanism of inhibition by lactic acids was related to the solubility of the non-dissociated form within the cytoplasm membrane, and the insolubility of the ionized acid form. This caused acidification of the cytoplasm, and the collapse of the motive force, resulting in inhibition of sugar utilization (Roukas and Kotzekidou, 1998). From this reason, it was strongly suggested that *in situ* removal of lactate ions from fermentation broth would be very beneficial for both bacterial growth and lactic acid production. Table 4.14 showed the fermentation parameters, including lactate concentration, dry cell weight, production yield, and volumetric productivity, respectively. It was noted that the volume of fermentation broth increased to 1.20 L after the first addition of feeding solution whilst the second addition resulted in the final volume of 1.50 L.

This experiment proposed the use of EDI technique for *in situ* lactate recovery coupled with the intermittent fed-batch system. The ultimate goal was to maximize the production of lactate compared with conventional batch, and fed-batch fermentation. An important issue for the cell was that of continuous removal of toxic lactate by EDI process. In order to avoid the excessive volume in the bioreactor, partial removal of 200 mL clarified broth was carried out at the end of each run followed by an addition of the same volume of feeding solution. As a result, the initial glucose concentration of each cycle was exactly 40 g/L. The time profiles of glucose, cell, lactate concentration in the

feed and concentrated solution during EDI process were shown in Figure 4.29(B). The largest impurities in fermentation broth (i.e., bacterial cells, high molecular weight residues) were first of all eliminated in a first step of clarification that could be done by filtration for instance in order to prevent the deposition of bacteria on the membrane (Habova *et al.*, 2004). During the lactic acid fermentation, as lactic acid was being produced by the cells, alkali would be pumped into the fermentation broth to prevent excessive reduction in pH. The potential benefits of *in situ* lactic acid removal were hence an increase in yield, plus separation and concentration of lactic acid and direct use of hydroxyl ions produced by water splitting to neutralize pH without the requirement for additional acid or base. The pH control was coupled to the power supply to enable lactic acid to be removed as it was produced.

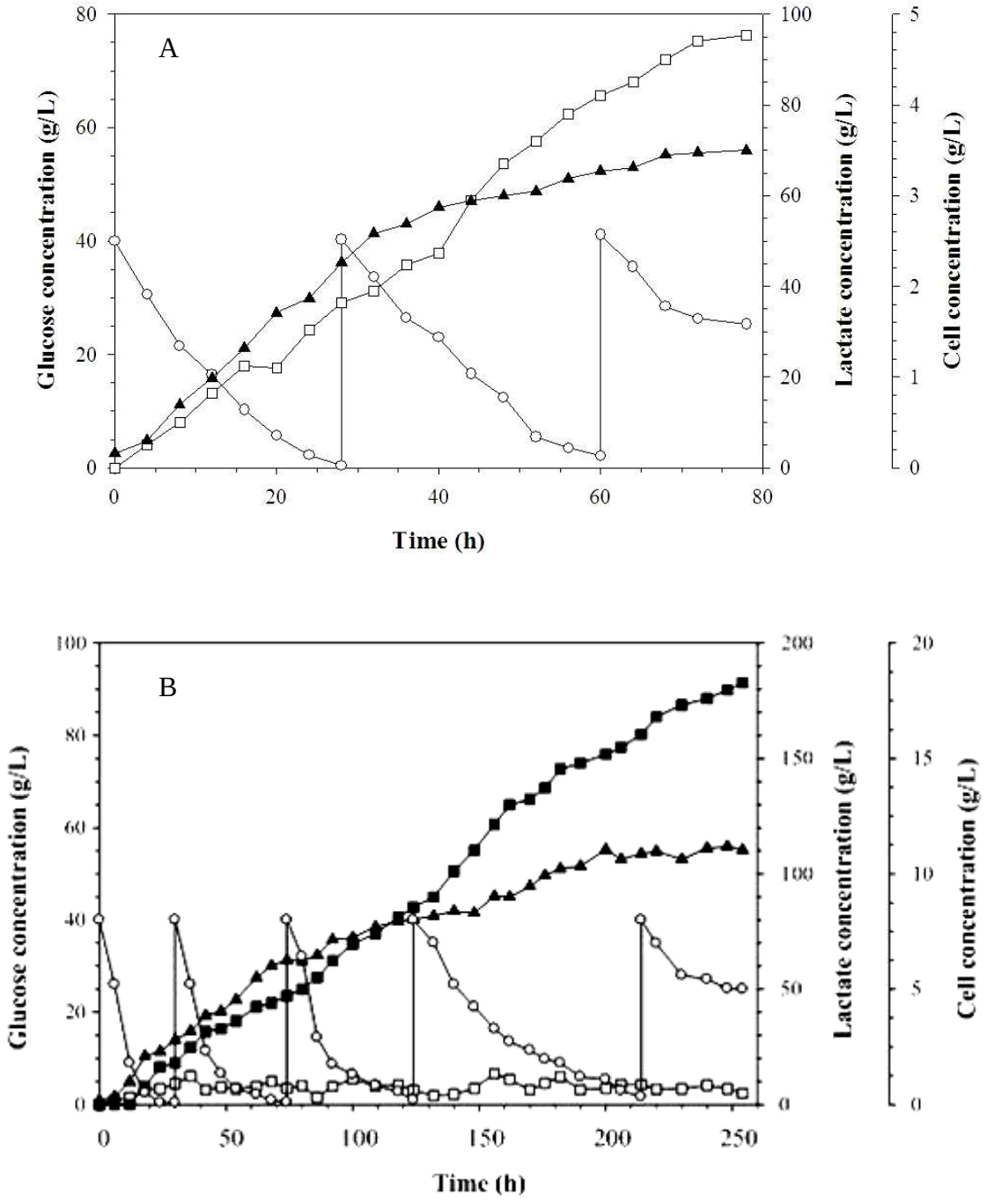


Figure 4.29 The time course of intermittent fed-batch fermentation (A) and extractive fermentation using intermittent fed-batch fermentation with partial broth removal (B) by *P. pentosaceus.* Symbols: glucose (—○—), cell (—▲—), lactate concentration in the feed side (—□—), and concentrate solution (—■—). Initial concentrate solution volume was 500 mL, applied voltage was set at 15 Volts.

The system showed that glucose was completely consumed after 4 cycles of feeding. However, the consumption time for each run was observed at 30, 75, 125, and 215 h, respectively. The 5^{th} run was not successful because glucose was utilized only 45%, and the fermentation was terminated after 250 h. During the first run, lactate concentration in fermentation broth linearly increased and the power supply was switched on at concentration approximately 15 g/L. Initially, concentrate solution contained no lactate ion, and EDI experiment was also carried out at the constant voltage mode (15 Volts). Lactate concentration in concentrate solution steadily increased with an average volumetric productivity of 0.78 g/L/h. The concentration of lactic acid was finally reached 184 g/L at the end of the operation which was almost 3 times higher than intermittent fed-batch fermentation. The concentrate solution volume increased from 500 mL at the beginning to approximately 850 mL at the end of the operation. The total amount of lactate accumulated in the concentrate solution was calculated at 156.4 g corresponding to conversion yield of 0.87 $g_{lactate}/g_{glucose}$. Moreover, the cell concentration of more than 11.0 g/L was the highest when compared with other fermentation processes. This system showed that a long continuation of fermentation activity was obtained, and the overall kinetic parameters were higher than in conventional fed-batch fermentation. In addition, 100% recovery of lactate product from fermentation broth was technically feasible which facilitated subsequent purification steps. It was concluded that these favorable results were obtained on the account of alleviating lactic acid inhibitory effect by EDI technique coupling with intermittent fed-batch strategy.

Therefore, for the purpose of alleviating this inhibitory effect, *in situ* lactic acid removal which could continuously remove produced lactic acid from the fermentation broth was applied to this fermentation process. The system could separated between biocatalyst (*P. pentosaceus*) and lactic acid in order to prevent the product inhibition. The fermentation continuously produced lactic acid while microorganism could be utilized

and grown well. In addition, lactic acid was simultaneously removed from the fermentation broth into the permeate side, so that the final product concentration increased dramatically. There was implemented together with controlled feeding of the nutrient or release of the substrate, which was also toxic to the cell, to also maintain low substrate concentration in the fermentation broth. This showed the growth associated with lactic acid production, recovery of the product allowed 95 %. This process indicated enhanced in all case in order to develop more-economic processes.

Table 4.14 The overall parameters of the intermittent fed-batch fermentation process.

Intermittent number	**Lactate concentration (g/L)**	**Dry cell weight (g/L)**	**Yield ($g_{lactate}/g_{glucose}$)**	**Volumetric productivity (g/L/h)**
0	42.08	2.26	0.91	2.50
1	70.11	2.87	0.85	1.17
2	87.24	3.10	0.71	1.12

Most of the efforts in the past were concentrated on increasing productivity and extending fermentation time, and it would not be necessary to take out fermentation broth in excess and, then, long-term continuous *in situ* lactic acid removal using EDI technique would be possible. As the result confirmed that, the EDI system could be applied reduce to the lactic acid concentration in the fermentation broth while the cell could grow and produced lactic acid as the main product of fermentation. Furthermore, having shown that the application of current could recover the pH in the fermentation chamber, leading to

increased lactic acid production, it was necessary to control the applied current such that the pH could be maintained at a suitable value. The coupling of a biological reaction with a membrane in only one unit was a very interesting configuration for the reactions where the continuous elimination of metabolites was necessary to maintain a good productivity (Moueddeb *et al.*, 1996). In addition, lowered the lactic acid concentration and therefore reduced the inhibition effect. As a result, bacteria growth and the lactic acid productivity improved correspondingly. From this finding, this study were useful for develop the lactic acid production process, which took us a step further towards creating a sustainable future.

4.8.3 Inhibition constant (k_d) studies using fluorescence techniques

Recently, a number of kinetic models for the fermentation of glucose to lactic acid have been proposed. Models including terms for both substrate and product inhibition have been suggested, as well as a model considering only product inhibition (Akerberg *et al.*, 1998). Limitation of growth and acid production by the end-product is well known. It would explain that accumulation of lactic acid in the broth inhibited fermentation, resulting in lower productivity. It is, therefore, very important to recover lactic acid from the fermentation broth to decrease inhibition of lactic acid on fermentation. Moreover, estimation of the cell viability, as opposed to the cell concentration, could be useful in the evaluation of microbial population during the fermentation processes. The viable, and non-viable discrimination of the strain was subjected to the present of inhibitory substances in the media.

The use of the fluorochrome primuline for microscopically discriminating between viable and nonviable microorganisms was investigated. In order to study the potential of the fluorescence staining technique for the enumeration of viable lactic acid

bacteria, the counts obtained by direct fluorescence microscopy were compared with those obtained using standard colony count procedure (for *P. pentosaceus*). The viable manual count using the staining technique showed good agreement with colony forming units (cfu). The plate count technique has long been a standard tool for enumeration of viable cell. The microbiological examination by this technique has depended on the dislodging of microorganism or homogeneous suspension for cultivation (Sigsgaard, 1989). For the fluorescence microscopy determination, a highly specific membranes permeable stain for DNA, 4',6-diamidino-2-phenylindole (DAPI), has been used, for example, for identifying and counting aquatic microflora, for the detection of bacteria in natural environments (Karwoski *et al.*, 1995). Moreover, estimation of the viable, as opposed to the total, number of microorganisms could be useful in the evaluation of microbial during the fermentation processes.

DAPI stained the DNA of the viable cells, making them easy to detect. DNA in cells is usually stained with DAPI for fluorescence microscopy. When stained with DAPI, the DNA appears as blue color under ultraviolet (UV) illumination, and the positions of cell nuclei and organelle nucleoids can therefore be determined (Suzuki *et al.*, 1992). On the other hand, PI was also suited for DNA staining. In this case, DNA in the cell nucleus appears red under blue excitation. Because PI binds to the nucleotide pair of guanine and cytosine, PI stains not only the DNAs but also the RNAs (the information shown in Table 4.15).

Table 4.15 Properties of the nucleic acid binding fluorescent dyes (Suzuki *et al.*, 1997).

Dye	Fluorescence	Cell nuclear DNA	Cytoplasmic RNA
Propidium iodide	Red	+++	++
DAPI	Blue	+++	-

A kinetic study of product inhibition in fermentation by *P. pentosaceus* showed that lactic acid inhibited cell growth and lactic acid production non-competitively. A kinetic study of the pH-dependence of the growth and death of *P. pentosaceus* has been reported (Figure 4.30). As a result showed staining characteristic of bacterial population at different fermentation time. Viable cells showed bright blue color whilst dead cells were spotted as red color. A more pronounced increase of the specific deactivation rate relative to lactate concentration was observed in this experiment. Hence, staining cells with fluorescent dyes was introduced because it was a powerful and reliable method. The samples were taken periodically and cell viability was assessed by counting the ratio of dead to living cells under the microscope.

The cell viables were correlated with the fermentation time showed in Figure 4.30. The cell deaths were changed linearly with time. From this Figure, the highest cell deaths were at 254 h, 156 h, 52 h, and 12 h, respectively. The accepted mechanism of inhibition by weak organic acids was related to the solubility of the non-dissociated form within the cytoplasm membrane and the insolubility of the ionized acid form. This caused acidification of the cytoplasm and the collapse of the motive force, resulted in inhibition of nutrient transport (Gatje and Gottschalk, 1991). Furthermore, cell death because of osmotic pressure caused by high lactic acid concentration also lowered the productivity of fermentation (Bulut *et al.*, 2004). It had been shown that, above a critical the concentration, the reduced water activity combined with plasmolysis caused a decrease in the rate of fermentation and sugar utilization (Roukas and Kotzekidou, 1998). The cells inability to deplete the residual lactose in the medium was an indication of lactic acid inhibition.

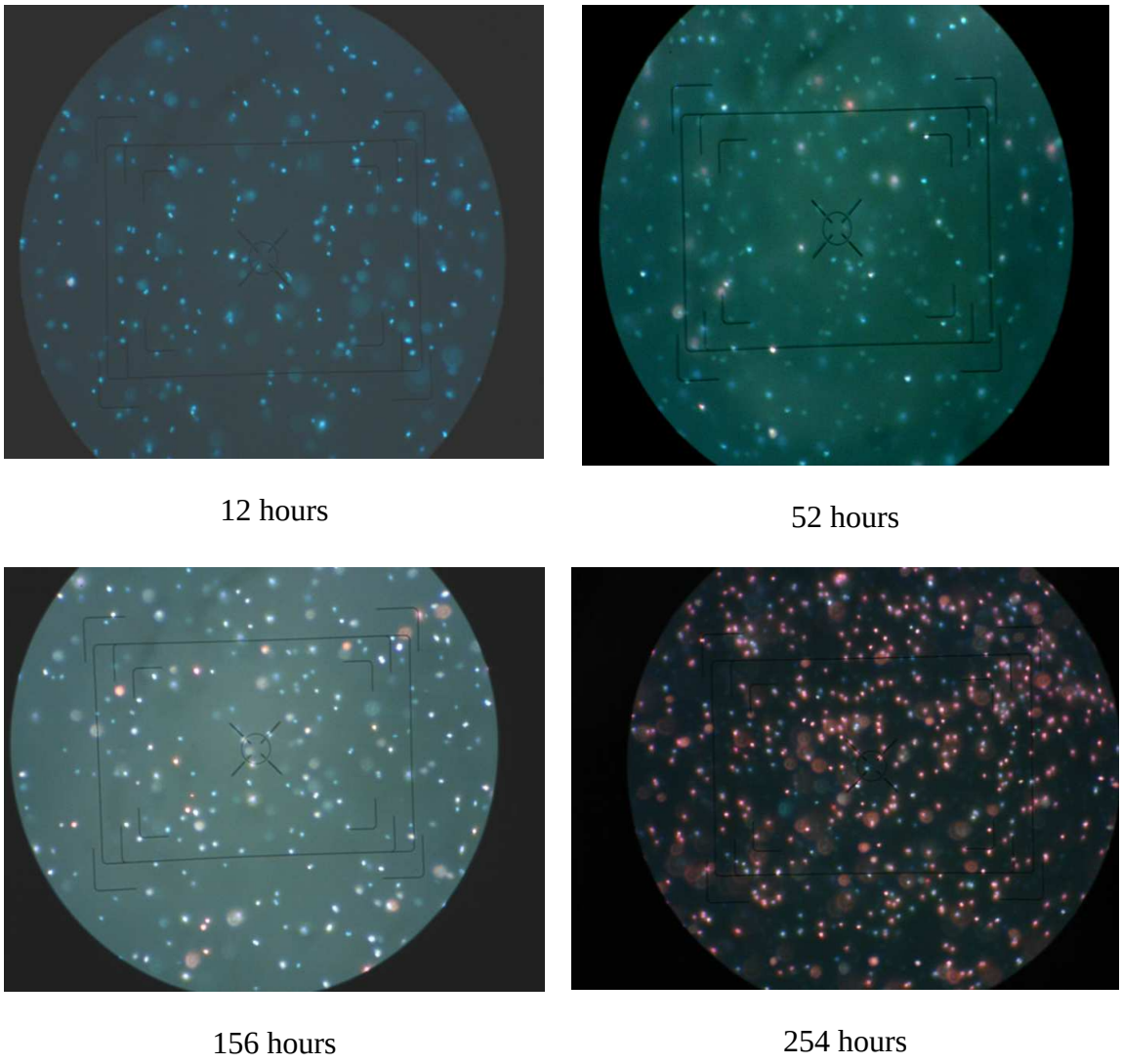


Figure 4.30 Analysis of cell viability of *P. pentosaceus* using fluorescence dyes under fluorescence microscope at different times during lactic acid fermentation.

A more pronounced decrease of the growth rate relative to the lactic acid concentration was observed during the fermentation. The inhibition effect shows that batch fermentation cannot growth was not suitable condition for growth of bacteria and production of lactic acid. This could be due to the fact that lactic acid added to the fermentation and lactic acids produced from the bacteria do not inhibit the cell growth to the same extent. A systematic investigation of the product inhibition is, therefore,

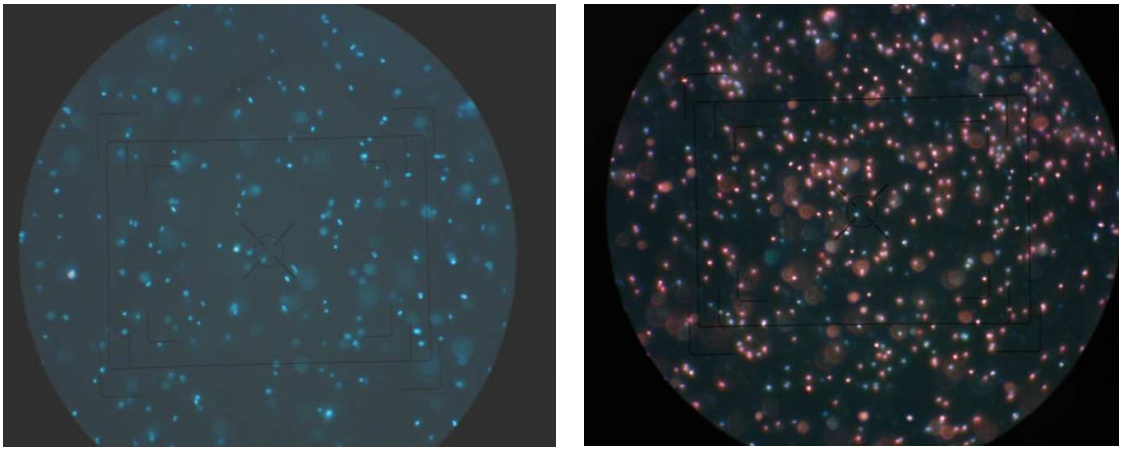
crucial for the development of an effective control strategy to improve lactic acid production and the modeling of inhibition kinetics. Furthermore, an inhibition of lactic acid is particularly important as it suggests the simultaneous removal of inhibitory product from the fermentation broth would facilitate lactic acid production. This confirmed that the possibility of using *in situ* product removal continuously for solve these problem. This system allows the continuous removal of lactic acid, and integration of this system could possibly lead to more efficient and cost-effective production of lactic acid.

4.8.4 Assessment of deactivation constant in fermentation processes

However, it is now undoubtedly proven that the undissociated form of the acid was involved in this inhibition. It is generally accepted that accumulation of lactic acid in the fermentation broth results in cell death. Previous experimental setup investigated *in situ* lactic acid removal, and found that higher production yield was obtained comparing with the fed-batch system. To quantify the viability of *P. pentosaceus*, turbidimetric methods were discounted because they did not distinguish between dead and living cells, and the presence of cell debris might interfere with the OD value. Cell viability, defined as the ability to reproduce and grow, was measured during lactic acid fermentation. Both live and dead cells were positively stained with DAPI/PI, and the bright red fluorescence was only associated with dead cells. However, bacteria which may have been kept in check during exponential and early stationary phase may "explode" as cell membrane integrity became progressively compromised or leaked and a rich carbon source for bacterial growth was released. Free pigment and bacterial growth were further considered reasons why measures of turbidity or fluorescence should not be used beyond early stationary phase as surrogate biomass indicators, or especially as indicators of culture health.

Figure 4.31 showed the dead and living cells at the beginning and the end of fermentation process. Therefore, estimation of the cell viability could be very useful in the evaluation of fermentation performance during the fermentation process. Figure 4.32 showed a kinetic study of product inhibition of *P. pentosaceus* at different fermentation processes. Experimental results showed that the strain severely suffered from undissociated lactic acid under the non-controlled pH condition. The deactivation constant (k_d) which was directly calculated from the slope of the graph revealed a high value of 0.01 h^{-1}. Activation of cells were completely stopped after 36 hours of operation. Batch fermentation with non-controlled pH was subject to either high substrate concentration or high lactic acid concentration in the fermentation broth. The main problem for this system was the severe contact between the biocatalyst with high osmotic pressure, high toxic substances especially lactic acid which resulted in a relatively short biocatalyst. In batch process with controlled pH and fed-batch process, the improvement of the fermentation performance was evidenced by a reduced value of deactivation constant resulting in prolonged fermentation time. Cell viability was almost 100% at the beginning of fermentation before the value gradually reduced after 15 h. However, the value sharply declined at the end of fermentation was probably due to substrate starvation. The experimental results of fed-batch showed a significant reduction in deactivation compared with batch processes. From the results, it was obvious that deactivation constant of the cells was at its minimum value since cells still possessed some growth. Repeated substrate feeding enabled the cells to multiply, and the substrate starvation was avoided. However, the system was still vulnerable to end-production inhibition. Experimental results showed that cell viability could be divided into 2 distinct phases. During the first 40 h of operation, cell viability remained almost 100%, therefore the value rapidly reduced until the end of fermentation. Compared with Figure 4.31, critical concentration of lactic acid which resulted in the severe deactivation was

observed at concentration exceeded 80 g/L. The application of this fed-batch system could greatly enhance economic viability of commercial lactic acid production.



Initial fermentation Final fermentation

Figure 4.31 Analysis of cell viability of *P. pentosaceus* using fluorescence dyes under fluorescence microscope during lactic acid fermentation.

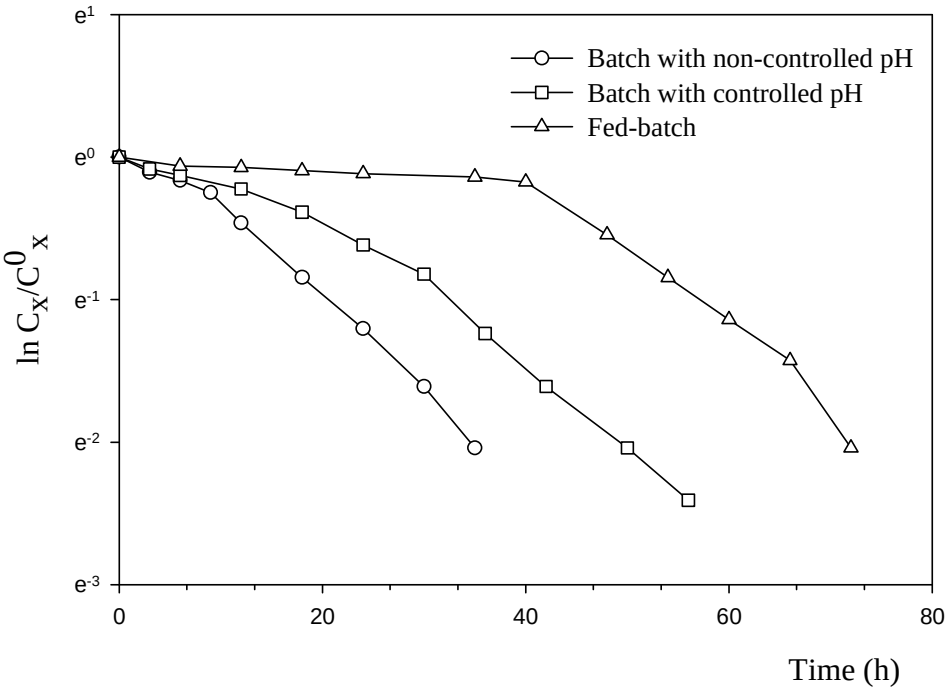


Figure 4.32 Time course of cell viability during different fermentation process of *P. pentosaceus*.

Figure 4.33 reveals the relative viability of *P. pentosaceus* during the intermittent fed-batch fermentation compared with intermittent fed-batch coupling with *in situ* lactate removal. This technique was successfully attempted to minimize the effect of product inhibition by reduce the deactivation constant to 0.0054 h^{-1} with the estimated biocatalyst half-life of approximately 128.4 h. During intermittent fed-batch fermentation, *P. pentosaceus* growth decreased when the concentration of acid in the medium increased but fermentative activity was not entirely inhibited until critical level of lactate concentration was reached. The rate of lactate accumulation within the bioreactor, and certain kinetic parameters were simultaneously determined in such fermentative processes. *In situ* lactic acid removal coupling with fermentation showed the improvement of the specific death rate decreased from 0.026 h^{-1} to 0.0054 h^{-1}, respectively. This process confirmed that the problems associated with product inhibition could be minimized resulting in a reduced specific death rate. Despite attempts on the reduction of product toxicity effect, the experimental result revealed approximately 40% of deactivation constant lower than intermittent fed-batch fermentation. Since one of the problems encountered in lactic acid fermentation was the short biocatalyst lifetime, the manipulation of direct feeding of the substrate effectively reduced substrate toxicity, whereas *in situ* product removal using the EDI technique demonstrates that this particular problem could be minimized, and thus the economical viability of lactic acid fermentation can be substantially enhanced. As a result, the application of this system could consequently enhance economic viability of commercial scale production.

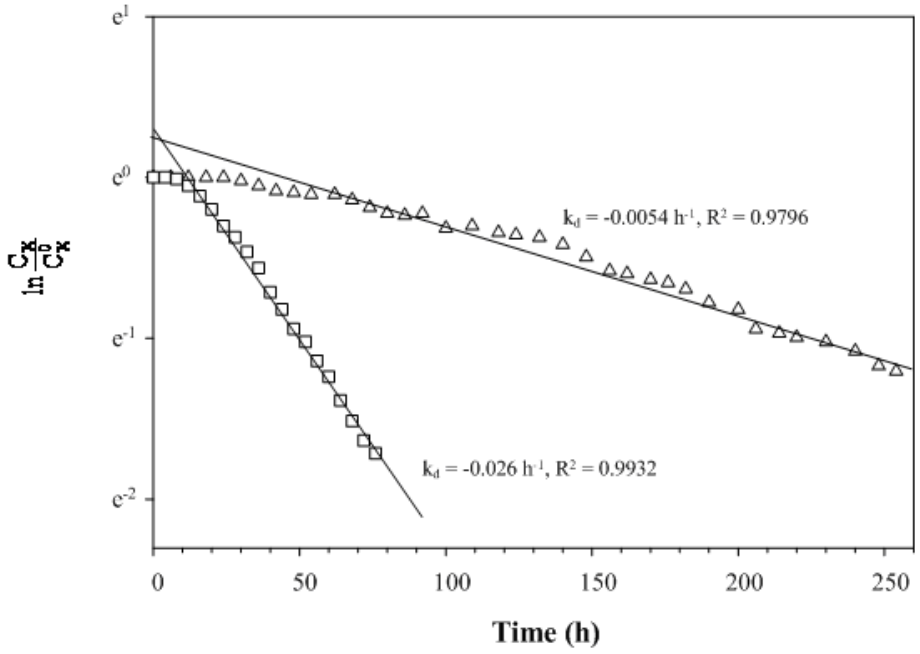


Figure 4.33 Comparison of deactivation constants of *P. pentosaceus* in intermittent fed-batch fermentations with (△) and without EDI technique (□).

Table 4.16 showed some of the kinetic parameters of the fermentation processes obtained from this study. The result of *in situ* removal coupling with fermentation showed the improvement of the profile that the specific growth rate, specific cell death rate increased from 0.37 to 0.47 h^{-1}, and 0.091 to 0.0054 h^{-1}, respectively. The maximum of cell number were 6.7 x 10^8 cells/mL during fed-batch process with continuously lactic acid removal. However, in the arrangement of cell cultivations presented enabling continuous growth during the cultivation. The parallel fermentations have been done. Moreover, optimal exploitation of them required specific conditions of use and further, more detailed studied of their technological properties.

Table 4.16 Some kinetic parameters of the fermentation processes.

Parameters	**Batch process**	**Fed-batch process**	***in situ* product removal**
Specific growth rate (μ), h^{-1}	0.37	0.41	0.47
Specific cell death rate (k_d), h^{-1}	0.091	0.026	0.0054
Initial cell number (cells/mL)	1.6×10^6	2.3×10^6	2.1×10^6
Maximum cell number (cells/mL)	1.8×10^8	2.0×10^8	6.7×10^8

This process demonstrated that the problems associated with product inhibition could be minimized resulting in a reduced death rate. Because lactic acid concentration in the fermentation broth was kept low, this results in higher final concentration, and production yield. As a result, the application of this system could consequently enhance economic viability for commercial production. Despite attempts on the reduction of substrate and product toxicity effect, the experimental result revealed only approximately 40% of deactivation constant lower than fed-batch fermentation. The experimental result was not as expected, and the reason for this was unclear. However, further investigation of such effect to the bacteria cells was far beyond the scope of this study. Since one of the problems encountered in lactic acid fermentation was the short biocatalyst lifetime (Boontawan and Stuckey, 2006), the manipulation of direct feeding of the substrate effectively reduced substrate toxicity, whereas *in situ* product removal coupling with the EDI technique demonstrated that this particular problem could be minimized, and thus the economical viability of lactic acid fermentation could be substantially enhanced.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Lactic acid obtained by biotechnological process is preferred for industrial applications, especially, bioplastic industry. Lactic acid bacteria are good organisms for lactic acid fermentation. *P. pentosaceus* is a homo-fermentative LAB used extensively in this study. It exhibited more than 90 % lactic acid production yield which is desirable for industrial application. The intention of the present investigation was to study growth and lactic acid production at different environments, temperatures, and pH values as well as media compositions, etc. The improvements of lactic acid production for industrial purposes were achieved in this research.

The nutrients traditionally used in most of the fermentative media, particularly yeast extract and peptone, are very expensive. Alternative nutrient sources are particularly interesting. The efficiency of this process could be improved within limits by varying culture conditions and medium composition. Cassava starch and its product as carbon source and spent brewer's yeast extract as nitrogen source were used in this work because they can be effectively used to reduce the nutrients cost by 80%, and readily available. Fermentation performances of lactic acid production in the presence of these nutrients were performed, and the results showed acceptable improvement. The use of renewable resources opens the ways in a dual working manner for value adding through an eco-friendly green technology. This study suggested that hydrolyzed cassava starch could be supplied as a useful basis for lactic acid production by *P. pentosaceus*.

Conventional batch processes for lactic acid production commonly suffered from reduced cell growth and productivity due to substrate and product inhibition which was estimated from the model at 210 g/L and 80 g/L, respectively. Exponential fed-batch mode provides better advantage over batch mode in term of alleviated substrate inhibition effect. The substrate concentration was maintained at the optimum level during cultivation resulting in a reduction of specific death rate. Traditional product recovery processes required high energy input. In this work, a complete downstream (from the fermentation) process based on combined EDI steps for purification/concentration and conversion was proposed. Generally, traditional processes were based on precipitation steps that generate large amounts of chemical effluents. Therefore, the environmental impact and the operating costs of traditional precipitation processes could be reduced by using to alternative technologies, such as EDI technique.

Recovery of lactate from fermentation broth was investigated in order to pave the way for applying in the real fermentation system. The influence of current density, and lactate concentration on separation performance were investigated. The performance of the EDI system was stable during 250 h of operation at the constant voltage of 15 Volts. This continuous EDI technique was successfully applied for lactate recovery from the clarified fermentation broth, and subsequent application for *in situ* product was introduced. The recovery ratio of nearly 100% was obtained at the end of the process. As a result, the application of this system could consequently enhance economic for efficient commercial lactic acid production.

The introduction of fluorescence microscopy was a suitable procedure for cell viability providing a useful tool for studying cell response to operating conditions. *In situ* lactic acid removal coupling with fermentation showed the 40% improvement of the deactivation constant decreased from 0.026 h^{-1} to 0.0054 h^{-1}, respectively. Because lactate

concentration in the fermentation broth was kept low, this resulted in higher final concentration, and production yield. In conclusion, *in situ* product removal methods address the limitations by selectively removal of lactic acid from the vicinity of the biocatalyst as soon as it was formed, and also provide further benefits for the subsequent downstream processes. It was hoped that this study, which provided preliminary insight into the efficiency of various operational modes of bioreactors, would lead to more comprehensive studies using different biological systems.

Recommendation for further studies

For purification of lactic acid, different techniques had been introduced; such as, solvent extraction, adsorption, direct distillation, electrodialysis, electrodeionization and esterification (Joglekar *et al.*, 2006). However, such purification procedures were difficult because of the low volatility of lactic acid (122 °C at 166.73 Pa), with its affinity to water, and its tendency to self-polymerize. The esterification was the only downstream process, which separated other organized impurities from lactic acid (Joglekar *et al.*, 2006). The lactic acid purification process using esterification and hydrolysis was highly encouraging. Esterification of lactic acid and ethanol could be studied in well-mixed reactors coupling with reactive distillation. The production process involved esterification of lactic acid with alcohols to produce ethyl lactate, distillation of lactate ester and hydrolysis of the distillated lactate ester to yield alcohol and lactic acid.

High purity lactic acid could be also produced by esterification of crude lactic acid with alcohols, distillation of ester, hydrolysis of the distillated lactate ester to yield the alcohol and lactic acid (Joglekar *et al.*, 2006). Esterification was the only downstream process, which separated other organic acids from lactic acid. Esterification gave esters of lactic acid, and further hydrolysis of esters was necessary to get the product as pure lactic

acid. Simultaneous distillation with esterification-hydrolysis was called reactive distillation. Fermented broth containing lactic acid needs to be pretreated to remove some impurities before reactive distillation using resin beads as catalyst as well as bead for distillation column. Expected results of this technique are:

1. High purity L-(+)-lactic acid would be produced using the combination of pervaporation and esterification technique and optimal operating conditions would be obtained.

2. This purification system could be applied for industrial lactic acid production with improved yield of lactic acid and reduced production cost.

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APPENDICES

APPENDIX A

1. The production cost of different nitrogen source used for lactic acid production.

Table 1A The production cost of different nitrogen source used for lactic acid production (Tellez-Luis *et al.*, 2003).

Nitrogen sources	Prices (Euro per kg)
Corn steep liquor	36.06
Yeast extract	76.74
Peptone	112.27
Sodium acetate	13.94
Sodium citrate	20.73
K_2HPO_4	30.29
$MgSO_4$	10.58
$MnSO_4$	15.03
Fe_2SO_4	11.54

APPENDIX B

Chromatograms of organic acid analysis by HPLC.

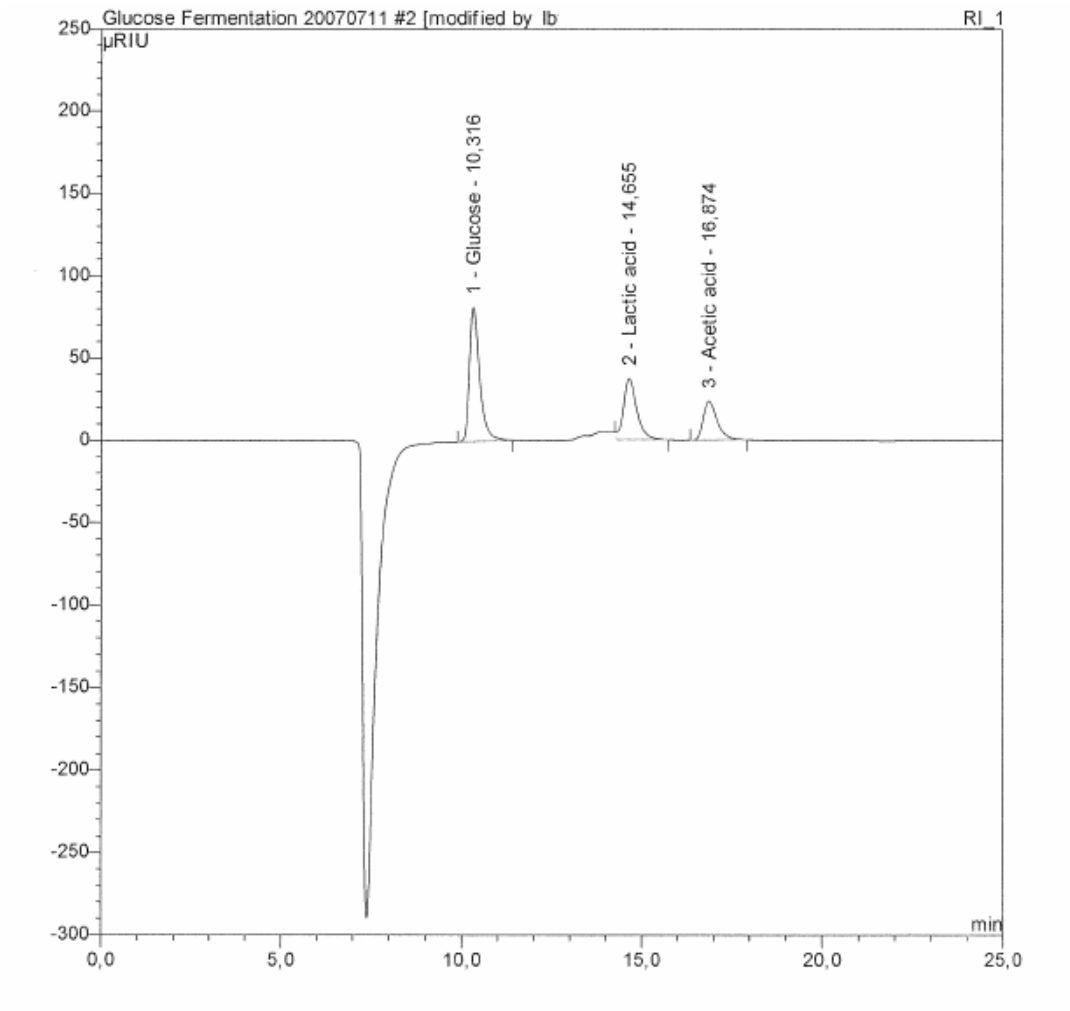

Figure 1B Representative chromatograms showing results of the standard of substances (glucose and organic acid which presence in the fermentation broth) obtained from HPLC. Negative result represented water content.

APPENDIX C

1. Standard curve of dry cell weight

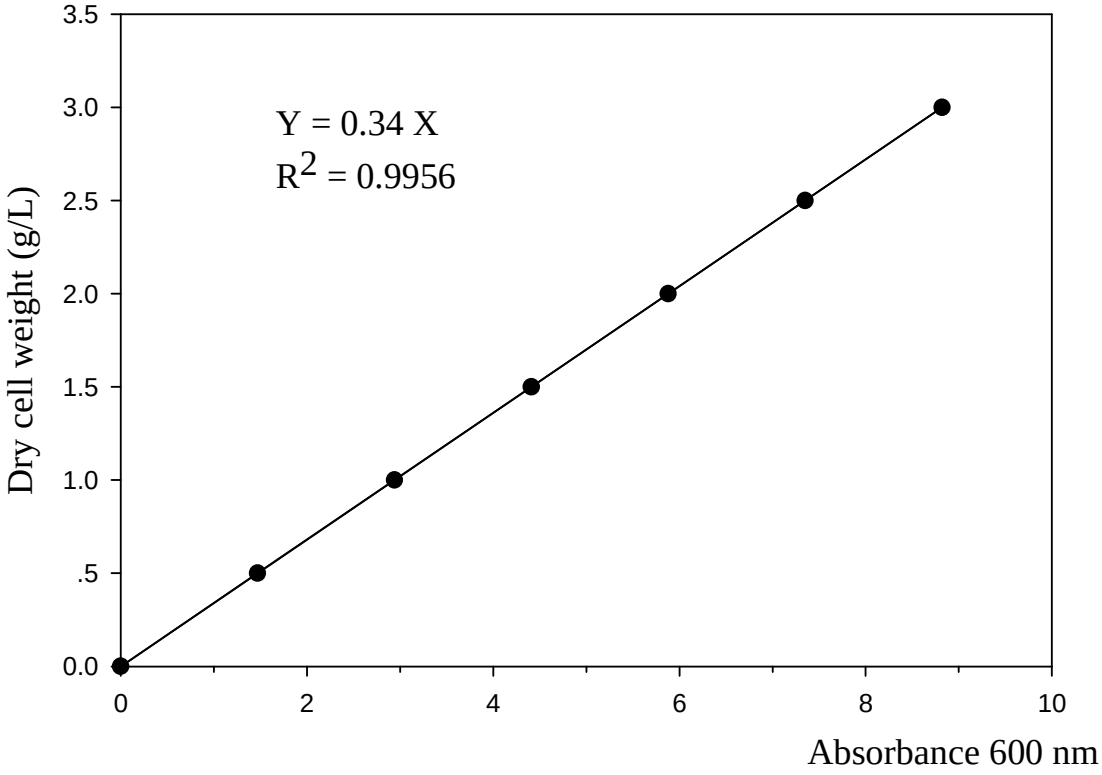

Figure 1C Standard curve of dry cell weight concentration of *P. pentosasceus* at a wavelength of 600 nm.

2. Standard curve of reducing sugar

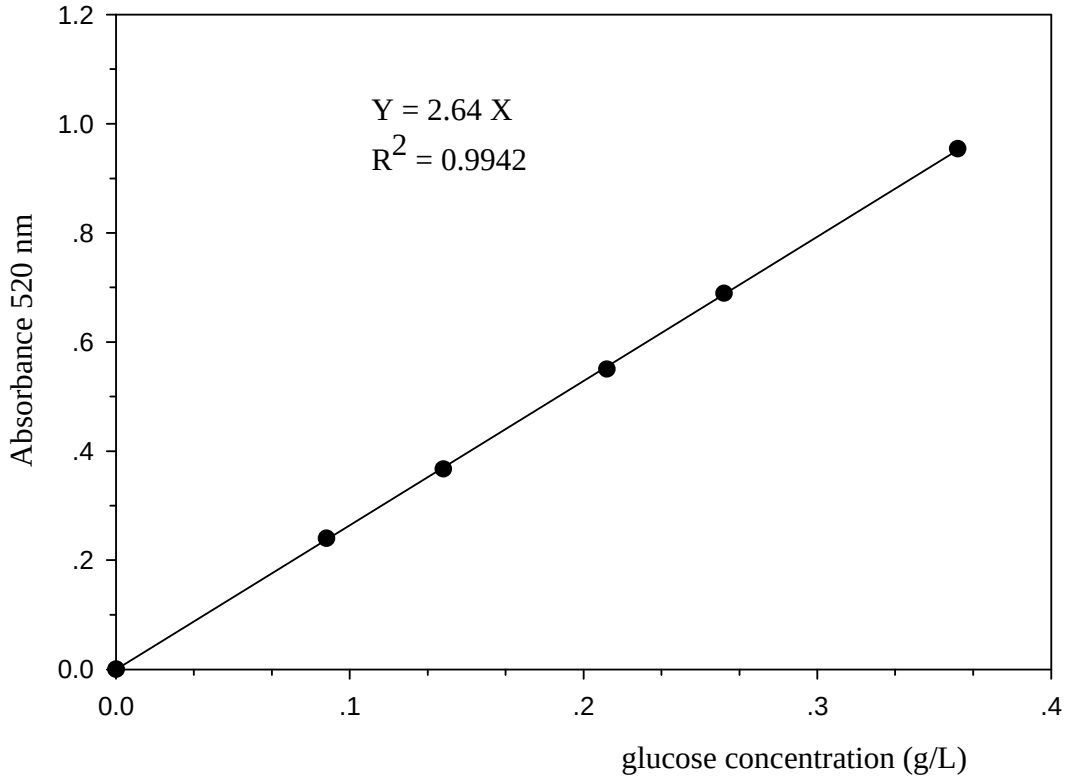

Figure 2C Standard curve of reducing sugar concentration by DNS method analysis.

3. Chromatogram for chiral separation by HPLC

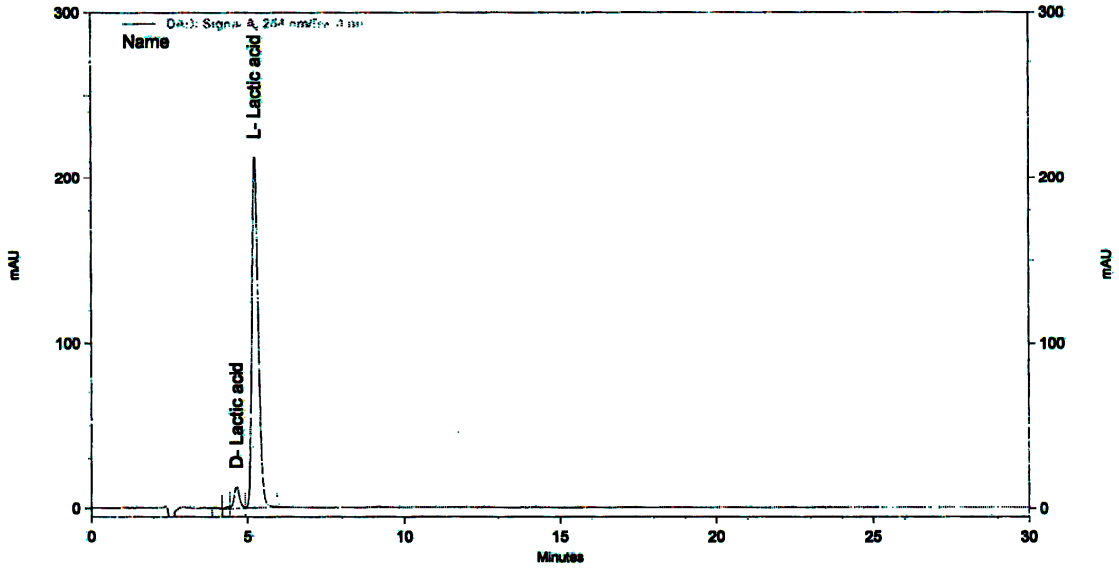

Figure 3C Image of HPLC chromatogram for chiral separation (DAD detector) shows more than 95% optical purity of purified L-(+)-lactic acid. No other organic acid impurities were detected**.**

APPENDIX D

Publication

Title: Extractive fermentation of L-(+)-lactic acid by *Pediococcus pentosaceus* using electrodeionization (EDI) technique

Authors: Pailin Boontawan, Sunthorn Kanchanathawee, Apichat Boontawan

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CURRICULUM VITAE

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Publication

Boontawan, P., Kanchanathawee, S., and Boontawan, A. (2011). Extractive fermentation of L-(+)- lactic acid by *Pediococcus pentosaceus* using electrodeionization (EDI) technique. **Biochemical Engineering Journal**, DOI: doi:10.1016/j.bej.2011.02.021**.**

Research presentation experience

Boontawan, P., Haltrich, D., and Kanchanatawee, S. (2008). Utilization of cassava starch supplementing with brewer's yeast extract as nutrient source for possible commercial L (+)-lactic acid production. **TSB: Biotechnology for Global Care.** 14^{th}-17^{th} October 2008, Taksila Hotel, Taksila Hotel, Mahasarakham, Thailand. (Oral Presentation).