Development of Chromatographic Methods to Investigate the Purification Process of Lactic Acid from Lactic Acid Bacteria

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ชื่อวิทยานิพนธ์ การพัฒนาเทคนิคโครมาโตกราฟี เพื่อใช้ติดตามกระบวนการทำบริสุทธิ์กรดแล็คติก จากน้ำหมักของเชื้อแบคทีเรียที่ผลิตกรดแล็คติก
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บทคัดย่อ

กรดแล็คติกเป็นผลิตภัณฑ์หลัก ที่ได้จากกระบวนการเมตาบอลิซึมของสารประกอบคาร์โบไฮเดรตในเชื้อแบคทีเรียสายพันธุ์ที่สามารถสร้างกรดแล็คติกได้ เช่น *Lactobacillus* และ *Streptococcus* กรดแล็คติกถูกใช้อย่างกว้างขวางในการเป็นสารตั้งต้นสำหรับการผลิตไบโอพอลิเมอร์ เพื่อใช้ในกระบวนการทางวิศวกรรมเนื้อเยื่อและการนำส่งยา ในกระบวนการเพาะเลี้ยงเชื้อแบคทีเรียเพื่อให้สร้างกรดแล็คติกนั้น อาจเกิดผลิตภัณฑ์กรดอื่นๆในปริมาณน้อยได้เช่นกัน ได้แก่ กรดอะซิติก และ กรดบิวทิ ริก ซึ่งเป็นปัญหา กับการทำบริสุทธิ์กรดแล็คติก ทำให้ไม่สามารถแยกกรดแล็คติกให้บริสุทธิ์โดยการกลั่นแยกแบบธรรมดาได้ จึงต้องนำน้ำหมักมาทำปฏิกิริยา esterification เพื่อเปลี่ยนผลิตภัณฑ์กรดทั้งหมดให้เป็นเอสเตอร์ของกรดก่อน ซึ่ง นอกจากจะช่วยให้ผลิตภัณฑ์ที่ได้มีจุดเดือดที่ต่ำลงแล้ว จุดเดือดของเอสเตอร์แต่ละชนิดยังแตกต่างกันมากกว่าเมื่ออยู่ในรูปของกรดด้วย เป็นผลให้การกลั่นแยกทำได้ง่ายขึ้น

ในการประเมินประสิทธิภาพของกระบวนการทำบริสุทธิ์กรดแล็คติกนั้น ได้ทำการพัฒนาวิธีวิเคราะห์เชิงปริมาณโดยอาศัยเทคนิคแก๊สโครมาโตกราฟี ที่สามารถวิเคราะห์ได้ทั้งกรดแล็คติก กรดอะซิติก กรดบิวทิ ริก และ เอธิลแล็คเตต โดยใช้คอลัมน์ SGE BP-20 ขนาดยาว 30 เมตรและมีเส้นผ่าศูนย์กลาง 0.53 มิลลิเมตร ความหนาของชั้นฟิลม์ 0.5 ไมครอน ใช้เครื่องตรวจวัดชนิด flame ionized detector ใช้ก๊าซฮีเลียมเป็นก๊าซตัวนำที่ความดัน 0.5 กิโลกรัมต่อตารางเซนติเมตร โปรแกรมอุณหภูมิที่ใช้ เริ่มต้นที่อุณหภูมิ 50 องศาเซลเซียส คงอุณหภูมินี้ไว้ 3 นาที ก่อนเพิ่มอุณหภูมิเป็น 110 และ 200 องศาเซลเซียส ด้วยอัตราการเพิ่มของอุณหภูมิที่ 10 และ 12 องศาเซลเซียสต่อนาทีตามลำดับ การประเมินความถูกต้องของวิธีวิเคราะห์ในหัวข้อ ความเป็นเส้นตรง ความแม่นยำ ความถูกต้อง และ limit of detection และ quantification (LOD และ LOQ) พบว่า % recovery ของการวิเคราะห์กรดทั้ง 3 ชนิด และเอธิลแล็คเตต อยู่ในช่วง 98.0 – 102.8%

กราฟมาตรฐานของสารทั้ง 4 ชนิด ให้ค่าความเป็นเส้นตรงที่ดี โดยมีค่า R^2 มากกว่า 0.999 ระบบดังกล่าวยังมีค่า LOD และ LOQ ของกรดอะซิติก และกรดบิวทิริก ที่ 0.01 และ 0.10 ไมโครกรัมต่อมิลลิลิตร ส่วน กรดแล็คติกที่ 0.10 และ 1.00 ไมโครกรัมต่อมิลลิลิตร และเอธิลแล็คเตตที่ 0.10 และ 0.50 ไมโครกรัมต่อมิลลิลิตร นอกจากนั้นระบบดังกล่าวยังมีความจำเพาะเจาะจง และความเที่ยงสูง โดยที่ค่า % RSD ของการวิเคราะห์ภายในวันเดียวกันและระหว่างวันน้อยกว่า 5%

ในส่วนของกระบวนการเพาะเลี้ยงเชื้อแบคทีเรียนั้น ได้ทำการพัฒนาวิธีวิเคราะห์โดยเทคนิค high-performance liquid chromatographic (HPLC) เพื่อใช้ในการวิเคราะห์หาปริมาณน้ำตาล ได้แก่ กลู โคส ฟรุ กโตส และ แล็คโตส เพื่อ ประเมินประสิทธิภาพของเชื้อแบคทีเรียในการสร้างกรดแล็คติก วิธีวิเคราะห์ที่พัฒนาขึ้นนั้นประกอบด้วยการใช้คอลัมน์ชนิดอะมิโนคอลัมน์ (Zorbax®) (250 × 4.6 มิลลิเมตร; ขนาดอนุภาค 5 ไมครอน; USA) ที่อุณหภูมิ 30 องศาเซลเซียส ใช้สารละลายผสมของ Acetonitrile และน้ำที่บริสุทธิ์สูงในอัตราส่วน 78 ต่อ 22 โดยปริมาตร เป็นเฟสเคลื่อนที่ มีอัตราการไหล 1.5 มิลลิลิตรต่อนาที ปริมาตรที่ฉีด 20 ไมโครลิตร และตรวจวัดสัญญาณโดยใช้ refractive index detector ที่อุณหภูมิ 35 องศาเซลเซียส จากการประเมินความถูกต้องของวิธีวิเคราะห์พบว่า % recovery ของการวิเคราะห์น้ำตาลทั้ง 3 ชนิด อยู่ในช่วง 91.5 – 109.4% กราฟมาตรฐาน ให้ค่าความเป็นเส้นตรงที่ดี โดยมีค่า R^2 มากกว่า 0.9980 ระบบดังกล่าวให้ค่า LOD และ LOQ ของน้ำตาลกลูโคสและฟรุกโตสที่ 0.0625 และ 0.25 มิลลิกรัมต่อมิลลิลิตร ส่วนน้ำตาลแล็คโตสที่ 0.50 และ 4.00 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้ ระบบดังกล่าวยังมีความจำเพาะเจาะจง และความเที่ยงสูงโดยที่ ค่า % RSD ของการวิเคราะห์ภายในวันเดียวกันและระหว่างวันน้อยกว่า 10%

วิธีวิเคราะห์ทั้ง 2 เทคนิคที่ได้พัฒนาขึ้นจะนำไปใช้เพื่อติดตามกระบวนการทำบริสุทธิ์กรดแล็คติก ตั้งแต่การวิเคราะห์หาปริมาณน้ำตาลที่เชื้อใช้เป็นอาหาร จนถึงการแยกได้กรดแล็คติกที่บริสุทธิ์ ซึ่งพบว่าเป็นวิธีที่เที่ยงตรง แม่นยำ และมีความจำเพาะเจาะจงสูง นอกจากนี้ยังได้นำวิธีวิเคราะห์ที่พัฒนาได้ไปใช้วิเคราะห์ หาปริมาณน้ำ ตาลที่ใช้ไปเพื่อผลิตกรดแล็คติกและกรดแล็คติกที่ถูกสร้างขึ้นจากเชื้อ *Streptococcus mutans* สายพันธุ์ดั้งเดิมและสายพันธุ์ปรับปรุง (SMP และ SML) จากผลการทดลองแสดงให้เห็นว่าวิธีวิเคราะห์ที่พัฒนาขึ้นนั้น สามารถใช้วิเคราะห์ และติดตามกระบวนการผลิตกรดแล็คติ กบริสุทธิ์ได้จริง และ เหมาะสมที่จะนำไปใช้ในระดับอุตสาหกรรมต่อไป

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ABSTRACT

Lactic acid is a main product from carbohydrate metabolism of lactic acid bacteria, including bacteria of the species *Lactobacillus* and *Streptococcus.* It is widely used as substrate of biopolymer production, applicable for tissue engineering and drug delivery systems. Besides lactic acid, minute amounts of other acids such as acetic acid and butyric acid are co-produced in the culture broth. This causes difficulty on lactic acid purification by normal distillation because they are slightly different in vapor pressure. Conversion of the acids into the esters form not only increases such the different intervals, but also lowers their boiling points as compared to those of the acids.

To evaluate process efficiency on lactic acid purification, a new method of gas chromatography (GC) was developed for monitoring yields of ester conversion and of lactic acid in the final product. The GC capillary column was SGE BP-20 (30 m × 0.53 mm; film thickness 0.5 μm) coupled with flame ionized detector using helium as a carrier gas at a flow rate of 0.5 kg/cm^2. The temperature program was initiated at 50 °C for 3 minutes, followed by increasing to 110 and 200 °C with the rates of 10 and 12 °C/min, respectively. Method validation parameters of linearity, precision, accuracy and limit of detection and quantification (LOD and LOQ) were determined. Results showed that the accuracy ranged between 98.0 – 102.8% with $R^2 > 0.9990$ for all of the acids tested and ethyl lactate. LOD and LOQ values 0.01 and 0.10 μg/mL for acetic acid and butyric acid, 0.10 and 1.00 μg/mL for lactic acid and 0.10 and 0.50 μg/mL for ethyl lactate, respectively. Precision values (% RSD) of each acid and ethyl lactate were not more than 5%. Very low concentrations of the acid could be detected with high degree of specificity.

In the part of culturing process, high-performance liquid chromatographic (HPLC) method was developed for determining of sugar utilization. The sugar resources were glucose, fructose and lactose. The developed HPLC conditions were as followed: Zorbax® NH_2 column (250 × 4.6 mm; 5 µm particle size; USA) at 30 °C, acetonitrile and Milli-Q grade water (78 : 22, v/v) as the mobile phase with a flow rate of 1.5 mL/min, 20 µL injection volume, and refractive index as a detector with a set temperature of 35 °C. Method validation parameters including linearity, precision, accuracy LOD and LOQ were determined. Results showed that the accuracy ranged between 91.5 – 109.4% with R^2 > 0.9980 for all of the sugars. LOD and LOQ were respectively of 0.0625 and 0.25 mg/mL for both glucose and fructose and 0.50 and 4.00 mg/mL for lactose. Precision value (% RSD) of each sugar was not more than 10%.

The developed GC and HPLC methods were useful for determining of acids, acid esters and sugar utilization during the lactic acid purification process. It was found that these methods exhibited high % recovery and resolution. The newly established methods were of capable to determine sugar utilization and lactic acid production from the wild type and the mutants of *Streptococcus mutans* as well. It might be concluded that, the newly established methods could be used for determining and monitoring lactic acid production and lactic acid purification process on large scale manufacturing.

CONTENT

CONTENT (continued)

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

AA	Acetic acid
ACN	Acetronitrile
ATP	Adenosine tri-phosphate
ζ-	alpha-
α	Selectivity factor
BA	Butyric acid
BHI	Brain Heath Infusion
bp	boiling points
η-	beta-
cm	centimeter
ºC	degree Celcius
EL	Ethyl lactate
EMP	Emden-meyerhof-Parnas pathway
FTIR	Fourier transform infrared spectroscopy
g	gram
GAP	Glyceraldehyde phosphate
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IS	Internal standard
kg	kilogram
kg/cm^2	kilogram per square centimeter
kV	kilovolt
L	liter
LA	Lactic acid
LAB	Lactic acid bacteria
LOD	Limit of detection
LOQ	Limit of quantification
m	meter

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

min	minute
mM	millimolar
M	molarity
σg	microgram
σL	microliter
σm	micrometer
mg	milligram
mL	milliliter
mm	millimeter
MS	Mass spectroscopy
N	normality
NADH	Nicotinamide adenine dinucleotide
ng	nanogram
nm	nanometer
Pa	Pascal
pKa	Acid dissociation constant
PLA	polylactic acid
PLGA	poly (lactic-co glycolic) acid
Rs	Resolution
R^2	Coefficient of determination
rpm	rounds per minute
RSD	Relative standard deviation
SD	Standard deviation
t_m	Elution time of solvent
t_r	Retention time
v/v	volume by volume
w/v	weight by volume

CHAPTER 1

INTRODUCTION

1.1 Background

Lactic acid (LA) is mainly produced by carbohydrate metabolism of lactic acid bacteria, including bacteria of the species *Lactobacillus* and *Streptococcus*. It can be produced naturally or synthetically. Commercial lactic acid is produced naturally by fermentation of carbohydrates such as glucose, sucrose, or lactose by *Lactobacillus* bacteria, among others. It is used in many applications such as meat and poultry preservation, cosmetics, oral and health care products and baked goods. In medicine, lactate is one of the main components of Ringer's lactate or lactated Ringer's solution. This intravenous fluid consists of sodium and potassium cations, and lactate and chloride anions, in solution with distilled water in isotonic concentration compared to human blood. It is most commonly used for fluid resuscitation after blood loss due to trauma, surgery, or burn injury (Siggaard-Andersen and Gothgen, 1995). Two molecules of lactic acid can be dehydrated to lactide, a biodegradable polyester with valuable medical properties and currently attracting much attention.

The genus of *Lactobacillus* is divided into three groups based on fermentation patterns: homofermentative, producing more than 85% lactic acid from glucose; heterofermentative, producing only 50% lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide; and the less well known heterofermentative species which produce DL-lactic acid, acetic acid and carbon dioxide. *Lactobacillus crispatus* 21L07 has been identified as homofermentative bacteria (Kaewsrichan *et al*., 2006). It produces lactic acid and minute amounts of other acids including acetic acid and butyric acid have been generally co-produced. For purification of lactic acid, the solvent extraction (Li *et al*., 2004), adsorption (Choi *et al*., 2002), direct distillation (Cockrem and Johnson, 1993) and electrodialysis (Saavedra and Barbas, 2002) have been conventionally adopted. However, such purification procedures have been challenged

by the low volatility of LA (122 °C at 1661.73 Pa), with its affinity to water, and its tendency to self-polymerization. Distillation is a popular method for purification of lactic acid in large scale manufacturing, but the boiling points of lactic acid and other acids which co-produced are too high and close to each other. Conversion of these acids into the ester forms by reacted with suitable alcohol makes them easily to separate from the others by direct distillation. This is because esterification lowers their boiling points and increases, such difference. This ensures to obtain high purity of lactic-ester, and after hydrolysis process the high purity of lactic acid will be obtained (Sanz *et al*., 2004; Wei and Tong, 2004).

In lactic acid production and purification, chromatographic methods have been often used to evaluate efficiency and monitor effectiveness of all processes involved. In this study, HPLC method was developed and used to determine sugar consumption by bacteria during lactic acid production via bacterial fermentation. GC method was established to quantify amount of acids and acid esters throughout the purification process. The developed chromatographic methods may be useful for routinely determining and monitoring lactic acid production and purification in industry.

1.2 Objectives

1) To develop HPLC analytical method for simultaneous determination of sugars in lactic acid bacteria culture broths.

2) To develop GC analytical method for simultaneous determination of acetic acid, butyric acid, lactic acid and ethyl lactate in lactic acid purification processes.

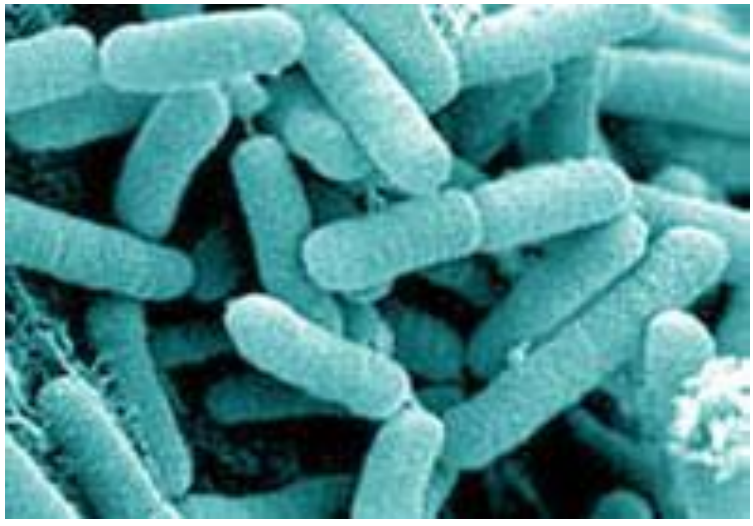
3) To evaluate the effectiveness of lactic acid producing bacteria by determining of sugar utilization and lactic acid production with the developed chromatographic methods.

CHAPTER 2

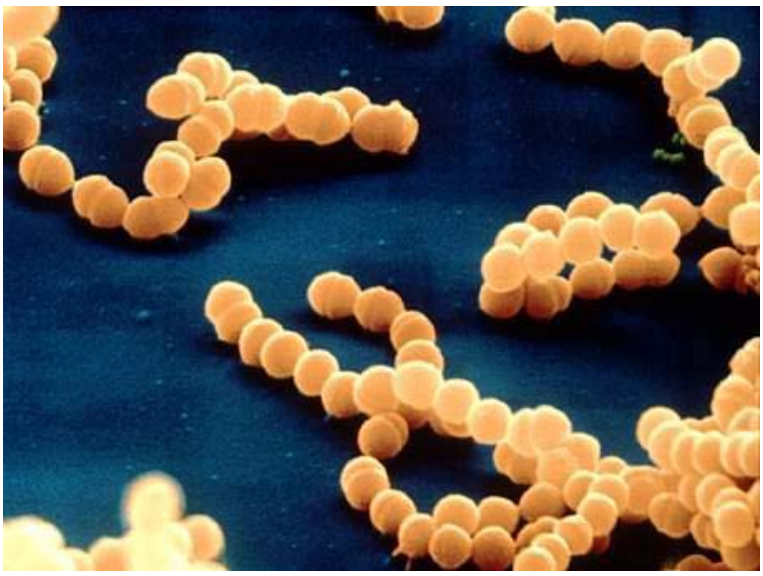
REVIEW OF LITERATURES

2.1 Description of lactic acid bacteria

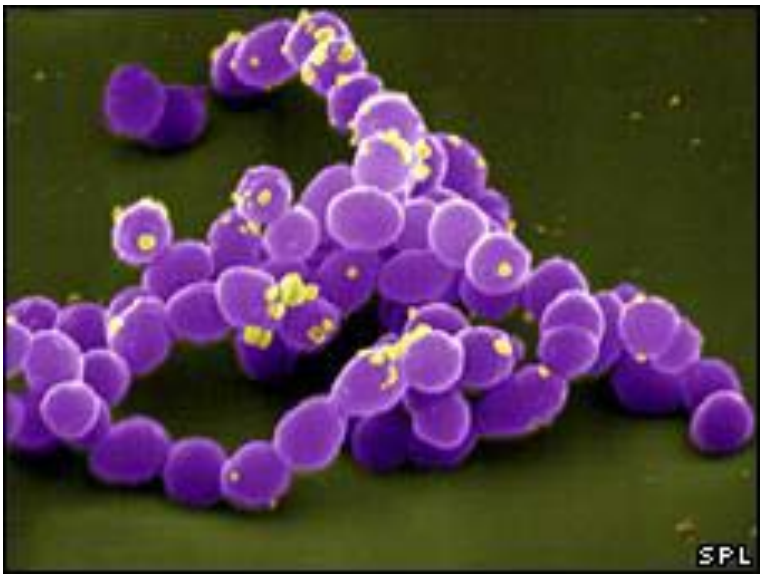
Lactic acid bacteria (LAB) is a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. These microbes are broadly used in the production of fermented food products, such as yogurt (*Lactobacillus* spp.; Figure 2-1 and *Streptococcus* spp.; Figure 2-2), cheeses (*Lactococcus* spp.), sauerkraut (*Leuconostoc* spp.) and sausage. 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 other bacteria residing in animal oral cavities and intestines (eg. *Enterococcus faecalis*; Figure 2-3), plant leaves (*Lactobacillus, Leuconostoc*) as well as decaying plant or animal matter such as rotting vegetables, fecal matter, compost, etc (Holzapfel and Wood, 1998).



Figue 2-1 Scanning electron micrograph of *Lactobacillus* spp. (http://bio-nin.com)



Figue 2-2 Scanning electron micrograph of *Streptococcus* spp. (http://microbewiki.kenyon.edu)

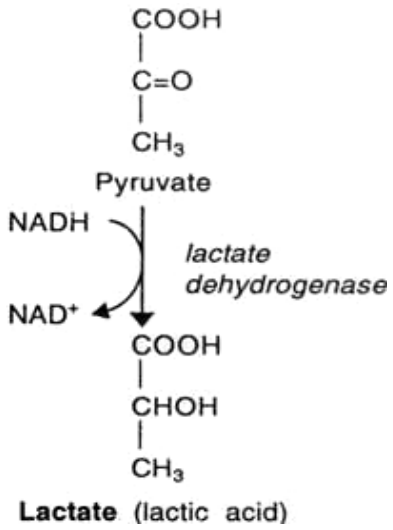


Figue 2-3 Scanning electron micrograph of *Enterococcus faecalis* (http://news.bbc.co.uk)

There are two main hexose fermentation pathways that are used to classify LAB genera; homofermantative and heterofermentative LAB. Under conditions of excess glucose and limited oxygen, homofermentative LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnas (EMP) pathway to yield two moles of pyruvate. Intracellular redox balance is

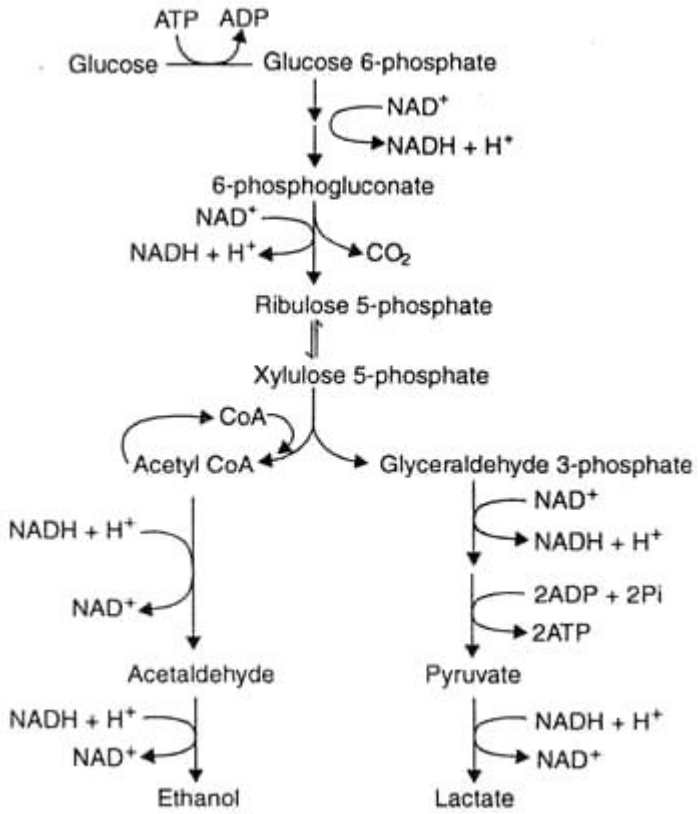
maintained through the oxidation of NADH coupled with lactate dehydrogenase enzyme, concomitant with pyruvate reduction to lactic acid (Figure 2-4). Organisms that form L(+) or D(-) isomer of lactic acid have one of two forms of lactate dehydrogenases, which differ in their stereospecificity. Accordingly, D or L isomer of lactic acid produced by LAB was specified by the stereospecificity of lactate dehydrogenase enzyme. Representative homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, and group I lactobacilli (Salminen *et al*., 2004).

Heterofermentative LAB use the pentose phosphate pathway, alternatively referred to as the pentose phosphoketolase pathway (Figure 2-5). In this pathway, one mole Glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole of glyceraldehyde phosphate (GAP) and one mole of acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. In this pathway, end-products (including ATP) are produced in equimolar quantities from the catabolism of one mole of glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and group III lactobacilli (Salminen *et al*., 2004).



Figue 2-4 Schematic displays the homolactic fermentation pathway.

(http://www.studentsguide.in)



Figue 2-5 Schematic displays the heterolactic fermentation pathway.

(http://www.studentsguide.in)

2.2 The advantage of lactic acid

Lactic acid is a three carbon organic acid: one terminal carbon atom is part of an acid or carboxyl group; the other terminal carbon atom is part of a methyl or hydrocarbon group; and a central carbon atom having an alcohol carbon group. Lactic acid exists in two optically active isomeric forms (Figure 2-6). Lactic acid is used as acidulant, flavouring, pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods. In contrast to other food acids it has a mild acidic taste. The addition of lactic acid aqueous solution to the packaging of poultry and fish increases their shelf life (Anon, 1992). Lactic acid has many pharmaceutical and cosmetic applications such as, substrates for biodegradable polymer (polylactic acid; PLA, poly (lactic-co glycolic) acid; PLGA), For example, PLGA microspheres containing gentamicin have been mixed with an apatite calcium phosphate bone cement to generate an antibiotic drug

delivery system for treatment of bone defects (Schniders *et al.,* 2006), PLA coupled with antibody for targeting drug delivery (Nakase *et al.,* 2000), was used for control drug release after coating on gauze (Xu *et al*., 2008).

L (+) Lactic acid D (-) Lactic acid

Figue 2-6 The chemical structure of L (+) Lactic acid and D (-) Lactic acid.

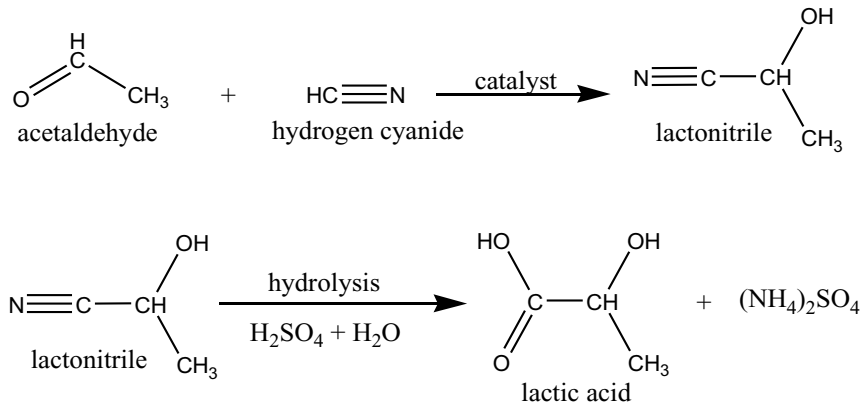
2.3 Lactic acid production

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 sulfuric acid to produce the corresponding ammonium salt and lactic acid. (Figure 2-7). Chemical synthesis produces a racemic mixture. Biodegradable polymer which produced from racemic mixture of lactic acid is amorphous, it is less stable than the specific isomeric polymers (poly L-lactic acid or poly D-lactic acid) which are semicrystalline. Since stereo specific lactic acid can only be made by carbohydrate fermentation, depending on the strain being used (Narayanan *et al*., 2004), the selection of strains specifically producing only one isomer is thus important.

The culturing technique is the most popular method in industry because of low cost with high yield of lactic acid acquired. However, the selection of bacterial strains and the development of culturing broths were most important, since they could reduce time and cost of lactic acid production. The highly effective analytical technique which is necessary in evaluation

of the quality of bacterial strains for producing lactic acid is by determining sugar utilization [step (1) and (2) in Figure 2-8].

Therefore, an appropriate analytical technique is needed for determination of sugars consumed by the bacterium. At present, high-performance liquid chromatography (HPLC) is the most popular technique for determination of complex samples and non-volatile substances. The advantages of this technique are of the capability to vary types of stationary phase, mobile phase and detectors for proper analysis (Swartz, 2005). Thus, HPLC technique was selected to develop and optimize for using in this state.



Figue 2-7 Schematic displays of the chemical reaction for synthesis lactic acid. (Narayanan *et al*., 2004)

2.4 Purification of lactic acid

To recover mixed acids from the fermentation process of LAB, the cultured broth was filtrated and subjected to anion exchange column, in using an ion exchange membrane based extractive fermentation system (Ziha and Kefung, 1995). The pre-purification process was set to remove other impurities, such as sugars, amino acid, vitamins etc. Then, the mixed acids were esterified to form acid esters which subsequently purify by direct distillation.

In esterification process, the complete of the reaction is monitored by determination of acid decreasing, while increasing of esters. Acid esters are determined before and after distillation to ensure the purity of ethyl lactate, which then subjected to hydrolysis. At the end, ethyl lactate is hydrolyzed to lactic acid. Quantitative analyses of ethyl lactate and lactic acid are needed to use for monitoring the completion of such reaction [step (3)-(7) in Figue 2-8].

Gas chromatography (GC) represents as a suitable analytical method for monitoring lactic acid during purification process, because it is rapid, easy to operate. Moreover, all compounds, including acetic acid, butyric acid, lactic acid as well as ethyl lactate from overall processes can be analyzed by this technique (Yang and Choong, 2001).

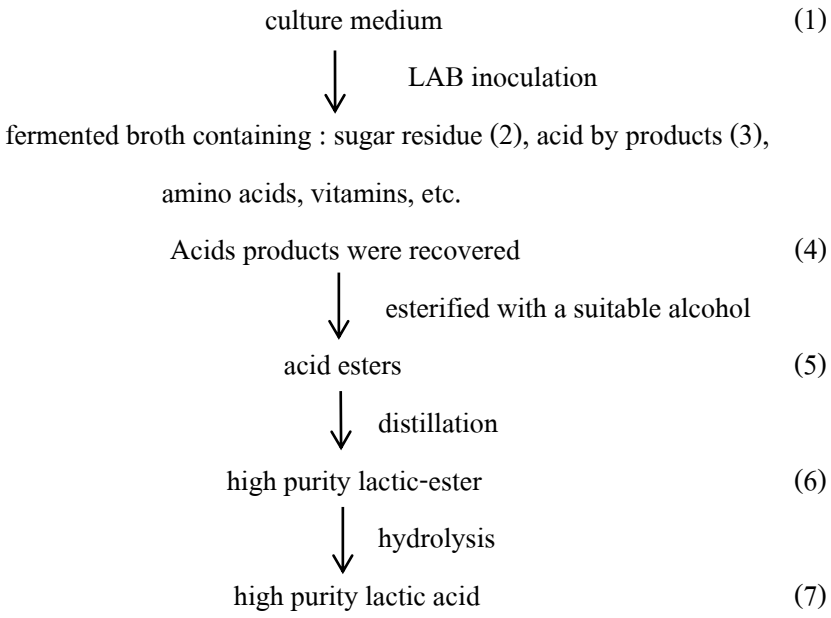


Figure 2-8 The diagram described lactic acid production and purification processes.

Previous reported analytical methods for determination of sugars (glucose, fructose and lactose) and organic acids (acetic acid, butyric acid and lactic acid) from various sources are summarized in Table 2-1

Table 2-1 Analytical techniques used for determining of sugars and organic acids

Samples	Analytes	Techniques	Conditions	References
Wines	Tartaric, malic, citric, lactic, succinic and acetic acids	Flow injection on-line dialysis coupled to HPLC	C_{18} column Injection volume: 20 µL Mobile phase: 1% acetonitrile in 99% of 0.05 M KH_2PO_4 buffer (pH 2.5) Flow rate: 1.25 mL/min UV detector 210 nm	Kritsunankul *et al.,* 2009
Orange juice and orange wine	Sugars ; - sucrose - glucose - fructose Organic acids; - citric acid - ascorbic acid - malic acid	HPLC	C_{18} column Gradient conditions mobile phase Solvent A, water/formic acid (95:5; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v) Flow rate: 1 mL/min Photo diode array detector	Hasim *et al.,* 2009
Brewed coffee	Acetic, citric, formic, malic, pyruvic, quinic and succinic acid	HPLC	C18 column Injection volume: 20 µL Mobile phase: 25 mM phosphate buffer, pH 2.4, with 1% methanol Flow rate: 1 mL/min UV detector 210 nm	Carla *et al.,* 2007

Samples	Analytes	Techniques	Conditions	References
Tobacco	Propionic, butanoic, 2-methylbutyric, 3-methylbutyric, pentanoic, 3-methylpentanoic, 4-methylpentanoic, caproic, 2-furoic acid, heptanoic, benzoic, octanoic, nonanoic, decanoic and dodecanoic acid	Needle-based derivatization headspace liquid-phase microextraction couple to GC-MS	Derivatizing agent: *N*,*O*-bis(trimethylsilyl)trifluoroacetamide DB-5MS capillary column Injector: 250 °C Transfer line: 280 °C Oven temperature programmed from 40 °C (for 3 min) to 210 °C at 4 °C/min, then to 280 at 10 °C/min Carrier gas was helium at 1 mL/min Injection of 1 µL; split ratio 1:10	Sun *et al.,* 2008
Fruit juices	Sucrose, glucose and fructose	HPLC	Bio-Rad Aminex HPX 87H Hydrogen form cation exchange resin-based column Mobile phase: 0.005 N phosphoric acid Injection volume: 20 µL Flow rate: 0.4 mL/min UV detector 210 nm	Fabio *et al.,* 2005
Coffee and tea	Acetic, malic, ascorbic, citric, malic and succinic acids, chloride and phosphate	HPLC	Anion-exchange column operated at 40 °C Mobile phase: 0.6 mM aqueous potassium hydrogenphthalate (pH 4.0) solution containing 4% (v/v) acetonitrile, Determination by conductivity detection	Alcázar *et al*., 2003

Samples	Analytes	Techniques	Conditions	References
Wines	Glucose, fructose, glycerol, ethanol, acetic, citric, lactic, malic, succinic and tartaric acid	HPLC couple with FTIR	An ion-exchange resin based column (counter ion: H+) Mobile phase: 0.005 M sulfuric acid. The FTIR detection in the spectral region from 1600 to 900 cm^{-1}	Vonach *et al.,* 1998
Grape juice and wines	Tartaric, malic, citric, lactic, succinic and acetic acids	Capillary zone electrophoresis	Sample injection was carried out in a hydrodynamic mode by elevating the sample at 10 cm for 30 sec. The running voltage was -25 kV at thermostated temperature of 25 °C. The detection mode was UV direct and the wavelength was 185 nm. The electrolyte composition was phosphate as the carrier buffer (7.5 mM $NaH_2PO_4$and 2.5 mM Na_2HPO_4), 2.5 mM tetradecyltrimethylammonium hydroxide (TTAOH) as electroosmotic flow modifier and 0.24 mM $CaCl_2$ as selectivity modifier, adjusting the pH at 6.40 constant value	Inés *et al.,* 2007

Samples	Analytes	Techniques	Conditions	References
Fruit juice and vinegar	Acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, heptanoic, caprylic, capric, lauric, lactic and levulinic acids	GC	Polyethylene glycol capillary column with temperature program initiate at 75°C for 1 min before increase to 180°C with rate of 6 °C/min and suddenly increase to 230°C with rate of 10°C/min	Yang *et al*., 2001
Urine	Oxalic, formic, malonic, fumaric, succinic, α-ketoglutaric, citric, acetic, pyruvic, lactic, isovaleric and hippuric acid	Capillary electrophoresis	All acids were separated in a fused-silica capillary (100 cm × 75 μm I.D.) filled with 50 mM borax buffer (pH 10.0) containing cationic surfactant as the electroosmotic flow modifier and detect the UV absorption at 185 nm	Mika *et al.,* 1994
Olive Plants	Sucrose, fructose, galactose, glucose and maniol	HPLC-RI	The column was (8 x 300 mm) Shodex Sugar SC 1011, maintained at 75°C. The mobile phase was water, Milli Q grade at 0.5 ml min^{-1} and detected with refractive index detector.	Romani *et al*., 1994
MRS broth	Phenyllactic acid	HPLC	C18 (150mm×4.6 mm, 5μm) at room temperature. Mobile phase: acetronitrile and water, the gradient used for elution. Detected at UV 210 nm	Armaforte *et al*., 2006

Samples	Analytes	Techniques	Conditions	References
Cultured broth	volatile acids: acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids; non-volatile acids: lactic, succinic, oxalic, fumaric and malonic acids	GC-FID	Volatile acids: 2 m x 1/8 in. stainless-steel column (Supelco) packed with 15%FFAP in 80/100 Chromosorb W/WA Non-volatile acids: 2 m x 1/8 in. stainless-steel column (Supelco) packed with 15%DGES in 80/100 Chromosorb WAVA Nitrogen (30 ml/min) was the carrier gas and air (300 ml/min) and hydrogen (30 ml/min) were used as the flammable gas mixture. Injection port, column and detector temperatures were 200, 160 and 250°C, respectively, for volatile acids and 270, 180 and 190°C, respectively, for non-volatile acids.	Urdaneta *et al.,* 1995

From the literature reviewed, the methods for determination of sugar mostly used HPLC technique with various analytical columns and detectors. Since there is no chromophore presenting in sugar structures (see in Appendix), detection of them with UV detector may be insufficiently sensitive. Refractive index detector is more appropriate for sugar analysis. The principle of this detector is based on refractive index of mobile phase changed when concentrated analytes pass the detector. For acids and ester determination GC method is mostly used, because acids and its esters are of low volatile compounds and stable at high temperature. In previous studies, derivatization has been used for the determination of acids which are easily (Urdaneta *et al.,* 1995). This procedure would be required more operating time and cost. Thus, it might be un suitable of routine analysis

CHAPTER 3

METERIALS AND METHODS

3.1 Materials and equipments for analysis of acids and ester by GC

3.1.1 Materials

- Milli-Q grade water was purified by Milli-Q system (Millipore, Bedford, USA)
- Ethanol, analytical grade (Lab scan Asia, Thailand)
- Acetic acid (Merck, Germany)
- Butyric acid (Sigma, USA)
- Caproic acid (Sigma, USA)
- Lactic acid (Sigma, USA)
- Ethyl lactate (Fluka, Switzerland)

3.1.2 Equipments

The equipments used in this study were listed in Table 3-1.

3.2 Materials and equipments for sugar analysis by HPLC

3.2.1 Materials

- Milli-Q grade water was purified by Milli-Q system (Millipore, Bedford, USA)
- Acetonitrile, HPLC grade (Lab scan Asia, Thailand)
- Glucose (Fluka, Switzerland)
- Fructose (Fluka, Switzerland)

- Lactose (Fluka, Switzerland)

3.2.2 Equipments

The equipments used in this study were listed in Table 3-1.

Table 3-1 General information of equipments

Instrument	Model	Company
GC system	GC 14A	Shimadzu, Japan
GC integrator	ChromatoPac C-R6A	Shimadzu, Japan
GC column	BP 20 30 m×0.53 mm, 0.5 µm	SGE Analytical Science, Australia
GC syring	Plunger-in-Needle Syringes 1 µL	SGE Analytical Science, Australia
HPLC	Agilent 1100 series	Palo Alto, USA
- System controller:	- SCL-10AVP model	
- Pump	- LC-10ADVP	
- Degasser	- DGU-14A model	
- Automatic Injector	- SIL-10ADVP model	
- Column oven	- CTO-10ASVP model	
- Reflextive index Detector	- RF-10AXL model	
HPLC column	Zorbax NH_2 250x4.6 mm, 5 µm	Agilent, USA
Micro pipette:		RAININ, USA
Precision pipette tips:		RAININ, USA
Microtubes:	MCT-175	Axygen® Scientific, USA
Vortex:		Scientific Industries, INC., USA
Sonicator		Ultrasonicator, Chest
Vial		Agilent, USA

3.3 Methods

3.3.1 Method development for determination of acetic acid, butyric acid, lactic acid and ethyl lactate by gas chromatography

3.3.1.1 Standard solutions

The stock solution of 10%v/v acetic acid, butyric acid, lactic acid or ethyl lactate was freshly prepared using Milli-Q grade water. Acid density was taken into account for preparing correct concentration. Caproic acid was used as the internal standard (IS) because it has not been produced by LAB and can be separated from other analyzed acids by the established GC conditions. It was used at a concentration of 1 µg/mL. To prepare working acid standard solution, the corresponding acid stock solution was diluted with the purified water to obtain a range of acid concentrations in the following, e.g., 0.01-0.10 µg/mL for acetic acid and butyric acid, 1.0-10.0 µg/mL for lactic acid and ethyl lactate. Each acid calibration curve was then constructed.

3.3.1.2 Gas Chromatographic conditions

In this study, the GC analytical method was developed from previously described method (Yang and Choong, 2001) which was used for quantitative analysis of 13 organic acids including, acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, heptanoic, caprylic, capric, lauric, lactic and levulinic acids. Polyethylene glycol capillary column was used with a temperature program initiated at 75°C and held for 1 min before stepped up to 180°C with a rate of 6°C/min, and then stepped up again to 230°C with a rate of 10°C/min and held at this temperature for 5 min.

Since acids produced by LAB in cultured broth contained not more than the acids types as mentioned, in this study, the temperature program was newly adapted to reduce retention times and improve peak resolution. However, the types of GC column and carrier gas were not changed. The analytical conditions developed are shown below.

Column	: BP20 (wax) polyethylene glycol capillary column 30 m × 0.53 mm diameter, film thickness 0.5 μm (SGE Analytical Science)
Carrier gas	: Helium (gas pressure 1 kg/cm^2)
Injection volume	: 0.2 μL
Injection temperature	: 230°C
Detector	: Flame ionization detector (FID) with temperature at detection port temperature of 250°C (gas pressure for hydrogen was 0.6 kg/cm^2 and air zero was 0.5 kg/cm^2)
Column temperature program	: initiated at 40°C, and held for 3 min before stepped up to 110°C with a rate of 10°C/min, then stepped up to 200°C with a rate of 12°C/min and held at this temperature for 5 min.

3.3.2 Method development for determination of sugars in culture broth using high-performance liquid chromatography

3.3.2.1 Standard solutions

The stock solution of glucose, fructose and lactose was separately and freshly prepared using the purified water as a diluent at a concentration level of 24 mg/mL. They were stored at 4°C until use. Working standard solutions were subsequently prepared in the water by obtaining within a concentration range of 2-12 mg/mL. The calibration curve was then constructed.

3.3.2.2 High-performance liquid chromatographic conditions

The HPLC analytical method for determination of sugars from *L. crispatus* 21L07 cultured broth was developed from the method described by Romani *et al*. (1994). Their analytical conditions were as followed.

Column	:	Shodex Sugar SC 1011 (Showa Denko Europe GmbH, Germany) equipped with a Guard-Pak Insert Sugar Pak II (Waters)
Column temperature	:	75°C
Mobile phase	:	Milli-Q grade water
Flow rate	:	0.5 mL/min
Detector	:	Refractive index detector (LC-30 RI, Perkin Elmer)

In newly developed method, the column used was Zorbax amino column (250 × 4.6 mm; 5 µm particle size). Chromatographic conditions were adapted to reduce retention times and improve method resolution. The improved analytical conditions were shown below.

Column	:	Zorbax NH_2 (250 × 4.6 mm; 5 µm particle size)
Column temperature	:	30°C
Mobile phase	:	Acetonitrile : Milli-Q grade water (78 : 22, v/v)
Flow rate	:	1.5 mL/min
Detector	:	Refractive index detector (temperature 35°C)
Injection volume	:	20 µL

3.3.3 Validation of analytical method

Method validation was followed the US FDA guidance for industrial bioanalytical method validation (2001). The validation criteria contains specificity, limit of detection (LOD) and quantification (LOQ), calibration curve linearity, accuracy and precision.

3.3.3.1 Specificity

Peak identification was carried out using the retention time of standards compared to those of fermentation broth, which do not have any peaks appearing at the same position when blank broth was conducted.

3.3.3.2 Limit of detection (LOD) and limit of quantification (LOQ)

Serial dilutions of working standard solutions were made within detection limit of instruments. LOD and LOQ were determined by means of signal to noise ratio of 3:1 and 10:1, respectively.

3.3.3.3 Linearity

Working standard solutions were prepared by diluting stock standard solutions in at least five concentrations in which the lowest concentration was set as the LOQ. Calibration curves were constructed by determining of peak area at least five times each and plotted the average area against the concentrations. The linearity of detector response was determined using regression equation, and coefficient of determination (R^2) was calculated which must be more than 0.99 with RSD (%) at each concentration not less than 15% (US FDA, 2001).

3.3.3.4 Accuracy

The accuracy was determined by recovery (%) of accurately prepared sample at high, medium and low concentrations of the calibration curve, spiking into blank broths. Prior to analyze fortification, the background levels of each sample were determined so as to calculate actual recoveries. The amount of each analyte was determined in triplicate and percentage recoveries were calculated by the following equation:

$$\% \text{ Recovery} = \frac{C_{obs}}{C_{actual}} \Delta 100$$

C_{obs} = observed (determined) concentration

C_{actual} = prepared concentration

3.3.3.5 Precision

Relative standard deviation (RSD) of samples was calculated for within-run (6 replicated within 24 hours) and between-run (3 samples, analyzed in 3 day) to ensure the precision of the analytical method and calculated by the equation:

$$\text{RSD } \% = \frac{sd}{mean} \Delta 100$$

3.3.3.5.1 Within-run analysis

Within-run analysis studies were performed by analyzing of 3 samples at high, medium and low concentrations of the calibration curve. Each sample was determined five times repeatedly within 24 hours. Repeatability of the analytical method was evaluated using RSD (%), which should be lower than 15% (US FDA, 2001).

3.3.3.5.2 Between-run analysis

Between-run analysis studies were determined by analyzing samples as same as within-run analysis. Each sample was determined five times repeatedly within 24 hours and repeated all in three different days. Reproducibility of analytical method was evaluated using RSD (%), which is considered if %RSD is lower than 15% (US FDA, 2001).

3.4 Use of the developed analytical method to determine lactic acid in purification process

3.4.1 Determination of lactic acid from cultured broth

L. crispatus 21L07 was cultured in Brain Heart Infusion (BHI) broth (LabScan, Thailand) at 37°C for 36 hours. *S. mutans* was cultured in LAPT-g broth (1 litre contains: casein tryptone, 10 g; yeast extract, 10 g; peptone, 15 g; Tween 80, 1 ml; glucose, 10 g), at 37°C for 24 hours. Bacterial cells were separated by centrifugation (8000 rpm, 10°C, 5 minutes). The clear supernatant was filtered through 0.22 μm sterile filter, 150 μL of each sample was added with 50 μL of 10%v/v caproic acid. 0.2 μL of the mixture was directly injected onto the developed GC system for quantification of lactic acid produced.

3.4.2 Determination of lactic acid and ethyl lactate in esterification process

Conversion of 3 organic acids to acid esters made them more difference in boiling points (Table 3-2) and more easily separated from each other by distillation (Filachione and Fisher, 1946). The effectiveness of esterification process was assessed by determination of lactic acid remaining and ethyl lactate generated using the developed GC system. Samples from esterification process were prepared by spiking technique. The characteristic of sample was referred from the study described by Chooklin *et al*. (2009). The mixture of acetic acid (0.05 μg/mL), butyric acid 0.05 (μg/mL), lactic acid 0.5 (μg/mL) and ethyl lactate 0.5 (μg/mL) in ethanol was prepared. The accuracy was determined by recovery (%) of the accurately prepared sample.

Table 3-2 Melting point and boiling point of lactic, acetic, butyric acids and its esters

	Melting point (^{o}C)	Boiling point (^{o}C)
L (+) lactic acid	53	122
Acetic acid	16.5	118.1
Butyric acid	-7.9	163.5
Ethyl lactate	-26	154
Ethyl acetate	-83.6	77.1
Ethyl butyrate	-93	121

3.5 Application of the developed analytical method for assessment of *S. mutans* quality by determination of sugar utilization

S. mutans, a lactic acid bacteria, was selected as a model for studying an effectiveness in lactic acid production in utilizing sugar resources, including glucose, fructose and sucrose. LAPT-g broth and other modified LAPT where glucose was replaced by fructose (LAPT-f) or lactose (LAPT-l) were sterilized by autoclaving. Its sugar contents were determined before and after incubation with *S. mutans* at 37°C for 24 hours. After that, the cultured broth was clarified by centrifugation (8000 rpm, 10°C, 5 minutes), and filtered through 0.22 μm sterile filter prior to be subjected onto the developed HPLC system for quantification of sugar remaining and assessment of *S. mutans* quality.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Method validation of the developed GC method

4.1.1 Specificity

Figure 4-1 shows the chromatogram of mixed acids standards. The retention times of ethyl lactate (5C; bp = 154°C), acetic acid (2C; bp = 118.1°C), butyric acid (4C; bp = 163.5°C), caproic acid (IS) (6C; bp = 205°C) and lactic acid (3C; bp = 122°C) were of 6.2, 7.8, 10.0, 12.2 and 15.1 min, respectively. In general, the retention of all acids and ethyl lactate are directly proportional to their carbon numbers and boiling point. The higher polarity of lactic acid greatly increased its retention as compared to butyric and caproic acids, by partitioning well into polyethylene glycol which was used as stationary phase. A few peaks after the lactic acid peak was typically observed although for the highly pure lactic acid standard.

Selectivity factor and resolution values of all acids and ethyl lactate were calculated from the chromatogram to confirm specificity of analytical methods, where t_r was the retention time of each sample, t_m was the elution time referred to the solvent peak, α was the selectivity factor explaining the distance difference between two sample peaks. Rs was the resolution value explaining the separation of two peaks. From the result (Table 4-1), the selectivity factor (α) values of all samples were more than 1, suggesting that all samples had enough retention time gap. The resolution values (Rs) of all samples were more than 1.5, meaning that the developed condition was sufficient for quantitative analysis. Thus, high selectivity of the GC system for determining acetic, butyric lactic acids and ethyl lactate was developed.

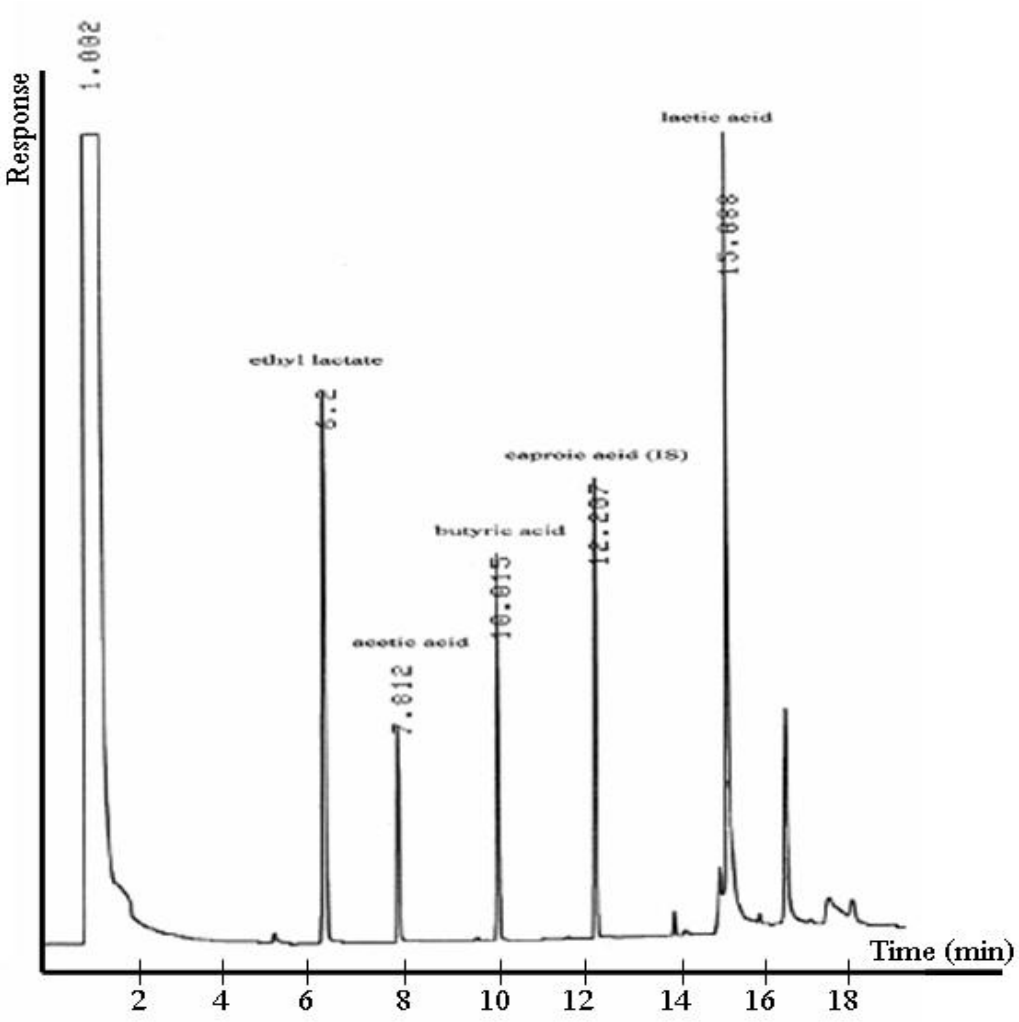


Figure 4-1 GC chromatogram of mixed standards solution of ethyl lactate, acetic acid, butyric acid, caproic acid (IS) and lactic acid.

Table 4-1 Selectivity factor and resolution values of all acids and ethyl lactate

	t_r	t_m	α	Rs
Ethly lactate	6.208		-	4.88
Acetic acid	7.818		1.310	10.20
Butyric acid	10.022	1.007	1.324	15.33
Caproic acid	12.210		1.243	15.33
Lactic acid	15.092		1.257	12.86

4.1.2 Limit of detection (LOD) and limit of quantification (LOQ)

The sensitivity of the developed conditions was assessed on sample concentrations, which gave a signal-to-noise ratio of approximately 3. This would be corresponding to the limit of detection (LOD) of the method. For the limit of quantification (LOQ), such the ratio was given to be approximately of 10. The LOD and LOQ values for acetic and butyric acids were of 0.001 and 0.010 µg/mL respectively. For lactic acid, the respective values were of 0.100 and 1.000 µg/mL and for ethyl lactate they were of 0.100 and 0.500 µg/mL (Table 4-2).

4.1.3 Calibration curves and linearity

The calibration curves were performed with acid standard solutions at concentrations ranging between 0.01 and 0.10 µg/mL for acetic and butyric acids (Figure 4-2) and 1.00 – 10.00 µg/mL for lactic acid and ethyl lactate (Figure 4-3). The linearity was observed over the evaluated ranges with the correlation coefficients of 0.9991, 0.9995, 0.9993 and 0.9996 for ethyl lactate, acetic acid, butyric acid and lactic acid, respectively (Table 4-2).

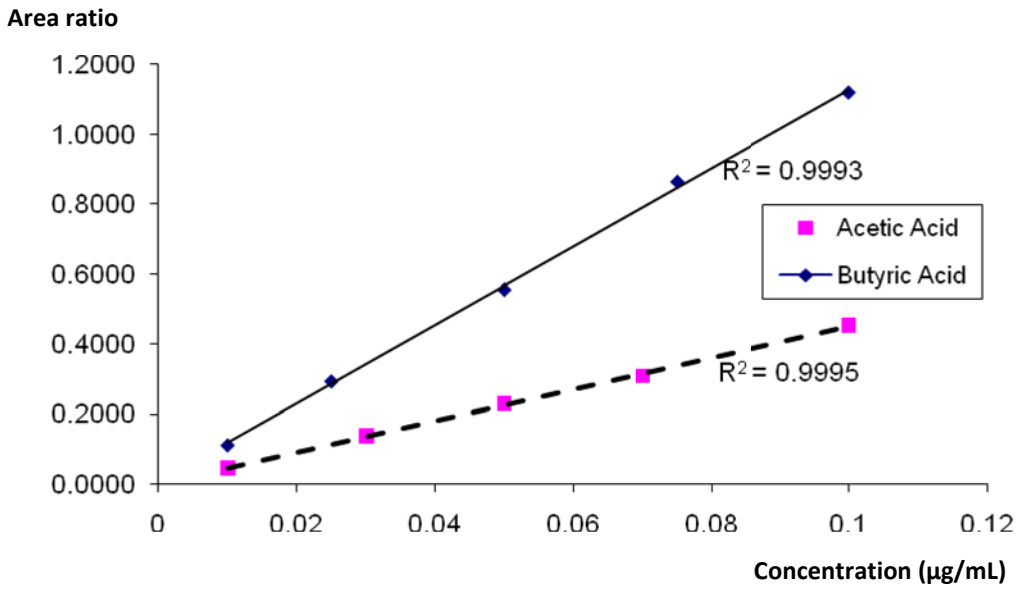


Figure 4-2 The calibration curves of acetic acid and butyric acid plotted by the ratio of acid peak area to that of the internal standard against concentrations

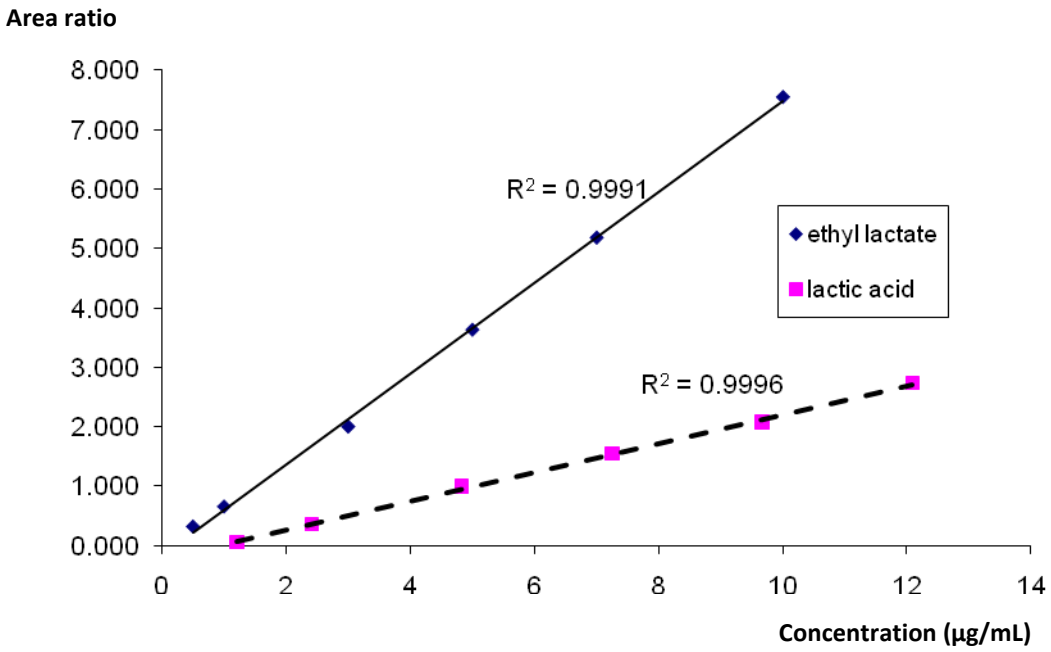


Figure 4-3 The calibration curves of lactic acid and ethyl lactate plotted by the ratio of acid peak area to that of the internal standard against concentrations

Table 4-2 The summary results of retention time, linearity and range, LOD and LOQ

	Retention time (min)	Linear Range (µg/mL)	Equation linear model*	Linearity (R^2)	LOD (µg/mL)	LOQ (µg/mL)
Ethyl lactate	6.2	1.00 – 10.00	y = 0.7644x – 0.1547	0.9991	0.100	0.500
Acetic acid	7.8	0.01 – 0.10	y = 4.4787x + 0.0023	0.9995	0.001	0.010
Butyric acid	10.0	0.01 – 0.10	y = 1.2627x – 0.0053	0.9993	0.001	0.010
Lactic acid	15.1	1.00 – 10.00	y = 0.2456x – 0.2256	0.9996	0.100	1.000

* y = peak area; x = concentration (µg/mL)

4.1.4 Accuracy

The accurately prepared standard solutions at high, medium and low concentrations of the calibration curve by spiking accurately amount of analytes into blank BHI broth, they were analyzed and calculated for the accuracy. Acetic acid, butyric acid, lactic acids and ethyl lactate showed good accuracy at all concentrations with %recovery ranging between 98 and 102 % (Table 4-3).

Table 4-3 The accuracy results (% recovery) of ethyl lactate, lactic acid, acetic acid and butyric acid

Acids	Spiked concentration (μg/ml)	Recovery (%)[a] (n = 3)
Acetic acid	0.01	98.72 ± 2.344
	0.05	99.72 ± 1.610
	0.10	101.21 ± 1.464
Butyric acid	0.01	98.77 ± 3.810
	0.05	100.26 ± 1.684
	0.10	101.87 ± 2.781
Lactic acid	1.00	100.61 ± 0.405
	5.00	101.20 ± 0.523
	10.00	100.15 ± 0.202
Ethyl lactate	1.00	102.81 ± 2.203
	5.00	98.02 ± 0.194
	10.00	98.90 ± 0.318

[a] All values were mean ± SD obtained by triplicate analyses.

4.1.5 Precision

The method precision was tested by performing between-run and within-run multiple injections of standard solutions. The percentage of relative standard deviation (%RSD) was determined. The %RSD values of between-run and within-run analysis for all acids were of less than 15% (Table 4-4), indicating the high precision of the developed chromatographic system.

Table 4-4 The precision results of between-run and within-run experiments

	Between-run				Within-run	
	content (μg/mL)[a]			% RSD	Content (μg/mL)[a]	% RSD
	day 1	day 2	day 3			
AA	0.0496 ± 0.0087	0.0495 ± 0.00277	0.0490 ± 0.00201	0.61	0.0496 ± 0.00187	1.91
BA	0.0499 ± 0.0013	0.0502 ± 0.00931	0.0494 ± 0.00128	0.87	0.0499 ± 0.00710	1.80
LA	4.97 ± 0.092	5.00 ± 0.184	4.98 ± 0.102	0.30	4.97 ± 0.016	0.08
EL	4.95 ± 0.078	5.06 ± 0.101	4.91 ± 0.089	1.55	4.95 ± 0.092	1.21

[a] All values were mean ± SD obtained by triplicate analyses.

4.2 Method validation of the developed HPLC method

4.2.1 Specificity

The specificity result was shown in Figure 4-4. The order of separation was based on the partition capacity differing between analytes in the stationary phase. NH_2 column was used as the stationary phase in this study, according to the previous report (Chavez-Servin *et al.,* 2004). The mixture of ACN:H_2O (78:22) was used as the mobile phase. Therefore, mode of separation was the reverse phase HPLC. Glucose and fructose have low molecular weight and are highly soluble in water than lactose. The chemical structure of fructose in closed ring is not stable. It prefers to open the chain by hydration. Accordingly, fructose was the first compound that passed the column. The last compound passing through the column was lactose, because its

molecule is bigger than fructose and glucose, and lower soluble in water (see in Appendix). Because of these, the retention times of fructose, glucose and lactose were apparent to be of 5.6, 6.3 and 11.2 min, respectively.

Selectivity factor and resolution values of all sugars were calculated from the chromatogram (Figure 4-4) to confirm the specificity of analytical methods (Table 4-5). t_r was the retention time of each sample, t_m was the elution time referring to the solvent peak, α was the selectivity factor explaining the distance difference between two sample peaks and Rs was the resolution value explaining the separation of two peaks. From the result, the selectivity factor (α) values of all samples were more than 1, indicating that they had enough retention time gap. The resolution values (Rs) of fructose and glucose were not greater than 1.5. Thus, the condition might be unsuitable for this separation. However, as only one type of sugars was present each time in the culture, this might not be the case. Accordingly, the conditions could be used for quantitative analysis, and the new HPLC method with high selectivity for determination of glucose, fructose and lactose was developed.

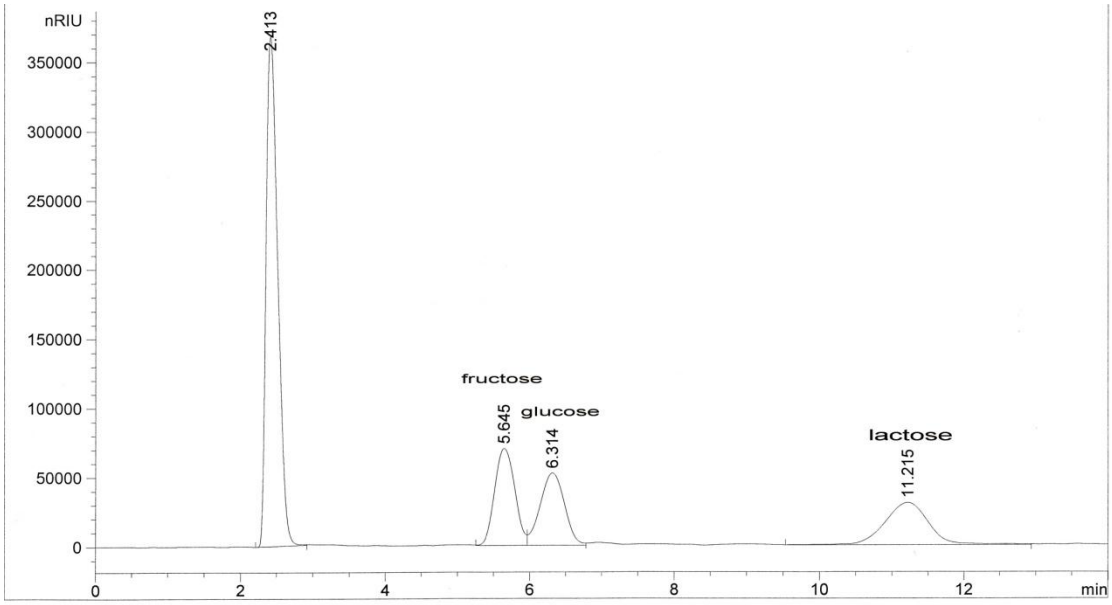


Figure 4-4 The HPLC chromatogram of standards solution containing 5 mg/ml each of fructose, glucose and lactose

Table 4-5 Selectivity factor and resolution values of fructose, glucose and lactose

	t_r	t_m	α	Rs
Fructose	5.645		-	5.84
Glucose	6.314	2.413	1.207	0.92
Lactose	11.215		2.256	4.36

4.2.2 Limit of detection (LOD) and limit of quantification (LOQ)

Sensitivity of the developed conditions was assessed on sample concentrations, which gave a signal-to-noise ratio of approximately 3. This concentration level is called the limit of detection (LOD). For the limit of quantification (LOQ), the ratio was given to be approximately of 10. The LOD and LOQ values for glucose and fructose were respectively of 0.0625 and 0.25 mg/mL. For lactose, it was calculated to be of 0.50 and 4.00 mg/mL, respectively (Table 4-6).

4.2.3 Calibration curves and linearity

The calibration curves were performed with sugar standard solutions at concentrations ranging between 2.0 and 12.0 mg/mL. The linearity was observed over the evaluated ranges with the correlation coefficients of 0.9983, 0.9984 and 0.9998 for glucose, lactose and fructose, respectively (Table 4-6; Figure 4-5).

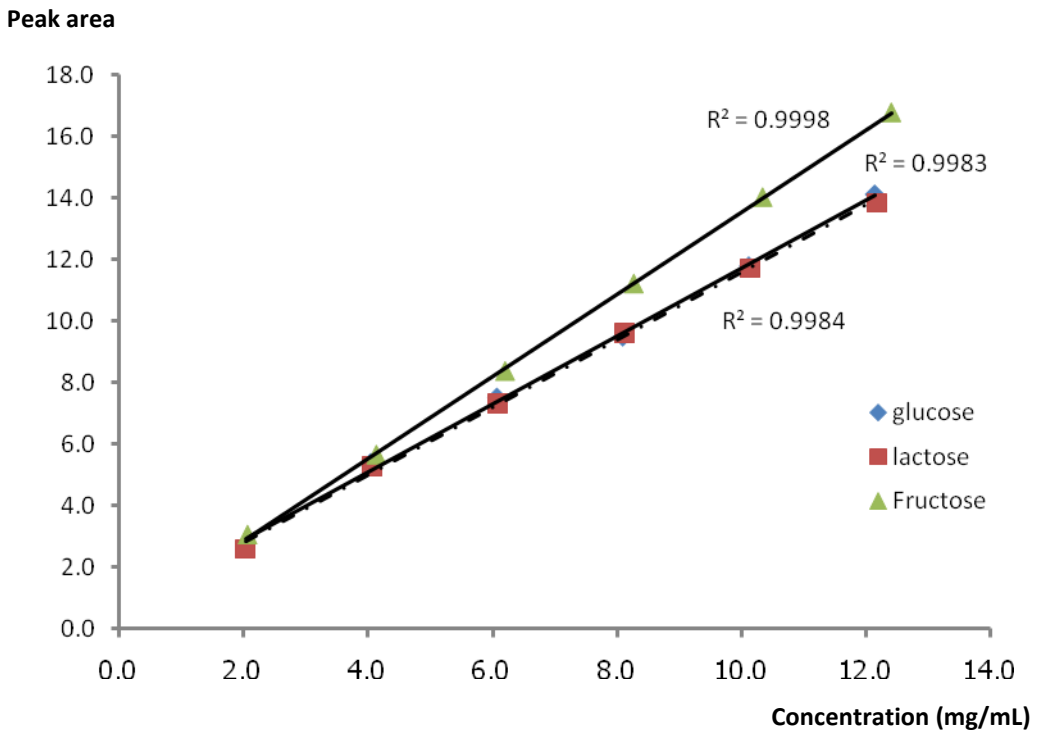


Figure 4-5 The calibration curves of glucose, lactose and fructose plotted by peak area against concentrations

Table 4-6 The summary results of retention time, linearity and range, LOD and LOQ

	Retention time (min)	Linear Range (mg/mL)	Equation linear model*	Linearity (R^2)	LOD (mg/mL)	LOQ (mg/mL)
Glucose	6.3	2.0 -12.0	y = 110665x + 65934	0.9983	0.0625	0.25
Lactose	5.6	2.0 -12.0	y = 109631x + 60347	0.9984	0.500	4.00
Fructose	11.2	2.0 -12.0	y = 133327x + 20104	0.9998	0.0625	0.25

* y = peak area; x = concentration (µg/mL)

4.2.4 Accuracy

The accurately prepared sugar standard solutions at high, medium and low concentrations of the calibration curve were spiked into blank LAPT broth. The mixed solutions were analyzed and calculated for the accuracy. Glucose, lactose and fructose showed good accuracy at all concentrations with % recovery ranging between 91 and 110 % (Table 4-7).

Table 4-7 The accuracy results (% recovery) of glucose, lactose and fructose

Sugars	Spiked concentration (mg/mL)	Recovery (%)[a] (n = 3)
Glucose	2.0	91.54 ± 0.883
	8.0	106.87 ± 0.870
	12.0	100.21 ± 0.304
Lactose	2.0	94.82 ± 0.994
	8.0	101.31 ± 1.269
	12.0	99.07 ± 0.187
Fructose	2.0	103.77 ± 1.093
	8.0	109.39 ± 0.039
	12.0	100.18 ± 0.412

[a] All values were mean ± SD obtained by triplicate analyses.

4.2.5 Precision

The method precision was tested by performing between-run and within-run multiple injections of standard solutions. The percentage of relative standard deviation (%RSD) was determined. The %RSD values of between-run and within-run analyses for all sugars were of less than 15% (Table 4-8), indicating the high precision of the developed chromatographic system.

Table 4-8 The precision results of between-run and within-run experiments

	Between-run				Within-run	
	content (mg/mL)[a]			% RSD	Content (mg/mL)[a]	% RSD
	day 1	day 2	day 3			
Glucose	5.79 ± 0.008	6.58 ± 0.044	6.14 ± 0.201	6.41	5.74 ± 0.057	1.00
Lactose	6.03 ± 0.023	5.64 ± 0.093	5.99 ± 0.288	3.67	5.98 ± 0.057	0.95
Fructose	5.48 ± 0.008	5.21 ± 0.193	5.11 ± 0.165	3.67	5.45 ± 0.036	0.65

[a] All values were mean ± SD obtained by triplicate analyses.

4.3 Utilization of the developed analytical method in lactic acid purification process

4.3.1 Determination of small organic acids produced in culture of LAB

The lactic acid purification process started from culturing of lactic acid bacteria in a well-defined medium. *L. crispatus* 21L07 and *S. mutans* were the selected bacteria to be used in lactic acid production. They were grown in BHI and LAPT-g broth, respectively. The developed GC method was used to determine small organic acids produced by the LAB. The method was advantage in that derivatization reaction was not necessary. Results revealed that *L. crispatus* 21L07 and *S. mutans* produced acetic acid, butyric acid and lactic acid (Figure 4-6-4-7). However, lactic acid was produced in abundant amounts. The acids produced by *L. crispatus* 21L07 fermentation broth were analyzed and shown in Table 4-9. It was estimated that the established GC method could be used for precise determination of acids metabolites produced by other LAB. All peaks were completely separated and the running time was only about 20 min for each injection. The GC method would be useful for monitoring of lactic acid production.

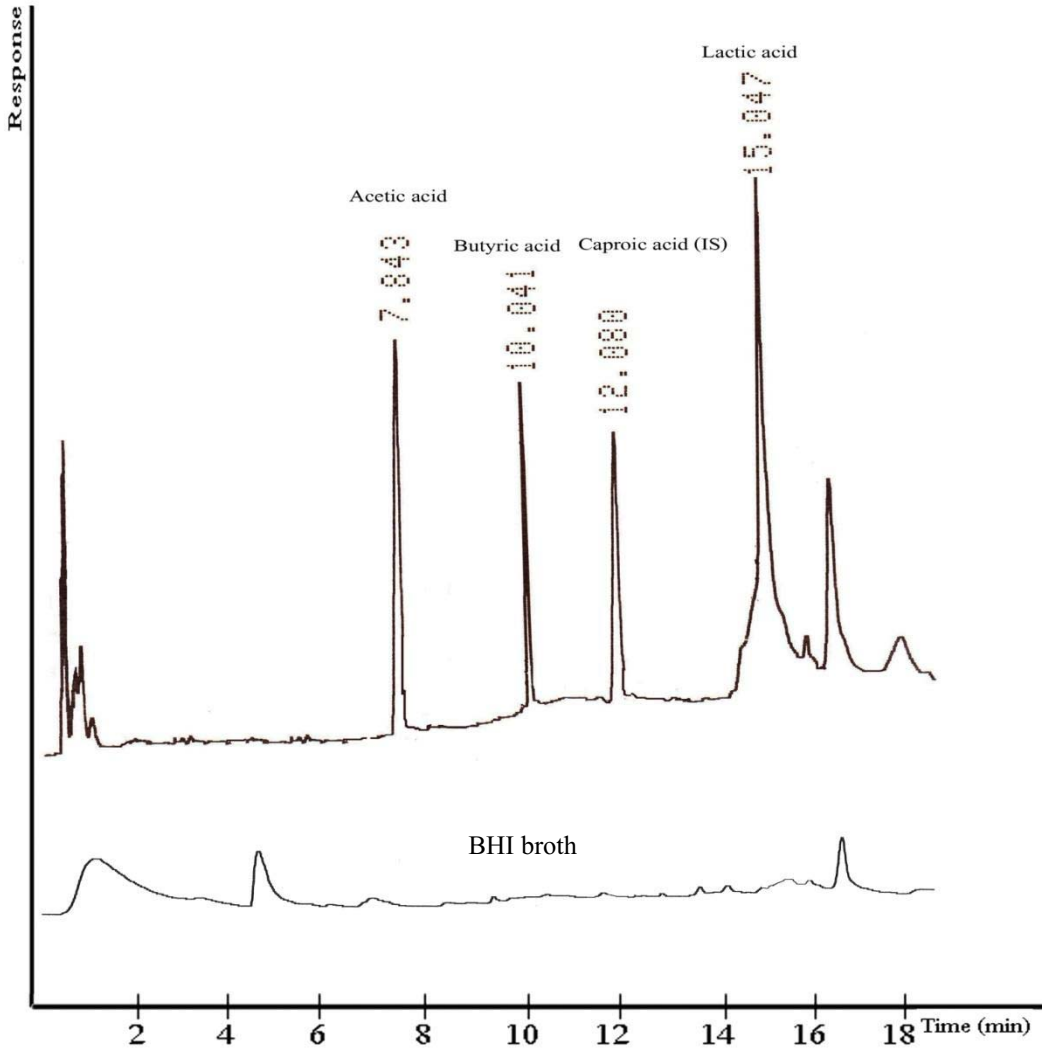


Figure 4-6 The GC chromatogram of acetic acid (t_r = 7.8), butyric acid (t_r = 10.0) and lactic acid (t_r = 15.0) produced by *L. crispatus* 21L07 in BHI culture. Caproic acid was spiked thereafter for precise determination of the acids produced. There were no peaks interfering from BHI blank broth.

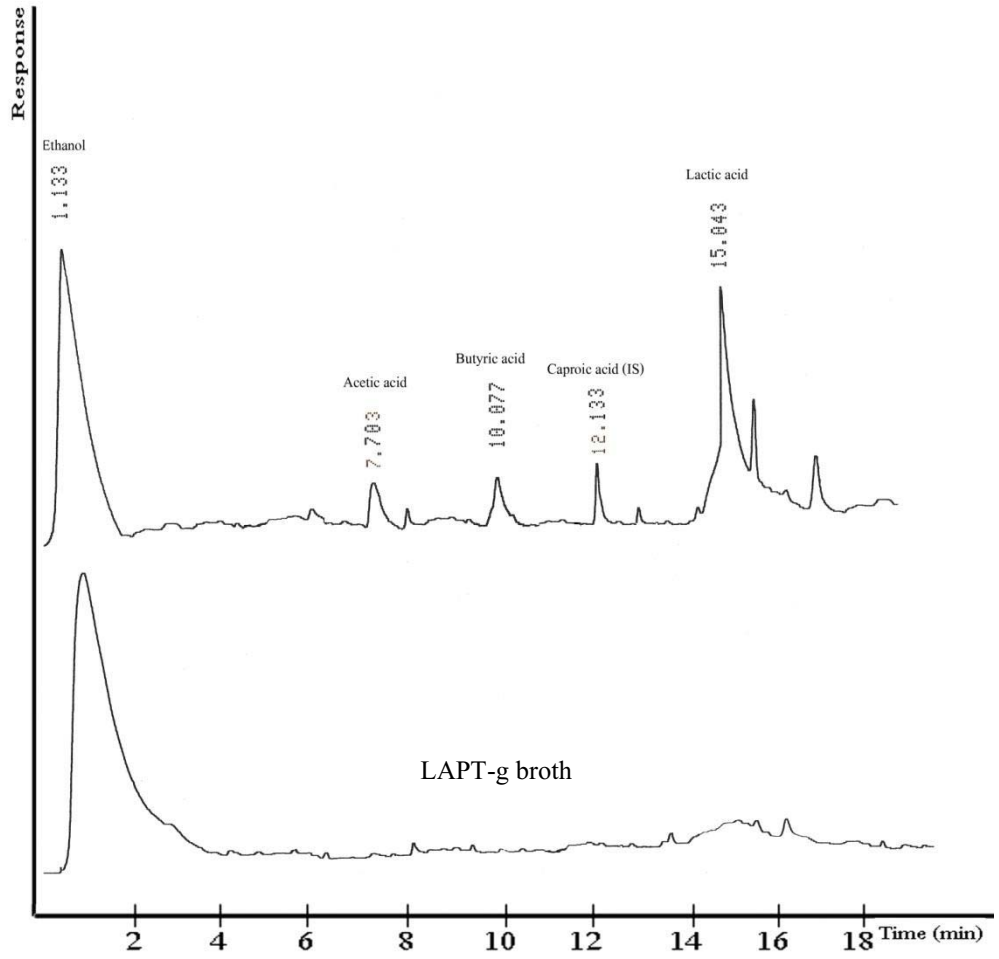


Figure 4-7 The GC chromatogram of acetic acid (t_r = 7.7), butyric acid (t_r = 10.1) and lactic acid (t_r = 15.0) produced by *S. mutans* in LAPT-g culture. Caproic acid was spiked thereafter for precise determination of the acids produced. There were no peaks interfering from LAPT-g blank broth.

Table 4-9 The acids concentrations produced by *L. crispatus* 21L07 grown in BHI broth after incubation at 37°C for 36 hours

Acids	Content (μg/mL)[a]
Acetic acid	0.0569 ± 0.09887
Butyric acid	0.0324 ± 0.11089
Lactic acid	6.6852 ± 0.15797

[a] All values were mean ± SD obtained by triplicate analyses.

4.3.2 Determination of lactic acid and ethyl lactate in esterification process

In this study, the simulated samples from esterification process were prepared. The accurate amounts of acetic acid, butyric acid, lactic acid and ethyl lactate were spiked in ethanol. The GC method was used to evaluate the separation power of the analytes. Results showed all acids could be well separate from ethyl lactate. The % recovery of the acids and ethyl lactate were calculated and shown in Table 4-10. It appeared that the developed GC method was appropriate for monitoring lactic acid in purification process with accepted precision and accuracy.

Table 4-10 Acids and ester contents simulated from esterification process by spiking technique

Acids	Spiking level	Content (μg/mL)[a]	Recovery (%)[a]
Acetic acid	0.05	0.0482	96.40 ± 0.932
Butyric acid	0.05	0.5120	102.40 ± 0.115
Lactic acid	5.00	4.9803	99.61 ± 0.564
Ethyl lactate	5.00	4.8695	97.39 ± 1.002

[a] All values were mean ± SD obtained by triplicate analyses.

4.4 Application of the developed HPLC method for assessment of *S. mutans* quality by determination of sugar utilization.

S. mutans was used as bacterial model for studying the effectiveness of lactic acid production. The wild type *S. mutans* and mutants SMP and SML strains cultured in media with different sugar sources including, glucose, fructose and lactose. The sugar utilization and lactic acid production by *S. mutans* were determined using the developed HPLC and GC methods. The sugar consumed by *S. mutans* strains and lactic acid produced were shown in Table 4-11, Results showed that the newly established methods might be suitable for determining and monitoring lactic acid production and lactic acid purification process on large scale manufacturing.

Table 4-11 Sugar consumed by *S. mutans* strains and lactic acid produced to assess the quality of bacterial stains.

Strains	Sugar used (mg/mL)[a]		
	Glucose	Fructose	Lactose
Wild type	4.185 ± 0.0425	3.655 ± 0.0549	2.912 ± 0.1937
SMP	5.830 ± 0.0010	3.456 ± 0.0331	1.223 ± 0.0444
SML	4.451 ± 0.0670	2.341 ± 0.0303	0.965 ± 0.0359
Strains	Lactic acid produced (μg/mL) [a]		
	Glucose	Fructose	Lactose
Wild type	3.587 ± 0.2587	3.586 ± 0.3501	3.501 ± 0.4673
SMP	1.300 ± 0.0352	1.271 ± 0.0117	1.782 ± 0.0525
SML	1.230 ± 0.0459	1.214 ± 0.0842	1.732 ± 0.1287

[a] All values were mean ± SD obtained by triplicate analyses.

CHAPTER 5

CONCLUSIONS

The quantitative analysis of acetic acid, butyric acid, lactic acid and ethyl lactate was developed and validated using gas chromatography coupled with flame ionization detector. The method was rapid, accurate, precise and simple, since direction injection without derivatization reaction was performed. The GC capillary column SGE BP-20 (30 m × 0.53 mm; film thickness 0.5 µm) using helium as a carrier gas at a flow rate of 0.5 kg/cm^2 was employed with the temperature program initiated at 50°C for 3 minutes, followed by increasing to 110 and 200°C with the rates of 10 and 12°C /min, respectively.

This method was sensitive with detection and quantification limits of 0.01 and 0.10 µg/mL for acetic and butyric acids, 0.10 and 1.00 µg/mL for lactic acid and 0.10 and 0.50 µg/mL for ethyl lactate. The method accuracy was evaluated by recovery (%) which ranged between 98.0 – 102.8% for all acids and ethyl lactate. The method exhibited good linearity for all samples with R^2 > 0.9990. The precision evaluated by RSD (%) was not more than 15%.

For the determination of sugar utilization, high-performance liquid chromatographic (HPLC) method was developed with refractive index detector, set at a temperature of 35°C. A mixture of acetonitrile and Milli-Q grade water (78 : 22, v/v) was used as a mobile phase with isocratic mode at a flow rate of 1.5 mL/min. Samples were injected at a volume of 20 µL through the Zorbax® NH_2 column (250 × 4.6 mm; 5 µm particle size; Agilent, USA) operated at 30°C.

The sensitivity of the method was determined by LOD and LOQ. It revealed that the LOD and LOQ values were 0.0625 and 0.25 mg/mL, for glucose and fructose and 0.50 and 4.00 mg/mL for lactose. Calibration curve was found to be linear in concentration ranges of 2.0 – 12.0 mg/mL with R^2 > 0.9980 for all sugars. The accuracy of the method was evaluated by

recovery (%), ranging between 91.5 – 109.4% and the precision evaluated by RSD (%) was not more than 15%.

The application of the developed chromatographic methods demonstrated in the study was that they were able to assess bacterial quality in lactic acid production and the acid purification processes. Also, they might be suitable for routine use on large scale manufacturing of lactic acid.

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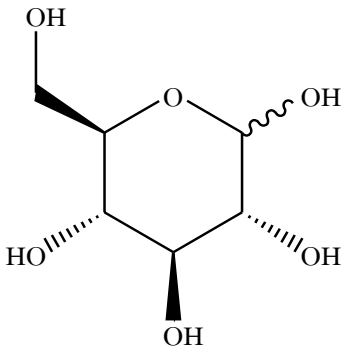
Appendix

Chemical and physical properties of substances used in this study

(The Merck index, 2000; British Pharmacopoeia, 2001)

Glucose



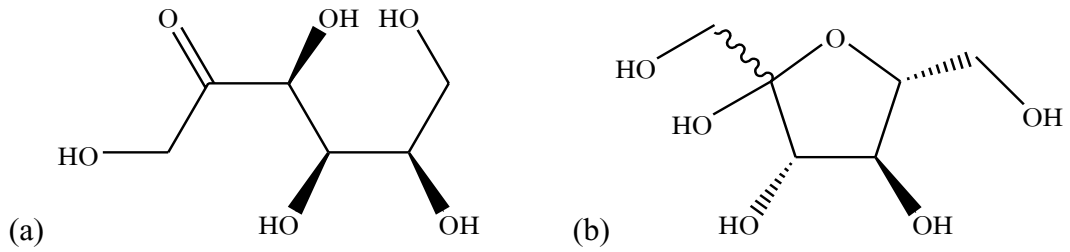
The chemical structure of (+)-D-glucose

Glucose is characterized as a white, crystalline powder, with a sweet taste. It is freely soluble in water, sparingly soluble in absolute alcohol, ether and acetone, soluble in heat glacial acetic acid, pyridine and aniline.

IUPAC name:	6-(hydroxymethyl)oxane-2,3,4,5-tetrol
Synonyms:	Dextrose, Blood sugar,
CAS number:	50-99-7
Molecular formula:	$C_6H_{12}O_6$
Molecular weight:	180.16 g/mole
Exact mass:	180.06339
Density:	1.54 g/mL
Melting points:	α-D-glucose 146°C
	β-D-glucose 150°C

Specific optical rotation: +52.5 to +53.3 at 20°C

Fructose

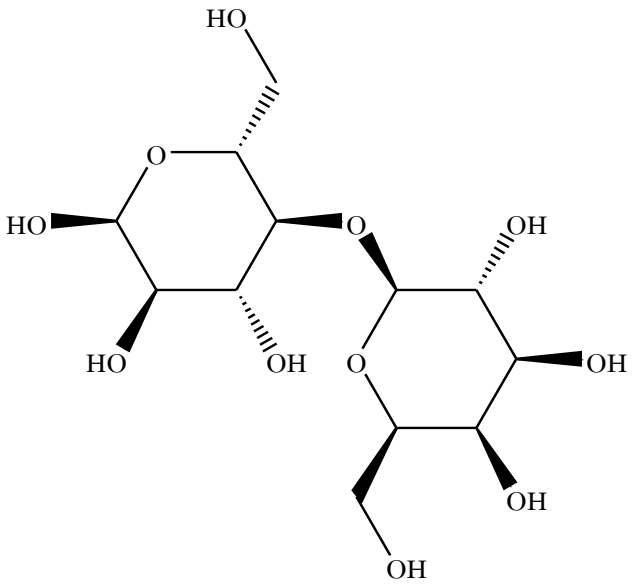


The chemical structure of (-)-D-fructose, (a) open chain formation and (b) ring formation

Fructose is characterized as a white, crystalline powder, with a very sweet taste. It is very soluble in water, soluble in alcohol, pyridine, ethylamine and methyl amine, freely soluble in hot acetone, sparingly soluble in cold acetone, heat glacial acetic acid, and aniline.

IUPAC name:	Tetrahydro-2,5-bis(hydroxymethyl)furan-2,3,4-triol
Synonyms:	Arabino-hexulose, Laevulose
CAS number:	57-48-7
Molecular formula:	$C_6H_{12}O_6$
Molecular weight:	180.16 g/mole
Exact mass:	180.06339
Density:	1.59 g/mL
Melting points:	103°C
Specific optical rotation:	-91.0 to -93.5 at 20°C

Lactose

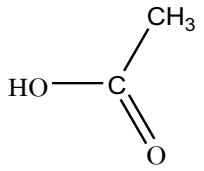


The chemical structure of lactose

Lactose is a disaccharide that consists of galactose and glucose fragments bonded through 2β-1->4 glycosidic linkage. It is characterized as a white or almost white, crystalline powder. It is freely but slowly soluble in water, very slightly soluble in alcohol, insoluble in chloroform and ether.

IUPAC name:	2-(hydroxymethyl)-6-[4,5,6-trihydroxy-2-(hydroxymethyl)oxan
Synonyms:	Milk sugar
CAS number:	63-42-3
Molecular formula:	$C_{12}H_{22}O_{11}$
Molecular weight:	342.30 g/mole
Exact mass:	342.11621
Density:	1.59 g/mL
Melting points:	201°C
Specific optical rotation:	+54.4 to +55.9 at 20°C

Acetic acid

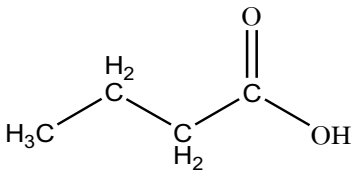


The chemical structure of acetic acid (glacial)

Glacial acetic acid (water free acetic acid) is a colorless liquid that absorbs water from environment (hygroscopy). Glacial acetic acid is characterized as a crystalline mass or clear, colorless, volatile liquid. It is miscible with water, alcohol, glycerol, ether, carbontetrachloride, and methylene chloride.

IUPAC name:	Ethanoic acid
Synonyms:	Acetyl hydroxide, Hydrogen acetate, Ethylic acid, Methane carboxylic acid
CAS number:	64-19-7
Molecular formula:	$C_2H_4O_2$
Molecular weight:	60.05 g/mole
Exact mass:	60.02113
Density:	1.053 g/mL (liquid)
	1.266 g/mL (solid)
Melting points:	16.5°C
Boiling points:	118.1°C
pKa:	4.76

Butyric acid

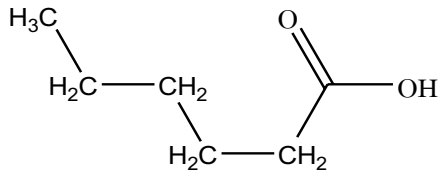


The chemical structure of butyric acid

Butyric acid is characterized as an oily colorless liquid. It has an unpleasant smell and acrid taste with a sweetish after taste. It is miscible in water, ethanol and ether.

IUPAC name:	Butanoic acid
Synonyms:	Propane carboxylic acid
CAS number:	107-92-6
Molecular formula:	$C_4H_8O_2$
Molecular weight:	88.11 g/mole
Exact mass:	88.05243
Density:	0.9595 g/mL
Melting points:	-7.9°C
Boiling points:	163.5°C
pKa:	4.82

Caproic acid

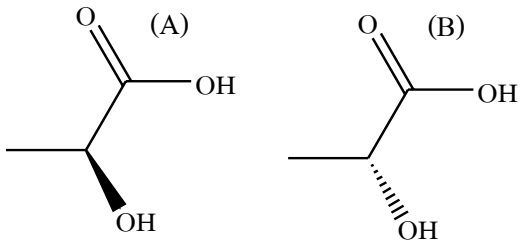


The chemical structure of caproic acid

Caproic acid is characterized as a colorless oily liquid with an odor reminiscent of goats or other barnyard animals. It is slightly soluble in water, soluble in ethanol and ether.

IUPAC name:	Hexanoic acid
CAS number:	142-62-1
Molecular formula:	$C_6H_{12}O_2$
Molecular weight:	116.16 g/mole
Exact mass:	116.08373
Density:	0.9265 g/mL
Melting points:	-3.4°C
Boiling points:	205°C
pKa:	4.88

Lactic acid



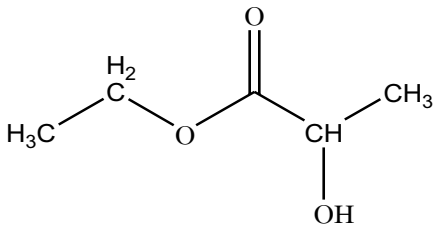
D-(-)-Lactic acid L-(+)-Lactic acid

The chemical structure of lactic acid (A) D-isomer and (B) L-isomer

Lactic acid is chiral and has two optical isomers. One is known as L-(+)-lactic acid or (S)-lactic acid and the other, it mirror image. Lactic acid is colorless or slightly yellow, syrupy liquid. It is soluble in water, alcohol, acetone, ether and glycerol, practically insoluble in chloroform.

IUPAC name:	2-hydroxypropanoic acid
Synonyms:	Milk acid
CAS number:	(L) 50-21-5
	(D) 79-33-4
	(D/L) 50-21-5
Molecular formula:	$C_3H_6O_3$
Molecular weight:	90.08 g/mole
Exact mass:	90.03169
Density:	0.9265 g/mL
Melting points:	(L) and (D) 53°C
	(D/L) 16.8°C
Boiling points:	122°C
pKa:	3.79

Ethyl lactate

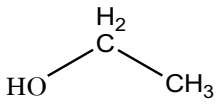


The chemical structure of ethyl lactate

Ethyl lactate is produced from biological source and can be either the *levo* (S) form or *dextro* (R) form, depending on types of lactic acid. Most biological source of ethyl lactate is ethyl (-)-L-lactate. It is miscible in water, alcohol and ether.

IUPAC name:	Ethyl 2-hydroxy propionate
Synonyms:	Ethyl lactate
CAS number:	97-64-3
Molecular formula:	$C_5H_{10}O_3$
Molecular weight:	118.13 g/mole
Exact mass:	118.06299
Density:	1.031 g/mL
Melting points:	-26°C
Boiling points:	154°C

2.5.1 Ethanol



The chemical structure of ethanol

Ethanol is characterized as a clear, colorless, very mobile, flammable liquid with a pleasant odor, burning taste and absorbs water rapidly from air. It is miscible in water and many organic acids.

IUPAC name:	Ethanol
Synonyms:	Ethyl alcohol, Grain alcohol, Hydroxy ethane, Drinking alcohol
CAS number:	64-17-5
Molecular formula:	C_2H_6O
Molecular weight:	46.07 g/mole
Exact mass:	46.04186
Density:	0.789 g/mL
Melting points:	-114.3°C
Boiling points:	78.4°C

VITAE

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Bachelor of Pharmaceutical Science	Prince of Songkla University	2007

List of Publication and Proceedings

Chooklin, S., Kaewsichan, L., Kaewsrichan, J. and Sura-apinan, P. (2009), "Ethyl lactate production and separation process: the esterification of lactic acid with wet ethanol by catalytic distillation", *The 3rd International Conference on Fermentation Technology for Value Added Agricultural Products*, August 26-28, Khon Kaen, Thailand.

Sura-apinan, P., Kaewsrichan, J. and Kaewsichan, L. (2010), "Development of gas chromatographic method for determination of lactic acid and its ester in the purification process", *The 1st Current Drug Development International Conference,* May 6-8, Phuket, Thailand.