SELECTION OF MALOLACTIC BACTERIA FOR WINE FERMENTATION

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การคัดเลือกแบคทีเรียมาโลแลคติกเพื่อใช้ในการหมักไวน์

นางสาวแก้วกัลยา วงศ์กาฬสินธุ์

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แบคทีเรียมาโลแลคติกมีบทบาทสำคัญในกระบวนการหมักไวน์แบบมาโลแลคติก เนื่องจากสามารถเปลี่ยนกรดมาลิกให้เป็นกรดแลคติกได้ งานวิจัยครั้งนี้มีวัตถุประสงค์เพื่อคัดเลือกแบคทีเรียมาโลแลคติกสำหรับนำไปใช้ในกระบวนการผลิตไวน์ได้อย่างมีประสิทธิภาพ แบคทีเรียจำนวน 9 ไอโซเลทที่คัดเลือกได้จากจำนวนทั้งสิ้น 70 ไอโซเลท (โดยพิจารณาจากความสามารถในการเปลี่ยนกรดมาลิกให้เป็นกรดแลคติกในอาหารสังเคราะห์ที่ปราศจากกลูโคสและฟรุคโตส) เพื่อนำมาทดสอบความสามารถในการเปลี่ยนกรดมาลิกให้เป็นกรดแลคติกในอาหารสังเคราะห์ที่มีแอลกอฮอล์ และ ปราศจากน้ำตาล พบหนึ่งไอโซเลท คือ CB5 ที่สามารถเปลี่ยนกรดมาลิกให้เป็นกรดแลคติก ได้สูงที่สุดและสามารถเจริญได้ดี ภายใต้สภาวะที่มีความเข้มข้นของเอทานอล 15% และที่อุณหภูมิ 20 และ 25 องศาเซลเซียส ไอโซเลท CB5 สามารถเปลี่ยนกรดมาลิกให้เป็นกรดแลคติกในสภาวะที่มีความเข้มข้นของเอทานอลสูงๆ ได้ดีกว่าแบคทีเรียสายพันธุ์ทางการค้า (*Oenococcus oeni* CH35) ดังนั้นจึงพิจารณาเลือกไอโซเลทดังกล่าว มาทดลองใช้ในการหมักไวน์แบบมาโลแลคติกที่ 25 องศาเซลเซียส จากการศึกษาพบว่าไอโซเลท CB5 สามารถเปลี่ยนกรดมาลิกและผลิตกรดแลคติกได้สูงกว่า *Oenococcus oeni* CH35 สำหรับการทดสอบคุณภาพทางประสาทสัมผัสของไวน์ที่หมักได้ พบว่าทุกคุณลักษณะ คือ ความเปรี้ยว กลิ่นรส ความสมดุลของความเป็นกรด ปริมาณแอลกอฮอล์ รสผลไม้และรสฝาด และคุณลักษณะโดยรวมของไวน์นั้นไม่มีความแตกต่างกันอย่างมีนัยสำคัญที่ระดับความเชื่อมั่น 95% สำหรับการระบุชนิดของไอโซเลท CB5 โดยใช้ลักษณะทางสัณฐานวิทยา สมบัติทางชีวเคมีและลำดับเบสของ 16S rDNA นั้นได้จัดไอโซเลท CB5 อยู่ในชนิด *Leuconostoc mesenteroides* เมื่อพิจารณาจากสมบัติทางชีวเคมี ซึ่งมีความเหมือน 99.9% กับแหล่งอ้างอิง แต่จัดอยู่ในชนิด *Leuconostoc pseudomesenteroides* เมื่อพิจารณาจากความเหมือนของลำดับเบส 16S rDNA (98%) ดังนั้นไอโซเลท CB5 อาจเป็น *Leuconostoc* ชนิดใหม่ หรือเป็น subspecies ใหม่ของ *Leuconostoc mesenteroides* และ *Leuconostoc pseudomesenteroides*

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MALOLACTIC BACTERIA/MALOLACTIC FERMENTATION/MALOLACTIC CONVERSION/WINE

Malolactic bacteria play an important role in malolactic fermentation of winemaking. They are capable of conversion of L-malic acid to L-lactic acid. This study aimed to select and apply malolactic bacteria for the potential production of wine. Nine isolates of malolactic bacteria were selected (from the total of 70 isolates) by considering their malolactic conversion capability in a synthetic medium without sugar. When these isolates were tested for their malolactic conversion in the synthetic medium containing ethanol, it was found that only the isolate CB5 had the highest malolactic conversion and a good growth under 15% ethanol condition. The CB5 could also convert L-malic acid to L-lactic acid in higher ethanol concentration than the commercial strain *Oenococcus oeni* CH35 at 20 and 25°C. Therefore, it was considered to be the best strain for malolactic fermentation at 25°C. The CB5 could convert L-malic acid to L-lactic in higher concentration than *O. oeni* CH35 did. Sensory evaluation of wine products was also conducted. It was found that all wine samples were no significantly different at 95% confidential in four characters including sour, flavor, balance of acidity, alcohol content, fruit flavor and astringency, and overall characteristics. For the identification of malolactic bacterium using morphology, biochemical properties, and 16S rDNA sequence, the CB5 belonged to

Leuconostoc mesenteroides (99.9% similarity of biochemical characteristics compared to reference source), but belonged to *Leuconostoc pseudomesenteroides* (98% similarity of 16S rDNA sequence). So, the isolate CB5 could be either a new species of *Leuconostoc* or a new subspecies of *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides.*

School of Microbiology

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Student's Signature KAEWKANLAYA WONGKALASIN

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Co-advisor's Signature

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Kaewkanlaya Wongkalasin

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LIST OF ABBREVIATIONS

a.m.	Ante meridiem
ANOVA	Analysis of varience
BLAST	Basic local alignment search tool
bp	Base pair
°C	Degree Celsius
CFU	Colony forming unit
cm	Centrimeter
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	Deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
et al.	et alia (and others)
FID	Flame ionization detecter
(m, μ) g	(milli, micro) Gram
GC	Gas chromatography
h	Hour
HPLC	High performance liquid chromatograph
(m, μ) l	(milli, micro) Liter

LIST OF ABBREVIATIONS (Continued)

(m, μ) M	(milli, micro) Molarity
min	Minute
MLF	Malolactic fermentation
(m, μ) mol	(milli, micro) Mole
N	Normality
%	Percentage
PCR	Polymerase chain reaction
QDA	Quantitative descriptive analysis
rpm	Round per minute
s	Second
SAS	Statistical analysis system
sp.	Species
supsp.	Subspecies
UV	Ultra violet
%v/v	Percentage volume by volume
%w/v	Percentage weight by volume

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Wine is an alcohol beverage obtained from the fermentation of grape juice or other fruit juices by yeast. Wine fermentation is one of the oldest forms of food preservation technologies in the world, and has taken place for thousands of years. Winemaking has become a global enterprise, which significantly affects the economic well being of several countries in present (Walker, 2000). The alcoholic fermentation (the primary wine fermentation) is carried out by yeast which added to the juice. The most beneficial yeast in terms of desirable wine fermentation is *Saccharomyces cerevisiae,* which is widely used to produce ethanol during the biodegradation of glucose and fructose in grape juice (Klingshirn, 2002).

Another group of microorganisms involved in wine fermentation is the lactic acid bacteria which plays an important role in the secondary wine fermentation or so called malolactic fermentation. The effect of malolactic fermentation depends on the grape variety (Bozoğlu and Yurdugül, 2000). The climatic conditions and soil features of the grape growing areas affect the chemical composition of grape, especially acidity in each of grape variety (Sieiro *et al.,* 1990). The grape juice contains mainly tartaric acid and malic acid. If the acidity is too high for the best flavor, malolactic fermentation is a necessary process for conversion of L-malic acid to L-lactic acid, resulting in the decrease in acidity in wine (Steinkraus, 1992).

The benefits of malolactic fermentation include the decreasing of acidity in high acid wines and enhancement of sensory characteristics though bacterial activity (Bozoğlu and Yurdugül, 2000). The lactic acid bacteria involved in malolactic fermentation are called malolactic bacteria belonging to the genera *Leuconostoc*, *Pediococcus* and *Lactobacillus* (Lonvaud-Funel, 2000; Van Vuuren and Dicks, 1993; Versari, Parpinello, and Cattaneo, 1999), which are capable of direct decarboxylation of malic acid to lactic acid and CO_2 by malate carboxylase or so-called malolactic enzyme (MLE) (Bozoğlu and Yurdugül, 2000; Fugelsang, 1997; Renault *et al.,* 1988; Salema *et al.,* 1996).

Thailand has been the strong potential country of producing wine. Therefore the selection of the efficient malolactic bacteria as local strains for wine fermentation becomes important for the improvement of flavour in grape wine. The purpose of this study is to select the high efficient malolactic bacteria, which are capable of conversion of L-malic acid to L-lactic acid in wine fermentation.

1.2 Research objectives

The purposes of this research are as follows:

1) to select malolactic bacteria for the potential production of wine,

2) to identify the selected isolates of malolactic bacteria,

3) to apply the selected isolates of malolactic bacteria for wine fermentation.

1.3 Research hypothesis

The malolactic bacteria isolates efficiently converting L-malic acid to L-lactic acid would be very useful for wine production. The genomic analysis could be used to combine with biochemical characterization to get the precise and identification of malolactic bacteria.

1.4 Scope and limitation of the study

Malolactic bacteria were isolated from wastewater of food industries, dairy and vineyard. All isolates of malolactic bacteria were selected by considering for the viability and malolactic conversion in synthetic medium without D-glucose and D-fructose. The selected isolates of malolactic bacteria were determined malolactic conversion in various ethanol concentrations added the synthetic medium. Ethanol concentrations used were (%v/v): 0, 5, 7.5, 10, 12.5 and 15. One isolate was selected for malolactic fermentation by considering for the highest malolactic conversion. All fermentation treatments were performed in duplicates in 5 L round flat bottom flasks, which were equipped with fermentation lock (air lock), and incubated at 25°C. Twenty milliliters of wine samples were taken for chemical analysis every week. The growth of bacteria were monitored by total viable count technique using MRS medium. And pH of wine were monitored by using pH meter. After the malolactic fermentation completed within 1 month, L-malic acid, L-lactic acid, D-glucose and D-fructose were determined by using enzymatic test kits (Boehringer Manneheim, R-biopharm, Germany). Ethanol was determined by Gas Chromatograph (GC). The sensory evaluation was performed by the trained panelists using Quantitative Descriptive Analysis (QDA). Data were statistically analysed by the SAS (Statistical

Aanalysis System) program. The selected isolates were identified by study of its morphological and biochemical characteristics. The gene sequence encoding 16S rRNA was also analysed.

1.5 Expected results

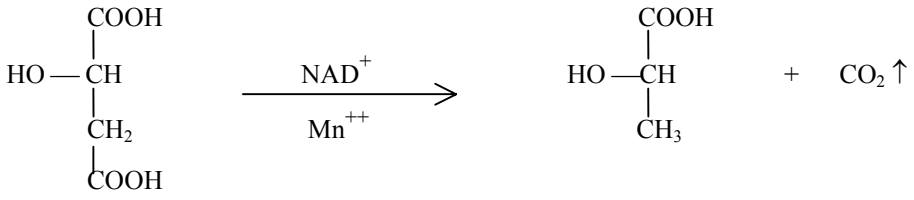
The selected malolactic bacteria are high efficient local strains which could be useful for wine industry. The data of malolactic bacteria identification by using phenotypic and genomic analysis will be the prerequisite data for study of gene encoding malolactic enzyme as well as gene cloning.

CHAPTER II

LITERATURE REVIEW

2.1 Malolactic bacteria

Malolactic bacteria are group of lactic acid bacteria, which are present in wines and grape musts. They play an important role in the secondary wine fermentation or so-called malolactic fermentation. Malolactic bacteria are capable of conversion of dicarboxylic L-malic acid to monocarboxylic L-(+)-lactic acid and carbon dioxide (CO_2) by decarboxylation (Figure 1). The reaction is called malolactic conversion, which is catalyzed by enzyme malate carboxylase (EC1.1.1.38) or so called malolactic enzyme (MLE), requires the coenzyme NAD^+ as well as Mn^{2+} (Bozoğlu and Yurdugül, 2000; Fugelsang, 1997; Selema *et al.,* 1996).



L(-)-malic acid L-(+)-lactic acid

Figure 1 Malolactic conversion: a direct decarboxylation of L(-)-malic acid to L-(+)-lactic acid.

Source: Bozoğlu and Yurdugül (2000).

Malolactic bacteria are Gram-positive cocci or rod, catalase negative, and microaerophilics or facultatively anaerobes, which require reducing (low oxygen) condition for normal growth. Furthermore, they are nutritionally fastidious microorganisms, requiring complex organic media for growth. (Zoecklein *et al.,* 1995). The genera of malolactic bacteria are show in Figure 2, which include *Lactobacillus* (homofermentative and heterofermentative lactobacilli), *Pediococcus* (homofermentative pediococci) and *Leuconostoc* (heterofermentative species) (Bozoğlu and Yurdugül, 2000; Fugelsang, 1997; Lonvaud-Funel, 1999). Malolactic bacteria are resistant to low pH condition such as in wine. Among them *Leuconostoc oenos*, which is more recently reclassified as *Oenococcus oeni* (Dicks *et al.,* 1995), is recognized as the most tolerant bacterium in wine conditions, such as low pH, high SO_2 and alcohol contents (Lonvaud-Funel, 2000; Van Vuuren and Dicks, 1993; Versari *et al.,* 1999).

General descriptions of malolactic bacteria in wine (Bozoğlu and Yurdugül, 2000) are as follows:.

Genus *Leuconostoc*

A simple identification procedure is applicable to *Leuconostoc* spp. If a *Leuconostoc* sp. is isolated from wine, it is automatically classified as *Leuc. oenos,* as only strains of this species can grow in the presence of 10% ethanol at pH values less than 4.2. Cells are spherical and usually occur in pairs or chains. Growth, compared to that of non-acidophilic *Leuconostoc* spp., is slow and takes 5-7 days at 22°C.

Genus *Pediococcus*

Four species of *Pediococcus* that can grow at pH 4.2 or below are related to winemaking: *P. damnosus, P. parvulus, P. pentoseus* and *P. acidilactici.*

Genus *Lactobacillus*

Species classification of the wine related lactic acid bacteria have not commanded great attention, since these organisms as a class show little resistance to the low pH values found in wine. Two species have been shown to be essentially important in the spoilage of high alcohol desert wine: *Lactobacillus bilgardii* and *L. fructivorans.*

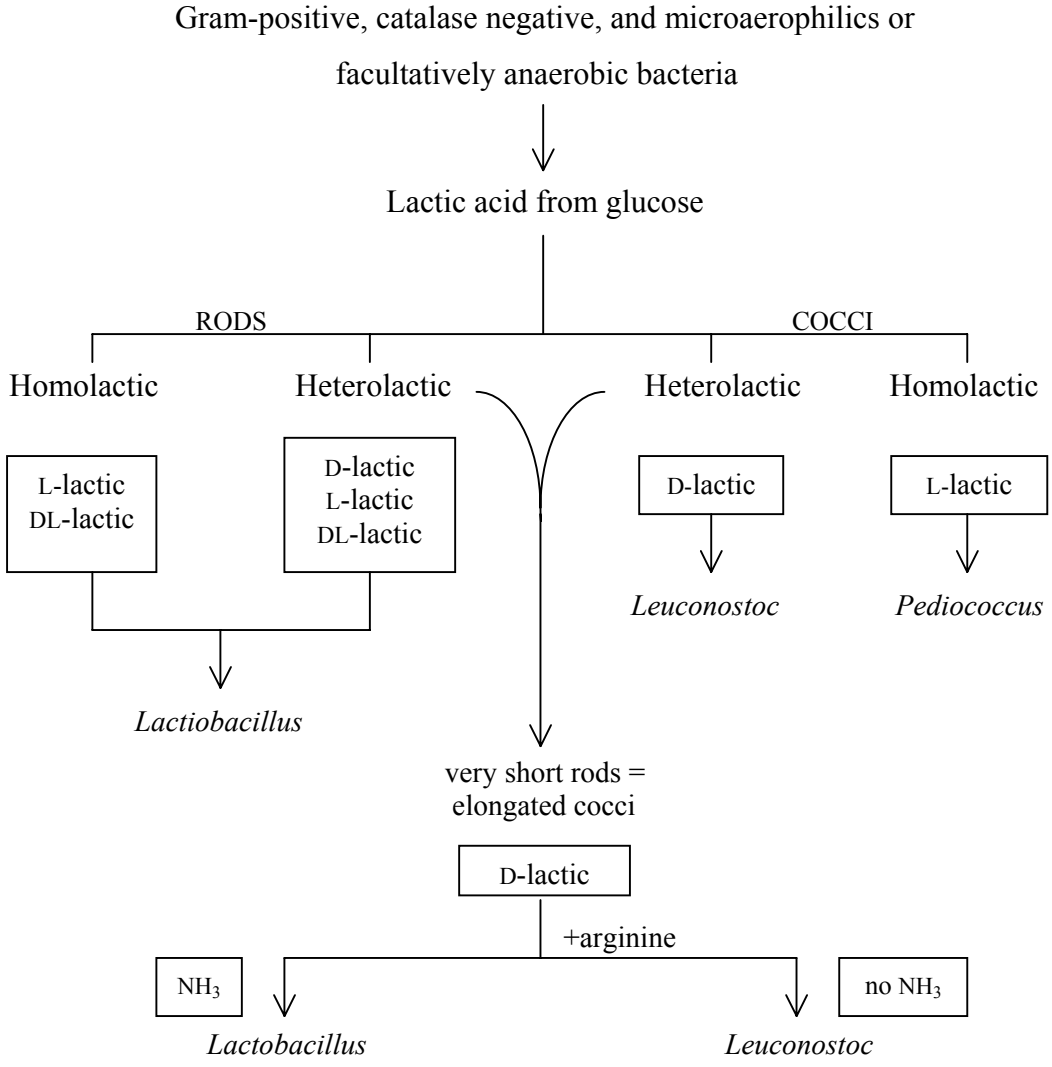


Figure 2 Genera of malolactic bacteria.

Source: Boulton *et al.* (1996).

Malolactic bacteria produce lactic acid as the major end-product during fermentation of carbohydrates. The amount of lactic acid (and other metabolites) formed from glucose and the pathway of its formation separate lactic acid bacteria into two groups, the hetero- and homofermenters (Figure 3). Homofermenters produce primarily lactic acid as the end-product of glucose metabolism. Lactic acid bacteria in this group use the Embden-Meyerhoff Parnas (EMP) pathway. Pyruvate is produced to lactic acid yielding 2 moles of lactic acid and 2 moles of ATP per mole of glucose. Heterofermenters, however, lack the aldolase (fructose-diphosphate aldolase) enzyme, which mediates cleavage of fructose-1,6-diphosphate. This group uses the pentose phosphate pathway (6-phosphogluconate/phosphoketolase pathway) yielding 1 mole of ATP per mole of glucose and other end-products such as ethanol, acetate, and CO_2 in addition to lactic acid (Fugelsang, 1997; Priest and Campbell, 1999; Axelsson, 2004; Zoecklein *et al.,* 1995).

The isomer of lactic acid produced from growth on glucose is unique to the species (shown in Figure 2). *Leuconostoc oenos* produces only D(-)-lactate whereas *Pediococcus* produces L(+)-lactate. Depending on the species, *Lactobacillus* sp. produces both D(-) and L(+) as well as D and L isomer (Fugelsang, 1997).

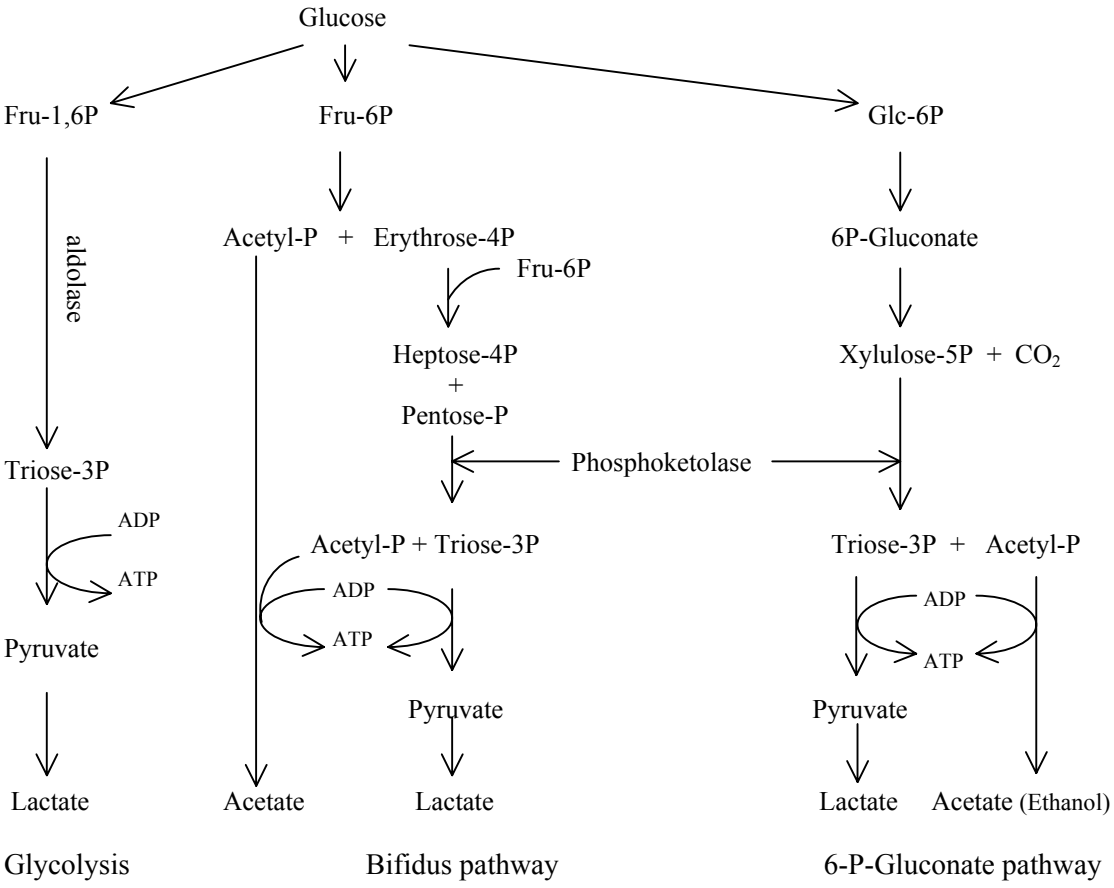


Figure 3 Metabolic pathway of glucose fermentation by malolactic bacteria.

Source: Wood and Holzapfel (1995).

2.2 Wine production

Wine is an alcoholic beverage made from fermented grape juice or other fruit juices. Wine fermentation is performed by yeast which play additional roles in the development of flavour and aroma of the wine (Walker, 2000). The alcoholic fermentation (the primary wine fermentation) is carried out by yeast which belongs to genus *Saccharomyces*, in particular species *S. cerevisiae.* The yeast utilizes glucose

and fructose in grape juice, and metabolize them via the Embden-Meyerhof-Parnas glycolytic pathway, to pyruvate. Under anaerobic conditions, the yeast decarboxylate pyruvate by pyruvate carboxylase, to yield acetaldehyde and CO_2. The final step in alcoholic fermentation is catalysed by alcohol dehydrogenase, and involves the reduced coenzyme NADH, resulting in the reduction of acetaldehyde to ethanol. The conversion equation of glucose to ethanol by yeast can be summarized as follows: (Steinkraus, 1992)

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + 2Pi + 2ADP + 2H^+ \rightarrow \underset{\text{Ethanol}}{2C_2H_5OH} + 2CO_2 + 2ATP + 2H_2O$$

In the secondary wine fermentation, a group of microorganisms involved is lactic acid bacteria. Due to grape juice contains mainly tartaric acid and malic acid. If the acidity is too high, to improve flavor, it may have to apply lactic acid bacteria to convert the malic acid to lactic acid, which decreases the total acidity in wine (Steinkraus, 1992). This conversion is occur in the malolactic fermentation, which are present in various genera of lactic acid bacteria, particularly *Leuconostoc*, *Pediococcus* and *Lactobacillus* (Lonvaud-Funel, 2000; Versari *et al.,* 1999).

Malolactic fermentation in wine is desirable for three reasons: (i) to decrease the acidity; (ii) to enhance the sensory characteristics and (iii) to increase the microbiological stability of wine. However, malolactic fermentation is not favorable for all wines (Versari *et al.,* 1999). Because wine produced in cold regions such as Germany, France and the Eastern United States, has a high acid content and may have benefit from the deacidification by malolactic fermentation. But wine from warmer viticultural regions such as South Africa, California and Australia, have a lower

acidity. Malolactic fermentation in these wines could reduce the acid level in the vary low acidity resulting in a flat, insipid wine and growth of spoilage bacteria (Davis *et al.,* 1985; Van Vuuren and Dicks, 1993).

2.3 Malolactic bacteria in wine production

Malolactic bacteria are present in all grape musts and wines. Depending on the stage of the winemaking process, environmental conditions determine their ability to multiply. When they develop, they metabolize numerous substrates. Malolactic bacteria therefore play an important role in the wine process. Their impact on wine quality depends not only on environmental factors acting at the cellular level but also on the selection of the best adapted species and strain of bacteria (Alexandre *et al.,* 2004; Ribéreau-Gayon *et al.,* 2000; Steinkraus, 1992).

During the first day of alcoholic fermentation, the bacteria and yeasts multiply. The latter, better adapted to grape must, rapidly invade the medium with elevated populations. During this time, the bacteria multiply but their growth remains limited, with maximum population of 10^4 to 10^5 CFU/ml. To large extent their behavior at time depends on the pH of the medium and the grape sulfating level (Ribéreau-Gayon *et al.,* 2000). Malolactic bacteria play an important role in the secondary wine fermentation, which usually occurs after yeasts have completed the primary alcoholic fermentation when the bacterial population is about 10^6 CFU/ml (Lonvaud-Funel, 1999; Osborn *et al.,* 2000; Versari *et al.,* 1999).

Moreover malolactic bacteria may be inoculated at the following stages of vinification: (i) simultaneous with yeast inoculation, (ii) during alcoholic fermentation, and (iii) after completion of alcoholic fermentation (Davis *et al.,* 1985).

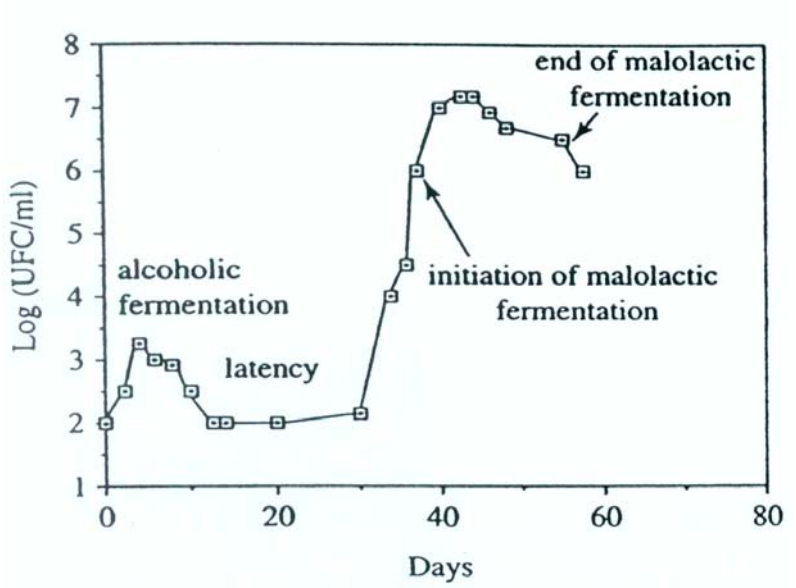


Figure 4 Evolution of malolactic bacterial population during alcoholic and malolactic fermentation.

Source: Ribéreau-Gayon *et al.* (2000).

The malolactic fermentation phase begins during the growth phase, as soon as the total population exceeds 10^7 CFU/ml. It continues and is completed during the stationary phase, or sometimes at the beginning of the death phase (Figure 4). In very favorable conditions with a limited concentration of malic acid, malolactic fermentation is often completed even before the end of the growth phase. The optimum population exceed 10^8 CFU/ml. As soon as a sufficient biomass is formed, malic acid is degraded. The malolactic bacterial activity is always present but depends on various conditions, especially temperature. The transformation of 2 g of malic acid per liter can take more time than 4 g/l if the population level attained is lower (Ribéreau-Gayon *et al.,* 2000).

2.4 Cultural characteristics of malolactic bacteria

The malolactic bacteria are generally grown in complex media, often containing peptone, tryptone or yeast extract at pH 5-6, and are usually stored in such media with agar, as stab cultures. MRS broth has been used for the maintenance of lactic acid bacteria (Bozoğlu and Yurdugül, 2000).

Moreover, oxygen can influence the multiplication of lactic acid bacteria in wine but its effect is not clear. In fact, the behavior of bacterial species present in wine can be diverse with respect to oxygen. They can be indifferent to its presence, adapt better in its absence (facultative anaerobiosis), tolerate oxygen at its partial pressure in air but be incapable of using it (aerotolerant), or finally can require a small oxygen concentration for optimal growth (microaerophiles). Furthermore, behavior of a given strain can vary with its environment. In a laboratory culture medium, growth is activated in an inert gas atmosphere: CO_2 and N_2 (Ribéreau-Gayon *et al.,* 2000).
Kelly *et al.* (1989) reported that growth of *Leuconostoc oenos* under strict anaerobic condition using a 100% CO_2 atmosphere found growth in culture media containing *Lactobacillus* MRS broth (Difco) plus 20% (v/v) apple juice (pH 4.7). Especially on agar plates added 1.5% (w/v) agar, its growth are much more rapid than when incubated in aerobic condition.

Saguir and De Nadra (1996) reported that *Leuconostoc oenos* M (isolated from Argentinian red wine) did not grow in the absence of glucose and it was clearly stimulated by presence of L-malic and citric acids in synthetic medium with different glucose concentrations. In basal medium, D-glucose and L-malic and citric acids were simultaneously consumed. L-malic acid was metabolized at a higher rate than glucose

and citric acid. When the organic acids were completely consumed only 50% of the glucose was utilized.

2.5 Identification of malolactic bacteria

Since the beginning of microbiology, the identification of bacteria has been based on their phenotypic characters. Besides by its morphology, which gives little information, a strain is identified essentially by the substrates and products of its metabolism. When more discriminating analytical methods appeared, the chemical composition of microorganisms (fatty acid and protein) also participated in their identification (Ribéreau-Gayon *et al.,* 2000).

For the analysis of biochemical characterization, the API 50 CH identification system (Bio-Mérieux) is commonly used for malolactic bacteria (Edwards *et al.,* 1998; Ribéreau-Gayon *et al.,* 2000). The unidentified strain is inoculated in a nutritive medium that contains all of the nitrogen based nutrients, vitamins and salts necessary for its growth. Different carbohydrate energy sources are represented in each microtube of the system. In this manner, 49 substances are tested, including hexoses, pentoses, disaccarides, etc. An indicator in the culture medium, which changes color, facilitates the reading of results. Fermentation in a microtube acidifies the medium, provoking the indicator to change color (Ribéreau-Gayon *et al.,* 2000).

For the identification based on genomic analysis, there are several molecular biological techniques exist which permit different levels of identification: strain, species, or genus. The study of the restriction polymorphism is based on the specific action of restriction enzyme. Sato *et al.* (2000) performed restriction fragment length polymorphism (RFLP) analysis using the *Acc*II or *Hae*III enzyme for 44 strains of

lactic acid bacteria isolated from red wine, and results indicated profiles identical to *Oenococcus oeni* type strain. *O. oeni* strains exhibited unique RFLP patterns by *Hae*III digestion of 12 reference strains.

Hybridization is a technique often used in molecular genetics, and it is very well adapted for the identification of species and even strains. This technique is based on the ability of single-strand DNA chains to reassemble in double-strand chains. Sohier and Lonvaud-Funel (1998) modified DNA hybridization to *in situ* hybridization method for detecting and identifying lactic acid bacteria in wine using total genomic DNA probes, which were labeled with digoxygenin (DIG) by random priming and hybridized with genomic DNA of the bacteria to be identified. The hybrids were detected with fluorescent anti-DIG Fab-fragments or with enzyme-conjugated anti-DIG Fab-fragments. And they discussed about *in situ* hybridization technique possible the control of the bacteria population in wine at different stages of vinification of storage, and the identification of lactic acid bacteria which can cause wine spoilage.

Finally, the polymerase chain reaction or PCR technique is more useful for classification purpose, and it is possible to amplify a genera or a part of gene from a very limited number of cells for subsequent DNA sequencing (Axelsson, 2004). Lee *et al.* (2000) developed PCR for rapid and reliable identification of *Leuconostoc* species, by using species-specific primers targeted to the genes encoding 16S rRNA. They reported that PCR technique can detect and differentiate *Leuconostoc* species from mixed populations in natural sources as well as from pure cultures, within 3 hours. And the results showed perfect correlation with the results of a polyphasic method, including 16S rRNA sequencing and DNA-DNA hybridization.

Furthermore, Jang *et al.* (2003) developed PCR-RFLP method to detect and identify typical *Leuconostoc* species. The size of the amplified products was 976 bp and the amplicons of the different species could be differentiated from each other with *Mse*I, *Hae*III and *Tsp*509I endonucleases. This PCR-RFLP method enables the rapid and reliable identification of *Leuconostoc* species and to distinguish them from the other phylogenetically related lactic acid bacteria in food samples.

2.6 Detection of malolactic conversion

Changes in titratable acidity and pH characterize the malolactic conversion, however these changes are variable in degree and may be made by or arise from other reactions in wine (Bozoğlu and Yurdugül, 2000). Moreover, malolactic fermentation can be induced in synthetic medium without any problem (Lonvaud-Funel, 1995). The measurement of disappearance of malic acid is the accepted means of determining whether malolactic conversion has occurred. On visual inspection of relatively clear and light coloured wines, an increase in turbidity can see from the increased concentration of bacteria. Increased effervescence may be evident from the formation of CO_2 and there may be a loss in color due to change in pH and available hydrogen ions, the latter, from NADH formed during the fermentation, can provide some indications of malolactic activity. The loss of color in red wine can be measured spectrophotometrically (Bozoğlu and Yurdugül, 2000).

Further, several techniques; paper chromatography, enzymatic analysis, liquid chromatography and high-performance liquid chromatography (HPLC) are used for the determination of malic acid.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals used for chemical reaction were analytical grades and some chromatographic grades for chemical analysis by HPLC, and were purchased from BDH, England; Fluka Chemika, Swizerland; Himedia, India; Merck, Germany; and Across, USA.

Reagents used for PCR amplification were PCR buffer, $MgCl_2$ solution, dNTPs, and *Taq* DNA polymerase (Finneyzme, F. Hoffman-La Roche Molecular Systems, Inc. and The Perkin-Elmer Corperation, U.S.A.). The oligonucleotide primers were ordered from the Invitrogen life technologies, Hong Kong.

3.1.2 Microorganisms

Saccharomyces cerevisiae strain 71B1122 were used for wine production and *Oenococcus oeni* CH35 (commercially as Vinifera) were used as reference strain for malolactic fermentation.

3.1.3 Wine

Wine used in this study was obtained from the completed alcoholic fermentation by *Saccharomyces cerevisiae* 71B1122, from Suranaree University of

Technology farm. The wine was prepared From Shiraz grape (*Vitis vinifera*) which was harvested in January, 2004.

3.14 Equipments

Equipments used were as follows: Gas chromatograph: GC (Hewlett Packard HP 6890 series, Agilent Technologies, Inc., England), High-Performance Liquid Chromatograph: HPLC (Thermo Separation Products Inc., USA), DNA sequencer (ABI PRISM^{TM} 310 Genetic Analyzer, Applied Biosystems, USA), DNA thermal cycler (Gene Amp® PCR system 9700, Perkin Elmer Corp., USA), Spectrophotometer (Pharmacia Biotech Ultrospec 2000, England), Microcentrifuge (Centrifuge 5415C, Eppendorf, Germany), Biological incubator, Hot air oven, Refrigerator (4°C), Freezer (-20°C), Laminar flow hood, pH meter, Compound microscope and basic microbiological equipment. All equipment are located at the Instrument Buildings of the Center for Scientific and Technological Equipment at Suranaree University of Technology.

3.2 Methods

3.2.1 Isolation of malolactic bacteria

Malolactic bacteria were collected from 2 wastewater samples of food industries (Doikham food industry, Sakon Nakhon and Malee food industry, Nakhon Panom), and 2 samples from dairy and vineyard (Suranaree university of technology farm, Nakhon Ratchasima). Ten grams of each samples were suspended in 90 ml sterilized phosphate buffer (pH 7.2) and serially diluted. The samples were also serially diluted with sterilized phosphate buffer (pH 7.2)

The bacteria were isolated by spread-plating 100 µl of each dilution onto MRS agar (Himedia, India). A duplicate set of culture plates were incubated at 30°C for 48 hours in under anaerobic condition (Booysen *et al*., 2002; Kelly *et al.,* 1989; Muyanja *et al.,* 2003). All cultures were stored in 15% glycerol, at -20°C.

3.2.2 Selection of malolactic bacteria for wine production

3.2.2.1 Screening of malolactic bacteria based on malolactic conversion in a synthetic medium without sugar

A. Medium preparation and malolactic bacterial cultivation

A synthetic medium used as basal medium was modified from Panangiotis (2001) by adding no sugars (D-glucose and D-fructose). The medium composed of 1 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 0.3 g of citric acid, 5 g of malic acid, 5 g of tartaric acid, 0.4 g of $MgSO_4$, and 5 g of KH_2PO_4 in 1 L. The pH of medium was adjusted to 3.5 with 10 N NaOH, and autoclaved at 121°C for 15 min The commercial strain of *Oenococcus oeni* CH35 was used as a positive control.

The sterilized medium was used to cultivate all isolates of malolactic bacteria. The initial population was 10^8 cell/ml. All cultures were incubated at 30°C for 7 days under anaerobic conditions.

B. Detection of malolatic conversion

Malolactic conversion was tested by the determination of L-malic acid and L-lactic acid by HPLC (Herjavac *et al.,* 2001). The cultured medium was filtered through 0.45 µm membrane filter. Five microliters of the sample was injected into the thermo separation product (tsp) HPLC system composed of

autoinjector AS3000 and refractive index RI1530 detector. The Phenomenex® Rezex ROA organic acid column (300 x 7.8 mm) was used, and heated to 70°C. The column supporter is sulfonated styrene divinyl benzene. The H_2SO_4 (0.01N) was used as mobile phase with flow rate of 0.6 ml/min.

3.2.2.2 Detection of malolactic conversion of selected isolates of malolactic bacteria in a synthetic medium containing ethanol

The malolactic bacterial strain were selected to test for malolactic conversion in a synthetic medium with the addition of ethanol and without either glucose or fructose. The composition of a synthetic medium was mentioned in section 3.2.2.1 (A). Various ethanol concentrations added to the medium were 0, 5, 7.5, 10, 12.5 and 15 % (v/v). The medium was sterilised by filtration through 0.22 μm membrane cellulose acetate membrane filter. (Saguir and De Nadra, 1996). The selected isolates of malolactic bacteria were inoculated with the initial cell concentration of 10^8 cell/ml. All culture were incubated at 20, 25 and 30°C for 2 weeks under anaerobic conditions. Malolactic conversion were tested as mentioned in section 3.2.2.1 (B).

3.2.3 Application of malolactic bacteria for wine fermentation

The selected isolates of malolactic bacteria was tested for malolactic conversion, and compared to the commercial strain of *Oenococcus oeni* CH35. The secondary fermentation of Shiraz wine which had completed alcoholic fermentation, was performed by adding 3.5 g/l of L-malic acid and 1 g/l of D-glucose. The inoculum of malolactic bacteria were prepared by the method modified from Herrero *et al.*

(1999). Middle-log phase cells of malolactic bacteria were prepared in MRS medium under anaerobic conditions, at 30°C. The initial population was 10^8 cell/ml. All cultures were incubated at 30°C for 7 days under anaerobic conditions.

Malolactic fermentations were carried out in 5 L sterilized round flat bottom flasks, which were equipped with fermentation lock (air lock), and incubated at 25°C. Twenty milliliters of wine samples were taken for chemical analysis every week. The growth of bacteria were monitored by total viable count technique using MRS medium. And pH of wine were monitored by using pH meter.

Ethanol was monitored by using GC. The sample was filtered through 0.45 μm membrane filter. The 0.5 microliter of sample was injected directly into a GC, which was equipped with FID detector (Wang *et al.,* 2003).

GC condition for ethanol determination; The HP-INNOWAX column (30 m x 3.2 mm x 1.5 μm film thickness) was used as stationary phase. GC condition was modified from the method of Clemente-Jimenez *et al.* (2005). Nitrogen gas was used as the carrier gas with the flow rate of 2 ml/min. The temperature of the FID detector and the injection port was set at 220°C. Oven temperature was set initially at 40°C for 5 minutes and ramped to 220°C by rate 40°C/min. The injection volume was limited to 0.1 μl.

After the malolactic fermentation was completed within 1 month, D-glucose, D-fructose, L-malic acid and L-lactic acid were determined by using enzymatic kits from Boehringer Manneheim (R-biopharm, Germany). (Nault *et al.,* 1995; Reguant *et al.,* 2000; Saguir and De Nadra, 1996).

3.2.4 Sensory evaluation

The Quantitative Descriptive Analysis (QDA) was used in this study. Wine samples were tested by trained panelists, who interested in wine tasting. Panelists were trained to identify volatile flavors and tastes of wine by using model substances (Gutierrez-Afonzo *et al.,* 1998; Kontkanen *et al.,* 2005). Then, all panelists were qualified by using the pair different test and triagle test. The panelists who had more than 75% total score were chosen to taste wine (Lawless and Heyman, 1998). The sensory evaluation system was appropriately set by following the method of Baldy (1993). The wine sample was served to panelists at 20°C, at eleven a.m.

The analysis of variance (ANOVA) was applied to the sensory data using the SAS (Statistical Analysis System) program, version 6.08 for windows (Copyright 1989 by SAS Institute Inc., Cary, North Carolina, USA) (Girard *et al.,* 1997; Mamede *et al.,* 2005).

3.2.5 Identification of selected isolates ofmalolactic bacteria

3.2.5.1 Morphological and biochemical tests

Nine of cultures grown on MRS agar at 30°C for 24 h. Cell morphology were observed by Gram staining. Catalase activity and gas production from glucose were determined according to the criteria established in Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Other biochemical characteristics were detected using API 50 CH strips (Bio-Mérieux, France) which contained tests for carbohydrate assimilation and fermentation of 49 different compounds (and one control). After inoculation with identical colonies of all cultures, The strips were incubated at 30°C for 48 h. Then the APILAB Plus software version

5.0 from bioMérieux and Analytab Products' computer database were used for comparison of carbohydrate assimilation and/ or fermentation patterns and bacterial identification. (Booysen *et al.,* 2002; Edwards *et al.,* 1998; Ennahar *et al.,* 2003; Muyanja *et al.,* 2003).

Some selected isolates of malolactic bacteria were confirmed for their cell morphology using scanning electron microscopy at the electron microscopy laboratory of the Instrument Buildings of the Center for Scientific and Technological Equipment at Suranaree University of Technology.

3.2.5.2 Genetics Analysis

A. DNA preparation

Chromosomal DNA was prepared from culture cells of each isolates grown for 3 days in stationary cultures at 30°C in 5 ml of the MRS medium. Bacterial cells were collected from the single colony before transferred into MRS medium. Then bacteria cells were centrifuged by using 1.5 ml microcentrifuge tubes, washed once in 500 μl of saline EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0), and suspended in 100 μl of saline EDTA. Then, 300 μl of Tris-SDS (0.1 M Tris-HCl, 1% SDS, pH 9.0) was added and cells were boiled in a hot water bath for 30s. Immediately after cooling, the lysates were treated once with phenol-chloroform (1:1). After precipitation with ethanol cooled at –20°C, the DNA was dissolved in 100 μl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) (Sato *et al.,* 2000). The DNA products were eletrophoresed at 100 V, for 30 min on 1.0 % (w/v) agarose gel stained with ethidium bromide in 0.5X TBE buffer (45mM Tris-borate, 1 mM EDTA, pH 8.0) and visualized by UV transillumination (Chagnaud *et al.,* 2001).

B. 16S rDNA PCR condition and DNA primers

The ingredient of PCR mixture was modified from the method of Jang *et al.* (2003) which consisting of 5 μl of 10x reaction buffer, 1 μl of a mixture of four deoxyribonucleotide triphosphates (10 mM each), 3 μl of 25 mM $MgCl_2$ solution, 2 μl (20 pmol) of PCR primer , 0.5 μl (2.5 units) of Taq DNA polymerase, and aliquot of DNA sample (10 ng) diluted to a final volume of 50 μl with DI water. The primer sequences were as follows: (i) POmod: 5'-AGAGTTTGATCGTGG-3' (*Escherichia coli* numbering system, position 8-22) for the forward primer, and (ii) PC5: 5'-TACCTTGTTACGACTT-3' (position 1492-1507) for the reverse primer.

PCR amplification was modified from the method of Lee *et al.* (2000) which was performed using a DNA thermal cycler. A total of 30 cycles of amplification were performed with template DNA denaturation at 94°C for 30 sec, primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min. The last cycle was followed by a post run at 72°C for 7 min. Then, the PCR products were purified by using QIAquick PCR Purification Kit (Invitrogen), before used for 16S rDNA sequencing.

C. Direct 16S rDNA sequencing

The direct sequences of 16S rDNA gene was performed by using a PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction kit (Applied Systems, USA) in combination with an automated sequencing system. The gene was amplified using DNA thermal cycler. An estimated amount of 100 ng of DNA was used for each reaction together with 1.6 pmol of primer, 4 μl of ready reaction mix and DI water to make up a 10 μl final volume. The same primers were used as for

previous PCR amplification. Cycle-sequencing PCR and DNA precipitation with ethanol and sodium acetate were done following the manufacturer's instructions (Applied Systems, USA).

The data of DNA sequences were assembled and checked with the autoassembler 1.4 program (Perkin Elmer) and transferred directly to a sequence analysis program. Sequence similarity searches were performed in the GenBank data library using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST) (Ennahar *et al.,* 2003; Jang *et al.,* 2003).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of malolactic bacteria

A total of 70 isolates of malolactic bacteria were isolated from waste water samples of food industries, dairy and vineyard. Eleven isolates were isolated from dairy, 13 isolates were isolated from vineyard, 28 isolates were isolated from Doikham food industry and 18 isolates were isolated from Malee food industry.

4.2 Selection of malolactic bacteria for wine production

4.2.1 Primarily determination of malolactic conversion

All isolates of malolactic bacteria and one commercial strain of *Oenococcus oeni* CH35 used as control, were used to detect the malolactic conversion occurred in synthetic medium without sugar (glucose and fructose). An amount of L-malic acid and L-lactic acid were determined by using HPLC. The conversion of L-malic acid to L-lactic acid by malolactic bacteria can be calculated as % yield of L-lactic acid production as equation:

$$\% \text{ Yield} = \frac{\text{Amount of L-lactic acid (g/l)}}{\text{Amount of L-malic acid (g/l)}} \times 100$$

The result was shown in Table 1.

Table 1 The yield of L-lactic acid produced by all isolates of malolactic bacteria.

No.	Sample name	% Yield of L-lactic acid production
1	AA1	40.32
2	AA2	51.85
3	AA3	1.47
4	AA4	1.75
5	AA5	0.00
6	AA6	5.20
7	AB1	8.03
8	AB2	2.70
9	AB3	11.16
10	AB4	2.71
11	AB5	28.79
12	BA1	17.77
13	BA2	18.67
14	BA3	1.66
15	BA4	30.63
16	BA5	1.93
17	BA6	16.32
18	BB1	44.99
19	BB2	21.38
20	BB3	2.56
21	BB4	17.64
22	BB5	2.15

Table 1 (continued)

No.	Sample name	% Yield of L-lactic acid production
23	BB6	1.32
24	BB7	41.50
25	CA1	1.36
26	CA2	0.00
27	CA3	13.50
28	CA4	1.57
29	CA5	0.00
30	CA6	1.66
31	CA7	2.74
32	CA8	1.48
33	CA9	1.58
34	CA10	0.00
35	CA11	1.62
36	CA12	0.00
37	CA13	2.36
38	CA14	1.65
39	CA15	2.24
40	CB1	0.00
41	CB2	2.65
42	CB3	1.57
43	CB4	6.49
44	CB5	5.93

Table 1 (continued)

No.	Sample name	% Yield of L-lactic acid production
45	CB6	1.64
46	CB7	1.63
47	CB8	4.42
48	CB9	1.62
49	CB10	1.44
50	CB11	0.00
51	CB12	3.29
52	CB13	2.70
53	DA1	1.60
54	DA2	1.63
55	DA3	2.12
56	DA4	1.85
57	DA5	1.77
58	DA6	6.48
59	DA7	43.34
60	DA8	2.19
61	DA9	0.00
62	DA10	1.65
63	DA11	2.33
64	DB1	1.63
65	DB2	3.46
66	DB3	41.10

Table 1 (continued)

No.	Sample name	% Yield of L-lactic acid production
67	DB4	1.96
68	DB5	2.52
69	DB6	23.07
70	DB7	3.08
Control	CH35	2.68

Results from Table 1 shown the isolate AA2 could produce highest of lactic acid (51.85%) whereas eight isolates could not produce lactic acid. Only 33 isolates which could produce CO_2 from glucose (Table 3), were classified as heterofermentative (or heterolactic) bacteria, resulting in the low of their % yield of lactic acid production. For the primarily selection, the seven isolates which had the highest of %yield were selected for the next experiments. Besides the two isolates which had highest %yield in group of heterolactic bacteria, were also selected too.

4.2.2 Determination of malolactic conversion in synthetic medium containing ethanol

Nine isolates of malolactic bacteria and one commercial strain of *Oenococcus oeni* CH35 used as control, were detected the malolactic conversion in a synthetic medium with the addition of ethanol and without glucose and fructose. Various ethanol concentrations added to the medium were 0, 5, 7.5, 10, 12.5 and 15 % (v/v). All cultures were incubated at 20, 25 and 30°C for 2 weeks. An amount of L-malic acid and L-lactic acid were determined and calculated as %yield of L-lactic acid

production. The yield of lactic acid production by nine isolates of malolactic bacteria which were inoculated in ethanol concentration varied in synthetic medium shown in Figures 5 to 7. The results shown that the commercial strain *Oenococcus oeni* CH35 (a positive control strain) was able to produce highest lactic acid when grown in all alcohol condition at 20°C and 25°C , except the medium added with high ethanol concentration (15 %v/v), lactic acid was not produced. Lactic acid was produced from all isolates when grown in all alcohol condition at 30°C, except the isolate BA4. When temperature were decreased to 25°C and 20°C, lactic acid production of all isolates were decreased too, except the CH35 was not.

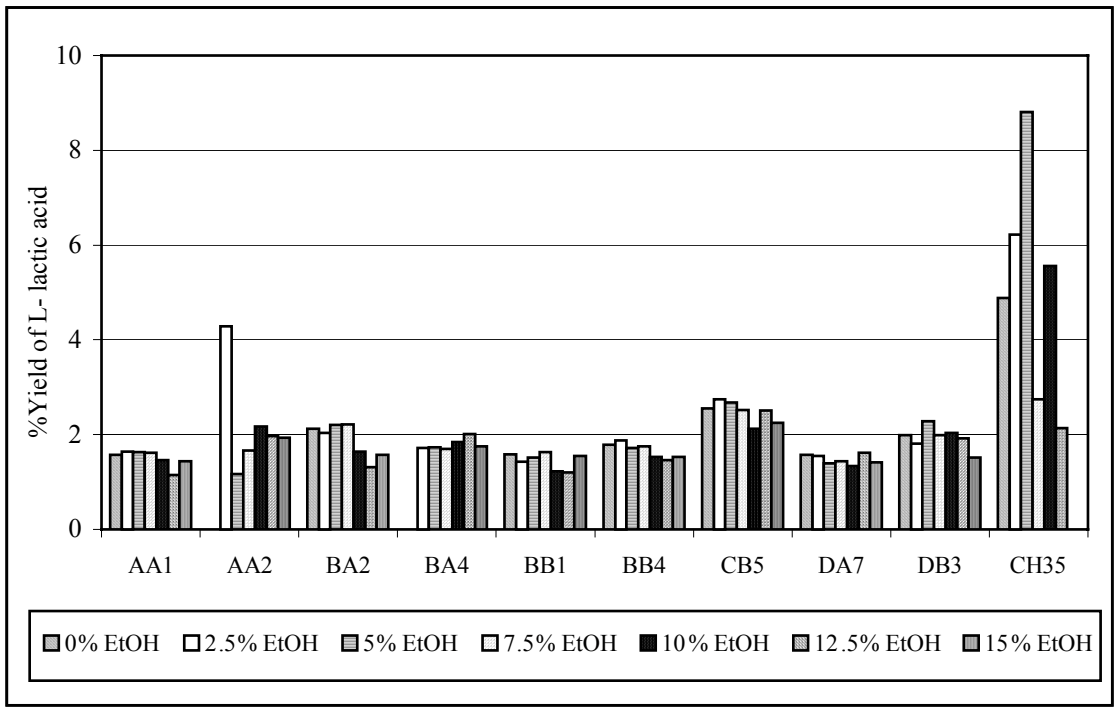


Figure 5 Lactic acid production from nine isolates of malolactic bacteria via malolactic conversion at 20°C.

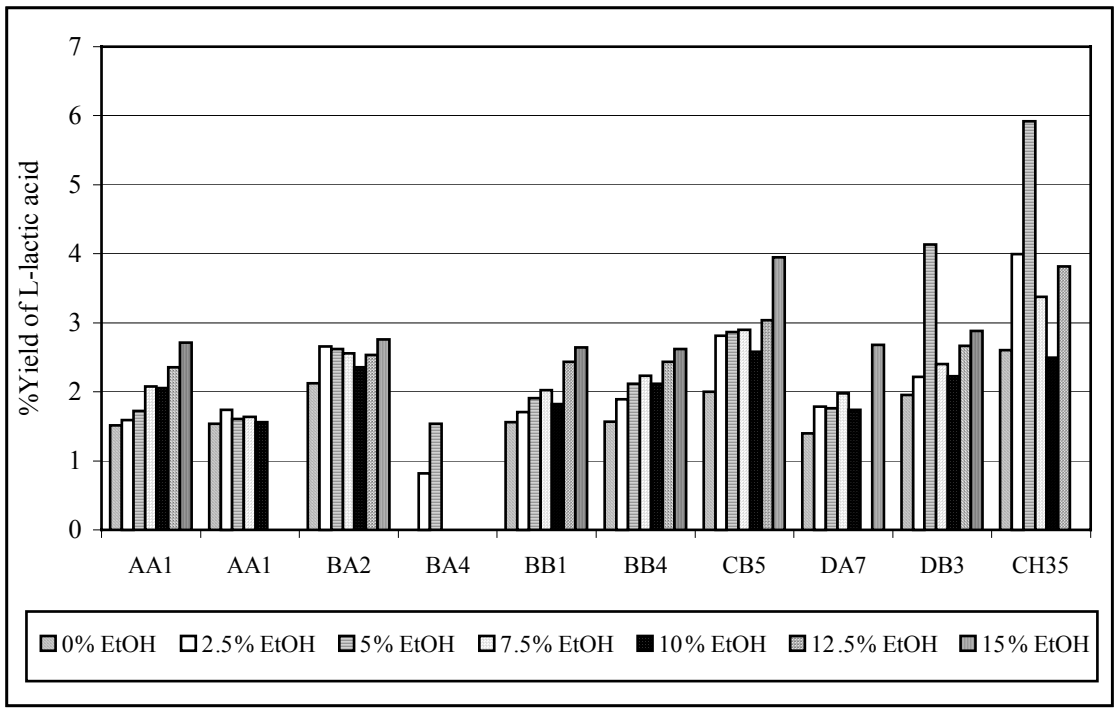


Figure 6 Lactic acid production from nine isolates of malolactic bacteria via malolactic conversion at 25°C.

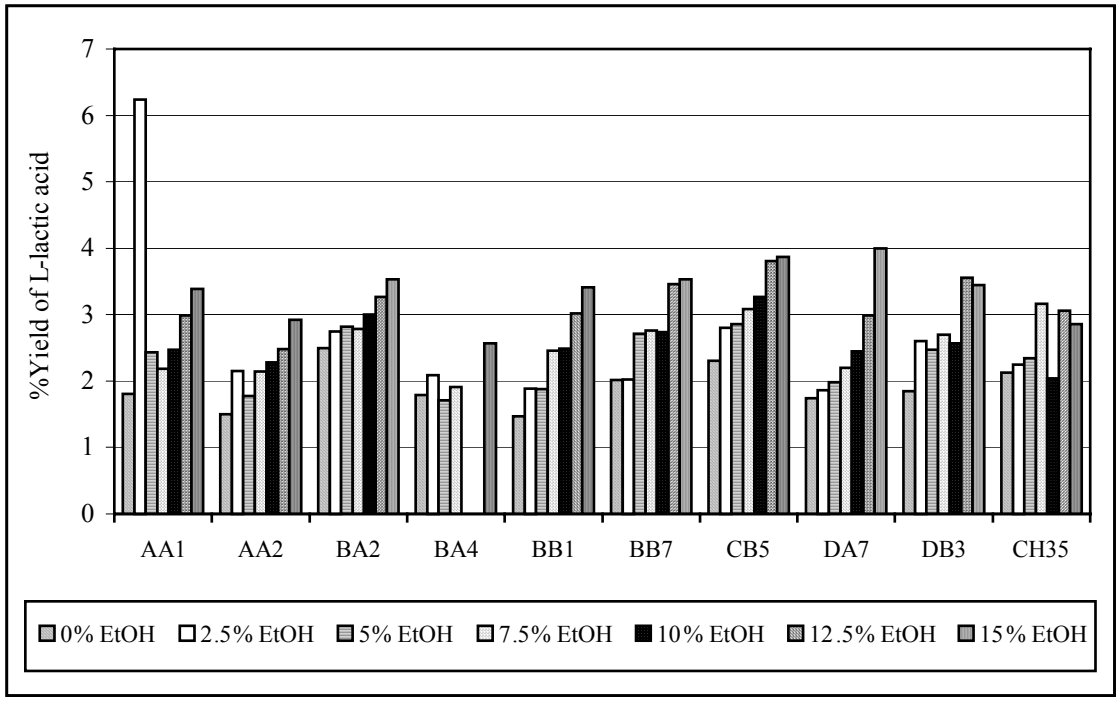


Figure 7 Lactic acid production from nine isolates of malolactic bacteria via malolactic conversion at 30°C.

Although the medium condition without sugar (as carbon source of bacteria), these isolates could grow and produce lactic acid which occurred by acid assimilation. There was other observation, which indicated the organic acids degradation of bacteria. Ribéreau-Gayon *et al*. (2000) had referred to two organic acids (malic acid and citric acid) degradation of wine by lactic acid bacteria. Other acids can of course be degraded but of less interest in enology with the exception of tartaric acid, which has rarely been studied. In the case of non proliferating cells during winemaking, lactic acid bacteria transform L-malic acid exclusively to L-lactic acid. This reaction involves a decarboxylation without an intermediary product capable of following another metabolic pathway.

From the results, the isolate CB5 could produce yield of lactic acid high when grown in all alcohol condition, at all temperature, when compared with the other isolates. So the isolate CB5 was considered to the best strain of selected isolates for wine fermentation at 25°C, because their lactic acid production was higher than at 20°C, and winemaking should be not performed at high temperature (30°C).

4.3 Application of malolactic bacteria for wine production

The isolate CB5 which was the best strain of selected isolates of malolactic bacteria was tested for malolactic conversion compared with the commercial strain *Oenococcus oeni* CH35 which was used as a positive control. Another factor was negative control without malolactic bacteria added. Fermentation was done at 25°C. Wine samples were taken for chemical analysis every weeks. The growth of bacteria were monitored by total viable count technique using MRS medium shown in Figure 8. And pH of wine were monitored by using pH meter

shown in Figure 9. The result of bacterial growth indicated the viable cell of the control was slowly increased whereas the CH35 and the isolate CB5 were rapidly increased in first week. Then growth of the CB5 and CH35 were stable and slowly decrease until completed fermentation. The reason might be these bacteria degraded some sugar or acids to maintain cell to survived under severe condition, so viable cell concentration was not much. For the pH value of wine sample inoculated with the CB5 and CH35, had higher than the control until fermentation completed. This result related to the bacterial growth, which was increased in the first week whereas pH value decreased because of acid production by bacteria. Then pH value was slowly increased due to occuring malic transformation.

Ethanol concentration of wine were monitored by using GC shown in Figure 10. The results shown that ethanol concentration of all treatments were slightly different among the first 2 weeks. Then ethanol concentration of wine inoculated with CH35 and control were incresed. The reason might be the CH35 produced ethanol among fermentation, and/or might be yeast (of the control treatment) produced ethanol from glucose added into wine before fermentation.

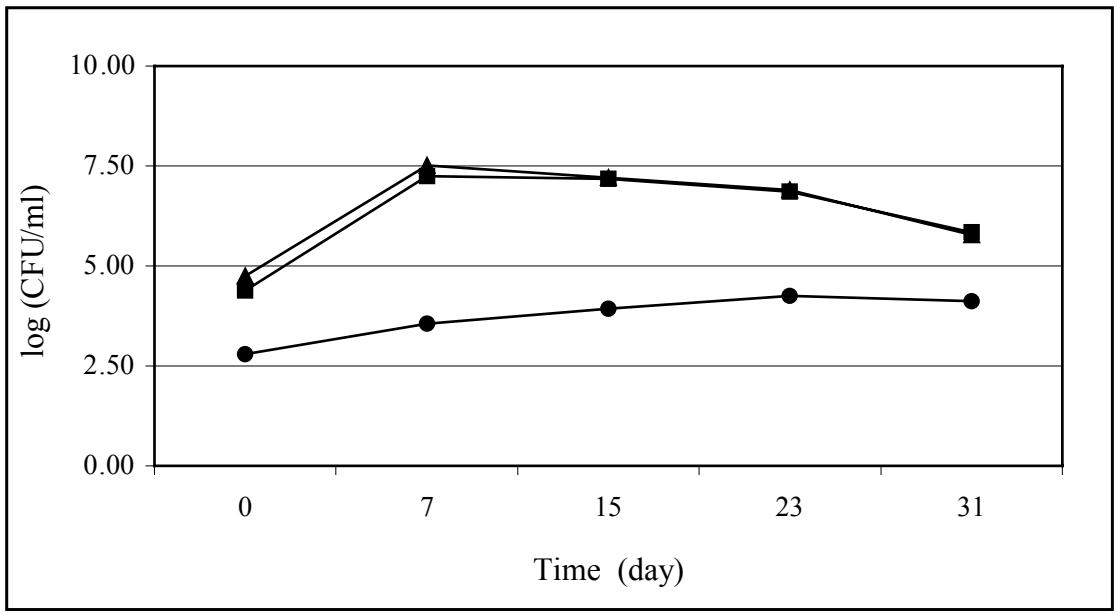


Figure 8 Total viable counts of malolactic bacteria in wine; the isolate CB5 (▲), the commercial strain of *Oenococcus oeni* CH35 (■), and control without malolactic bacteria added (●).

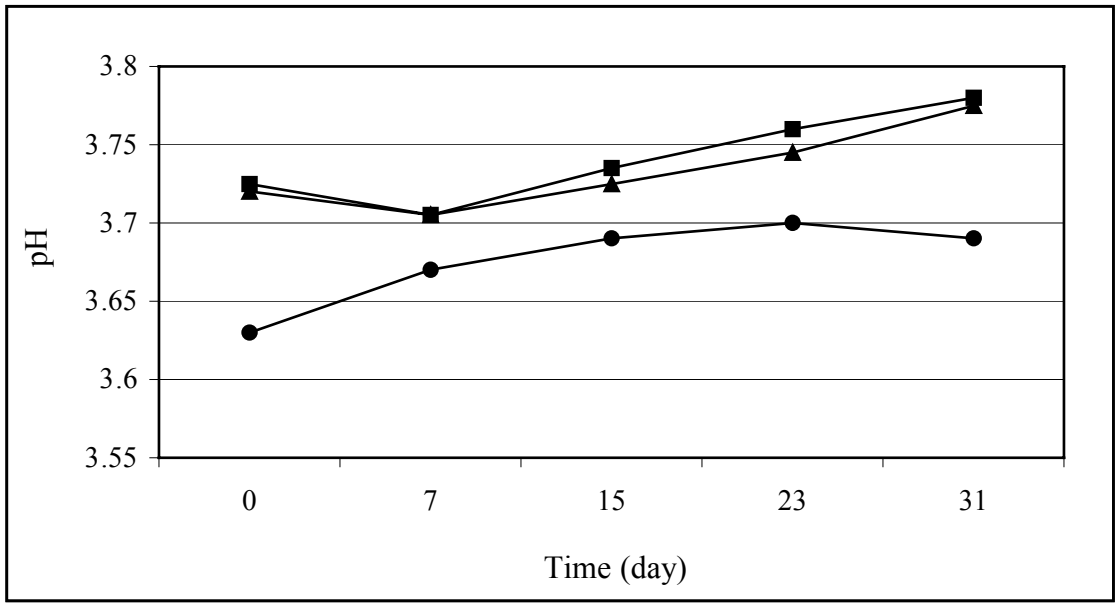


Figure 9 pH value of wine during malolactic fermentation by using the isolate CB5 (▲), the commercial strain of *Oenococcus oeni* CH35 (■), and control without malolactic bacteria added (●).

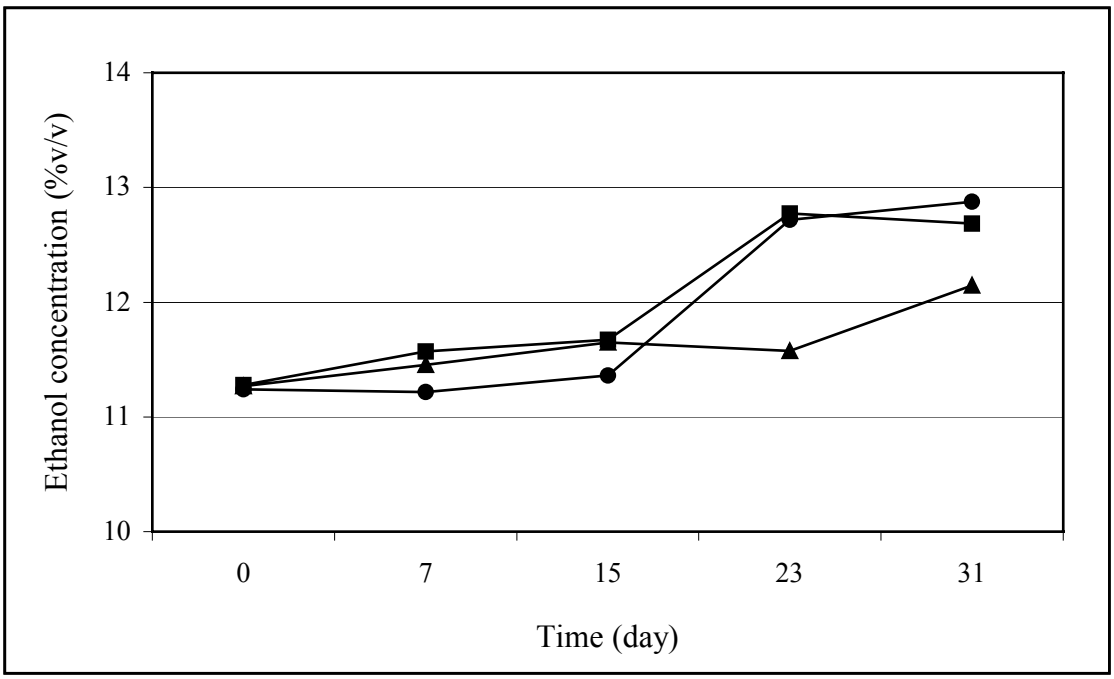


Figure 10 Ethanol concentration in wine during malolactic fermentation by using the isolate CB5 (▲), the commercial strain of *Oenococcus oeni* CH35 (■), and control without malolactic bacteria added (●).

After the completed malolactic fermentation, concentration of L-malic acid, L-lactic acid, D-glucose, and D-fructose in wine sample were determined by using enzymatic test kits from Boehringer Manneheim (Germany). The results were shown in Figures 11 to 14, respectively. The result shown that L-malic acid degradation was slightly different among the CH35 and the isolate CB5 (Figure 11). When the malolactic fermentation was completed, the CB5 and CH35 could degrade 0.671 and 0.572 g/l L-malic acid, respectively. For L-lactic acid concentration in wine inoculated with the CB5 and CH35 were also increased. This result indicated that both CB5 and CH35 could produce L-lactic acid increasingly as 0.537 g/l for the CB5 and 0.253 g/l for the CH35 (Figure 12).

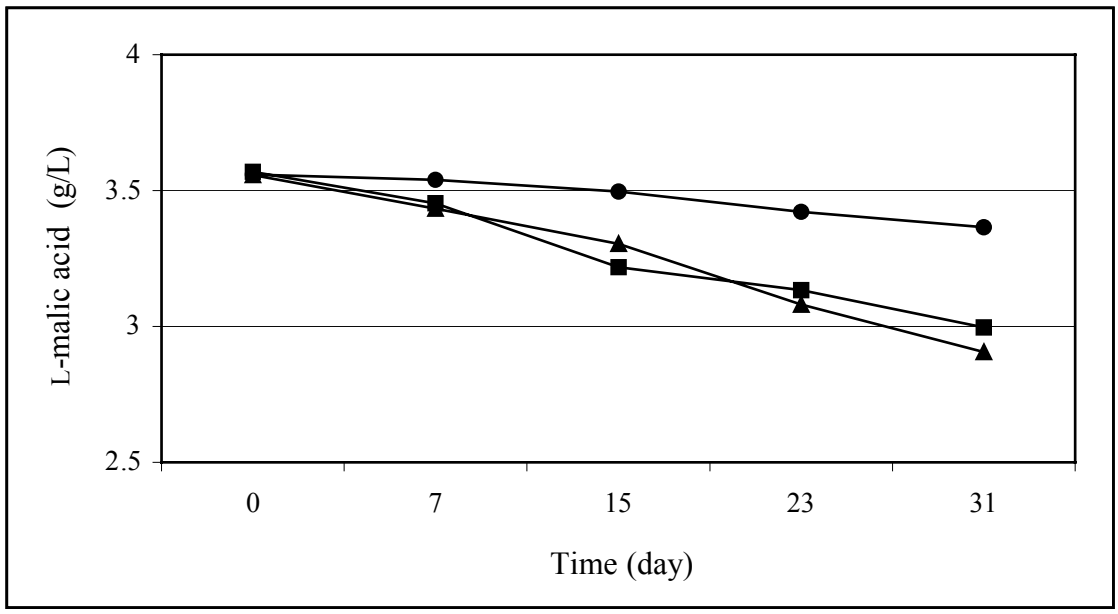


Figure 11 L-malic acid concentration in wine during malolactic fermentation by using the isolate CB5 (▲), the commercial strain of *Oenococcus oeni* CH35 (■), and control without malolactic bacteria added (●).

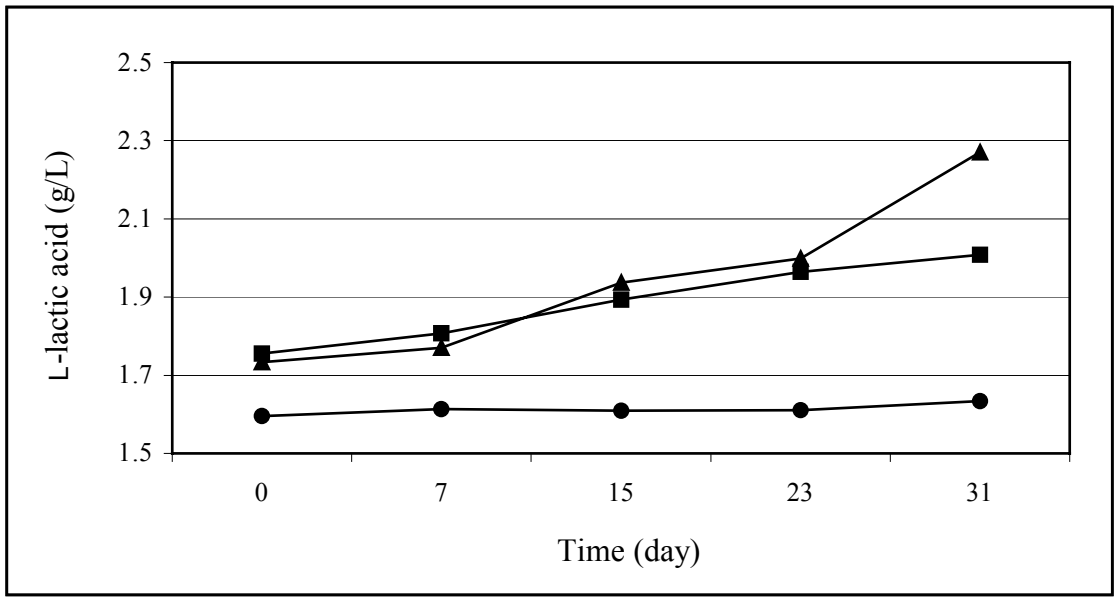


Figure 12 L-lactic acid concentration in wine during malolactic fermentation by using the isolate CB5 (▲), the commercial strain of *Oenococcus oeni* CH35 (■), and control without malolactic bacteria added (●).

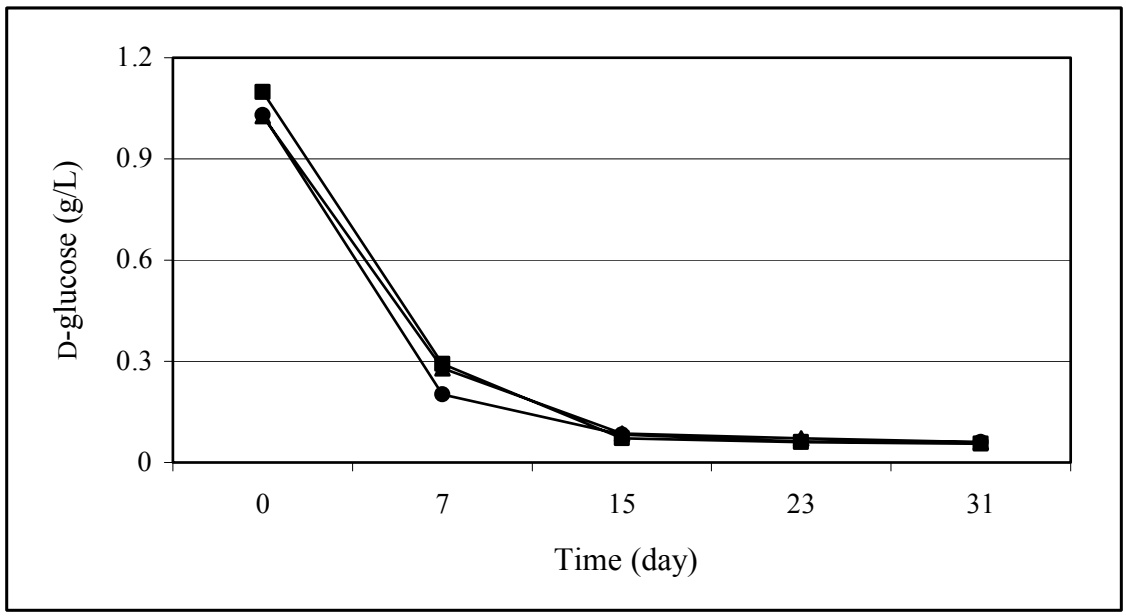


Figure 13 D-glucose concentration in wine during malolactic fermentation by using the isolate CB5 (▲), the commercial strain of *Oenococcus oeni* CH35 (■), and control without malolactic bacteria added (●).

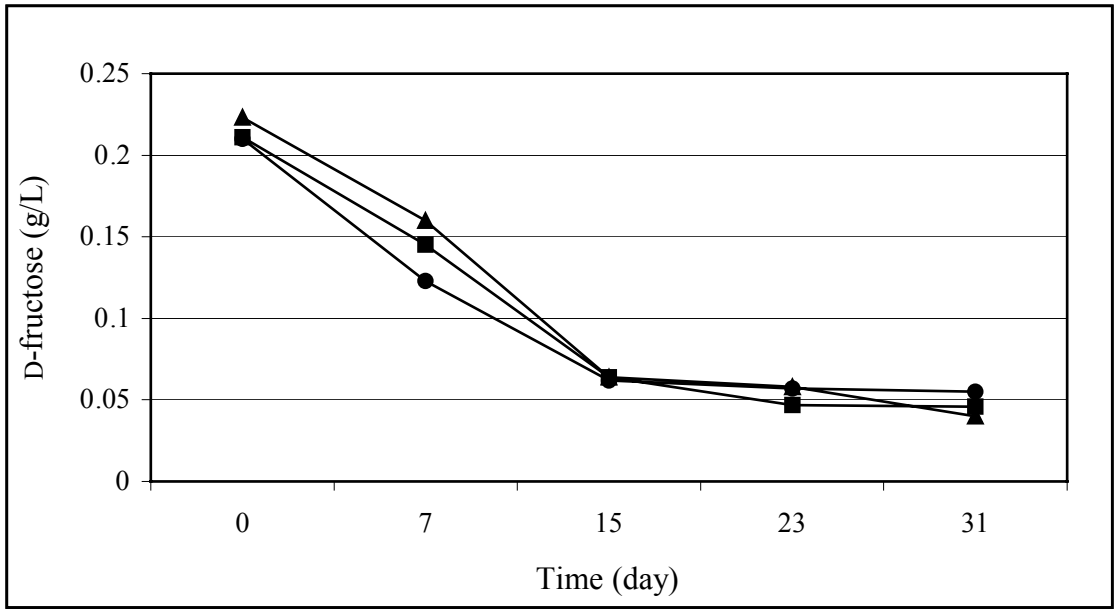


Figure 14 D-fructose concentration in wine during malolactic fermentation by using the isolate CB5 (▲), the commercial strain of *Oenococcus oeni* CH35 (■), and control without malolactic bacteria added (●).

For the concentration of D-glucose and D-fructose in wine sample found that both D-glucose and D-fructose of all samples were rapidly decreased among first week (Figures 13 and 14). This result related to the bacterial growth both the isolate CB5 and the CH35, which was rapidly increased whereas the D-glucose and D-fructose were rapidly consumed in this time too. The reason might be bacteria used sugar as carbon source (especially glucose) for their growth, so they utilized these sugars rapidly. For the D-glucose and D-fructose reduction of the control might be yeast which remained in wine, and utilized these sugar to maintain its cell.

4.4 Effect of malolactic fermentation by using different malolactic bacteria on wine characters evaluated by the sensory test

The finished products of malolactic fermentation were tested by trained panelists and evaluated characters of wine. The objective of the sensory test was to determine wine characters after fermented with the isolate CB5, the commercial strain *Oenococcus oeni* CH35, and the control which no added with malolactic bacteria. Mean intensity ratings for the wine made by three conditions were plotted on polar coordinate or radar graph, the center of the graph represented low intensity with respect to each character increasing to an intensity of 10 at the ends of axes (Figure 15). Four characters of wine include sour, flavor, balance of acidity, alcohol content, fruit flavor and astringency, and overall characteristics which were significantly different among three conditions ($p \geq 0.05$). The Least Significant Different (LSD) was calculated to determine where the different occurred and denoted by letters (Table 2). Three wine samples was evaluated the characters by nine panelists. The results shown that all wine samples were no significantly differences in all characters

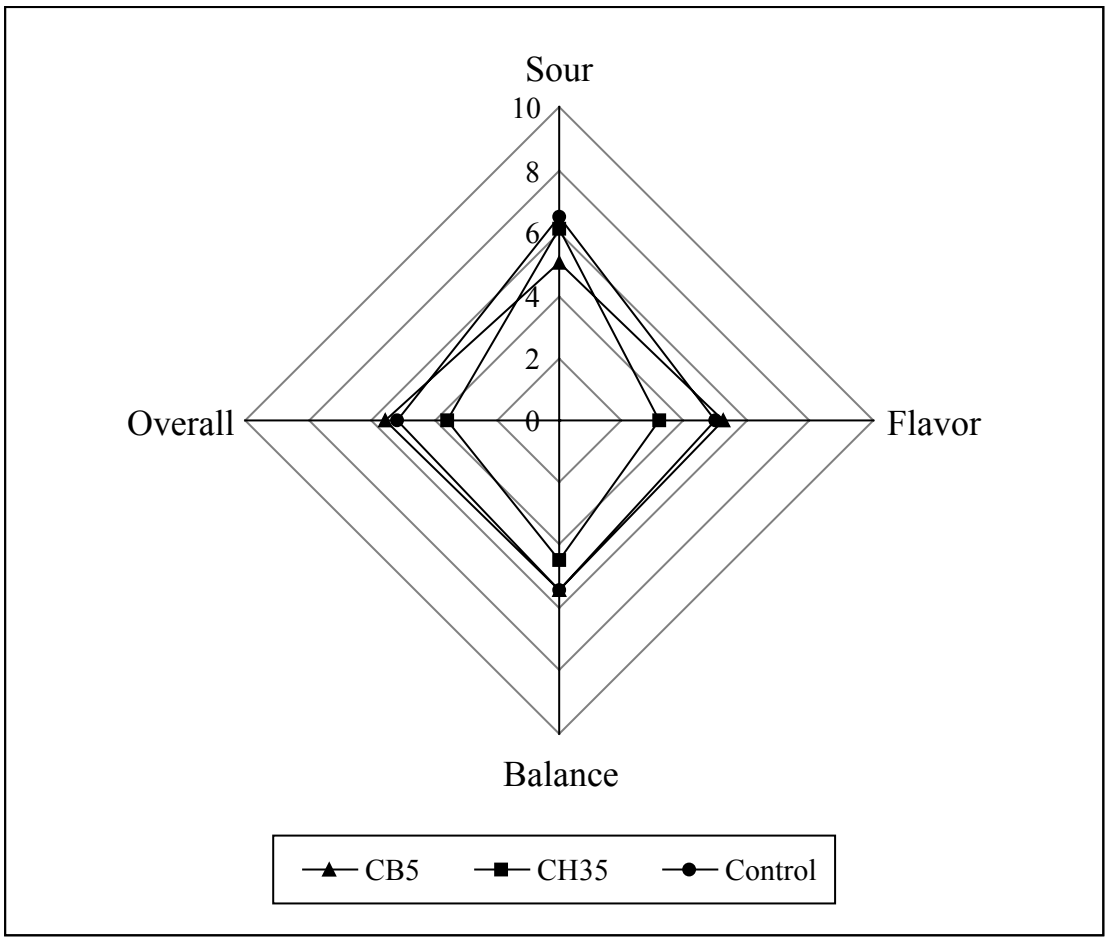


Figure 15 Polar coordinate graph of the mean intensity rating of three wine samples in term of sour, flavor, balance and overall ($p<0.05$).

Table 2 Mean rating and Least Significant Differences (LSD).

Characters	**Sample**		
	CB5	CH35	Control
Sour	5.03a	6.14a	6.52a
Flavor	5.24a	3.16a	4.99a
Balance	5.43a	4.47a	5.42a
Overall	5.53a	3.58a	5.14a

Means in a column followed by the same letter are not significantly different ($p\geq0.05$)

4.5 Identification of malolactic bacteria

4.5.1 Morphological and physiological characteristics

Total of 70 isolates of malolactic bacteria were tested for the CO_2 production from glucose in MRS broth (Table 3). The results shown that only 33 isolates could produce CO_2 from glucose. These isolates were classified as heterofermentative bacteria which could produce other end-products such as ethanol, acetate, and CO_2 in addition to lactic acid. Another group which produce primarily lactic acid as the end-product of glucose metabolism and non CO_2, production, so called homofermenters (Fugelsang, 1997; Ribéreau-Gayon *et al.*, 2000).

Table 3 Carbon dioxide production from glucose by 70 isolates of malolactic bacteria cultured in MRS broth.

No.	Sample name	CO_2 production
1	AA1	-
2	AA2	-
3	AA3	-
4	AA4	+
5	AA5	-
6	AA6	-
7	AB1	-
8	AB2	+
9	AB3	-
10	AB4	+
11	AB5	-

Table 3 (continued)

No.	Sample name	CO_2 production
12	BA1	-
13	BA2	+
14	BA3	+
15	BA4	-
16	BA5	+
17	BA6	-
18	BB1	-
19	BB2	-
20	BB3	+
21	BB4	-
22	BB5	+
23	BB6	+
24	BB7	-
25	CA1	+
26	CA2	-
27	CA3	-
28	CA4	+
29	CA5	-
30	CA6	+
31	CA7	+
32	CA8	+
33	CA9	+

Table 3 (continued)

No.	Sample name	CO_2 production
34	CA10	-
35	CA11	-
36	CA12	-
37	CA13	-
38	CA14	-
39	CA15	+
40	CB1	+
41	CB2	+
42	CB3	-
43	CB4	-
44	CB5	+
45	CB6	+
46	CB7	+
47	CB8	-
48	CB9	-
49	CB10	-
50	CB11	-
51	CB12	-
52	CB13	+
53	DA1	+
54	DA2	+
55	DA3	+

Table 3 (continued)

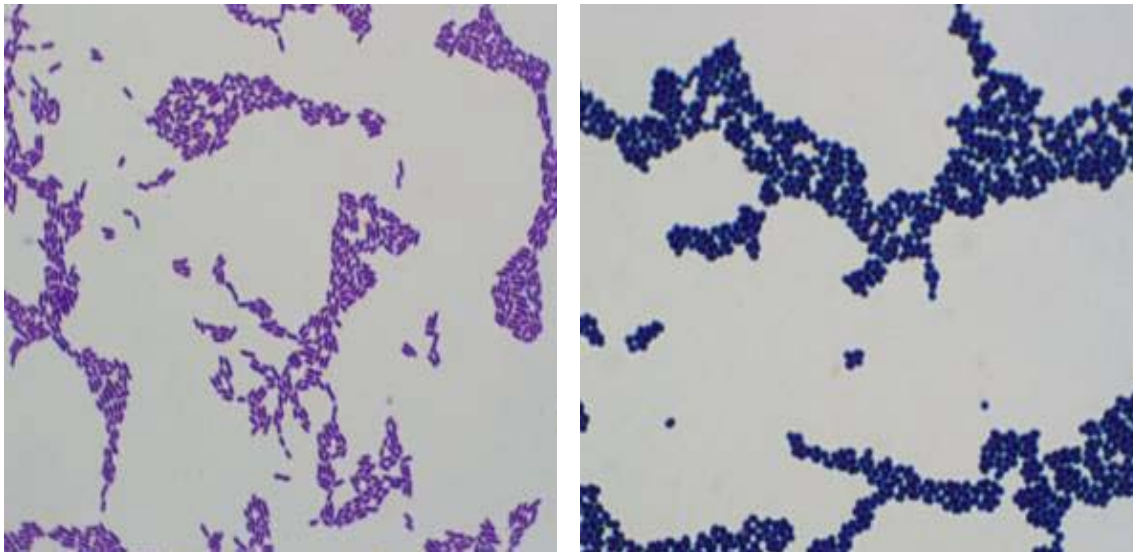
No.	Sample name	CO_2 production
56	DA4	+
57	DA5	+
58	DA6	-
59	DA7	-
60	DA8	-
61	DA9	-
62	DA10	-
63	DA11	+
64	DB1	-
65	DB2	+
66	DB3	-
67	DB4	+
68	DB5	+
69	DB6	-
70	DB7	+
Control	CH35	+

Symbol: +, positive; -, negative.

Nine of selected isolates of malolactic bacteria were tested by catalase activity and Gram staining which was also recoded the shape and size of cell (Table 4). The results shown that these isolates were positive gram and negative catalase test. The cell shape of all isolates were ovoid, except the isolate CB5 was coccoid-rod

(Figure 16). The isolate BA2 and CB5 were determined to heterofermentative species (only genus *Leuconostoc*) because they could produce CO_2 (Bozoğlu and Yurdugül, 2000; Fugelsang, 1997; Lonvaud-Funel, 1999).

All strains were identified based on carbohydrate assimilation and/or fermentation by using API 50 CH system from Bio-Mérieux (Table 5). The results which were compared the similarity percentages of carbohydrate assimilation and/or fermentation patterns with reference strain of the APILAB Plus software (version 5.0) and shown in Table 5.



(a) (b)

Figure 16 Gram stain of the isolate (a) CB5, and (b) BA2, bright field microscopy (x1000).

Table 4 Different characteristics of nine isolates of malolactic bacteria.

Characteristics	Isolates of malolactic bacteria								
	AA1	**AA2**	**BA2**	**BA4**	**BB1**	**BB7**	**CB5**	**DA7**	**DB3**
Cell shape	ovoid	ovoid	ovoid	ovoid	ovoid	ovoid	coccoid-rod	ovoid	ovoid
Cell size (μm)	0.71	0.70	0.61	0.75	0.80	0.56	0.5x1.2	0.88	0.86
Gram	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-
CO_2 production	-	-	+	-	-	-	+	-	-

Symbols: +, positive; -, negative.

Table 5 Carbohydrate fermentation of nine isolates of malolactic bacteria.

Carbohydrate source	Isolates of malolactic bacteria								
	AA1	**AA2**	**BA2**	**BA4**	**BB1**	**BB7**	**CB5**	**DA7**	**DB3**
Glycerol	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+	+	+	-	+	+
D-Ribose	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	+	-	-	-	+	-	-
L- Xylose	-	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	-	-	-	-	-	-
D-Galactose	+	+	+	+	+	+	+	+	+

Table 5 (continued)

Carbohydrate source	Isolates of malolactic bacteria								
	AA1	**AA2**	**BA2**	**BA4**	**BB1**	**BB7**	**CB5**	**DA7**	**DB3**
D-Glucose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	+	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-
Methyl-αD-Mannopyranoside	+	+	+	+	+	+	+	+	+
Methyl-αD-Glucopyranoside	-	-	-	-	-	-	+	+	+
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+
Amygdaline	-	-	-	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+	-	+	+
Esculin	+	+	+	+	+	+	-	+	+
Salicin	+	+	+	+	+	+	-	+	+
D-Celiobiose	+	+	+	+	+	+	-	+	+
D-Maltose	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	-	+	+
D-Melibiose	+	+	+	+	+	+	+	+	+

Table 5 (continued)

Carbohydrate source	Isolates of malolactic bacteria								
	AA1	**AA2**	**BA2**	**BA4**	**BB1**	**BB7**	**CB5**	**DA7**	**DB3**
D-Saccharose (sucrose)	+	+	+	+	+	+	+	+	+
D-Trehalose	-	-	-	-	-	-	+	-	-
Inulin	-	-	-	-	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-	-
D-Rafinose	-	-	-	-	-	-	+	-	-
Amidon (Starch)	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-
Gentiobiose	+	+	+	+	+	+	-	+	+
D-Turanose	-	-	-	-	-	-	+	+	+
D-Lyxose	-	-	-	-	-	-	-	-	-
D-Tagatose	+	+	+	+	+	+	-	+	+
D-Fucose	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-
Potassium Gluconate	-	-	-	-	-	-	-	-	-
Potassium 2-KetoGluconate	-	-	-	-	-	-	-	-	-
Potassium 5-KetoGluconate	-	-	-	-	-	-	-	-	-

Symbols: +, positive; -, negative.

Table 6 The similarity of carbohydrate assimilation and/or fermentation patterns compared with reference strains of the APILAB Plus software (version 5.0) from BioMérieux.

Isolated malolactic bacteria	% Similarity	Identification from carhydrate fermentation
AA1	75.4%	*Lactococcus lactis ssp. lactis*
AA2	75.4%	*Lactococcus lactis ssp. lactis*
BA2	75.4%	*Lactococcus lactis ssp. lactis*
BA4	75.4%	*Lactococcus lactis ssp. lactis*
BB1	75.4%	*Lactococcus lactis ssp. lactis*
BB7	75.4%	*Lactococcus lactis ssp. lactis*
CB5	99.9%	*Leuconostoc mesenteroides*
DA7	88.3%	*Lactobacillus plantalrum*
DB3	88.3%	*Lactobacillus plantalrum*

The result shown that all isolates were morphologically similar to genus *Leuconostoc*, but their carbohydrate fermentation profile were different. Only the isolate CB5 was biochemically identified to *Leuconostoc mesenteroides* at 99.9% similarity. Two isolates were similar with *Lactobacillus plantalrum* at 88.3%. And six isolates were similar with *Lactococcus lactis ssp. lactis* at 75.4%. Following morphological and biochemical analysis, most of the isolates of malolactic bacteria (six isolates) characterized belonged to the genera *Lactococcus* which were not clearly related to malolactic species. And only three isolates (CB5, DA7 and DB3) were classified to malolactic bacteria. For the isolate CB5 which was high efficient bacteria for malolactic conversion, and selected to apply for wine fermentation. So the

isolate CB5 was detected to comfirm the cell morphology by using scanning electron microscopy (Figure 17).

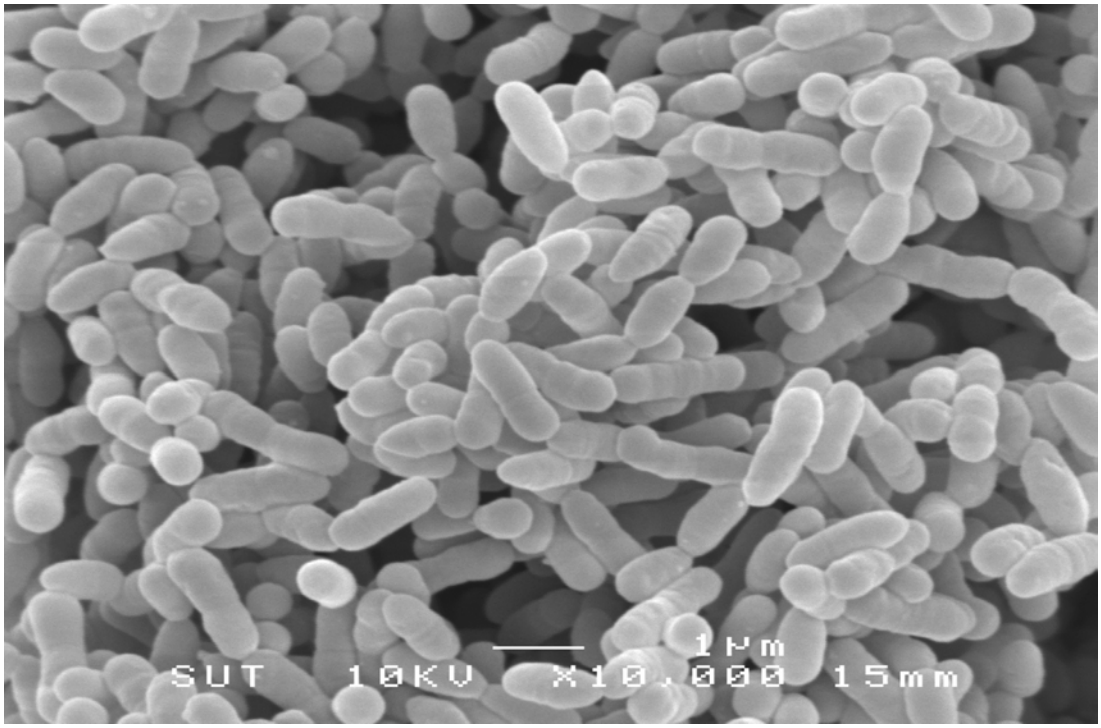


Figure 17 Scanning electron micrograph of the isolate CB5 grown in MRS medium. Scale bar = 1 μm.

4.5.2 Genetis analysis

The isolated malolactic bacteria were determined the strains based on 16S rDNA analysis. Genomic DNA of all isolates were extracted and used as DNA template for PCR amplification. The PCR reaction were performed by POmod (forward) and PC5 (reverse) primer. The results were generated the single amplified DNA fragment in size about ±1,650 bp (Figure 19).

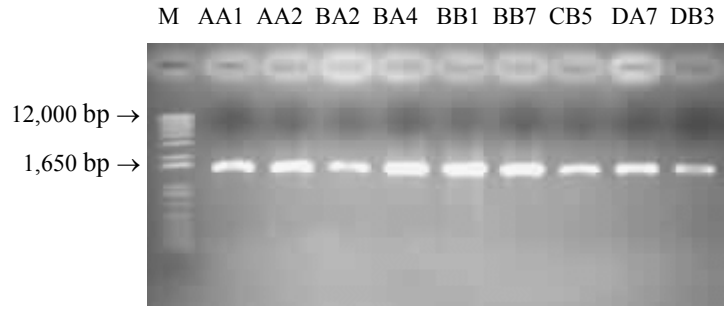


Figure 18 Agarose gel electrophoresis of 16S rDNA-PCR fragment obtained from the amplification of genomic DNA of nine isolates of malolactic bacteria.

4.5.3 16S rDNA sequence analysis

To confirm the strain of nine isolates of malolactic bacteria, the sequence analysis of 16S rDNA was conducted. The genomic DNA from PCR products were detected the partial nucleotide sequences for eight isolates. For the isolate CB5 was detected the full length of nucleotide sequences of 16S rDNA (Figure 19). The sequences of 16S rDNA were compared with other 16S rDNA from database. The results of %similarity and strain homology were shown in Table 7. When these results were compared with their biochemical characteristics, it found that all isolates were different among their carbohydrate fermentation profile and 16S rDNA sequence. Only the isolate CB5 was biochemically identified to genus *Leuconostoc* same the result of 16S rDNA sequence analysis, but the species level was different.

Table 7 The similarity of 16S rDNA sequence of nine isolates compared with stains from databae.

Isolates	Compared with strains from database		
	Strains homology	**Acession No.**	**%Similarity**
AA1	*Enterococcus faecium* strain SF 16S ribosomal RNA gene, complete sequence	AY675247	98%
AA2	*Enterococcus faecium* strain SF 16S ribosomal RNA gene, complete sequence	AY675247	98%
BA2	*Lactobacillus kefiri* strain JCM 5818 16S ribosomal RNA gene, partial sequence	AY579584	96%
BA4	*Enterococcus faecium* strain SF 16S ribosomal RNA gene, complete sequence	AY675247	97%
BB1	*Bacterium Te6A* 16S ribosomal RNA gene, partial sequence	AY587777	98%
BB7	*Enterococcus faecium* strain SF3 16S ribosomal RNA gene, complete sequence	AY735408	95%
CB5	*Leuconostoc pseudomesenteroides* 16S ribosomal RNA gene, partial sequence	AF468002	98%
DA7	*Enterococcus faecium* strain SF 16S ribosomal RNA gene, complete sequence	AY675247	95%
DB3	*Enterococcus mundtii* 16S ribosomal RNA gene, partial sequence	AF061013	97%

From these identification results could not certainly point to the strain of isolate CB5 which was shown very closed similarity to *Leuconostoc mesenteroides* (99.9%) and *Leuconostoc pseudomesenteroides* (98%) by API 50 CHL system and 16S rDNA sequence comparison, respectively. Among them, only the species from the genus *Leuconostoc* that has been isolated from wine is *Leuconostoc mesenteroides* (Lonvaud-Funel, 1999; Ribéreau-Gayon *et al.,* 2000) whereas *Leuconostoc pseudomesenteroides* has not been reported that found in wine (Plessis du *et al.,* 2004).

Although the isolate CB5 was very closed similarity to *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*. However, both of them have been reported the negative result (for *Leuconostoc mesenteroides*) and not determination (for *Leuconostoc pseudomesenteroides*) of growth in 10% ethanol in the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Moreover, Plessis du *et al.* (2004) reported that *Leuconostoc mesenteroides* can be found only the first day of alcoholic fermentation. After the third day of the alcoholic fermentation, *Oenococcus oeni* was the only species recovered from the base wine. As this result, indicated that the isolate CB5 was probable to be either a new *Leuconostoc* species or subspecies of *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*. Because it can grow in high ethanol concentration (15% ethanol) whereas *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* can not.

```
   1  GACACCTGCC TCAAGGCTGG GGATAACATT TGGAAACAGA TGCTAATACC
  51  GAATGAAACT TAGTGTCGCA TGATACGAAG TTAAAAGGCG CTTTGGCGTC
 101  ACCTAGAGAT GGATCCGCGG TGCATTAGTT AGTTGGTGGG GTAAAGGCCT
 151  ACCAAGACAA TGATGCATAG CCGAGTTGAG AGACTGATCG GCCACATTGG
 201  GACTGAGACA CGGCCCAAAC TCCTACGGGA GGCTGCAGTA GGGAATCTTC
 251  CACAATGGGC GAAAGCCTGA TGGAGCAACG CCGCGTGTGT GATGAAGGCT
 301  TTCGGGTCGT AAAGCACTGT TGTATGGGAA GAACAGCTAG AATAGGGAAT
 351  GATTTTAGTT TGACGGTACC ATACCAGAAA GGGACGGCTA AATACGTGCC
 401  AGCAGCCGCG GTAATACGTA TGTCCCGAGC GTTATCCGGA TTTATTGGGC
 451  GTAAAGCGAG CGCAAGACGG TTGATTAAGT CTGATGTGAA AAGCCCGGAG
 501  CTCAACTCCG GGAATGGCAT TGGGAAACTG GTTACTTGAA GTGCAGTAGA
 551  GGTAATGGAA CTCCATGTGT AGGGTGGAAT GCGTAGATAT ATGGAAGAAC
 601  ACCAATGGCG AAGGCGGCTT ACTGGACTGT AACTGACGTT GAAGCTCGAA
 651  AGTGTGGTTA GCAAACAGGA TTAAGATAAC CCTGGTAGTC CACCCCCTTA
 701  AACGATGAAC ACTAGGTGTT AGGAGGTTTT CCGCCTCTTA GTGCCGAAGC
 751  TAACGCATTA AGTGTTCCGC CTGGGGAGTA CGACCGCAAG GTTGAAACTC
 801  AAAGGAATTG ACGGGGACCC GCACAAGCGG TGGAGCATGT GGTTTAATTC
 851  GAAGCAACGC GAAGAACCTT ACCAGGTCTT GACATCCTTT GAAGCTTTTA
 901  GAGATAGAAG TGTTCTCTTC GGAGACAAAG TGACAGGTGG TGCATGGTCG
 951  TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
1001  CCCTTATTGT TAGTTGCCAG CATTCAGATG GGCACTCTAG CGAGACTGCC
1051  GGTGACAAAC CGGAGGAAGG CGGGGACGAC GTCAGATCAT CATGCCCCTT
1101  ATGACCTGGG CTACACACGT GCTACAATGG CGTATACAAC GAGTTGCCAA
1151  CCCGCGAGGG TGAGCTAATC TCTTAAAGTA CGTCTCAGTT CGGATTGTAG
1201  TCTGCAACTC GACTACATGA AGTCGGAATC GCTAGTAATC GCGGATCAGC
1251  ACGCCGCGGT GAATACGTTC CCGGGTCTTG TACACACCGC CCGTCACACC
1301  AAGGGAGTTT GTAATGCC
```

Figure 19 Nucleotide sequence of PCR fragment (1,318 bp) from the amplification of genomic DNA of the isolate CB5 using 16S rDNA primer.

```
CB5: 4    acctgcctcaaggctggggataacatttggaaacagatgctaataccgaatgaaacttag 63
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 103  acctgcctcaaggctggggataacatttggaaacagatgctaataccgaatgaaacttag 162

CB5: 64   tgtcgcatgatacgaagttaaaaggcgctttggcgtcacctagagatggatccgcggtgc 123
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 163  tgtcgcatgatacgaagttaaaaggcgctttggcgtcacctagagatggatccgcggtgc 222

CB5: 124  attagttagttggtggggtaaaggcctaccaagacaatgatgcatagccgagttgagaga 183
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 223  attagttagttggtggggtaaaggcctaccaagacaatgatgcatagccgagttgagaga 282

CB5: 184  ctgatcggccacattgggactgagacacggcccaaactcctacgggaggctgcagtaggg 243
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 283  ctgatcggccacattgggactgagacacggcccaaactcctacgggaggctgcagtaggg 342

CB5: 244  aatcttccacaatgggcgaaagcctgatggagcaacgccgcgtgtgtgatgaaggctttc 303
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 343  aatcttccacaatgggcgaaagcctgatggagcaacgccgcgtgtgtgatgaaggctttc 402

CB5: 304  gggtcgtaaagcactgttgtatgggaagaacagctagaatagggaatgattttagtttga 363
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 403  gggtcgtaaagcactgttgtatgggaagaacagctagaatagggaatgattttagtttga 462

CB5: 364  cggtaccataccagaaagggacggctaaatacgtgccagcagccgcggtaatacgtatgt 423
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 463  cggtaccataccagaaagggacggctaaatacgtgccagcagccgcggtaatacgtatgt 522

CB5: 424  cccgagcgttatccggatttattgggcgtaaagcgagcgcaagacggttgattaagtctg 483
          |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Leu: 523  cccgagcgttatccggatttattgggcgtaaagcgagcgca-gacggttgattaagtctg 581

CB5: 484  atgtgaaaagcccggagctcaactccgggaatggcattgggaaactggtta-cttgaagt 542
          |||||||| |||||||||||||||||| |||||||||| |||||||||| ||||| ||
Leu: 582  atgtgaaa-gcccggagctcaactccgg-aatggcattgg-aaactggttaacttga-gt 637

CB5: 543  gcagtagaggtaa-tggaactccatgtgtag-ggtggaatgcgtagatatatggaagaac 600
          |||||||||||||| |||||||||||||||| ||||||||||||||||||||||||||||
Leu: 638  gcagtagaggtaagtggaactccatgtgtagcggtggaatgcgtagatatatggaagaac 697
```

Figure 20 Nucleotide sequence alignment between the isolate CB5 (upper line) and *Leuconostoc pseudomesenteroides* (AF468002; below line) which were performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST).

```
CB5: 601  accaatggcgaaggcggcttactggactgtaactgacgttgaagctcgaaagtgtggtta 660
          ||||  ||||||||||||||||||||||||||||||||||  |||||||||||||| ||
Leu: 698  accagtggcgaaggcggcttactggactgtaactgacgttgaggctcgaaagtgtgggta 757

CB5: 661  gcaaacaggattaagataaccctggtagtccacccccttaaacgatgaacactaggtgtt 720
          ||||||||||||| |||| |||||||||||||| || | ||||||||||||||||||||
Leu: 758  gcaaacaggatta-gata-ccctggtagtccacaccgt-aaacgatgaacactaggtgtt 814

CB5: 721  aggaggttttccgcctcttagtgccgaagctaacgcattaagtgttccgcctggggagta 780
          ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 815  aggaggttt-ccgcctcttagtgccgaagctaacgcattaagtgttccgcctggggagta 873

CB5: 781  cgaccgcaaggttgaaactcaaaggaattgacggggacccgcacaagcggtggagcatgt 840
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 874  cgaccgcaaggttgaaactcaaaggaattgacggggacccgcacaagcggtggagcatgt 933

CB5: 841  ggtttaattcgaagcaacgcgaagaaccttaccaggtcttgacatcctttgaagctttta 900
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 934  ggtttaattcgaagcaacgcgaagaaccttaccaggtcttgacatcctttgaagctttta 993

CB5: 901  gagatagaagtgttctcttcggagacaaagtgacaggtggtgcatggtcgtcgtcagctc 960
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 994  gagatagaagtgttctcttcggagacaaagtgacaggtggtgcatggtcgtcgtcagctc 1053

CB5: 961  gtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttattgttagttgccag 1020
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 1054 gtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttattgttagttgccag 1113

CB5: 1021 cattcagatgggcactctagcgagactgccggtgacaaaccggaggaaggcggggacgac 1080
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 1114 cattcagatgggcactctagcgagactgccggtgacaaaccggaggaaggcggggacgac 1173

CB5: 1081 gtcagatcatcatgccccttatgacctgggctacacacgtgctacaatggcgtatacaac 1140
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 1174 gtcagatcatcatgccccttatgacctgggctacacacgtgctacaatggcgtatacaac 1233

CB5: 1141 gagttgccaacccgcgagggtgagctaatctcttaaagtacgtctcagttcggattgtag 1200
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 1234 gagttgccaacccgcgagggtgagctaatctcttaaagtacgtctcagttcggattgtag 1293

CB5: 1201 tctgcaactcgactacatgaagtcggaatcgctagtaatcgcggatcagcacgccgcggt 1260
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Leu: 1294 tctgcaactcgactacatgaagtcggaatcgctagtaatcgcggatcagcacgccgcggt 1353

CB5: 1261 gaatacgttcccgggtcttgtacacaccgcccgtcacaccaagggagtttgtaatgcc 1318
          |||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Leu: 1354 gaatacgttcccgggtcttgtacacaccgcccgtcacaccatgggagtttgtaatgcc 1411
```

Figure 20 (continued)

CHAPTER V

CONCLUSIONS

Seventy isolates of malolactic bacteria were primarily tested for their malolactic conversion in a synthetic medium without sugar. For the primarily selection, the seven isolates which had the highest of %yield of lactic acid production (from L-malic acid) were selected for the next experiments. Besides the two isolates which had the highest %yield in group of heterolactic bacteria, were also selected.

Nine isolates which had high activity of malolactic conversion were selected to determine the malolactic conversion in various ethanol concentrations added to a synthetic medium (without glucose and fructose) and incubated at 20, 25 and 30°C. Only the isolate CB5 was found to produce lactic acid too high when grown in all alcohol conditions, at all temperatures. So the isolate CB5 was selected as the potential strain of selected isolates of malolactic bacteria for the secondary wine production at 25°C. During the malolactic fermentation, L-malic acid degradation was slightly different among the isolate CB5 and the commercial strain of *Oenococcus oeni* CH35 until fermentation completed. Whereas L-lactic acid production of the isolate CB5 was higher than the CH35.

Sensory evaluation was conducted after the secondary wine fermentation completed. There were no significantly differences in all characters (including sour, flavor, balance of acidity, alcohol content, fruit flavor and astringency, and overall characteristics of products) of all wine samples ($p \geq 0.05$). From results of this study,

shown the isolate CB5 could efficiently convert L-malic acid to L-lactic acid compared to a commercial strain *Oenococcus oeni* CH35. And it was able to apply for malolactic fermentation which would be useful for Thai wine industry.

For bacterial identification, all nine isolates of malolactic bacteria were morphologically similar to the genus *Leuconostoc*, but their biochemical characteristics were different. The isolate CB5 was biochemically identified to *Leuconostoc mesenteroides* as 99.9% (similarity) whereas the sequence analysis of 16S rDNA indicated the isolate CB5 to *Leuconostoc pseudomesenteroides* at 98% similarity. Although the CB5 was very closed similarity to *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides,* the isolate CB5 could grow in higher ethanol concentration (15% ethanol) than the two references (*Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*). As these results, indicated that the isolate CB5 could be either a new *Leuconostoc* species or subspecies of *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*.

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APPENDICES

APPENDIX A

METHODS

1. Sensory evaluation

The sensory evaluation of wine was done by using 12 volunteers and one expert of wine making. All twelve volunteers participated in round table discussion session. They were given representative wines, termiology of wine descriptors and tentative reference standards. These standards were prepared from attributes reported in the literature (Noble *et al.,* 1987). After group discussion, the panelists were evaluated performance before wine tasting. Panelists had score lower than 75% were rejected.

2. Evaluation of panelist performance by using Duo-Trio test and ranking test

The three cups of two flavors were constructed for the Duo-Trio test. Panelists had to identify th cup, which had the same flavor to reference cup by smelling. For ranking test, four cups of different concentration of L-malic acid were used. Panelist had to consequence the sour taste in ascending order of acidity. The work sheet were shown in Figures 1A to 3A.

DUO-TRIO TEST

Name_____Code_____Date_____

In front of you are three samples, one marked R and the other two, coded; evaluate the samples starting from left to light, first R and then the other two. **Circle the code of the sample different from R.** You may reteste the samples. You must make a choice. Thank you.

R **……..** **……..**

Figure 1A Work sheet of Duo-Trio test for sensory evaluation.

Ranking test

Name_____Code_____Date_____

Rank the sour taste wines in the coded cup in ascending order of acidity.

Least sour **Most sour**

Code _____ _____ _____

Figure 2A Work sheet of ranking test for sensory evaluation.

QDA sheet for wine tasting

Please evaluate the sour taste, flavor, balance and overall of the sample in sequence. Place a vertical line across the horizontal line at the point that best describes each property in the sample.

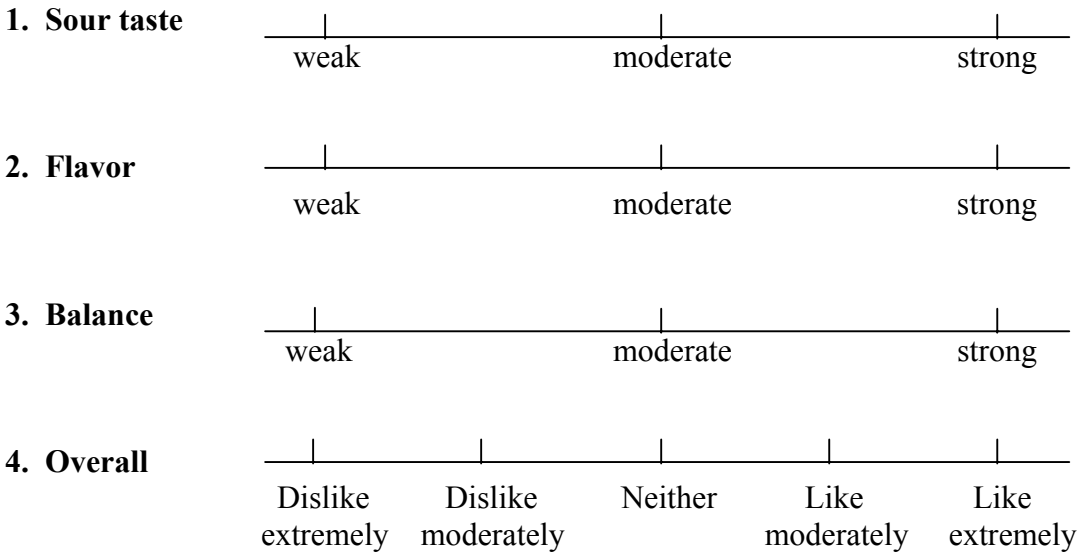
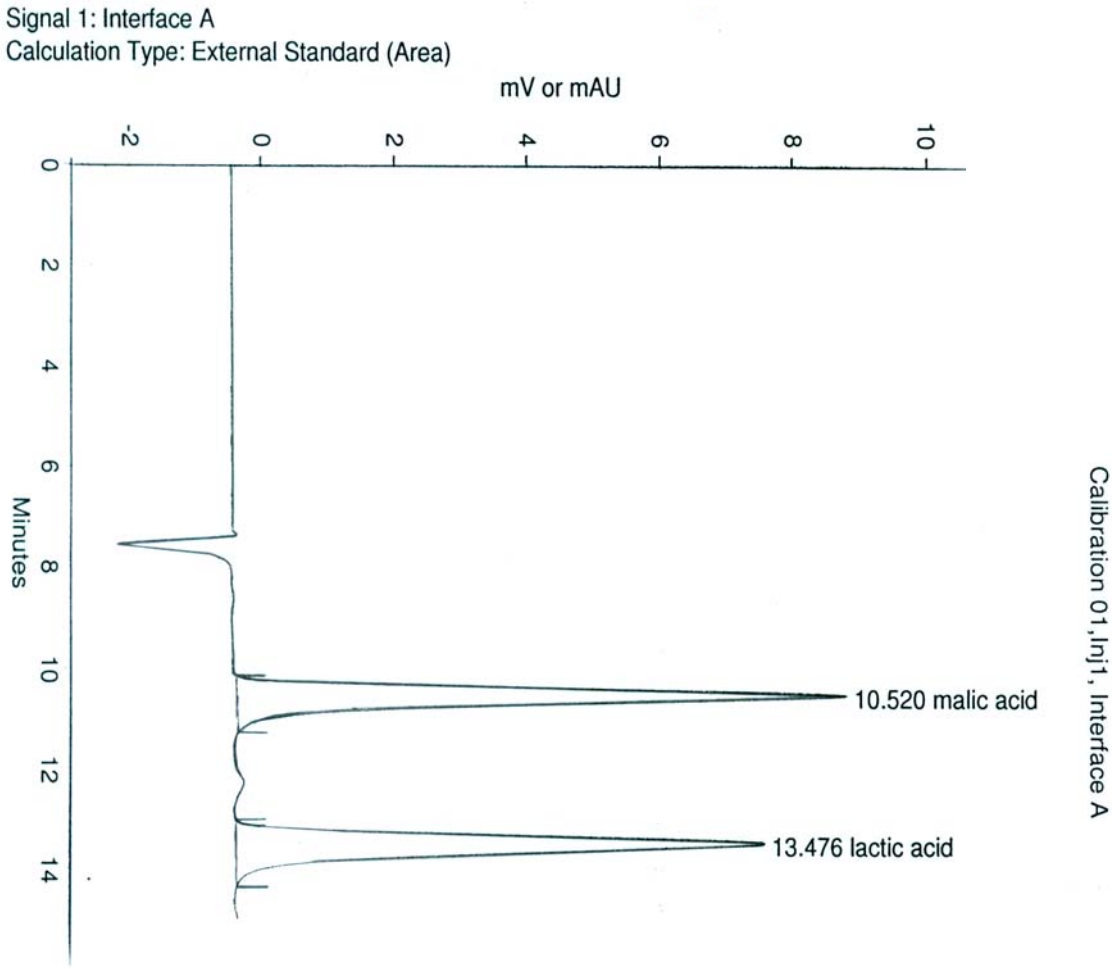


Figure 3A Work sheet of QDA for wine tasting

APPENDIX B

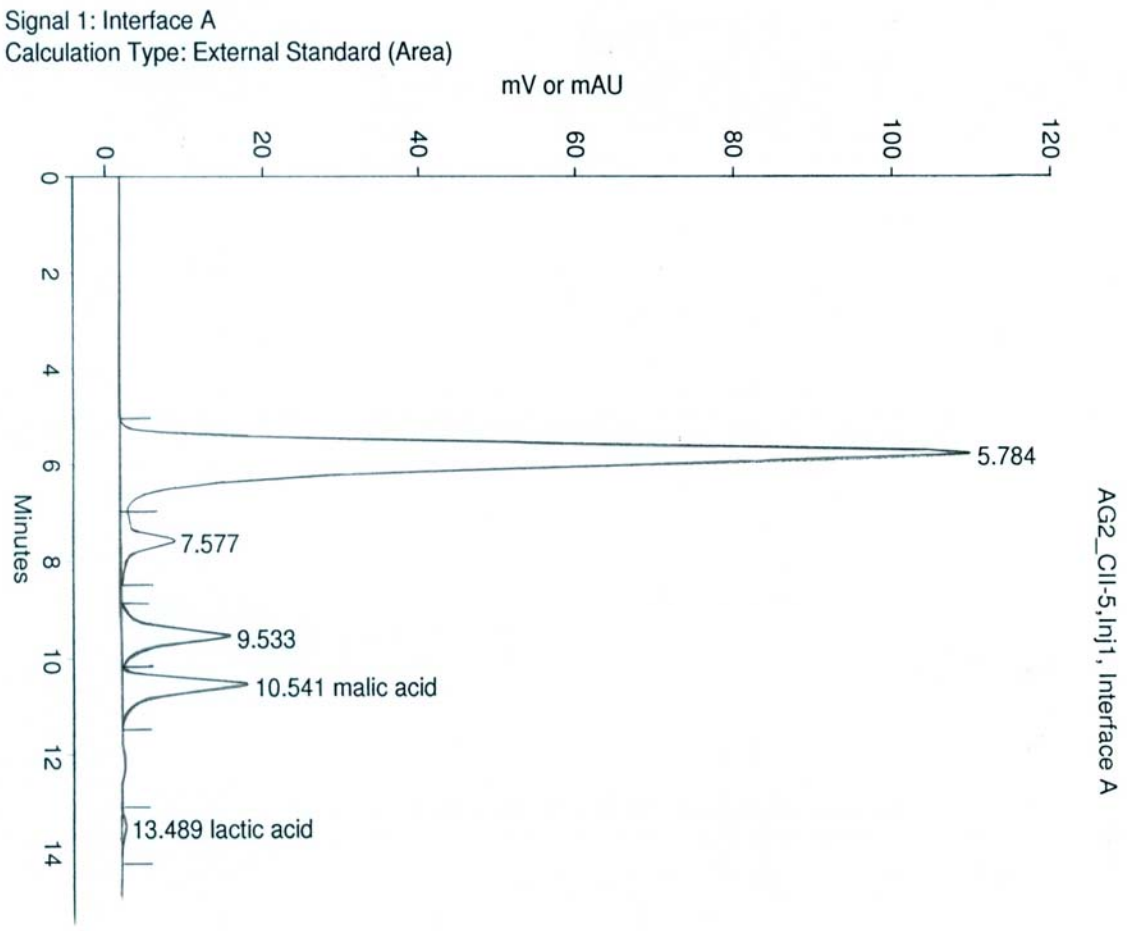
RESULTS

1. Chromatograms of malic acid and lactic acid analysis by HPLC



Component	RT(min)	Area	Height	g/L	Peak Type
malic acid	10.520	203421	9205	2	Resolved
lactic acid	13.476	197921	7976	2	Resolved
Totals		401342	17181	4	

Figure 1B Chromatograms of standard malic acid and lactic acid.

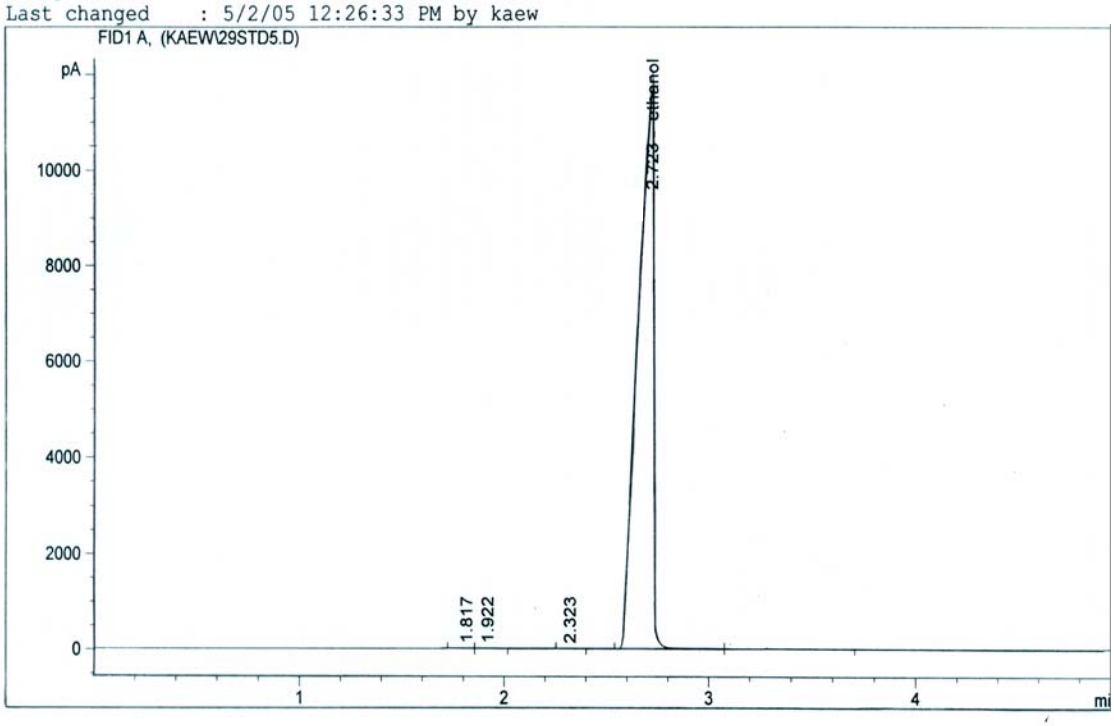


Component	RT(min)	Area	Height	g/L	Peak Type
Unident0001	5.784	3795640	107980	0.0	Fused
Unident0002	7.577	166267	6752	0.0	Fused
Unident0003	9.533	316059	13833	0.0	Fused
malic acid	10.541	359191	15985	4.899	Fused
lactic acid	13.489	10760	363	0.15205	Resolved
Totals		4647917	144913	5.0511	

Figure 2B Chromatograms of malic acid and lactic acid in wine sample.

2. Chromatogram of ethanol analysis by GC

```
Injection Date  : 4/29/05 1:16:19 PM
Sample Name     : ethl                                  Vial :   1
Acq. Operator   : kaew                                   Inj :   1
                                                  Inj Volume : Manually
Acq. Method     : C:\HPCHEM\1\METHODS\BUTANE.M\MLB.M
Last changed    : 4/29/05 1:15:35 PM by kaew
                  (modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\BUTANE.M\MLB.M
Last changed    : 5/2/05 12:26:33 PM by kaew
```




```
=====================================================================
                      External Standard Report
=====================================================================

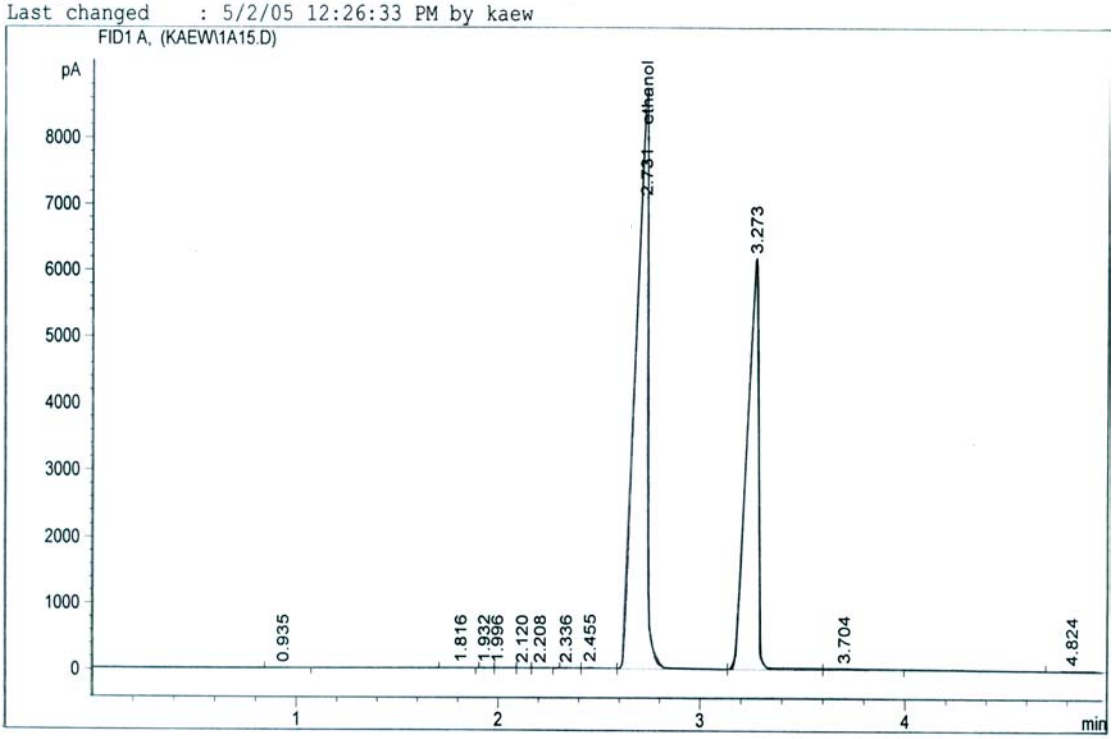
Sorted By             :      Signal
Calib. Data Modified  :      Monday, May 02, 2005 12:26:32 PM
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,

RetTime  Type     Area      Amt/Area    Amount   Grp   Name
 [min]          [pA*s]                  [%]
-------|------|----------|----------|----------|--|------------------
  2.723 PV     6.01954e4  3.34841e-4   20.15587    ethanol
```

Figure 3B Chromatogram of standard ethanol.

```
Injection Date  : 4/29/05 3:22:12 PM
Sample Name     : ethanol                                  Vial :   1
Acq. Operator   : kaew                                      Inj :   1
                                                       Inj Volume : Manually
Acq. Method     : C:\HPCHEM\1\METHODS\BUTANE.M\MLB.M
Last changed    : 4/29/05 1:49:10 PM by kaew
Analysis Method : C:\HPCHEM\1\METHODS\BUTANE.M\MLB.M
Last changed    : 5/2/05 12:26:33 PM by kaew
```

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=====================================================================
                      External Standard Report
=====================================================================

Sorted By             :      Signal
Calib. Data Modified  :      Monday, May 02, 2005 12:26:32 PM
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,

RetTime  Type     Area      Amt/Area    Amount   Grp   Name
 [min]          [pA*s]                  [%]
-------|------|----------|----------|----------|--|------------------
  2.731 VV     3.63921e4  3.31552e-4   12.06584     ethanol
```

Figure 4B Chromatogram of ethanol in wine sample.

APPENDIX C

LIST OF PRESENTATION

Wongkalasin, K., Wanapu, C., and Rodtong, S. (2004). **Selection of malolactic bacteria for wine fermentation.** The 4th National sysposium on Graduate Research, August 10-11, 2004, Lotus Pang Suan Kaew Hotel, Chiangmai, Thailand. (Oral Presentation).

O-ST-107

ORAL PRESENTATION

การคัดเลือกแบคทีเรียมาโลแลคติกเพื่อใช้ในการหมักไวน์

SELECTION OF MALOLACTIC BACTERIA FOR WINE FERMENTATION

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Abstract: Malolactic bacteria (MLB) are group of lactic acid bacteria which play an important role in the secondary wine fermentation or so-called malolactic fermentation. They are capable of conversion of L-malic acid to L-(+)-lactic acid. This study, MLB were isolated from industries and vineyards. All isolates were selected by considering the malolactic conversion and viability of MLB in ethanol added synthetic medium (pH 3.5). Isolated CII-5 had a highest of malolactic conversion and they can grow in 15% ethanol condition. For the identification, isolated CII-5 were tested morphology and biochemical profile using API 50 CHL system recoded as *Leuconostoc mesenteroides* (99.9% of similarity).

Methodology: MLB were isolated from industries and vineyards. All isolates were selected by considering the viability in ethanol added synthetic medium. The selected MLB were determined for malolactic conversion using HPLC technique (Herjavac *et al.* 2001). Identification of MLB, morphology and biochemical test were detected by using API 50 CHL galleries.

Results and Conclusion: A total of 70 isolates of MLB that isolated from industrial and vineyard wastewater samples. Of these, isolated CII-5 had a highest activity of malolactic conversion and they can grow in 15% ethanol added synthetic medium whereas the control (the commercial strain of *Oenococcus oeni*, CH35) cannot (Fig.1). For the result of MLB identification, all strains were morphologically similar to genus *Leuconostoc*, but their carbohydrate fermentation profile were different (Table.1). However, the identification will be confirmed again by the genomic analysis, and application of the selected MLB is currently being determined.

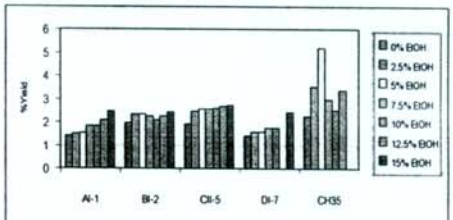

Fig.1 Lactic acid production of malolactic bacteria via malolactic conversion

Table 1 Differential characteristic of the selected malolactic bacteria

Characteristics	AI-1	BI-2	BII-7	CII-5	DI-7
Cell shape	ovoid	ovoid	ovoid	coccoid-rod	ovoid
Gram	Positive	Positive	Positive	Positive	Positive
Catalase test	-	-	-	-	-
Gas production	-	+	-	+	-
Growth at pH 4.8	+	+	+	+	+
Growth with 10% ethanol	+	+	+	+	d
Carbohydrate fermentation:					
L-Arabinose	+	+	+	-	+
D-Xylose	-	+	-	+	-
Esculin	+	+	+	-	+
L-Sorbose	-	+	-	-	-
D-Lactose	+	+	+	-	+

Symbols: +, 90% or more of strains are positive; d, 11-89% of strains are positive; -, 90% or more of strains are negative

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