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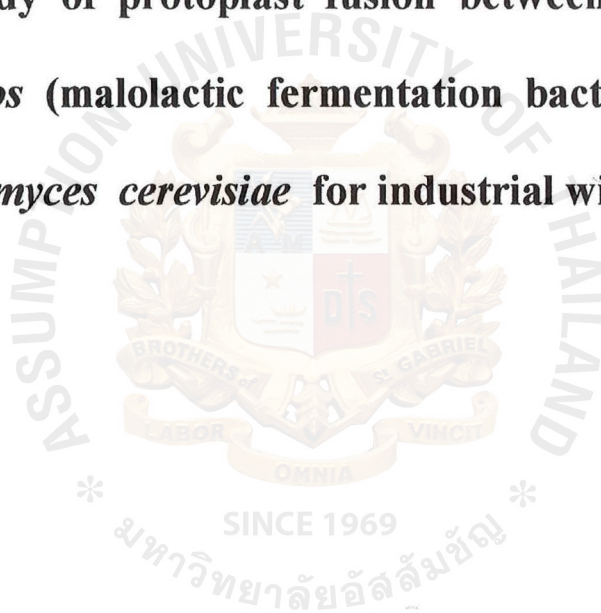
By

Ms. Haritchanan   Suvachananond

A special project submitted to the Faculty of Biotechnology,
Assumption University in part fulfillment of the requirement
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December 1999

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Advisor   : Dr. Churdchai Cheowtirakul

Level of study   : Bachelor of Science

Department   : Agro-Industry   AI 4490

Faculty   : Biotechnology

Academic Year   : 1999

..............................Advisor

(Dr. Churdchai Cheowtirakul)

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ABSTRACT

In this study the techniques of protoplast fusion between *Saccharomyces cerevisiae* (yeast) and *Leuconostoc oenos* (lactic acid bacteria) were explored. More than 200 fusants were obtained. This fusants had a characteristic of yeast-like organisms which is much easier to grow and maintain than lactic acid bacteria. 50 fusants were chosen to study the properties of alcoholic and malolactic fermentation. The result showed that only one fusant that we obtained maintained the property of malolactic fermentation. The fusant can not produce any alcohol. *S. cerevisiae* wild type (W.T.) were added together with the fusant to explore the possibility of simultaneously alcoholic and malolactic fermentation. The result showed that the fusion which we obtained, we named it *S. cerevisiae* var. MLF 11 Haritchanan, due to the ability of MLF and morphologically similar to *S. cerevisiae* and can be used together with *S. cerevisiae* (W.T.) to perform a complete alcoholic and malolactic fermentation.

<u>ACKNOWLEDGMENT</u>

I am deeply indebted and express my sincere appreciation to Dr. Churdchai Cheowtirakul for his continued support, motivation, inspiration, and precious guidance during the course of this research and writing.

I extend my appreciation to all Biotechnology teachers, laboratory technicians, and all faculty members for their helpful suggestions and supportive ideas during the entire preparation of this special project.

I wish to offer my heartfelt thanks to all my good friends for their help that leading me to achieve this special project. Their encouragement was immeasurable when everything seemed so dark.

Last but not least, I would like to express my deep gratitude to my family, whom I have the greatest admiration for their inspiration and support during my undergraduate study. I also wish to express my very special appreciation to my father and my mother who provided the opportunity of studying at this university.

Haritchanan Suvachananond

December 1999

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INTRODUCTION

During the past several decades, considerable interest has been developed for the wines that are produced in wineries across many nations. This interest continues to intensify, especially for the truly good wines that are reasonably priced. Consumers are unforgiving. Second class wines will not be acceptable just because a vintner may be newly established.

During the aging process, as time passes, wines start to improve, a softer and smoother wine are produced by malolactic fermentation which is considered to be a secondary fermentation and is quite different from the primary fermentation. During this process, alcohol does not increase but malic acid is converted into lactic acid and carbon dioxide. Malic acid and tartaric acid are the principal acids found in grapes. The malic acid can be overwhelming for the wine so it is converted to lactic acid, which is more pleasant in both taste and flavor.

The wine microorganisms suitable for use, as starter cultures are the yeast *Saccharomyces cerevisiae* for alcoholic fermentation and the bacteria *Leuconostoc oenos* for malolactic fermentation. According to the both fermentation properties, new and genetically improved strains could be programmed and constructed by classical techniques, including the formation of hybrids from strains with different desirable qualities and mutagenesis followed by screening for selection of the desired phenotype. But a better yet technique that shows the greatest promise as an aid in the genetic manipulation of wine yeast strains is spheroplast fusion (protoplast fusion.)

The method of genetic improvement offer a means of programming and constructing new strains which represent both of alcoholic fermentation of *Saccharomyces cerevisiae* and malolactic fermentation of *Leuconostoc oenos*. And also play an important role in the control of wine during production process and the production investment cost can be reduced substantially.

<u>OBJECTIVE</u>

To develop the procedure of isolating the stable fusant strains which represent both properties of alcoholic fermentation of *Saccharomyces cerevisiae* and malolactic fermentation of *Leuconostoc oenos.*

LITERATURE REVIEW

During the last decade, the subjects of wine improvement have been the focus of national and international researchers. Strain improvements have been recognized widely as the ability to produce some influence on the better taste and flavor of the wine.

A. Study of Malolactic Fermentation

Prahl et al. (1991) studied the method of inducing the decarboxylation of malic acid in must or fruit juice (known as malolactic fermentation). Must or fruit juice is directly inoculated with a culture of viable malolactic bacteria of the genus *Lactobacillus*. The bacteria being capable of decarboxylating malic acid at a pH below about 3.2 without any significant consumption of sugar present in the must or fruit juice and substantially without any production of volatile acidity. The malolactic bacteria may be added before or at the commencement of alcoholic fermentation. One malolactic strain suitable in the method is *Lactobacillus plantarum* DSM 4361.

Snow et al. (1984) studied the malolactic gene. DNA sequences, which are capable of converting L-malate into L-lactate, are incorporated into suitable vectors and used to transform both prokaryotic and eukaryotic hosts. The DNA sequences and vectors are particularly useful in conferring the ability to perform malolactic fermentation on wine producing yeast. *Saccharomyces cerevisiae* 2514-10C/ysw1 and *E. coli* RRI/ysw1 were deposited at the A.T.C.C. on August 16, 1982, and given accession Ser. Nos. 20651 and 39176, respectively.

Labarre et al. (1998) studied the using of specific polyclonal antibodies to study the malolactic enzyme from *Leuconostoc oenos* and other lactic acid bacteria. Despite the homologies between the malolactic enzymes from *L. oenos* and *Lactococcus lactis*, no immunological relationship was detected with the *L. lactis* malolactic enzyme, suggesting differences in their structural organization. The use of the antiserum also demonstrated that the problem of heterologous expression occurring in the recombinant *E. coli* strain resulted in a low synthesis of the malolactic enzyme from *L. oenos*. Moreover, a small amount of the protein was found to be peripherally associated to the membrane of *L. oenos*.

D'amico et al. (1995) studied the preparation and use of a malolactic ferment biomass. A malolactic ferment is produce from at least one malolactic bacterium strain, which is cultured in a culture medium containing an assimilable nitrogen source, malic acid and alcohol. The ferment biomass is separated from the culture medium. The biomass may be concentrated and may be frozen with a cryoprotective agent or dehydrated. The biomass may be added directly to wine to effect malolactic fermentation.

Daeschel et al. (1991) studied the process for deacidifying wine. A method is disclosed for deacidifying wine using malolactic fermentation (MLF). Under conditions inhibitory for spoilage lactic acid bacteria (LAB). Making possible a "pure culture" MLF in which a winemaker is provided with a way to control the timing of onset and the outcome of wine MLF. The method comprises adding nisin to the wine at a concentration lethal to nisin-sensitive LAB. Adding to the wine an inoculum of nisin-resistant LAB mutants capable in the presence of nisin

of converting malic acid to lactic acid, and maintaining the inoculated wine under conditions in which the MLF can occur. Afterward, nisin can be removed from the wine by contacting the wine with a nisin-removing substance, such as bentonite. The nisin can be added either before or simultaneously with addition of the nisin-resistant LAB, at any time during winemaking, including primary yeast fermentation.

King et al. (1985) studied the method for converting malic acid to lactic acid in wine. Wine having a reduced malic acid content is produced by inoculating wine or must with an activated bacterial culture that converts malic acid to lactic acid. The activated bacterial culture is produced by inoculating a concentrate of the bacteria into fruit juice to provide a high number of bacterial cells in the juice and holding the inoculated juice for a period without significant increase in cell population of the bacteria. The bacteria concentrate is a lyophilized or frozen culture concentrate of the bacteria that has been grown on a medium containing malic acid. When producing the frozen concentrate, a freeze-stabilizing agent may be mixed with the bacteria.

Prahl et al. (1997) studied about composition for inducing malolactic fermentation using *Leuconostoc oenos* strains accession numbers DSM 7008-DSM 7015. A method of inducing malolactic fermentation in wine or fruit juice by the direct inoculation of a concentrate of a starter culture containing a selected malolactically active bacterial strains having the accession numbers DSM 7008, DSM 7009, DSM 7010, DSM 7011, DSM 7012, DSM 7013, DSM 7014, and DSM 7015. Further, the strains are characterized as having a survival rate of at least 80% when introduced into a wine having a pH of 3.2 or lower and

containing at least 25 mg. SO.sub.2 per L and at least 12 % ethanol by volume. Also the strains are capable of starting malolactic fermentation when added directly to the wine or fruit juice at a concentration of less than 10.sup.7 colonies forming units per ml.

Further, from the studied of Gestrelius et al. (1983) studied about method of deacidifying wine and composition, therefore, deacidifying wine by passage through an alginate gel containing living cells of *Leuconostoc oenos*, therein. To ensure maximum viability, the alginate gel is stored in a resting medium, preferably sterile grape juice containing 5-12 % ethanol. Before deacidifying wine, the immobilized cells are conditioned to a wine milieu.

We can see from the already mentioned works that there are still plenty of opportunity to improve wine microorganisms. However, all researchers came to the same agreement that malolactic fermentation is the one of the very important strategy that has concerned in the quality development of wine.

B. Genetic Improvement by Protoplast Fusion

Svoboda et al. (1976) developed the technique of spheroplast (protoplast) fusion on yeasts. Technique that shows the greatest promise as an aid in the genetic manipulation of wild yeast strains which sporulates poorly or does not sporulate at all. Protoplasts are the reforms resulting from the removal of the yeast cell wall with lytic enzymes and can be induced to fuse if they are mixed in polyethylene glycol solution. After fusion the product must be induced to regenerate its cell wall in suitable media and to begin cell division.

Russell and Stewart et al. (1981) have successfully fused a number of brewer's yeast strains. Genetic improvements of industrial yeasts have already been achieved at this moment. Unfortunately, the fusion product is often very different from both original partners because the genome of both donors becomes integrated. Consequently, this technique is not specific enough to selectively introduce a single character into a yeast strain.

Ferenczy and Kucsera (1985) have taken this technique one step further and developed a method whereby the hybrid products of a protoplast fusion event may be selected even when neither of the parental strains contains a genetic marker. This method which called the Lazarus technique, kills both parental strains by using of metabolic poisons. Poisons that attack a different class of enzymes are issued for each parent, e.g., N-ethylmaleimide for one and myconazole for the other. This result in the death of both parents, which may be brought back to life on fusion by phenotypic

complementation. Each parent supplies a functional set of enzymes, which the other lacks.

A number of workers have employed protoplast fusion to confer amylolytic activity on either brewer or distillery yeasts. Hockney and Freeman (1980) exploited both the dead-donor technique and the natural biotin auxotrophy of brewing strains to hybridize *S. diastaticus* and *S. cerevisiae*.

Wilson et al. (1982) used complementation of auxotrophies between two haploid strains in the fusion of *Schwanniomyces alluvius* and *Saccharomyces uvarum*.

Two groups of Brazilian researchers (Galembeck et al., 1982; Echeverrigaray, 1982) have used protoplast fusion to form hybrid between the starch-degrading yeast *Lipomyces konoenkoae* and *S. cerevisiae*. Hybrids were selected as being able to grown on starch at 37°C since *S.cerevisiae* can not utilize starch and *L. kononenkoae* is unable to survive at elevated temperatures. While successful fusants, capable of converting starch to ethanol were obtained in all cases, the instability of the hybrids was a universal problem.

Protoplast fusion affords a means of transferring cytoplasmic organelles, plasmid DNA or viral nucleic acid between microbial cells. Fusion of mini protoplasts, derived from anucleate fungal cells with normal (nucleated) protoplast enables transfer of cytoplasmic components in the absence of nuclei. This mini-protoplast fusion technique has facilitated intergeneric

mitochondrial transfer from *Hansenula wingel* to petite (respiration deficient) strains of *S. cerevisiae*, the research of Yamashita et al. (1981.)

Plasmid DNA can be transferred from bacteria to eukaryotic cells by protoplast fusion. Schaffner (1980) has transferred a recombinant plasmid of pBR 322 containing *Simian virus* (SV40) DNA from *E. coli* to mammalian cells by PEG-induced fusion.

Kohtaro et al. (1986) introduced the intraspecific protoplast fusion of citric acid-producing strains of *Aspergillus niger*. Fusants were heterokaryons and in some cases formed sectors of prototroph. Heterodiploids were induced from a heterokaryon by d-camphor treatment citric acid productivity of one heterodiploid was intermediate between those of parent strains in both shaking and solid cultures.

Fusions involving nonviable protoplasts find application particularly where counterselection of one (or both) parental type (s) in the fusion is required in order to enrich for recombinants that can not be selected directly. One strategy is to inactivate one of the partners in the fusion by, for example, heat treatment or UV irradiation. The use of heat does not bias the genotypes of recombinants against the treated parent (Fodor et al. 1978), whereas the use of UV irradiation does (Hopwood and Wright, 1981.)

Nowadays, many researchers have managed to develop better strain of microorganisms and since alcoholic beverage especially wine product play an important role in human civilization, better wine's qualities and the reasonable production investment are the reasons indeed. It is the goal of this work to isolate stable fusant strains of *Saccharomyces cerevisiae*, which are able to represent both abilities of alcoholic and malolactic fermentation in the commercial wine production.

EXPERIMENTAL MATERIALS AND METHODS

A. Organisms

The cultures used for transformation experiments were diploid strains of *Saccharomyces cerevisiae* and *Leuconostoc oenos*. These diploid strains were received from laboratory's culture stock of Faculty of Biotechnology Assumption University, Bangkok, Thailand.

B. Medium and Growth Conditions.

The mediums, which are used most frequently in this study, are listed below. All medium were sterilized by autoclaving at 121°C for 15 minutes.

YEPD agar was used as a maintenance media for yeast cultures and fusant cultures. The cultures were transferred to fresh media every month. YEPD agar was prepared from 10g of yeast extract, 20g glucose, 20g peptone and 20g agar and the volume was adjusted to one liter with distilled water. After 48-72 hours incubation at 37- 40°C, the tubes were store in refrigerator.

ATM agar (acid tomato medium) was used as a maintenance medium for bacteria cultures. ATM agar was prepared from 15g agar, 10g glucose, 10g peptone, 5g of yeast extract, 0.2g $MgSO_4 . 7H_2O$, 0.05g $MnSO_4 . 4H_2O$ and 250 ml. tomato juice and the volume was adjusted to one liter with distilled water. After 48-72 hours incubation at 27-30°C, the tubes were stored in refrigerator.

C. Experimental procedures

1. Bacteria's cell wall isolation

1.1 Wild type strains of *Leuconostoc oenos* were transferred from slant to acid tomato media and after 15-18hours, cells are ready to be used.

1.2 Transfer 1% of strain into another acid tomato media after 6 hours of growth in culture is in log phase (check by spectphotometer at OD 550). The following procedures were carried on.

1.3 Harvest cell by centrifuge at 5000 rpm / 10 minutes.

1.4 Resuspend with TE (Tris base buffer) without sucrose (5ml per tube).

1.5 Centrifuge at 5000 / 5 minutes.

1.6 Resuspend with TE with sucrose (1 ml / tube).

1.7 Precipitation, keep the supernatant.

1.8 Adding lysozyme.

1.9 Incubate at 37°C, 60 minutes.

NOTE: Stock lysozyme: 10mg / ml of TE

2. <u>Yeast's cell wall isolation</u>

2.1 Grow yeast culture at 30°C in 200 ml of YPD to concentration of 2×10^7cells/ ml. or until log phase.

2.2 Centrifuge the culture for 5 minutes at 5000 rpm.

2.3 Resuspend in 20 ml of SED.

2.4 Incubate for 10 minutes at 30°C.

2.5 Centrifuge the culture for 5 minutes at 5000 rpm.

2.6 Wash the cells once in 20 ml. of 1M Sorbitol.

2.7 Resuspend in 20 ml of SCE.

2.8 Add 0.2 ml of Glusulase.

2.9 Incubate for 30 minutes to 2 hours at 30°C, with occasional gentle agitation.

2.10 Assay spheroplast formation by diluting 10 ml of cells into 2 drops of 5% SDS on a microscope slide and observing "ghosts" at 400× magnification with 2 phase-contrast microscope.

2.11 When 95% or more of the cells have been converted to spheroplasts, harvest them by centrifugation for 3 minutes at 2500 rpm.

2.12 Wash the spheroplasts twice in 20 ml of 1M Sorbital. Wash once in STC.

3. <u>Protoplast Fusion</u>

3.1   Add 1 ml of STC and then divided into 100 µl aliquots in 10-ml disposable tubes.

3.2   Add bacteria, which is in 10 µl of TE. For controls, prepare two tubes of cells with no added bacteria's cells.

3.3   Store at room temperature for 20 minutes.

3.4   Add 1 ml of PEG to each tube.

3.5   Store at room temperature for 10 minutes.

3.6   Centrifuge at 2500 rpm for 5 minutes.

3.7   Discard the supernatant and resuspend in 150 µl of SOS.

3.8   Incubate at 30°C for 20 minutes.

3.9   At this point, cells may be plated or stored at 4°C for as long as a few days.

3.10 Preincubate the plates at 37°C until they are warm. Keep the molten top agar at 45°C.

3.11 Add 6 ml of top agar (YPD) to each of the tubes, mix gently, and immediately pour onto the appropriate selective plates (YPD plates.)

3.12 Incubate the plates a 30°C for 2-4 days.

4. <u>Alcohol Determination by Ebulliometer</u>

4.1 Rinse all inside surfaces of the ebulliometer with distilled or deionized water. Drain Value and close.

4.2 Fill upper reflux condenser jacket with cold tap water.

4.3 Measure 50 ml of distilled or deionized water into clean 100-ml graduated cylinder, and then carefully pour into lower chamber inlet.

4.4 Very carefully insert thermometer into lower chamber inlet, holding top of thermometer in one hand in a pendulum effect and holding rubber stopper portion in the other hand. Slowly and gently twist rubber stopper into position for 2 snug fit.

4.5 Ignite ethanol burner and carefully position under lower chamber in the proper position.

4.6 Observe thermometer mercury rising until it stop and holds for 15-20 seconds at the same temperature. Remove ethanol burner and close carefully to extinguish flame.

4.7 Remove thermometer carefully in reverse manner to step 4 above. Hold in vertical position until the mercury drops from the capillary. Dry with towel carefully and place upright in a safe place.

4.8 Adjust circular slide scale to indicate the boiling point temperature of the water.

4.9 Empty the ebulliometer carefully and rinse inner surfaces with a few milliliters of the wine sample to be analyzed. Drain the instrument and fill upper reflux condenser with cold tap water. Ensure that no water goes down the inner tube.

4.10 Rinse the 100ml graduate with a few milliliters of the wine sample to be analyzed. Empty and refill with 50ml of the wine.

4.11 Repeat step 4-7.

4.12 Compare reading of the thermometer to the corresponding alcohol percentage on the circular slide scale. For example, with water boiling at 99.8°C and a wine boiling at 91.1°C, the alcohol would be 12.1 % by volume.

5. Malolactic Fermentation Determination by Paper Chromatography

5.1   A pencil line (baseline) is drawn approximately 2.5 cm parallel to the long edge of the filter paper. The wine sample (or standard) is spotted from the micropipet on this line about 2.5 cm apart. Each spot is repeated for four times (allowed to dry in between) at a volume of 10 µl form the micropipet.

5.2   A cylinder is made from the paper by stapling the short ends, without overlapping.

5.3   Place solvent constituents in separatory funnel and mix. After about 20 minutes, the lower aqueous phase is drawn off and discarded.

5.4   Transfer 70 ml of the upper layer into the wide-mouth jar. Stand the paper cylinder up in the jar such that the spotted edge (baseline) is in the solvent, and then cover the jar.

5.5   The chromatogram should develop in about 5-8 hours.

5.6   Remove chromatogram (now yellow in color) and store in a ventilated area until dry and the formic acid has vaporized, leaving a blue-green background with yellow spots of acid having the following approximate Rf values:

Tartaric acid	0.28
Citric acid	0.45
Malic acid	0.51
Lactic acid	0.78

5.7   Standards and wine samples should be run simultaneously or one immediately following the other.

5.8   Solvent may be used repeatedly if care is taken to remove any aqueous layer, which may have separated after each run.

6. <u>Brix Determination by Refractometer</u>

6.1 Adjust juice sample to room temperature or to that required by operating instruction of the instrument.

6.2 Open prism cover and rinse prism surface with several drops of sample. Gently wipe dry with absorbent lens paper.

6.3 Apply several drops of sample again and close prism cover. Point refractometer toward bright light source and hold in the same manner as a telescope. Adjust focus and read Brix at light-dark dividing line. Record result.

6.4 Rinse refractometer prism surface and prism cover three times with distilled or deionized water and wipe dry with absorbent lens paper. Avoid scratching prism surface.

B. Malolactic Fermentation Determination by Paper Chromatography (part 1: wine added only fusant strains)

After 6 – 8 hours of the chromatogram development, it showed,

Figure 3: Malolactic Fermentation Determination of wine adding fusant strain number 1, number 2 and number 3 by Paper Chromatography.

Note: Y is the wine samples, which show malolactic fermentation.

    - Sample 1: A_1 (control, without adding fusant strains)

    - Sample 2: B_1 (wine adding fusant strain no.1)

Figure 4: Malolactic Fermentation Determination of wine adding fusant strain number 4, number 5 and number 6 by Paper Chromatography.

Figure 5: Malolactic Fermentation Determination of wine adding fusant strain number 7, number 8 and number 9 by Paper Chromatography.

Figure 6: Malolactic Fermentation Determination of wine adding fusant strain number 10, number 11 and number 12 by Paper Chromatography. Notice, the disappear of the malic acid spot on fusant strain number 11.
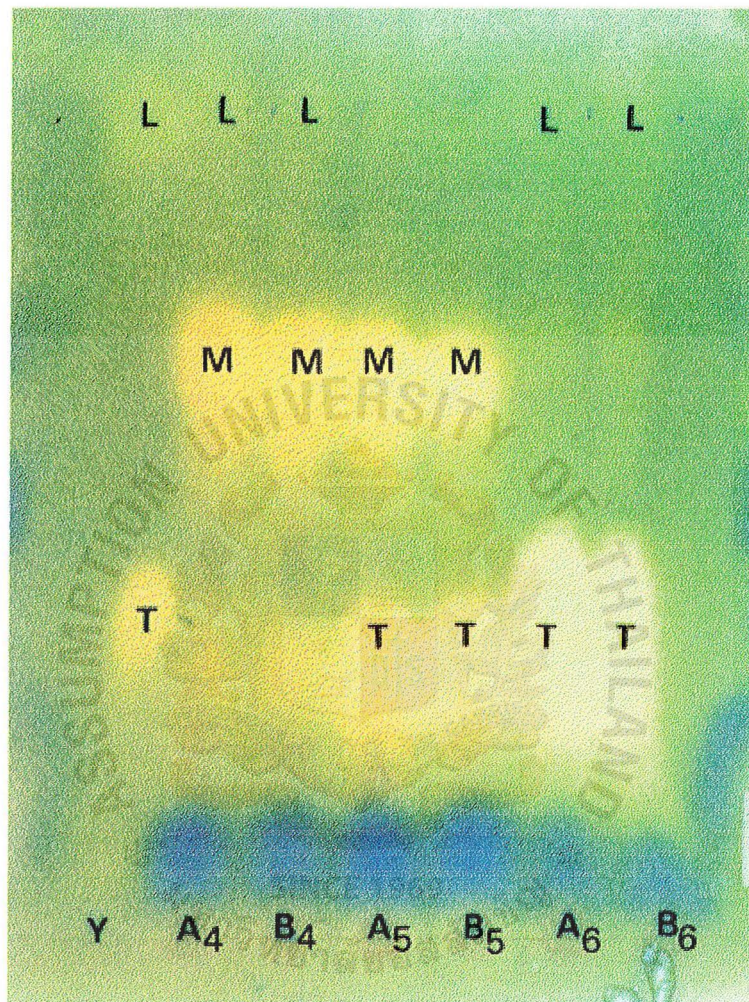
Figure 7: Malolactic Fermentation Determination of wine adding fusant strain number 13, number 14 and number 15 by Paper Chromatography.

Figure 8: Malolactic Fermentation Determination of wine adding fusant strain number 16, number 17 and number 18 by Paper Chromatography.
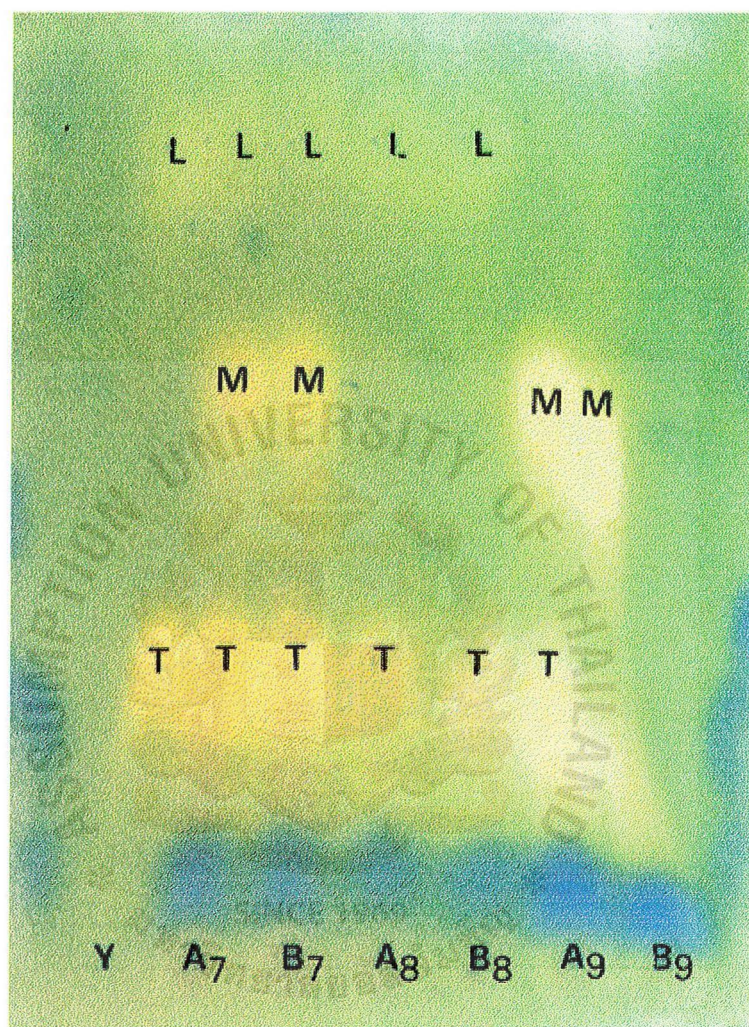
Figure 9: Malolactic Fermentation Determination of wine adding fusant strain number 19, number 20 and number 21 by Paper Chromatography.

Figure 10: Malolactic Fermentation Determination of wine adding fusant strain number 22, number 23, and number 24 by Paper Chromatography.

Figure 11: Malolactic Fermentation Determination of wine adding fusant strain number 25, number 26 and number 27 by Paper Chromatography.
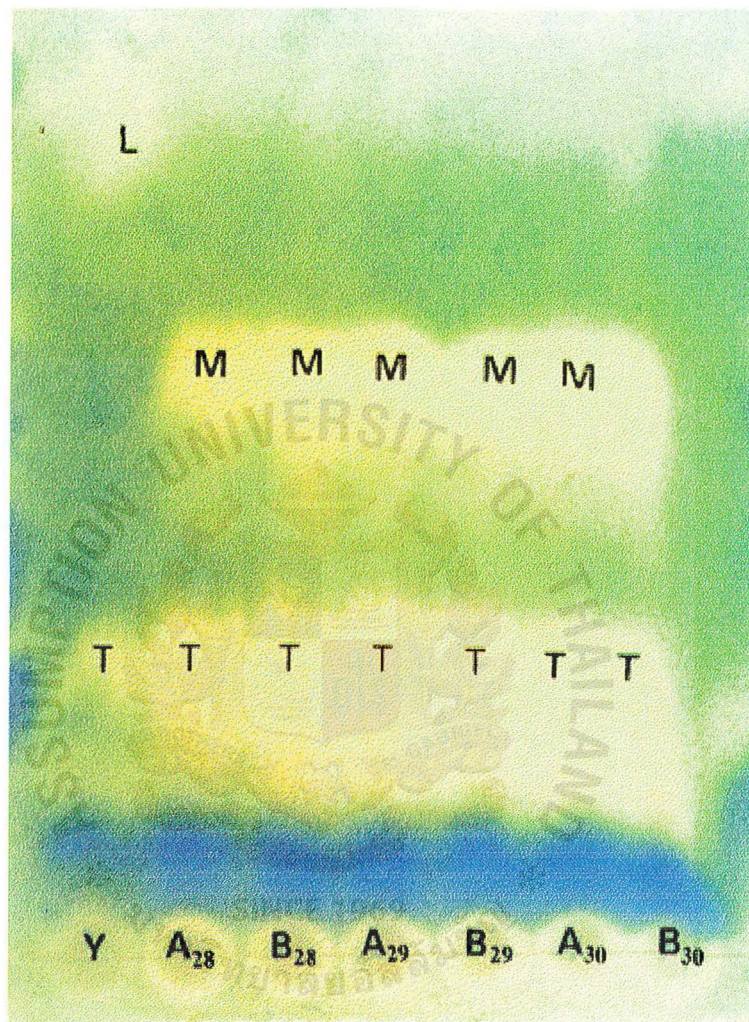
Figure 12: Malolactic Fermentation Determination of wine adding fusant strain number 28, number 29 and number 30 by Paper Chromatography.
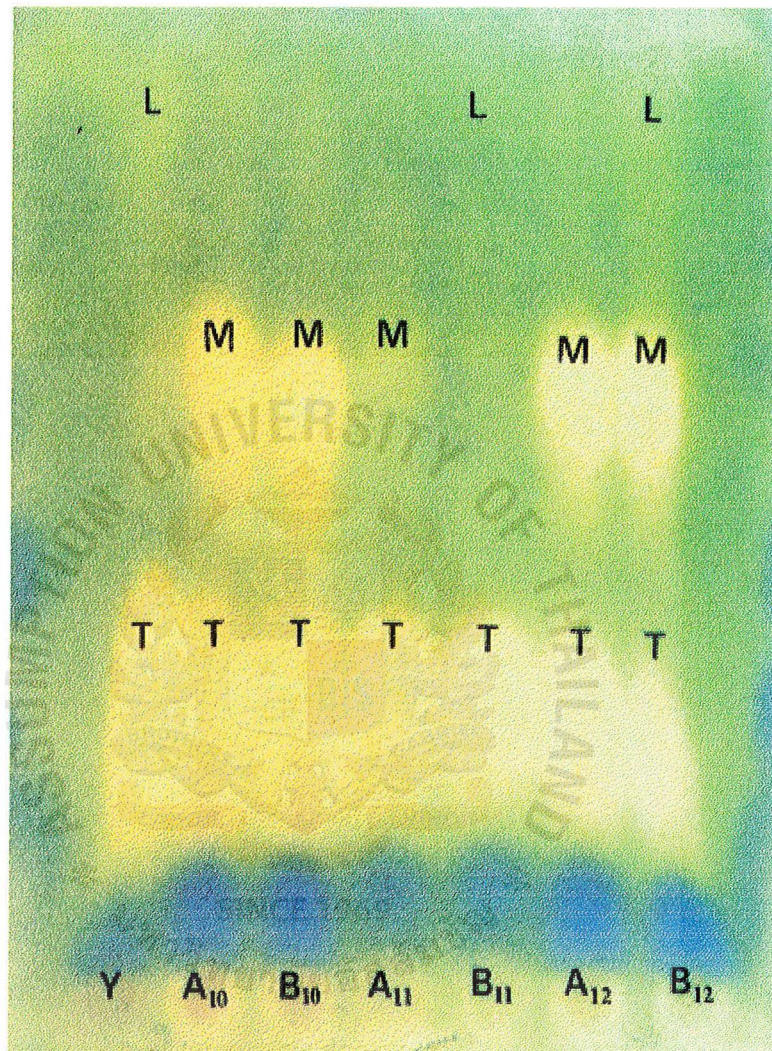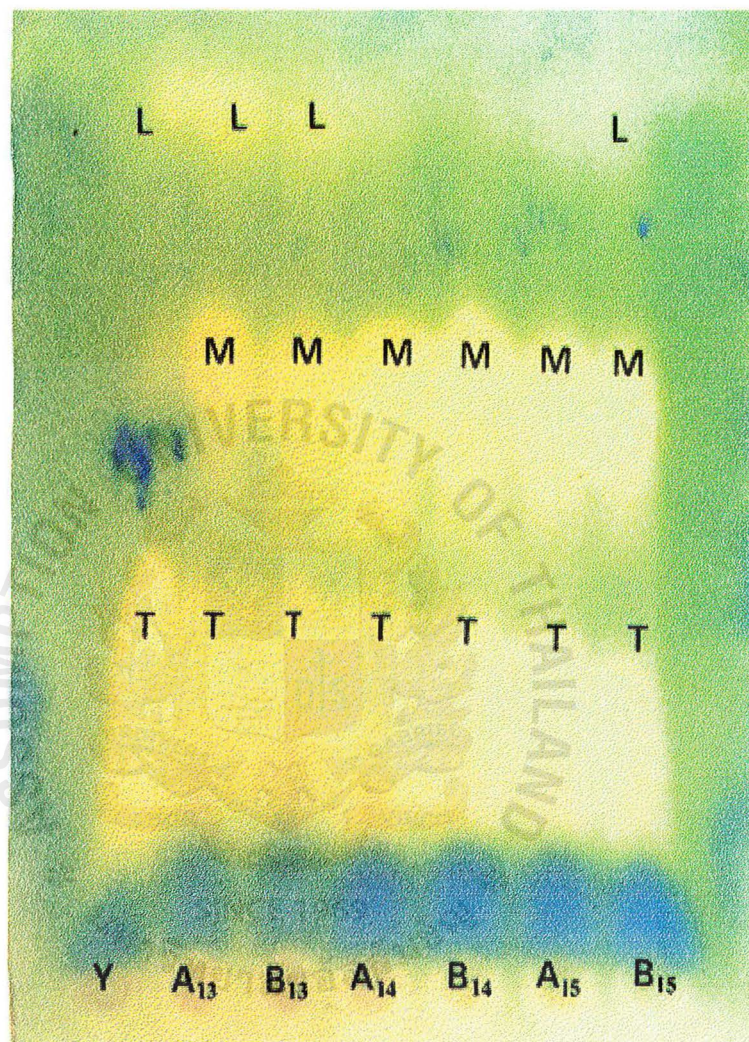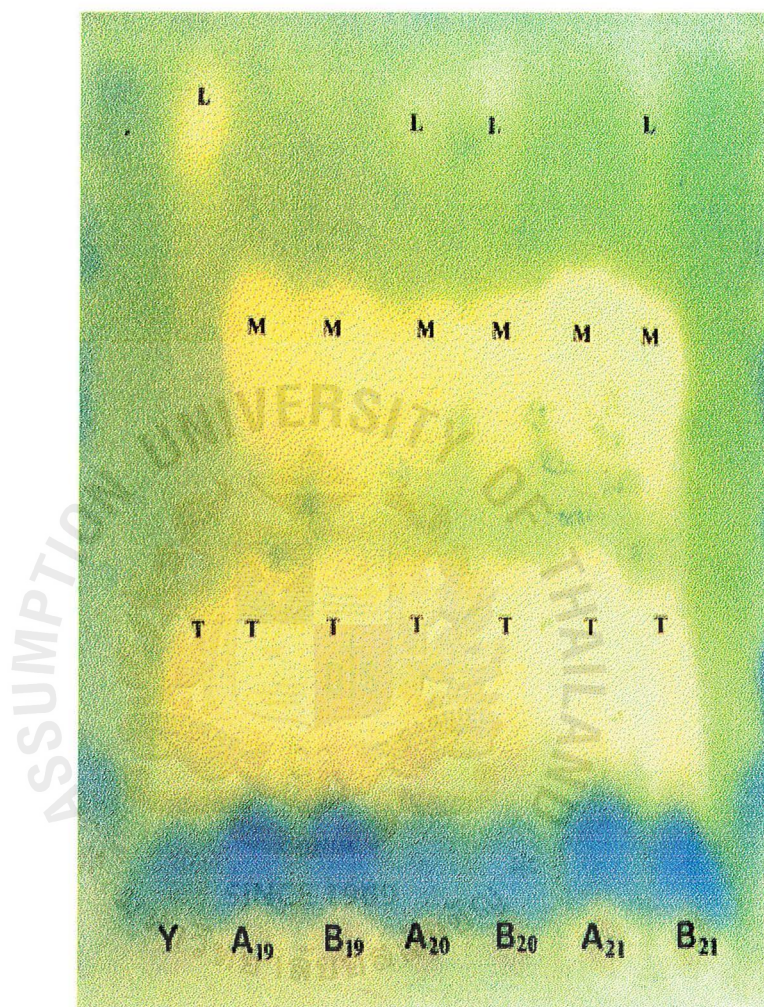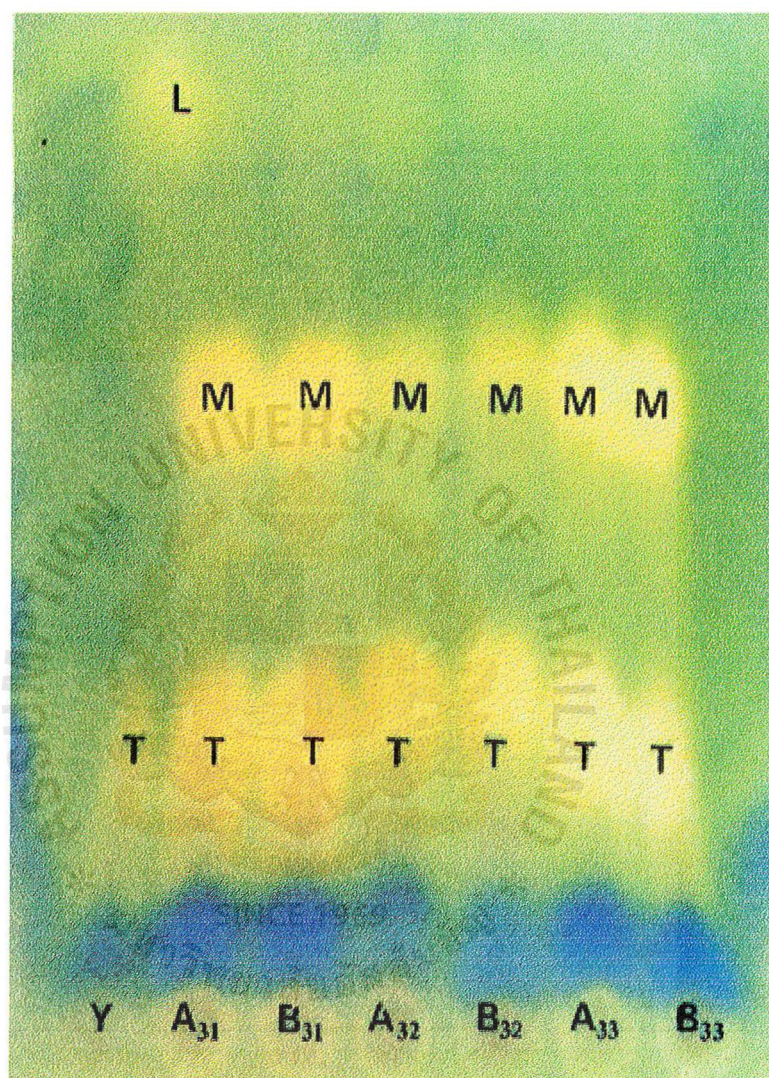
Figure 13: Malolactic Fermentation Determination of wine adding fusant strain number 31, number 32 and number 33 by Paper Chromatography.
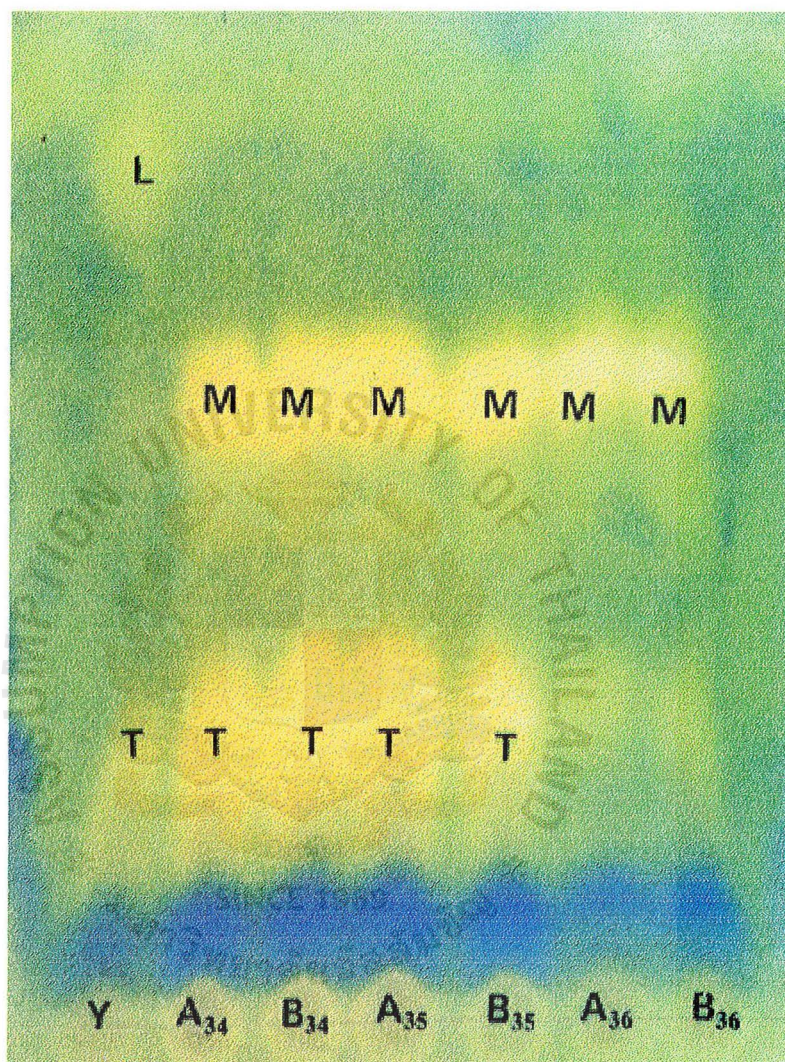
Figure 14: Malolactic Fermentation Determination of wine adding fusant strain number 34, number 35 and number 36 by Paper Chromatography.
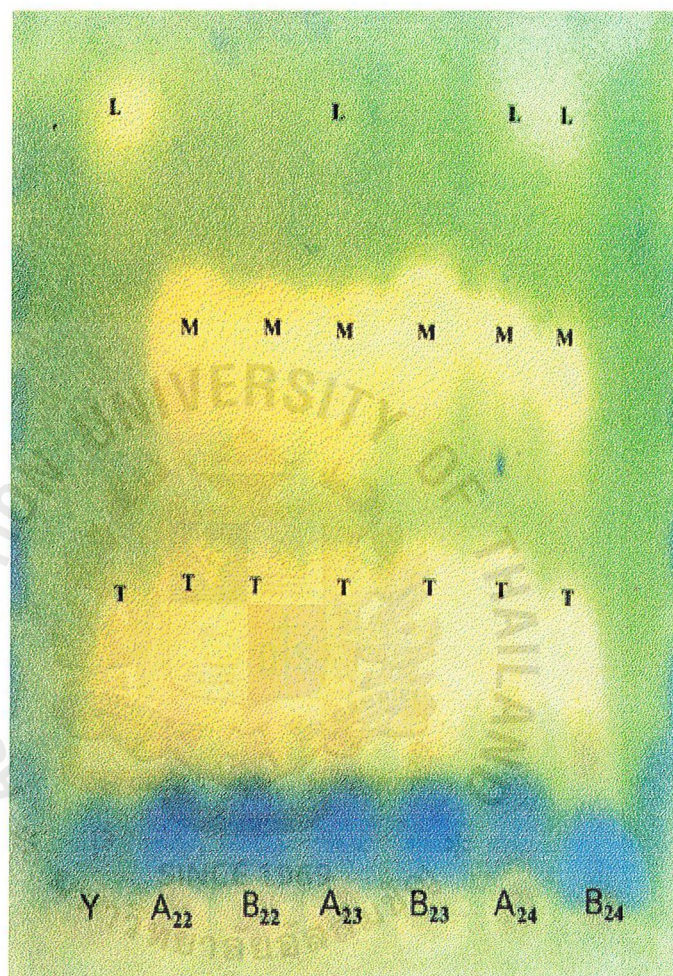
C. Brix and Alcohol Determination

1. Must which added *S. cerevisiae.*
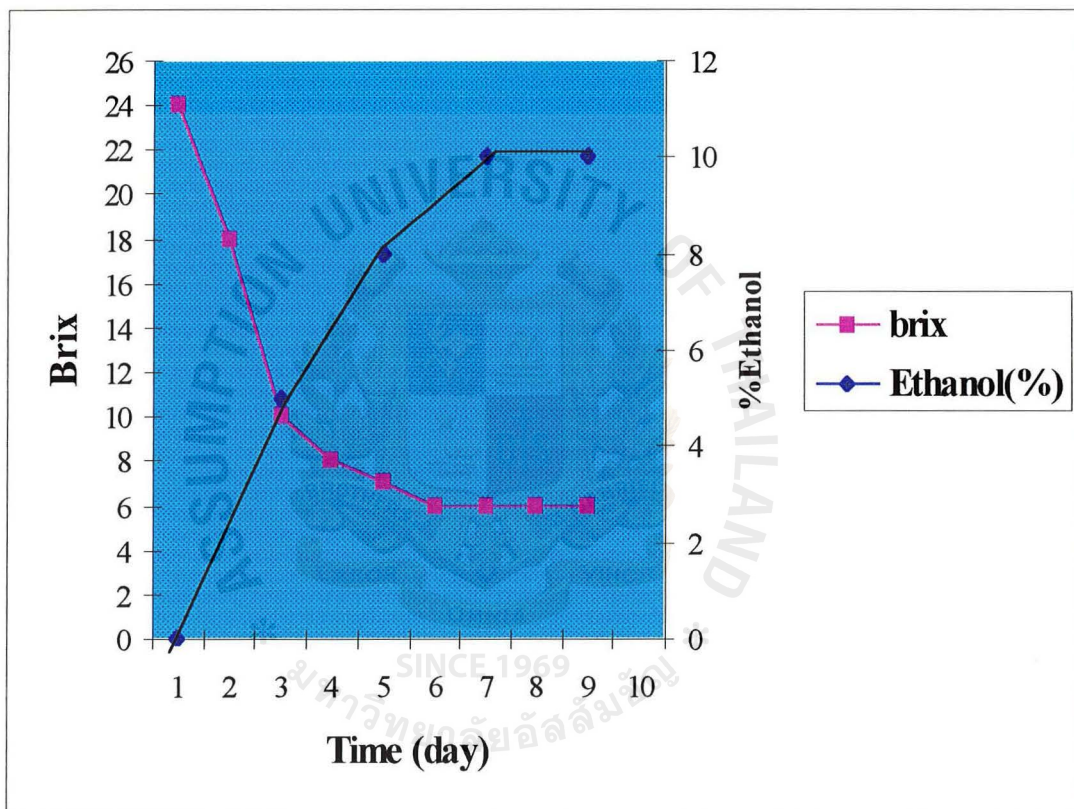
Figure 15: Brix and Alcohol Determination in the must sample which added wild type yeast (*S. cerevisiae*)

2. <u>Must which added fusant strain (number 11)</u>
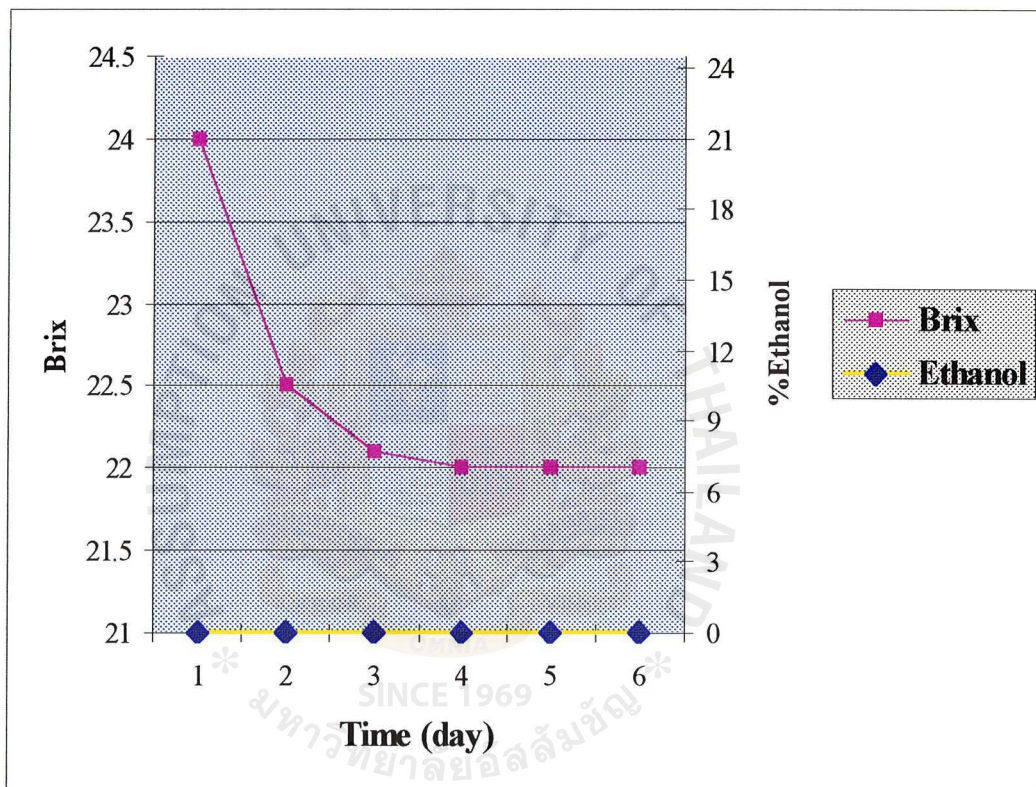
Figure 16: Brix and Alcohol Determination in the must sample which added fusant strain (no.11)

3. Must which added both of *S. cerevisiae* and fusant strain (no. 11)
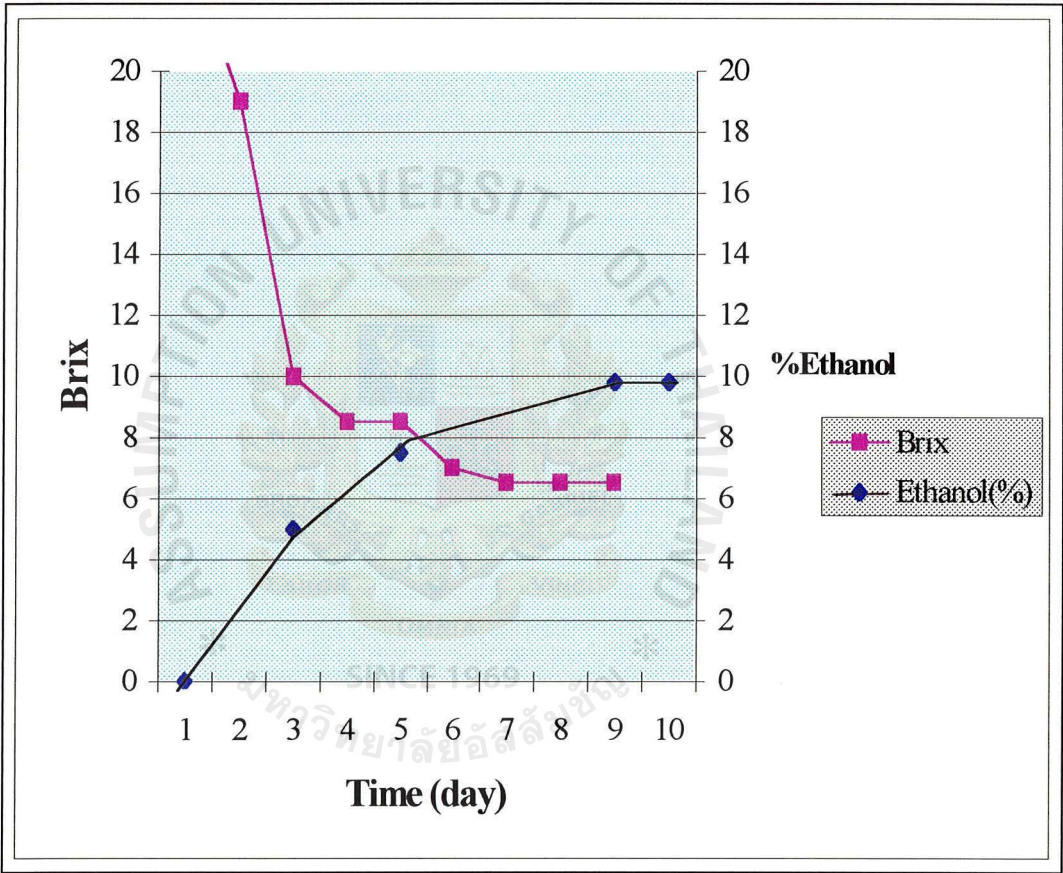
Figure 17: Brix and Alcohol Determination in the must sample which added both of wild yeast (*S. cerevisiae*) and fusant strain (number 11)

D. Malolactic Fermentation Determination by Paper Chromatography (part 2: wine added both of *S. cerevisiae* and fusant strain no. 11)
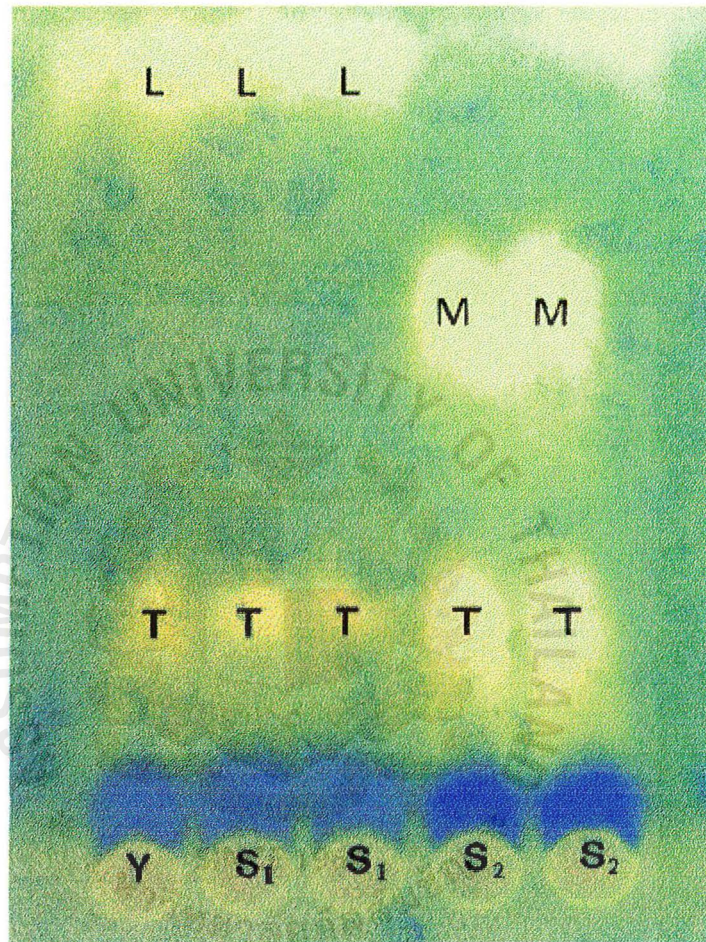
Figure 18: Malolactic Fermentation Determination of production of wine by adding both of wild type yeast (*S. cerevisiae*) and fusant strain no.11, compare with wine sample which added only yeast

Note: Y is the wine sample which show the absolutely malolactic fermentation.

S1 is the wine sample added both yeast and fusant strain no.11.

S2 is the wine sample added only yeast.

DISCUSSION

In the process of winemaking, all the equipment and container for juice and must fermentation provided excellent environmental condition for the growth of yeast. For the proper fermentation, the starting population of *Saccharomyces cerevisiae* in the must should exceed 1-3 $x10^6$ cells per ml. This is due to the prolonged growth of yeast during semi-anaerobic fermentation condition, will lead to the reducing of steroid and fatty acid components of the cell membrane, thereby making the yeast more sensitive to the effect of alcohol.

The malolactic fermentation (MLF) is a catabolic pathway in which L-malic acid is enzymatically oxidized to L-lactic acid and carbon dioxide. Depending on the strain(s) of lactic acid bacteria (LAB) involved; several by-products may be produced that impact the sensory properties of the wine.

Chemically, the most significant change observed during the course of MLF is increase in pH of 0.3. Decrease in total acid (TA) is generally on the order of 1-3 g/l.

Therefore, successful induction of MLF in high acid and low pH wines is, potentially, a useful technique for acid and pH adjustment. The occurrence of MLF is common to all wine-producing areas of the world. Studies have shown that LAB probably originate on the grape, where they may be isolated from the berry surface and grape leaves. Their numbers, however, are rather low in most instances, less than 10^2 CFU/ml. Other study suggested that winery equipment is an important source of infection.

Rapid onset and successful completion of MLF requires preparation of LAB starters of high cell density ($>10^6$ CFU/Ml) and vigor. *Leuconostoc oenos* is usually related for this task.

Over the past several years, a number of commercially available high liters lyophilize and cultures have been developed. Their advantage is that the lag time needed to prepare sufficient volume of active starter is reduced significantly from that needed to bring up cultures stored on laboratory media.

LAB used for this study can be described as hetero-fermentative, and recommended to be added in young wine with pH approximately 3.35 at 65-70°F.

Although, MLF bacteria are available commercially, but due to the fastidious property of these organisms, it would be some merit to construct yeast that has the MLF property and also can carry on the conversion of sugar into alcohol.

The method of protoplasm isolation by cell wall lysis was described in the material and method. Lysozyme is added to the protocol and incubated for 60 minutes for complete disruption of the cell wall. The ghost (cell without cell wall) can be detected by phase contrast microscope at the magnification of 400x.

Russell and Stewart et al. (1984) suggested that using polyethylene glycol (PEG) with a relative molecular weight of 4000 to 6000 could

induce protoplast fusion. But for this study we used PEG of relative molecular weight at 1000 instead.

After incubating for 3 days, the morphology of the fusants are similar to those aging yeast, which are round shape and either tan or gray in color.

The screening procedures used for the isolation of targeted organisms are determination of ethyl alcohol production and MLF properties by the fusants.

MLF are determined by using paper chromatography. The results were shown in the figures 3-14. Difference acids are represent by different spots on the paper chromatography. The absence of the spot represent malic acid is a good indicator of the organism carrying the property of MLF. Hence the malic acid is completely changed to lactic acid. The formation of lactic acid itself is not a valid evidence for MLF, as this may result from other microbial activity.

In this study, 50 strains of fusants are chosen for adding to the individual wine sample, only one strain (number 11) showed the absent of malic acid on the paper chromatography experiment. For certain reason, we named this strain *Saccharomyces cerevisiae* var. MLF 11 Haritchanan.

Next step is to determine the alcoholic fermentation property of this new variety. The fusant was added in fruit juice and let fermented for a period of 10 days. The juice was used for the determination of alcohol residue. The result was a very disappointed that this new strain of yeast

lost the property in alcohol production. No alcohol was detected in the juice.

We conducted a further experiment by adding both wild type *S. cerevisiae* and the new strain together in fruit to explore whether this new strain can work together simultaneously with wild type yeast to produce alcohol and at the same time has the characteristic of MLF.

The result in figure 17 and 18 has shown that *S. cerevisiae* var. MLF 11 Haritchanan can be added together with *S. cerevisiae* (W.T.) and performed both alcoholic and mololactic fermentation.

CONCLUSION

In this study, even though we did not obtain any new strains of organisms the can performed both alcoholic and malolactic fermentation but we obtained an organism that has the characteristic of *S. cerevisiae* which is much more easier to grow and maintain than lactic acid bacteria. This new strain of yeast carries the property of malolactic fermentation, which was not found in the wild type.

Further more, this new strain of yeast can be added together with *S. cerevisiae* (W.T.) to perform the complete stages of wine production process which are alcoholic and malolactic fermentation simultaneously.

SUGGESTION FOR FUTURE STUDY

In this study yeast like organism which can performed the property of malolactic fermentation was obtained. But the result still not fulfill with my projection at the beginning of the project. Therefore I would like to make the following suggestion for the further study.

A. Technique for obtaining fusant strains.

Sasaki et al. (1984) had successfully developed a fusion technique by mating a respiratory-deficient strain of bacteria with yeast. This respiratory-deficient strain can be obtain easily by exposed the bacteria with UV light and the petite colonies obtained will have the respiratory-deficient property. The suggestion for improving the method of fusant screening is by expose of *Leuconostoc oenos* with UV light with the proper intensity and screen for petite strain before performing of protoplast fusion with yeast.

B. Observation of spheroplast formation

In the experiment of isolating spheroplast, the detect of cell without cell wall was very difficult to observe, the control which was the cells without adding any lysis enzyme had almost the same character of the cell added with enzyme. The suggestion for the future study is to change the chemical used for the staining of cell wall (the one in this study is methyl green stain). Aldehyde bisulfite-toluidine blue may be the another attractive stain in the observation of yeast's cell wall.

C. <u>Method of protoplast fusion</u>

In this study, the method of protoplast fusion should have used the fusogenic agent as polyethylene glycol (PEG) which has the molecular weight around 3000 to 6000. But I had used PEG with 1000 molecular weight in stead, due to the availability of the substance. Therefore the question is, if I use PEG with the right molecular weight, would I get a better fusant? For the future work, it is worth while to try fusogenic agent which has the molecular weight in the range of 3000-6000.

After finishing the fusion procedure, more than 200 fusants were obtained but due to the limitation of time, I have selected only 50 colonies to determine the characteristic of alcoholic and malolactic fermentation. As the result, only one colony showed the capability of malolactic fermentation property. I suspect that if all the colonies were used for further study, would I be able to obtain any colony that has the ability of both alcoholic and malolactic fermentation?

Another point that I would like to suggest is a series of fusion can also be tried to increase the possibility of obtaining the fusant I targeted. Taya et al. (1984) recommend that the mechanism of a single or part of chromosome transfer from which I have postulated for construction of a new strain microorganism hybrid by protoplast fusion, though it has advantages in maintaining the genome of the recipient strain in its original states. With the addition of a few characters, does make it unlikely that a large number of characters will be transferred during any one fusion. However, once one desired character has been integrated into the genome, it may be possible to introduce other characters through further fusion, without losing the first. On the other hand, the probable

transfer of both the β-galactosidase and the lactose permease of *Kluyveromyces lactis* to *Saccharomyces cerevisiae*, to yield a strain of the later species which ferments lactose to ethanol, has been reported recently (Galeotti and Clark-Walker 1983.) From the above statement I can see that the development of this method will likely to increase the usefulness of protoplast fusion as a technique for producing improved strains of my purpose.

D. <u>Morphological study</u>

To study the morphological aspect of the new fusant would be interesting and useful for the manipulation of this fusant in industrial application.

E. <u>Study of the genetic mapping of microorganisms</u>

It would be interesting to know exactly how the gene in the yeast and bacteria I use in this study transform into the fusant. The study of gene mapping of malolactic gene into the yeast protoplasm and how the malolactic gene disturb the pathway of alcoholic production would be another aspect of interest.

SUMMARY

The process of wine making, from wine yard to bottled product, reflects not only the unique contribution of the graphs and winemaker, but the combine activities of resident wine yard and winery flora as well. There are quite few microorganisms that contribute to the quality of the making of premium wine. *Saccharomyces cerevisiae* contribute to the alcohol composition in wine. But what make the taste of wine could really be considered as a superior wine is the work of malolactic fermentation microorganisms. This secondary fermentation is accomplished by unique strains of bacteria *(L. oenos)* which can convert the sharp taste of malic acid into creamier taste of lactic acid, and the characteristic of wine will be much more pleasant. MLF happens more readily at warmer temperature (above 60 ° F) and in wines that have little or no SO_2 (sulfite). Some strains of *S. cerevisiae* can produce large amount of sulfite (more than 20 ppm) which become inhibitory to growth and metabolism of lactic acid bacteria. Some of lactic acid bacteria may produce substances (bacteriocin) inhibitory to yeast and may thus cause stuck fermentation and spoiled wines. According to this limited factor, new and genetically improved strains could be constructed to isolate stable strain which is able to represent both abilities of alcoholic and malolactic fermentation. The technique that shows the greatest promise for genetic manipulation of wine yeast strains and malolactic fermentative bacteria fusion which could be obtained by the use of polyethylene glycol (PEG) as a fusogenic agent. The fusant, which can grow on YPD agar, show tan-gray in color colonies which same as the aging *S. cerevisiae*. The fusant strains obtained were used for the determination of malolactic fermentation property by using technique of

paper chromatography. Only one strain of the 50 strains tested showed the property of malolactic fermentation but incidentally, this strain lack of the property of alcoholic production. Nevertheless, the fusant obtained that in this study has the characteristic of yeast-like organisms which can maintain in the same media (YEPD) with wild type yeast (*S. cerevisiae*) and can be used together with *S. cerevisiae* (*W.T.*) to produce wine with malolactic fermentation property. From this reason, I conclude that the advantage of this fusant is the much easier to maintain and manipulate for using as a microorganism for the industrial premium wine production.
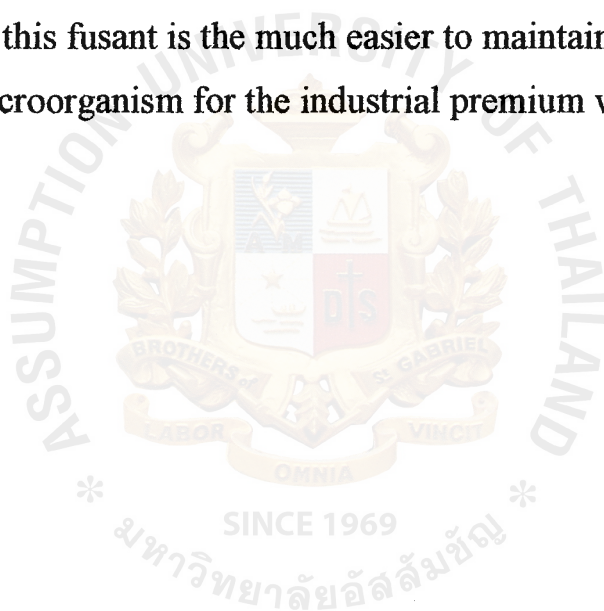
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APPENDIX

Appendix A: Media

Media for petri plates are prepared in 2-liter flasks, each flask containing 1 liter of media, which is sufficient for 30-40 plates. Unless otherwise stated, all components are autoclaved together for 15 minutes at 250 °F (121°C) and 15-lb./sq. inch of pressure. The plates should be allowed to dry at room temperature for 2-3 days after pouring. The plates can be stored in sealed plastic bags for over 3 months. The agar is omitted for liquid media (For convenience, The final concentration of each component in the medium is listed in parentheses below.)

YPD (YEPD)

YPD is a complex medium for routine growth.

Bacto-yeast extract (1%)	10 g
Bacto-peptone (2%)	20 g
Glucose (2%)	20 g
Bacto-agar (2%)	20 g
Distilled water	1000 ml

<u>ATM</u> (Acid Tomato Medium)

ATM is a complex medium for routine growth of
Leuconostoc oenos

Bacto-yeast extract (0.5%)	5	g
Bacto-peptone (1%)	10	g
Glucose (1%)	10	g
Bacto-agar (1.5%)	15	g
$MgSO_4.7H_2O$ (0.02%)	0.2	g
$MnSO_4.4H_2O$ (0.005%)	0.05	g
Distilled water	1000	ml

Appendix B: Stock Preservation

Culture strains can be stored indefinitely in 15% (v/v) glycerol at a temperature of -60°C or less (culture strains tend to die if stored a temperatures above -55°C). Many workers use 2-ml vials (35x12 mm) containing 1ml of sterile 15% (v/v) glycerol. The strains are grown on the surfaces of appropriate plates. The strains are then scraped up with sterile applicator sticks or toothpicks and suspended in the glycerol solution. The caps are tightened and the vials shaken before freezing. Transferring a small portion of the frozen sample onto an appropriate medium can revive the strains.

Strains can also be stored at 4°C for up to 6 months on slants prepared with appropriate medium. This method of storage is convenient since the slants take up little space; do not dry out. Slants are also a useful means of sending strains to colleagues.
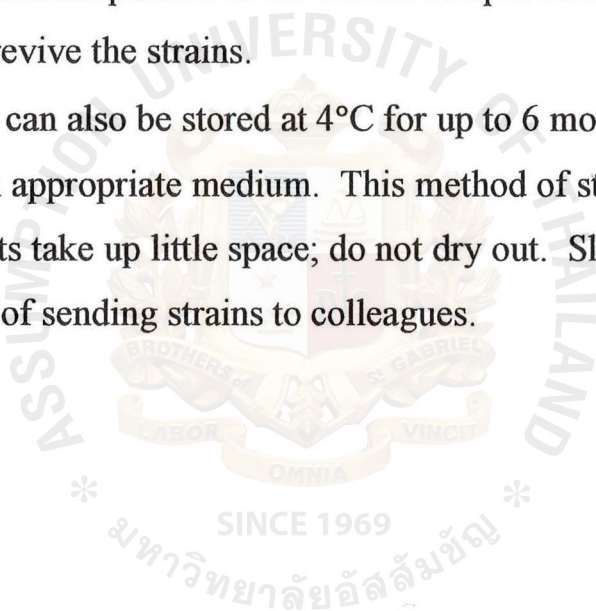
Appendix C: Reagents

Reagents for Strain Transformation

The following materials are needed for each 200-ml culture. All solutions should be steriled (i.e., autoclaved under standard conditions unless otherwise started.)

YPD

Distilled water

SED (20 ml):

- 1 M Sorbitol
- 25 mM Na_2EDTA (pH 8)
- 50 mM Dithiothreitol

1 M Sorbitol (200 ml)

SCE (20 ml):

- 1 M Sorbital
- 0.1 M Sodium citrate (pH 5.8)
- 0.01 M Na_2EDTA

Glusulase (0.2 ml)

5% SDE

STC (40 ml):

- 1 M Sorbitol
- 10 mM $CaCl_2$
- 10 mM Tris – Cl (pH 7.5)

10-ml disposable tubes (Falcon 2059)

DNA (0.1-5 μg/tube) in TE (pH 7.5)

    TE (pH 7.5)

    -   10 mM Tris-Cl (pH 7.5)
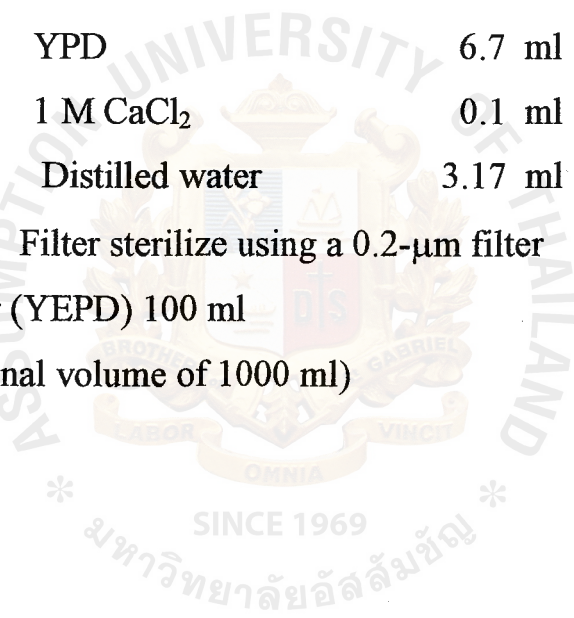
    -   1 mM Na_2EDTA

PEG (20 ml):

    -   20% Polyethylene glycol 3300

    -   10 mM $CaCl_2$

    *   Filter- sterilize using a 0.2-μm filter.

SOS:

- 2 M Sorbitol	10	ml
- YPD	6.7	ml
- 1 M $CaCl_2$	0.1	ml
- Distilled water	3.17	ml

    *   Filter sterilize using a 0.2-μm filter

Top agar (YEPD) 100 ml

Plates (final volume of 1000 ml)

Reagent for Malolactic Fermentation Determination by Paper Chromatography

The following materials are needed for one paper chromatogram; the larger volume can be prepared in the same ratio. Solvent may be used repeatedly over a period of two or three months if care is taken to remove any aqueous layer, which may have separated after each run.

Chromatographic grade filter paper cut in to 20x30 cm rectangles

1.2x75 mm micropipets

Separatory funnel

One- gallon wide-mouth glass jars with covers

Solvent constituents

- 100 ml distilled water
- 100 ml n-butyl alcohol
- 10.7 ml concentrated formic acid
- 15 ml 1% water-soluble Bromcresol Green

Standard solutions

- 0.3% tartaric acid
- 0.3% citric acid
- 0.3% malic acid
- 0.3% lactic acid

NOTE: If you are not trained in working with hazardous chemicals, do not even think of working with glacial formic acid by yourself. Get someone competent to do it for you.