CHAPTER III

RESULTS AND DISCUSSION

1. Isolation of yeasts from various sources

Yeasts are widely distributed in nature. They are found on fruits, flowers and other sugar containing foods. One of the most important uses of yeast is the production of ethyl alcohol as beverages from carbohydrate materials e.g.wine.

In order to survey wild yeasts, samples from various foods were collected from Sunday Market, Suan Jatujuk, Bangkok. Basically, the collected samples were suspended in sterile water and spreaded on the selective media contained 0.01 % chloramphenicol to eliminate the growth of bacteria. The colonies appearing after incubation were yeasts. The result was shown in Table 1. Fourty yeast isolates were obtained from twenty-two out of twenty-five of collected food samples . It was found that there were 28 strains from 12 samples of fruits, 7 strains from 7 samples of flowers, 3 strains from 3 samples of juices and 2 strains from 3 samples of fermented foods.

Table 1 List of isolated yeasts from various sources collected from Sunday Market, Suan Jatujuk, Bangkok.

Name of sample	No. of sample	Isolated strains
1. Fruits	12 samples	28 yeast strains
1.1 White grape		1.01, 1.02, 1.03, 1.04
1.2 Red grape		1.05, 1.06
1.3 Jack fruit		1.07, 1.08, 1.09, 1.10
1.4 Lancet fruit		1.11, 1.12, 1.13
1.5 Carambola		1.14
1.6 Sapota		1.15, 1.16, 1.17
1.7 Mango		1.18, 1.19
1.8 Pineapple		1.20, 1.21
1.9 Papaya		1.22
1.10 Coconut		1.23
1.11 Tamarind		1.24, 1.25
1.12 Star-gooseberry		1.26, 1.27, 1.28
2. Flowers	7 samples	7 yeast strains
2.1 Jasmine		2.01, 2.02
2.2 Jum-pee		2.03
2.3 Jum-pa		2.04, 2.05
2.4 Chinarose		-
2.5 Rose		2.06
2.6 Marigold		-
2.7 Doak khem		2.07

Name of sample	No. of sample	Isolated strains
3. Juices	3 samples	3 yeast strains
3.1 Cane juice		3.01
3.2 Palm juice		3.02
3.3 Coconut juice		3.03
4. Fermented Foods	2 samples	2 yeasts strains
4.1 Kao-mark		4.01
4.2 Fermented soybean		-
4.3 Fermented bamboo shoot		4.02

2. Isolation of killer Yeasts

Isolation of yeasts from natural sources in Thailand enable one to collect thermophilic strains, such as high temperature tolerant killer yeasts which were the desirable yeast to be used in this experimental work; 1,190 yeast strains were tested for killing ability. The sources of the yeast strains were divided into two groups. One group from the isolation and the other from private collection. The isolation group contained 40 isolates from various sources (Table 1) and 729 isolates of KU-NRIB joint research. The latter were isolates from collected samples in Thailand that had been done during 1981-1986. These yeast strains were isolated from fruits, flowers and fermented foods for the alcohol fermentation research. From private collections, there were 20 tested strains from Pradit collection, 173 tested strains from Patoomporn collection, 119 tested strains from Boontiem collection including to 109 tested strains from IFRPD stock culture.

All the strains under investigation were tested for different phenotype, i.e. killer, neutral and sensitive with respect to the killer character as shown in Figure 2. The methylene blue agar technique (Somers and Bevan, 1969) was used to determine the ability of various strains to kill the sensitive tester strain, Torulopsis glabrata IFO 0622, on the media buffered at pH 4.7. The killer colonies formed zones of suppression of growth of the tester sensitive strain. The colonies of the sensitive strains were blue, which is a specific strain for dead yeast cells (Lindegren, 1949). The colony that did not form zone and did not turn blue on the layer of lawn-plates was

defined as neutral. The phenotypes of the tested strains were shown in Table 2. It was found that out of 1,190 tested strains, 25 strains were killers, 871 strains were neutrals and 294 strains were sensitives. The killer phenotype was found from KU-NRIB isolation, Patoomporn collection and IFRPD stock culture. These killer yeasts were selected for the stable killing activity at high temperature. Twenty-five tested killer strains were assayed for killer activity at different temperature, i.e.20 °C, 25-28 °C, 37 °C and 40 °C (Table 3). The difference of killing ability at 37 °C and 40 °C was observed and it was found that one strain had weak killing ability, 2 strains had strong killing ability and 3 strains had very strong, while the others were the killers only in the range of 20-28 °C .

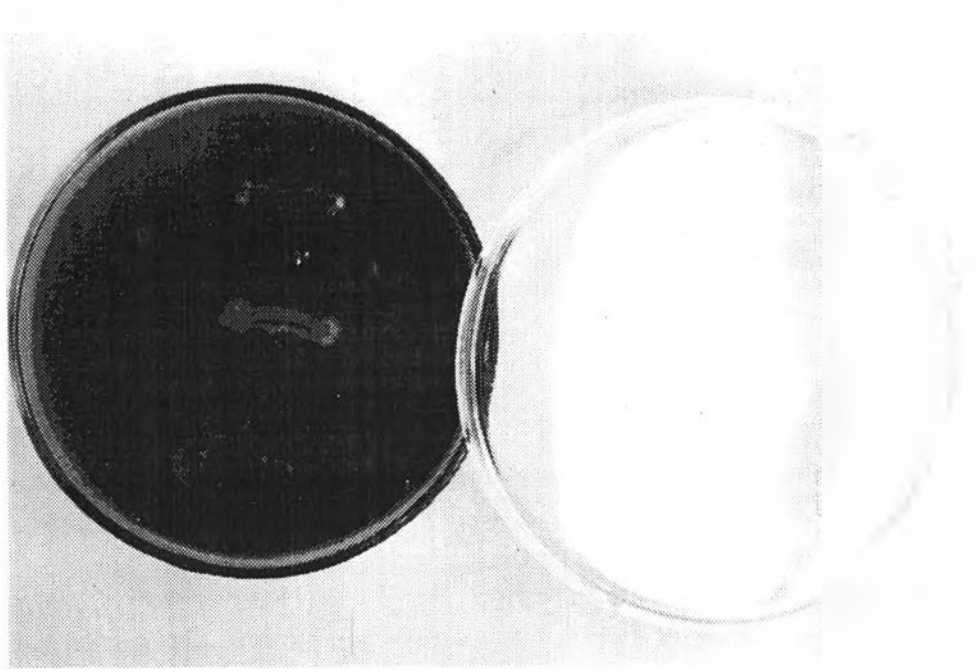

Figure 2 The killer phenomenon of Saccharomyces cerevisiae; A seeded lawn of a sensitive strain on YEPD-MB was streaked with a killer strain (on the bottom), a sensitive strain (on the top) and a neutral strain (in the middle).

Table 2 Phenotypes of the tested yeasts

Tested strains	Phenotype			Sources
	killer	neutral	sensitive	
109	1	69	39	IFRPD stock culture
20	–	12	8	Pradit collection
173	6	109	58	Patoomporn collection
119	–	90	29	Boontiem collection
729	18	554	157	KU-NRIB isolation
40	-	37	3	Various foods isolation
Total 1,190	25	871	294	

IFRPD : Institute of Food Research and Product Development,Thailand

KU : Kasetsart University, Thailand

NRIB : National Research Institute of Brewing, Japan.

Table 3 Killing reaction of the tested killer strains at different temperature.

Strain	Code	Killing ability at 20 °c	25-28 °c	37 °c	40 °c	Nomenclature	Source
Standard type killer strains							
1	K-1	+	-	-	-	Saccharomyces cerevisiae KL 88	NRIB
2	K-2	+	-	-	-	Saccharomyces cerevisiae NCYC 738	NRIB
3	K-3	+	-	-	-	Saccharomyces cerevisiae NCYC 761	NRIB
4	K-9	+	-	-	-	Hansenula mrakii NCYC 500	NRIB
5	K-10	+	-	-	-	Kluyveromyces drosophilarum NCYC 575	NRIB
6	K-11	+	-	-	-	Torulopsis glabrata ATCC 15126	NRIB
Tested killer strains							
1	KY 78	+++	+++	+++	+++	Hansenula saturnus IFO 0117	IFRPD stock culture
2	17	+	+	-	-	non-identified	KU-NRIB Isolation
3	21	+	+	-	-	"	KU-NRIB Isolation
4	23	++	++	++	++	"	KU-NRIB Isolation
5	31	+	+	-	-	"	KU-NRIB Isolation
6	41	+	+	-	-	"	KU-NRIB Isolation
7	44	+	+	-	-	"	KU-NRIB Isolation
8	50	++	++	++	++	"	KU-NRIB Isolation
9	72	+	+	-	-	"	KU-NRIB Isolation
10	94	+	+	-	-	"	KU-NRIB Isolation
11	98	+	+	-	-	"	KU-NRIB Isolation
12	123	+	+	-	-	"	KU-NRIB Isolation
13	176	+	+	-	-	"	KU-NRIB Isolation
14	190	+	+	-	-	"	KU-NRIB Isolation
15	192	+	+	-	-	"	KU-NRIB Isolation
						"	

Table 3 (cont.)

Strain	Code	Killing ability at 20 °C	25-28 °C	37 °C	40 °C	Nomenclature	Source
16	265	+++	+++	+++	+++	non-identified	KU-NRIB Isolation
17	266	+++	+++	+++	+++	"	KU-NRIB Isolation
18	370	+	+	-	-	"	KU-NRIB Isolation
19	469	++	+	+	÷	"	KU-NRIB Isolation
20	OK24	+	-	-	-	"	Patoomporn collection
21	OK48	+	-	-	-	"	Patoomporn collection
22	OK60	+	-	-	-	"	Patoomporn collection
23	OK74	+	-	-	-	"	Patoomporn collection
24	OK109	+	-	-	-	"	Patoomporn collection
25	OK111	+	-	-	-	"	Patoomporn collection

- \- no killing ability
- ÷ very weak killing ability
- \+ weak killing ability
- ++ strong killing ability
- +++ very strong killing ability

3. Identification of the isolates

Four selected high temperature tolerant killer yeast strains and the best wine strain were examined morphologically and physiologically. Identification was made by methods mentioned by Lodder (1970). The results were presented in Table 4 and 5 along with the identified Saccharomyces cerevisiae, strain Sc.90. as the comparison strain. Details of morphological and physiological characters of the selected strains were used to key out that they were Saccharomyces cerevisiae. All examined strains had spherical vegetative cells with multipolar budding as the vegetative reproduction. The ascospores were also spherical and the fermenting patterns were typical of Saccharomyces cerevisiae. No identification was needed for KY 78, Hansenula saturnus IFO 0117, since it was a culture preserved by Institute of Fermentation Osaka (IFO), culture collection which guaranteed by the World Data Centre for Microorganisms.

4. Interaction between killer yeasts

The examined yeast strains; No.23, 50, 265, 266 and KY 78 were streaked on the YEPD-MB plates, eight colonies per plate, using the methylene blue agar technique (Somers and Bevan, 1969). The sensitive phenotype was determined only as a background lawn and the killer as a streak on the same plate. Each of the examined strains was observed for its ability to kill and for its sensitivity to the standard killing activity of other killer strains. In this experimental work, the killer type strains, K-1, K-2, K-3, K-9, K-10 and K-11, were also interacted with these killers including the sensitive type strain, IFO 0622. These standard type strains were obtained from National Research

Table 4 Morphological characteristics of yeasts

Characteristics	Comparison strain	Wine strain	Killer strains			
	Sc90	No.2	No.23	No.50	No.265	No.266
colony colour	cream	cream	cream	cream	cream	cream
Vegetative growth	multipolar	multipolar	multipolar	multipolar	multipolar	multipolar
Cell shape	round	round	round	round	round	round
Pseudomycelium	-	-	-	-	-	-
True mycelium	-	-	-	-	-	-
Ballistospores	-	-	-	-	-	-
Arthrospores	-	-	-	-	-	-
Chlamydospores	-	-	-	-	-	-
Endospores	-	-	-	-	-	-
Teliospores	-	-	-	-	-	-
Ascospores	+	+	+	+	+	+
Ascospore shape	round	round	round	round	round	round
Ascospores No./ascus	4	3-4	4	4	4	3-4
conjugation	-	-	-	-	-	-
Asci rupture readily	-	-	-	-	-	-
Fermentation	+	+	+	+	+	+
Growth with KNO_3	-	-	-	-	-	-
Growth with inositol	-	-	-	-	-	-
Growth without vitamins	-	-	-	-	-	-

Table 5 Physiological characteristics of yeasts.

Characteristics	Comparison strain	Wine strain	Killer strains			
	Sc90	No.2	No.23	No.50	No.265	No.266
Fermentation of						
glucose	+	+	+	+	+	+
galactose	+	+	+	+	+	+
sucrose	+	+	+	+	+	+
maltose	+	+	+	+	+	+
melibiose	-	-	-	-	-	-
raffinose	+1/3	+1/3	+1/3	+1/3	+1/3	+1/3
soluble starch	-	-	-	-	-	-
Assimilation of						
glucose	+	+	+	+	+	+
galactose	+	+	+	+	+	+
sucrose	+	+	+	+	+	+
maltose	+	+	+	+	+	+
melibiose	-	-	-	-	-	-
raffinose	+	+	+	+	+	+
soluble starch	-	-	-	-	-	-
ethylamine hydrochloride	-	-	-	-	-	-
urease activity	-	-	-	-	-	-
cycloheximide resistance	-	-	-	-	-	-
vegetative phase	diploid	diploid	diploid	diploid	diploid	diploid
acid production	+	+	+	+	+	+

Institute of Brewing (NRIB), Japan and they are all classified as Saccharomyces cerevisiae. The results were shown in Table 6.

In common all killer yeasts were immuned to the action of their own toxins, i.e. KY 78 was inactivated against itself even though it was able to kill almost the tested killer strains. Wickner (1974) demonstrated that killer cells belonging to the genus Saccharomyces grown at temperatures of 37 to 40 °C and plated on assay medium yield almost exclusively non-killer colonies. In this experimental work, the killer type strains showed very low level of killing activity so they were considered to be cured by the climatic temperature (approximate 25-32 °C) in tropical countries, i.e. Thailand. The standard killer type strains were unable to kill the selected killer strains but KY 78 was able to kill all of these killer type strains. Strain No. 266 was able to kill almost these standard killer type strains except K-2. Strain No.265 was able to kill K-3, K-9, K-10 and K-11 and both strains of No.23 and No.50 were able to kill only Torulopsis glabrata, such as K-11 and IFO 0622. The strains No.23, 50 and 265 were unable to kill the other tested killer strains and itself, but strain No.266 was able to kill all of the tested killer strains except KY-78 and itself. From these results, strain No.266 was one of the Saccharomyces tested killers that had strong killing ability and immunity system. Hara and coworker (1983) demonstrated that yeast species with saturn-shaped ascospores or the yeast in Hansenula species like strain KY 78 in the experiment had a strong killer activity against various species and the killer toxin was stable. Unfortunately, Hansenula species is one of the undesirable yeast in winemaking, they have an ability to grow on the surface of wine in

Table 6 Interaction between killer yeasts at 20 °C

killer strains \ Seeded Strain	No.23	No.50	KY-78	No.265	No.266	K-1	K-2	K-3	K-9	K-10	K-11	IFO 0622
No.23	-	-	-	-	-	-	-	-	-	-	+	+
No.50	-	-	-	-	-	-	-	-	-	-	+	+
KY-78	+	+	-	+	+	+	+	+	+	+	+	+
No.265	-	-	-	-	-	-	-	+	+	+	+	+
No.266	+	+	-	+	-	+	-	+	+	+	+	+
K-1	-	-	-	-	-	-	+	+	-	-	-	+
K-2	-	-	-	-	-	+	-	-	-	-	-	+
K-3	-	-	-	-	-	-	-	-	-	-	-	+
K-9	-	-	-	-	-	+	+	+	-	-	-	+
K-10	-	-	-	-	-	+	+	+	-	-	-	+
K-11	-	-	-	-	-	-	-	-	-	-	-	+
IFO 0622	-	-	-	-	-	-	-	-	-	-	-	-

\+ Killing activity

\- No killing activity

a film-like growth and give rise to higher concentrations of aldehyde in the wine than normal (Kunkee and Amerine, 1970). Thus the investigation would omit strain KY-78 which was Hansenula saturnus and would be done only in Saccharomyces strain.

5. Killing reaction of selected killer yeasts against various yeasts

This experiment showed the killing action of the Saccharomyces tested strains against various yeasts which might be expected to contaminate in winemaking. The work was carried out with a streak of the tested killers on the buffered methylene blue medium and lawn of various yeasts as seeded strain. From Table 7, the tested killer strains killed chiefly yeasts belonging to the genus Saccharomyces. Strain No.23, 50 and 265 killed Saccharomyces cerevisiae, Saccharomyces bailii, Saccharomyces italicus, Saccharomyces rosei, Saccharomyces rouxii, Saccharomyces uvarum also killed Candida krusei, Kloeckera sp. and Kluyveromyces sp. while Saccharomyces chevalieri, Saccharomyces fermentati, Saccharomyces marxianus, Candida mycoderma, Candida lypolytica, Candida valida and Pichia membranefaciens were not killed. Strain No.266 also killed the yeasts belonging to genus Saccharomyces, except Saccharomyces italicus. In addition, it could kill Candida krusei, Hansenula anomala, Pichia membranefaciens Kloeckera sp. and Kluyveromyces sp. but Candida mycoderma, Candida lypolytica, Candida valida and Hansenula saturnus were not killed. Candida, Hansenula, Pichia, etc. were defined as film-forming yeasts and Saccharomyces fermentati was defined as pseudo-film-forming yeasts (Kunkee and Amerine, 1970). Strain No. 266 was thus considered to prevent contamination from some strains of film-forming and pseudo-film-forming.

Table 7 Killing reaction of selected killer yeasts against various yeasts at 20 °C

\Killer strain / Seeded strain\	No.23	No.50	No.265	No.266	K-1	K-2	K-3
Saccharomyces cerevisiae Sc 90	+	+	+	+	+	+	+
Saccharomyces bailii OK 94	+	+	+	+	+	+	+
Saccharomyces chevalieri J-6	-	-	-	+	+	+	+
Saccharomyces fermentati TC 19	-	-	-	+	+	-	+
Saccharomyces italicus KY 18	+	+	+	-	+	-	+
Saccharomyces marxianus TC 21	-	-	-	+	+	+	+
Saccharomyces rosei KY 74	+	+	+	+	+	+	+
Saccharomyces rouxii KY 75	+	+	+	+	+	+	+
Saccharomyces uvarum KY 14	+	+	+	+	+	-	+
Candida krusei KY 73	+	+	+	+	-	-	-
Candida mycoderma MIRCEN	-	-	-	-	-	-	-
Candida lypolytica NRRL Y1095	-	-	-	-	-	-	-
Candida valida MIRCEN	-	-	-	-	-	-	-
Hansenula anomala KY 79	-	-	-	+	-	-	-
Hansenula saturnus KY 78	-	-	-	-	-	-	-
Pichia membranefaciens KY 81	-	-	-	+	-	-	-
Kloeckera sp. KY 21	+	+	+	+	-	-	-
Kluyveromyces sp. KY 111	+	+	+	+	-	-	-

\- no killing reaction

\+ killing reaction

6. Extraction and analysis of ds RNA

Double-stranded RNA was extracted from the stationary phase culture of the tested yeast strains No.23, 50, 265, 266, K-1, K-2 and K-3 were analysed by agarose gel electrophoresis. Vodkin and Fink (1973) described that there was a ds RNA that was presented only in Saccharomyces killer yeasts but absented from Saccharomyces non - killer derivatives. The ds RNA from killer strains has been characterized and shown that standard killer strains had two ds RNA differing in size. When subjected to gel electrophoresis the higher molecular weight species of ds RNA (L) migrates slowly, but the smaller (M) migrates more rapidly. The ds RNA bands were visualized by the fluorescence ds RNA ethidium bromide complex under UV light (Bevan et.al., 1973). It was found in this experiment that all of the tested strains had L and M ds RNA but M ds RNA of K-3 was not clear (Figure 3). The large species (L ds RNA) in each strain had nearly the same molecular weight, but the small species (M ds RNA) was different. The M ds RNA of K-2 type was smaller than K-1, but K-3 was the smallest. These results corresponded to the investigations of Young and Yagiu (1978) and Kitano, et.al. (1984). Molecular weights of plasmids of the strain No.23, 50, 265 and 266 were in agreement with those of the K-1 strain. All of the tested strains had the length of L ds RNA in the range 4.4-6.6 kb and M ds RNA in the range 2.1-2.3 kb when they were compared with the DNA marker (λ-DNA digested with Hind III).

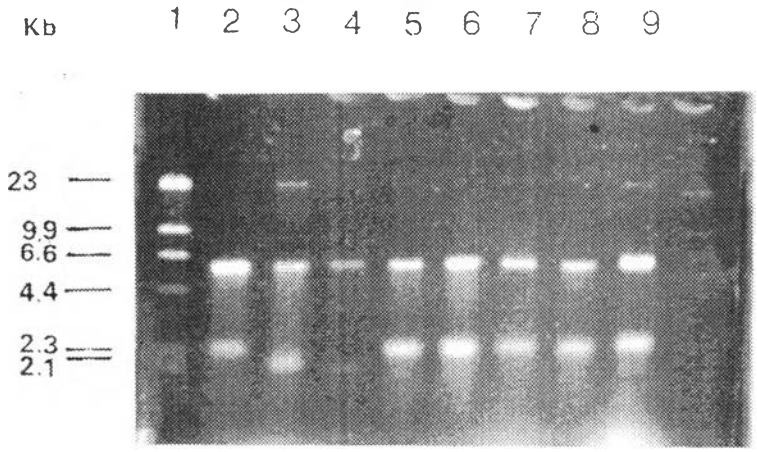

Figure 3. ds RNA of killer yeasts on agarose gel electrophoresis. ds RNA prepared by the method described by Fried and Fink (1978) was precipitated by ethanol and dissolved in a small amount of electrophoresis buffer. A 40 ul amount of each sample (absorbance at 260 nm) was applied to lanes 1 through 9 into 50 ml slab gel. Lane 1, λDNA marker digested with Hind III;) lane 2, K-1; lane 3, K-2; lane 4, K-3 (lane 2-4 used as standard killer type strains); lane 5, No.23; lane 6, No. 50; lane 7-8, No.265; lane 9, No.266.

7. Curing the killer yeasts

Only killer yeast strains belonging to the genus Saccharomyces were treated with either cycloheximide or incubated at elevated temperatures. When killer cells were treated with increasing concentration of cycloheximide they yielded either mixed killers and sensitives or only sensitive colonies as percentage curing. The concentrations of cycloheximide in the range of 0.1-0.2 ppm just permitted the growth of yeast strains and the degree of curing was an inhibition of killing property. The mechanism of curing by heat or cycloheximide is not known, but cycloheximide also affects secretion of the killer toxin (Fink and Styles, 1972). Sweeny and coworker (1976) showed that cycloheximide cured strains that lacked M ds RNA and retained L ds RNA also an increased amounts of L species. From the result in Table 8, shown that strains No.23, 50 and 265 readily cured by cycloheximide treatment,in contrast strain No.266 showed very low levels of curing. When incubation at elevated temperature (37 and 40 C), all of the tested strains were not cured, this might be the result of screening high temperature tolerant killer yeasts at the beginning of this experiment. The temperatures for curing treatment were only at 37 and 40 C and should not increased more than this because the temperature during fermentation should be maintained below 41.6 C (75 F) for winemaking (Ough and Amerine, 1961).
If the temperature increases, the fermentative activity of yeast decreases. More bouquet is formed in a wine by a long, slow fermentation at low temperature than by a short, rapid fermentation at higher temperature (Amerine et.al. 1979) . In addition ,Palfree

and Bussey (1979) had examined that the toxin from superkiller was more stable to heat (at 30 °C) and other treatments (including cycloheximide treatment) than the wild-type toxin. It might be assumed that strain No.266 was a superkiller.

Table 8 Curing of killer yeasts

Strain	Methods of curing			
	Cycloheximide		Temperature	
No.	conc (ppm)	% curing	°C	% curing
23	0.1	73	37	0
	0.2	100	40	0
50	0.1	100	37	0
	0.2	100	40	0
265	0.1	100	37	0
	0.2	100	40	0
266	0.1	0	37	0
	0.2	12	40	0

8. Effect of pH on killer toxin production and the effect of temperature on the killer toxin stability of killer yeast.

Crude toxin activity was determined by the Well-test method in agar plates seeded at 10^6 cells/ml with the sensitive tester strain. Toxin is produced during the log phase of growth of killer strains, and small amount of it is accumulated during stationary phase. Killing activity in the extracellular medium of growing killer yeast culture was examined and shown that activity of toxin increased as the cell titre increased, and leveled off as the culture reached stationary phase (Palfree and Bussey, 1979). In this experimental work, the stability of killing activity of the selected strain No.266, from the same count of cells in the pH range of 2.0-7.6 and incubating the cell-free toxin at 20, 25-28, 37 and 40 °C for 1 day and 2 days was studied. Increasing of the toxin activity was observed by measuring the width of inhibition zone around each well. The results were presented in Figure 4 that the toxin activity of cell-free culture filtrates of killer yeast strain No.266 was stable in the range of pH 3.8-7.6 with substantial inactivation occuring at pH values higher than 6.0. The optimum pH for killing of this strain was in the range of pH 6.0-6.4. In addition, its toxin production was stable in the same level of killing activity at 1 and 2 days of incubation time. Especially, the toxin activity was very stable at temperatures 20, 25-28, 37 and 40 °C. It has been reported that the killing was stable only within a narrow pH range (4.6-4.8) and quickly lost at temperatures above 25 °C (Woods and Bevan, 1968). In contrast the toxin activity of this strain differed from the other reports in its pH optimum for killing and was still stable at 40 °C. Thus, the

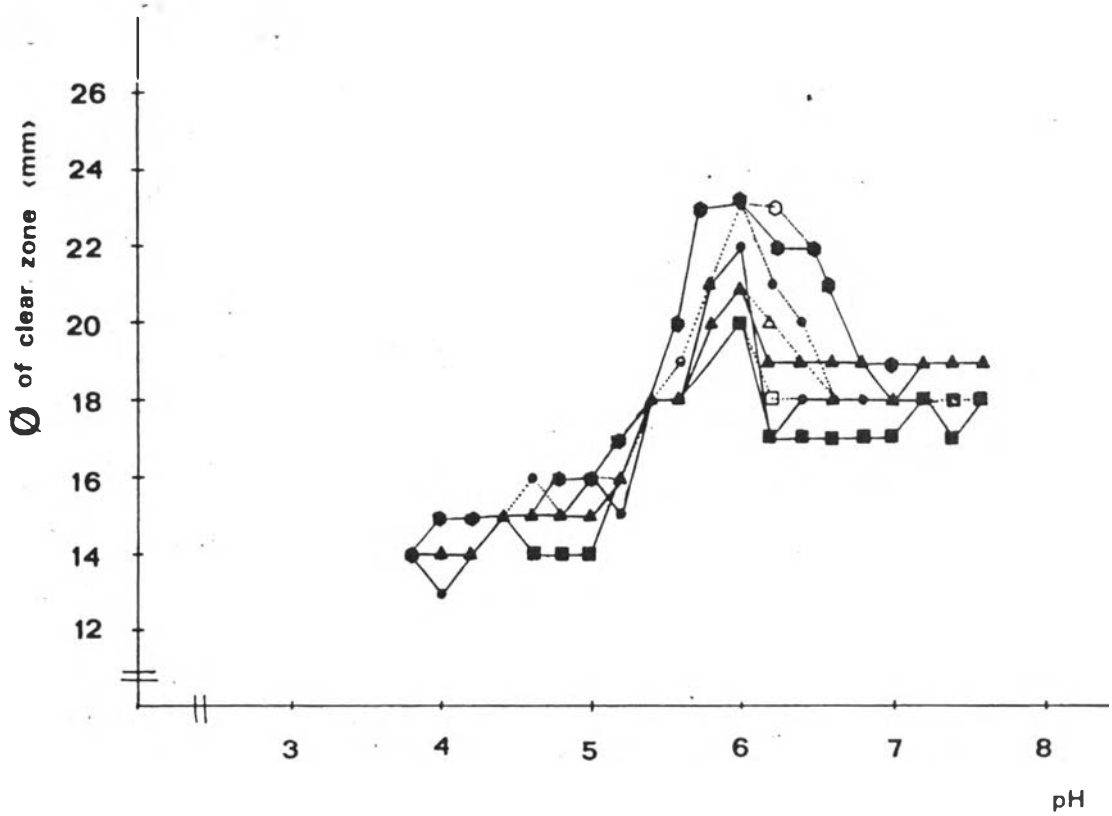

Figure 4 Effect of pH on the activity of killer yeast culture filtrates

○	incubated at 20 °c for	1 day
●	" "	2 "
○	incubated at 25-28 °c for	1 day
	" "	2 "
△	incubated at 37 °c for	1 day
	" "	2 "
□	incubated at 40 °c for	1 day
■	" "	2

mechanism of action of the killer toxin of this strain should be studied further.

9. The desirable properties of the parental strains for hybridization

The contamination by undesirable yeasts is one of the major problems in winemaking, because it results in a slow fermentation and inferior quailty of the product. Another problem is contamination by killer yeast that is able to kill the inoculated strain of desirable wine yeast if it is sensitive to the killer toxin of the contaminant. It is possible to construct a killer wine yeast for wine fermentation by mating technique. When strains of opposite mating type are mixed, mating reaction occurs. In this study the selected high temperature tolerant killer yeast was used to mate with the laboratory wine yeast to produce the hybrids. The parental strains and their characteristics were listed on Table 9.

Table 9 Characters of the parental strains used

Strain No.	Phenotype for killer	Genotype for killer	Sporulation ability (%)	SO_2 tolerance	Note
2	sensitive	mm(0)	11	+	one of the best laboratory strains of wine yeast.
266	killer	MM(k)	82	-	an isolate from this experiment, high temperature tolerant strain.

Notation of genotype for killer M/m : gene determining the presence (k)/ absence (o) of killing cytoplasmic determinants.

Symbol for SO_2 tolerance

+ that initiated to ferment within 30 hours.

± that initiated to ferment during 30-48 hours.

- that initiated to ferment over 48 hours or non-growth.

The genotype notation used is that of Somers and Bevan (1969). The killer phenotype is determined by the presence of cytoplasmic genetic determinants (k), which are maintained or expressed only in the presence of the dominant nuclear allele M. Absence of the (k) determinants, (o), confers the sensitive phenotype, regardless of the nuclear genotype. Hence the genotype of killer strains is M(k), and of sensitive strains is m(o). Strain 2 had fermenting ability in grape juice containing 100 ppm of SO_2 as mentioned in the experimental methods, while strain 266 did not grow under the same conditions. When vegetative cells of the parent were incubated in sporulation medium at 30 °C for 3 days, the sporulating ability was around 11 % for strain No.2, and 82 % for strain No. 266, Both strains could be improved by direct genetic manipulation of their specific characteristics.

10. Determinaion of killing curve and induction of mutants (Mutagenesis)

Essentially, hybridization used haploids of opposite mating type to produce the diploid hybrids. In this experiment, the genetic markers were introduced to the parental strains by mutagenic procedures and then the marked strains were crossed with different auxotrophic haploids. A survival curve of mutagen treatment was required before the optimal conditions for mutagenesis was able to be determined (Figure 5). The auxotrophic mutants by ethylmethane sulfonate (EMS) treatment at the condition that gave about 1% mutants at survival rates in the region of 50 % was preferred in many laboratory (Woods, 1980). The results shown in Figure 5 that strain No.2 and No.266 were induced to auxotrophic mutants by 3% ethylmethane sulfonate in the region of 50% survival rate to be 1.5 and 2 hours respectively.

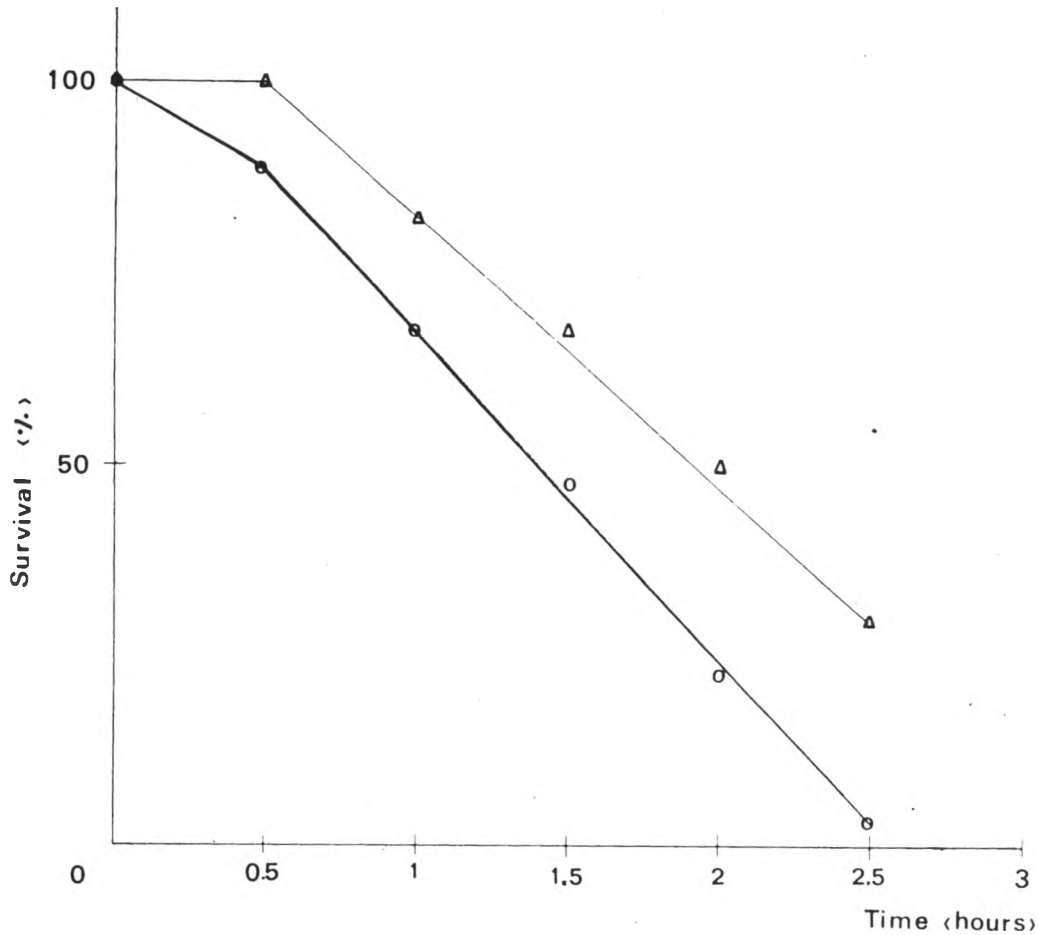

Figure 5. Effect of 0.3% ethylmethane sulfonate on the viability of parental cells at variable time.

(o) , strain No.2

(Δ) , strain No.266

When the parental cells were treated with ethylmethane sulfonate as mutagen at the optimal condition, the rate of induction of genetically stable auxotrophic mutants was examined.

11. Mutants selection from killing curve

To facilitate the genetic study of these parental yeasts, nutritional growth requirement had to be induced in these strains. After mutagenic treatment, cultures were serially diluted and plated on complete medium plates. Auxotrophic mutants were detected by lack of growth on a minimal medium when compared with the complete medium. The percentage of auxotrophic mutants was observed to be 0.23 % and 0.01 % for strain No.2 and No.266 respectively (Table 10). These auxotrophic mutants were screened for specefic nutritional requirements. The mutants might require lysine or histidine or arginine or tryptophane for growth (Table 11). Out of 23 wine mutants, the auxotrophic mutants for lysine, histidine, arginine and tryptophane were 15, 5, 2 and 1 respectively. The wine strain mutants 2-28, lysine auxotroph ; and 2-36, histidine auxotroph were selected to be a wine parental cell for hybridization because they were SO_2 tolerance and capable efficiently to convert sugar to alcohol. For the killer parental strain there were 2 mutants, one required lysine (strain No.266-1) and another one required tryptophane (strain No.266-2). The selected parental strains were dissected and the mating types of their spores were isolated.

Table 10 Recovery mutant from parental strains after mutagenesis with EMS

Strain	Total tested strains	Auxotrophic mutants	Recovery mutant (%)
2	10,038	23	0.23
266	18,880	2	0.01

Table 11 Characters of mutants

A. No.2 : the wine strain

Strain	Characters of wine strain		
	Auxotrophic requirement[a]	Fermentation ability[b]	SO_2 tolerance[c]
2-1	lys	+	±
2-2	lys	+	±
2-3	lys	+	±
2-4	lys	+	±
2-5	lys	+	±
2-6	lys	+	+
2-7	lys	+	±
2-8	his	+	-
2-9	trp	+	±
2-10	lys	+	-
2-11	arg	+	-
2-13	lys	+	±
2-21	his	+	±
2-22	arg	+	±
2-25	lys	+	±
2-27	his	+	±

Table 11 (cont.)

Strain No.	Characters of wine strain: Auxotrophic requirement[a]	Fermentation ability[b]	SO_2 tolerance[c]
2-28	lys	++	+
2-30	lys	+	±
2-34	lys	+	-
2-36	his	+++	+
2-37	lys	+	-
2-38	lys	+	+
2-40	his	+	±

B. No.266 : the killer strain

Strain No.	Characters of killer strain: Auxotrophic requirement[a]	Killing ability[d]
266-1	lys	+++
266-2	trp	++

[a] Symbols for auxotrophic requirement arg (arginine), his (histidine), lys (lysine), trp (tryptophane)

[b] Symbols for fermentation ability which were measured by refractometer

- +++ capable to convert sugar from 24 to 18° Brix
- ++ capable to convert sugar from 24 to 20° Brix
- + capable to convert sugar from 24 to 22° Brix

[c] Symbols for SO_2 tolerance

- + that initiated to ferment within 30 hours.
- ± that initiated to ferment within 30-48 hours.
- - that initiated to ferment over 48 hours.

[d] Symbols for killing ability

- +++ very strong killing ability
- ++ strong killing ability
- + normal killing ability

12. Isolation of spores

The selected mutants, strain No.2-28, 2-36, 266-1 and 266-2 were induced to sporulate after 5 days incubation on sporulation agar. It was observed that strain No. 2 produced spore (11 %) less than strain No.266 (82 %). After sporulation, the asci was split opened by using micromanipulation technique and the spores were seperated. Rupture of selected asci and isolation of spores were carried out on the agar slab secured to the inside of a sterile plate. After incubation, the single-spores were restreaked on complete medium plates and tested for mating type and sporulating ability (Table 12). The spore viability of strain 2-28 was only 12.5 % and the mating type could not be detected. For strain No.2-36, 10 viable spores were found from 57 spores tested and their mating type were 5 of a mating type, 2 of mating type and 3 of non-detectable mating type. On the other hand, spore viability of strain No.266-1 was 100 % and strain No.266-2 was 66.6 %. For mating type it was found that all spores of strain No.266-1 and 266-2 were non-mater because they did not show zygote formation with either a or standard cultures and they were identified as homothallic clones by their ability to sporulate. From these characters, the strain No.266 was designated as homothallic strain while the strain No.2 as the heterothallic.

It was considered that strain No.2-36 was suitable as the original wine yeast for hybridization. And its isolated spores were screened for the desirable properties (Table 13). The suitable spore was 2-36-2.3 which was still SO_2 tolerance which similar to the original, strain No.2-36. On the other hand, both strain No.266-1 and 266-2 were observed from determination of their killing ability that strain

Table 12 Percentage of ascospore-germination of selected auxotrophic mutants and determination of Mating type of single-spore colonies.

Strain No.	Total spore isolated	Viable spore isolated	Spore viability (%)	Mating type			
				a	∝	a/∝	n
2-28 (lys)	24	3	12.5	-	-	-	3
2-36 (his)	57	10	17.5	5	2	-	3
266-1 (lys)	36	36	100	-	-	36	-
266-2 (trp)	18	12	66.6	-	-	12	-

Note The spores in this experiment were isolated from three-single-spore and four-single-spore per ascus of the tested strains.

Table 13 The selected characters of haploid clones.

Strain	Mating type	Auxotrophic requirement	Killing activity[a]	SO_2 tolerance[b]
Haploid of 2-36				
2-36-1.4	a	his	-	-
2-36-2.3	a	his	-	+
2-36-3.2	a	his	-	-
2-36-3.3	α	his	-	±
2-36-6.1	α	his	-	-
2-36-13.1	a	his	-	-
2-36-15.1	a	his	-	-
Haploid of 266-1				
266-1-1.3	a/α	lys	+	-
266-1-1.4	a/α	lys	+	-
266-1-2.3	a/α	lys	+	-
266-1-2.4	a/α	lys	+	-
266-1-3.1	a/α	lys	-	-
266-1-4.3	a/α	lys	+	-
266-1-4.4	a/α	lys	+	-
266-1-5.1	a/α	lys	-	-
266-1-5.4	a/α	lys	+	-
266-1-6.1	a/α	lys	-	-
266-1-6.4	a/α	lys	-	-
266-1-7.3	a/α	lys	-	-
266-1-8.1	a/α	lys	+	-

Table 13 (cont.)

Strain	Mating type	Auxotrophic requirement	Killing activity[a]	SO_2 tolerance[b]
266-1-8.2	a/α	lys	+	-
266-1-8.3	a/α	lys	+	-
266-1-8.4	a/α	lys	+	-
266-1-9.1	a/α	lys	-	-
266-1-9.2	a/α	lys	-	-

a
Symbols show killing activity

+ present

- none

b
Symbols show growth and non-growth in grape juice containing 200 ppm of potassium metabisulfite

+ growth within 30 hours

± growth within 48 hours

- growth over 48 hours or non-growth

No.266-1 had stronger killing activity than No.266-2. Thus strain No.266-1 was selected to be the original killer yeast for hybridization. Unfortunately strain No.266 was homothallic strain, mass mating could not be used in this case. Thus, spore-to-cell mating technique was introduced to construct the killer wine hybrids.

13. Spore-to-cell mating

Spore-to-cell mating was carried out under a microscope with the aid of micromanipulator. Spore-cell hybrids were prepared using the technique developed by Oshima and Takano (1970). A sporulated culture of strain 266-1 was treated with the enzyme Zymolyase -100 T and the four spores of the ten asci were isolated on an agar slab. Haploid cells from a fresh culture of 2-36-2.3 was placed near to each spore of the four tetrads. The viabilities of the crosses of strain No.266-1 and 2-36-2.3 were determined on the slab agar (Table 14). Twelve in fourty crosses gave viable colonies and they were restreaked on minimal medium for selection of desirable hybrids. Hybrids were isolated using the prototrophy recovery method (Fink, 1970). Three in twelve viable crosses were prototrophic hybrids i.e., capable to grow on minimal medium. Three colonies, 266-1-1.2x2-36-2.3, 266-1-2.2x2-36-2.3 and 266-1-3.2x2-36-2.3, were tested for killing ability and SO_2 tolerance (Table 15). All three hybrids were capable of sporulating, eventhough strain No.266-1-1.2x2-36-2.3 sporulated very poorly. The result shown that 266-1-1.2x2-36-2.3 was the only hybrid enable both the killing ability and SO_2 tolerance. It was considered that strain 266-1-1.2x2-36-2.3 contained the desirable properties from both parental strains. Afterward, it would be tested for wine fermentation of grape juice compared with the parental strains, strain No.2 and 266.

Table 14 Appearance of cross combination

Cross combination	Viable colony on slab agar[a]	Growth on minimal medium[b]
266-1-1.1x2-36-2.3	+	-
266-1-1.2x2-36-2.3	+	+
266-1-1.3x2-36-2.3	+	-
266-1-1.4x2-36-2.3	+	-
266-1-2.1x2-36-2.3	+	-
266-1-2.2x2-36-2.3	+	+
266-1-2.3x2-36-2.3	-	n
266-1-2.4x2-36-2.3	-	n
266-1-3.1x2-36-2.3	-	n
266-1-3.2x2-36-2.3	+	+
266-1-3.4x2-36-2.3	-	n
266-1-4.1x2-36-2.3	-	n
266-1-4.2x2-36-2.3	-	n
266-1-4.3x2-36-2.3	-	n
266-1-4.4x2-36-2.3	+	-
266-1-5.1x2-36-2.3	+	-
266-1-5.2x2-36-2.3	+	-
266-1-5.3x2-36-2.3	-	n
266-1-6.1x2-36-2.3	-	n
266-1-6.2x2-36-2.3	-	n
266-1-6.3x2-36-2.3	-	n
266-1-6.4x2-36-2.3	-	n

Table 14 (cont.)

Cross combination	Viable colony on slab agar[a]	Growth on minimal medium[b]
266-1-7.1x2-36-2.3	-	n
266-1-7.2x2-36-2.3	-	n
266-1-7.3x2-36-2.3	+	-
266-1-7.4x2-36-2.3	-	n
266-1-8.1x2-36-2.3	-	n
266-1-8.2x2-36-2.3	-	n
266-1-8.3x2-36-2.3	-	n
266-1-8.4x2-36-2.3	-	n
266-1-9.1x2-36-2.3	-	n
266-1-9.2x2-36-2.3	+	-
266-1-9.3x2-36-2.3	-	n
266-1-9.4x2-36-2.3	-	n
266-1-10.1x2-36-2.3	-	n
266-1-10.2x2-36-2.3	-	n
266-1-10.3x2-36-2.3	-	n
266-1-10.4x2-36-2.3	-	n

[a] symbols show viability

+ viable colony

- nonviable colony

[b] symbols show growth on minimal medium

+ growth

- non-growth

n not determined

Table 15 Characteristics of parental strains and hybrids reviewed from spore-to-cell mating.

Strain	Killing ability	SO_2 tolerance	Sporulation ability (%)
The parental strain			
No.2	-	+	11
No.266	+++	-	82
The selected cross combination			
266-1-1.2x2-36-2.3	++	±	<0.1
266-1-2.2x2-36-2.3	+	-	1.2
266-1-3.2x2-36-2.3	+	-	0.7

[a] Symbols for killing ability

+ normal killing ability

++ strong killing ability

+++ very strong killing ability

[b] Symbols for SO_2 tolerance

+ that initiated to ferment within 30 hours

± that initiated to ferment during 30-48 hours

- that initiated to ferment over 48 hours or non-growth

14. Fermentation test

Fermentation was performed at 20 °c in flasks containing 500 ml of sterile grape juice.Agitation of the content was done during sampling. The fermentation rate of killer hybrid was compared with the parental strains, No.2 and No.266 as shown in Figure 6 and 7. The rate of fermentation of the individual pure yeast cultures was determined. The result shown that the ethanol production rate of the tested strains was not different. Therefore, among the three strains, strain No.2 and the hybrid fermented the grape juice faster than the killer strain No.266. Strain No.2 took about 120 hours to complete the fermentation with 0% reducing sugar, while No.266 was not able to do so but leaving 2.3 % reducing sugar.

Chemical analysis of the finished wines was shown in Table 16. There appears to be no significant differece of wine fermentation by each strain in pH, total acidity and volatile acidity. Fermentation of the grape juice in this experimental work resulted with dry wines, (strain No.266 leavening 2.3 % reducing sugar). Strain No.2 gave the highest amount of ethanol (11.01 % w/v) while the killer strain No.266 gave the lowest (8.71 % w/v) but the hybrid gave the amount between both parental strains. It was able to conclude that the killer strain was improved by genetic manipulation since the fermentation ability of the killer wine hybrid was better than the killer parental strain No.266.

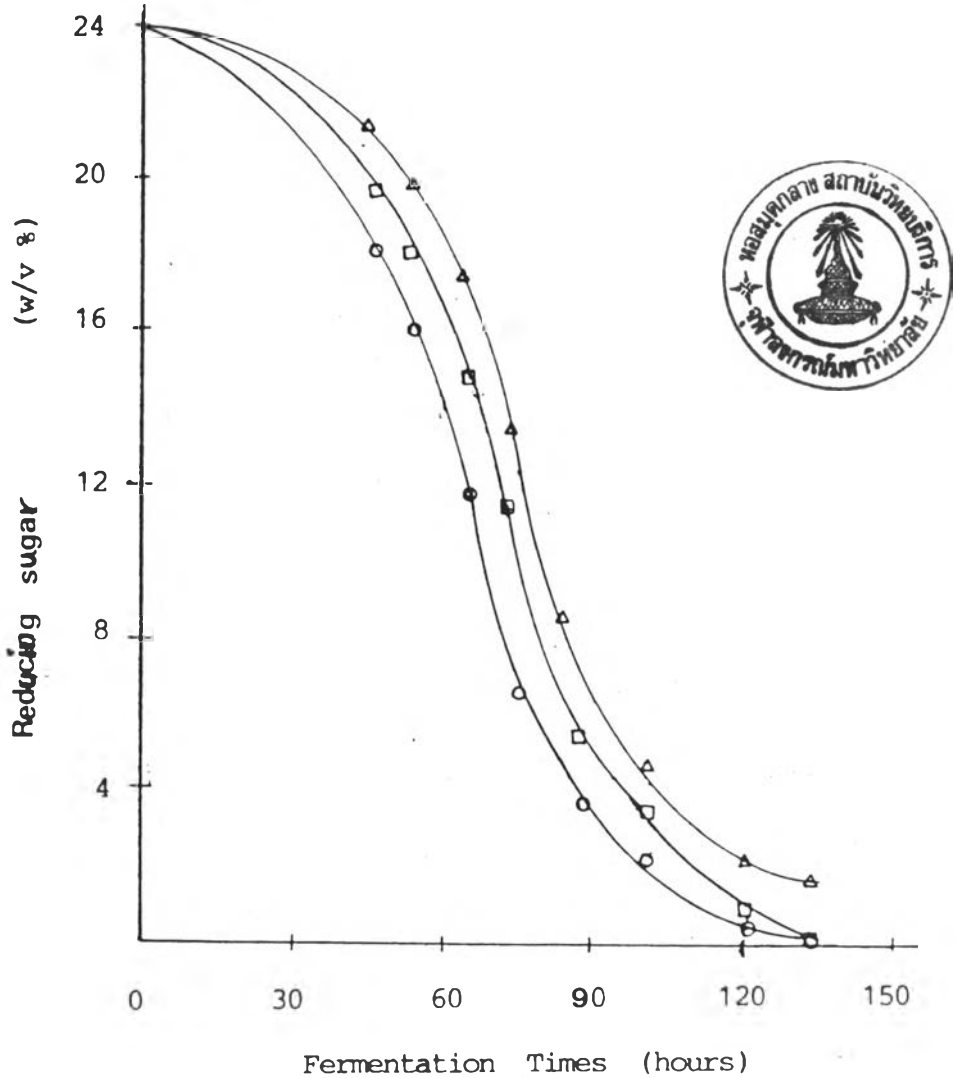

Figure 6 Decrement of reducing sugar in grape juice during fermentation (○), strain No.2; (△), strain No.266; and (□), hybrid 266-1x2-36-2.3-1.2

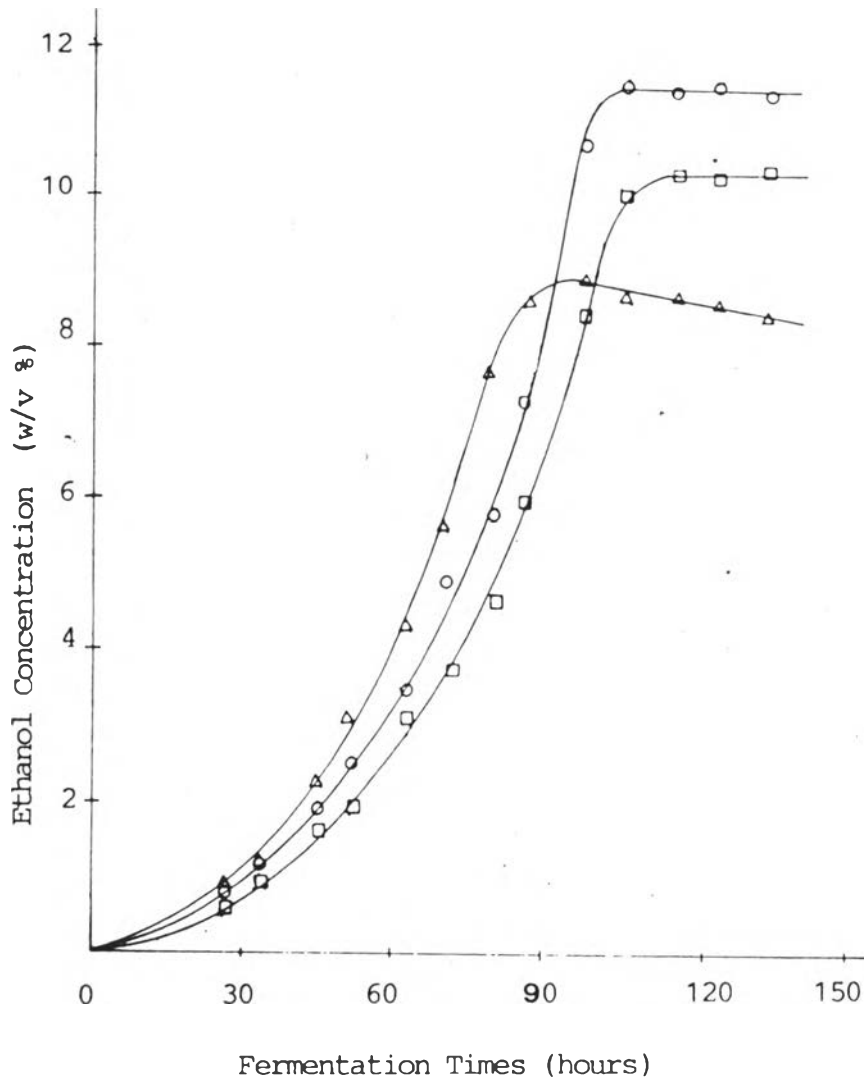

Figure 7 **Concentration of ethanol in grape juice during fermentation (o), strain No.2; (△), strain No.266; and (□), hybrid 266-1x2-36-3.3-1.2**

Table 16 Chemical analysis of the finished wines at 135 hours

Inoculating strain No.	pH	Reducing sugar(%)	Total acidity g/100 ml as tartaric acid	Volatile acidity g/100 ml as acetic acid	Ethanol g/100 ml (% w/v)
2	4.0	0	0.42	0.09	11.01
266	4.3	2.3	0.49	0.07	8.71
266-1-1.2x2-36-2.3	4.1	0	0.43	0.06	10.25