PN-ABA-955

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D.C. 20523

DATE: 9/2/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5, 6 & 8
PKTL

Attachment

5.238

Progress Report No. 4

Hybridization of Halotolerant Yeast for Alcohol Fermentation

A Research Project

USAID/PSTC Program

Grant No.936-5542-G-00-5080-00

Asst. Prof. Dr. Savitree Limtong

Project Leader

Rec'd on SEP 2 1986

Kasetsart University

Bangkok Thailand

Project Profile

Country	: Thailand
Grant No.	: 936-5442-G-00-5080-00
Program	: Program on Science and Technology Cooperation
Project Title	: Hybridization of Halotolerant Yeast for Alcohol Fermentation
Project Leader	: Asst. Prof. Dr. Savitree Limtong
Organization	: Kasetsart University
Co-investigator	: Mr. Wichien Yongmanitchai Mr. Pramote Tammarate Miss Manee Tantirungkit
Project Advisor	: Dr. Jaroon Kumnuanta
Project Consultants	: Prof. Dr. G.R. Fink Prof.Dr. J.B. Hicks
Authorized Officer	: Prof. Dr. Sutharm Areekul, Rector
Total Project Budget	: 150,000 US $
Project Duration	: 3 years, August, 1985 to November, 1988
Reporting Period	: September 1, 1987 to August 1988
Bugget Allocation for This Period	: 860,303.52 Baht

Background/Introduction

In Thailand and other LDCs alcohol distilleries are small and scattered all over the country. It is being considered as one of the few local agro-industries which has economic impact on the community in terms of employment and utilization of local raw materials, since 75-80% of the production cost comes from raw material. From our own investigation and personal communication with local alcohol distilleries in northeastern and southern areas where fresh water is scarce and brackish water is used in the mashing process, fermentation proceeds slowly and efficiency is reduced by about half. This is due to many complications such as the intolerance of the yeast strain employed to high salt concentrations which sodium chloride is included, and the high salt concentrations of brackstrap molasses itself, the combined effects of which are detrimental to fermentation activities. The inhibiting effect on yeast growth enables other microorganisms present in the mash to over grow and retard fermentation by yeast, since the main mash is unpasteurized.

To solve such problem of alcohol distilleries improvement of yeast strain employ in mashing process to be halotolerant yeast is one promising method. Protoplast fusion technique is a novel technique in constructing hybrids of microbial strains currently used in industry. Although the concept and techniques are well known, hybridization of yeasts from the two distinct

characteristics of halotolerance and high ethanol production has not yet been reported.

Protoplast fusion has proved to be a highly efficient procedure for accomplishing genetic recombination in microorganisms, including yeast. Successful protoplast fusion has been reported in many industrial strains of yeast, for example, by Spencer et al. (1980) on Saccharomyces diastaticus, which improved starch hydrolysis ability. Wilson et al. (1982) obtained hybrids from Schwanniomyces alluvious and Saccharomyces spp. which ferment starch directly to ethanol. Morishita and Yamaguchi (1983) fused Torulopsis halonitratophila, a salt-tolerant yeast which required vitamins for growth, with Phototheca zopyfii, salt-sensitive fungus and obtained a hybrid which grew in 1.5 M sodium chloride and did not require vitamins for growth. Seki et al.(1983)obtained high ethanol producing and flocculent hybrids from a flocculent strain of S. cerevisiae isolated in Thailand and an industrial ethanol producing strain from Japan.

Halotolerant yeast, a kind of osmophilic yeast, is usually present in various kinds of fermented food where high concentration of sodium chloride is used in the process, such as soysauce, miso etc. Predominant strains usually found in these products are S. rouxii which can tolerate 20-22% salt concentration (Lee and Lee, 1978). However, the efficiency of alcohol fermentation by these strains is very low; S. rouxii var. halomembranis, for example, produced only 1.9% W/V ethanol in the presence of

18% sodium chloride and 3.2% W/V without salt (Onishi, 1963). Yamagata and Fujita (1970) isolated and identified several strains of halotolerant yeasts from miso and soy mash. They found that the tolerant rang of yeast varied widely between 0-22% sodium chloride. Wickerham and Burton (1960) reported heterothallism in S. rouxii, and various strain of this species are either diploid or haploid with opposite mating types. Heterothallism may be useful for the purpose of mating desirable strains of opposite mating types by conventional method. Mori and Onishi (1967) were successful in breeding two strains of S. rouxii and obtained a diploid hybrid of high salt tolerance. Dhevahuti (1978) investigated S. rouxii in fermenting soy mash and found that this species was involved in soysauce fermentation in Thailand as well. A single mating type was predominant in most of the soysauce factories in Bangkok, while opposite mating type was isolated from only one sample in Uthai-Thani province, north of Bangkok.

A total of 413 strains of halotolerant yeast were isolated from 285 samples of brine fermented products, products of high sugar content, molasses etc. which collected from many provinces in Thailand. Two hundred and sixty one strains were chosen from 690 strains in stock cultures. All 674 halotolerant strains obtained were screened at two level of sodium chloride, 5 and 17%. The temperature used for screening was 40 C. At 5% sodium chloride good ethanol fermenting strains in salted molasses medium were expected to obtain. While at 17% sodium chloride the strains which showed good growth in high concentration

of sodium chloride were selected. At each level of sodium chloride the screening was divided into 3 steps. The results of final screening showed that at 5% sodium chloride strain S013, S037, S065, S090 and 251 were selected. While at 17% podium chloride strain 126, 130, 356 and 370 were obtained.

From 9 strains of high ethanol producing Saccharomyces cerevisiae strain M30, AM12 and Sc90 were selected to be used for hybridization with halotolerant strains.

Each of selected halotolerant strain is intended to hybridized with each of high ethanol producing strain of S. cervisiae using protoplast fusion.

To facilitate the selection of hybrids halotolerant parental strain is induced to be auxotrophic mutant while high ethanol producing parental strain to be used is respiratory deficient mutant. In the last reporting period auxotrophic mutants of halotolerant strains selected at 5% sodium chloride were obtanied by treatment with ethylmethan-sulfonate (EMS). However, no auxotrophic mutant of the strains selected at 17% sodium chloride was able to be constructed by EMS, UV or both EMS and UV. Respiratory deficient mutants of all selected high ethanol producing strains were successfully isolated by treatment cells with ethidium bromide. Few crosses of protoplast fusion were carried out and some hybrid fusants were obtained.

In this reporting period the isolation of auxotrophic mutant of halotolerant strains selected at 17% sodium chloride was repleated by using N-methyl N'-nitro-N-nitrosoguanidin (NTG) instead of EMS, UV or both EMS and UV. All mutants obtained from the last and this reporting periods were tested for their cabability of ethanol production compared with their wild types. This is in order to obtain the mutant which could produce ethanol the same or almost the same amount as produced by their wild type. Hybridization between halotolerant strain and high ethanol producing strain was carried out at this stage 11 crosses were successfully completed The selection of potent fusant from the fusants obtained were carried out.

Works which were carried out at the laboratory of Dr.J.B. Hicks, Department of Molecular Biology, Research Institute of Scripps Clinic, San Diego, California by Dr. Savitree Limtong during her visit will be discussed.

Objectives

Overall objectives

The project is aimed at constructing a new hybrid from two different foundation strains, namely halotolerant strain and an industrial ethanol producing strain, through a protoplast fusion technique. The halotolerant strain of S. rouxii, currently used for fermentation of soysauce, which tolerates salt concentrations over 18% but produces low ethanol concentration (usually less than 5% V/V) will be used as the parental strain. The industrial alcohol producing strain, S. cerevisiae, which can ferment cane molasses at high rate and produce high ethanol concentration will be used as the other parental strain. The hybrid from both strains mentioned will incorporate the characteristics of producing high ethanol concentration in high salinity molasses mash, due to recombination of desirable characteristics from the parental strains. This result is impossible by conventional mating techniques because the parental strains required are from different yeast species, but a protoplast fusion technique can be used successfully for the combining of two different species, as reported by many authors. The performance of new hybrid strains obtained will be tested under laboratory conditions.

It is expected that the selected hybrid will be used for industrial alcohol production, especially for distilleries where brackish water or high salinity well

water is the only available resource. There will be no doubt that the new hybrid can be used in other LCDs under similar environmental conditions. Furthermore, the new hybrid will be useful for fuel alcohol production from cellulose based feed stock using an acid treatment process which also generate high salinity mash and requires a special yeast strain for that purpose.

Objectives of this reporting period

1. To construct the selective (genetic) marker of the remaining parental strains which could not construct in the last reporting period.

2. To construct new hybrids by hybridization high ethanol producing strain with halotolerant strain by mean of protoplast fusion.

3. To select the potent fusants which can produce higher ethanol concentration than their parental strains in high salinity molasses mash at 40 C.

Materials and Methods

1. Isolation of auxotrophic mutant

Cells were mutagenized by treatment with N-methy, N'-nitro-N-nitrosoguanidine (NTG) following the method recommended by Fink (1986) with variation on NTG concentration.

2. Isolation of respiratory deficient (RD) mutant

Cells were treated with ethidium bromide following the method described by Seki et al. (1983).

3. Protoplast fusion

Protoplast was prepared using enzyme Zymolyase (5,000 or 20,000 U) following the method of Arima and Takano (1979). Protoplast fusion was performed in polyethylene glycol(PEG) and calcium ion as the method described by Seki et al. (1983).

4. Selection for the potent fusant

Selection for the potent hybrid was carried out many steps at 40 C. At first fusants picked from selective regeneration medium (minimal medium containing glycerol as sole source of carbon) were isotaled into pure cultures. The purified fusants were tested for their

capability to grow on molasses agar (molasses 5% sucrose equivalent sugar concentration, 0.05% ammonium sulfate and 1.5% agar) containing 0 and 1.5% sodium chloride at 40 C. Growth of the fusants which could grow well on 1.5% sodium chloride molasses agar at 40 C were investigated on 3% sodium chloride molasses agar at 37 and 40 C. The fusants which could grow on this medium were selected to be investigated for ethonol fermentation in 3% sodium chloride molasses fermentation medium (molasses 15% sucrose equivalent, 0.05% ammonium sulfate and 3% sodium chloride) at 40 C both by standing tube and shaking flask cultivations.

5. Analysis

Ethanol concentration was determined by using Gasliquid Chromatograph (Shimadzu GC-9A) with propanol as internal standard. Cell concentration was measured as optical density of cell suspension at 660 nm after washing with 0.1 N HCl by Spectrophotometer (Shimadzu UV-240). Sugar concentration was determined by phenol sulfuric acid method and High Performance Liquid Chromatograph (HPLC).

6. Works had been done at Research Institute of Scripps Clinic

6.1 Investigation of growth on YPD agar containing various concentration of sodium chloride

Growth of all selected yeasts to be used for protoplast fusion were investigated after 3 days cultivation at 30 C on YPD medium(yeast extract 1%, peptone 1%, dextrose 2% and agar 1.5%) containing various concentration of sodium chloride, 3-17%.

6.2 Chromosome separation

Chromosome of the selected strains were separated by orthogonal field-alteration electrophoresis (OFAGE) following the method dercribed in Experiment XIV "Chromosome separation" in, Manual for a course "Methods in Yeast Genetics" of Cold Spring Harbor Laboratory. (Fink et al. 1986).

6.3 DNA preparation

DNA of all selected strains were prepared using the method of "Yeast DNA mini-preparation (40 ml) described in Manual for a course "Method in Yeast Genetics" of Cold Spring Harbor Laboratory (Fink et al. 1986) with some modification. The DNA was investigated by mini-gel electrophoresis using 1 x TBE buffer at 96-100 V for 30 minutes.

6.4 Transformation of plasmid BT131 into high ethanol producing strains.

a) Growth of high ethanol producing strains on minimal medium containing 3 mg/l of sulfometuron methyl(SM), a herbicide. This medium will be used as selective medium for transformation.

b) Lithium transformation of plasmid BT 131 into Saccharomyces cerevisiae M 30, AM12 and Sc90 were performed by the method recommended by Dr. Hicks and his colleague as follow. Inoculated 2 ml of a saturated overnight culture into 100 ml YPD and grew shaking at 30 C for four hours. Pelletted cells in two 50 ml polypropylene tubes in centrifuged at 2,200 rpm for 5 minutes. Poured off supernatant. Resuspended cells in a total of 10 ml of TE (pH 7.5) and combined in one tube. Spun at 2,200 rpm for 5 minutes. Poured off supernatant. Resuspended in 5 ml of 0.1 M LiOAc in TE (pH 7.5). Shaked gently at 30 C for 30 minutes. Spun at 2,200 rpm for 5 minutes. Be careful when pouring off this supernatant since some strains form a slippery pellet at this stage. If necessary the cells can be spun for a longer time to form a better pellet. Resuspended in 1.0 ml of TE (pH 7.5). Transferred 0.1 ml aliquots into microfuge tubes containing 5 ul of 10 mg/ml sonicated salmon sperm DNA and 5 to 20 ug of plasmid DNA in a maximum volume of 10 ul. Remember to do a no plasmid DNA control. Incubated on ice for 30 minutes. Then transferred to 37 C water bath for 5 minutes. Vortexed briefly to resuspended cells and

add 1 ml of 40% PEG (average MW 8,000), 0.1 M LiOAc, TE (pH 7.5). Mixed by inverting the tube several times. Inucbated on ice for 30 minutes, then at 30 C for 30 minutes. Spun in microfuge at 10,000 rpm for 15 seconds. Resuspended in 200 ul of SOS and spreaded on two selective plates (Minimal medium containing SM).

SOS (to make 20 ml) : 10 ml 2 M sorbitol, 6.7 ml YPD, 3.2 ml H_2O, 13 ml 1 M $CaCl_2$

PEG : Dissolved 22 g of PEG (average MW 8,000) in 31 ml of H_2O and autoclaved. Measured volume of this solution needed for your experiment and added one-tenth volume of sterile 1 M LiOAC, 100 mM Tris-HCL (pH 7.5), 10 mM EDTA (pH 8.0).

Results and Discussion

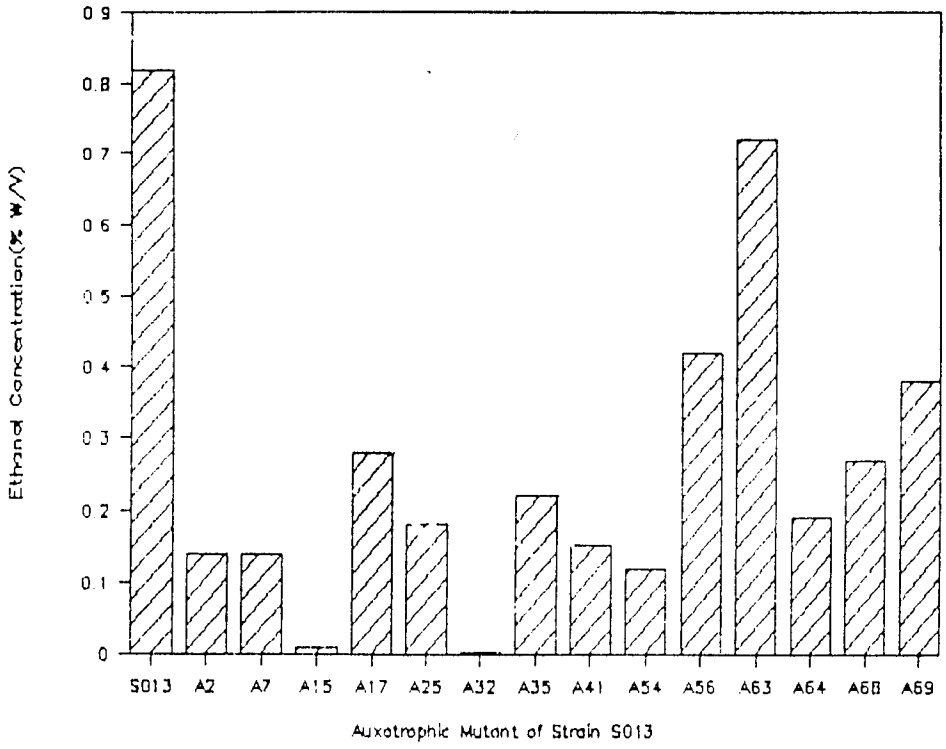
1. Isolation of auxotrophic and respiratory deficient mutants and selection for the potent mutants to be used as parental strains for protoplast fusion.

In the last reporting period auxotrophic mutagenesis of halotolerant strains were carried out by traetment with EMS. Unfortunately, no auxotrophic mutant of the strains selected at 17% sodium chloride, strain 126, 130, 356 and 370 was obtained. However, in the late of last reporting period auxotrophic mutagenesis was repeated but mutagen used was changed to NTG. The result obtained in this reporting period showed that only 2 auxotrophic mutants of strain 126 and 1 mutant of strain 356 were able to be isolated when NTG 100 mg/ml was used for 20 minutes. No mutant was obtained from strain 130 and 370 even cells were treated with the same concentration of NTG for the same period. Isolation of auxotrophic mutant of strain 130 and 370 were repeated many times, however, no mutant was obtained.

All auxotrophic mutants obtained in the last and this reporting periods were investigated for ethanol production compared with their wild type cultures in order to select the strain which had no or little change in ethanol fermentation capability from wild type.

Ethanol fermentation of auxotrophic mutants of strain S013, S037, S065, S090 and 251 were carried out in

molasses fermentation medium containing 5% sodium chloride (molasses 20% sucrose equivalent,0.05% ammonium sulfate and 5% sodium chloride), at 40 C by standing tube cultivation. The result of ethanol fermentation by auxotrophic mutants of strain S013 was shown in Figure 1. Among 42 mutants obtained 15 mutants could not produce ethanol while the other mutants could. Among the mutants which could produce ethanol mutant S013-A63 produced highest ethanol concentration, 0.72% W/V. Therefore mutant S013-A63 was selected to be used for protoplast fusion. From the total of 23 mutants of strain S037, 7 mutants could not produce ethanol in this condition. The other mutants produced 0.1-0.79% W/V while their wild type produced 0.64% W/V (Figure 2). Mutant S037-A73 which produced 0.79% W/V was selected. A total of 24 mutants of strain S065 were tested for ethanol fermentation in 5% sodium chloride molasses fermentation mediumm using standing tube cultivation, at 40 C. The result showed that 5 mutants could not produced ethanol. The other mutants produced 0.05-0.39% W/V of ethanol while wild type produced 0.32% W/V (Figure 3). From the result mutant S065-A25 and S065-A44 which produced 0.39% W/V were selected. Three auxotrophic mutants of S090 were tested for ethanol production in 5% sodium chloride molasses fermentation medium. The result was shown in Figure 4. Mutant S090-A1 and S090-A3 were selected since they produced same amout of ethanol as their wild type, 0.80% W/V. Ethanol production of 7 mutants obtained from strain 251 were investigated in 3% sodium chloride molasses fermentation medium using shaking flask cultivation at 40 C for 3 days. Mutant 251-A17 produced highest ethanol concentration, 1.92%



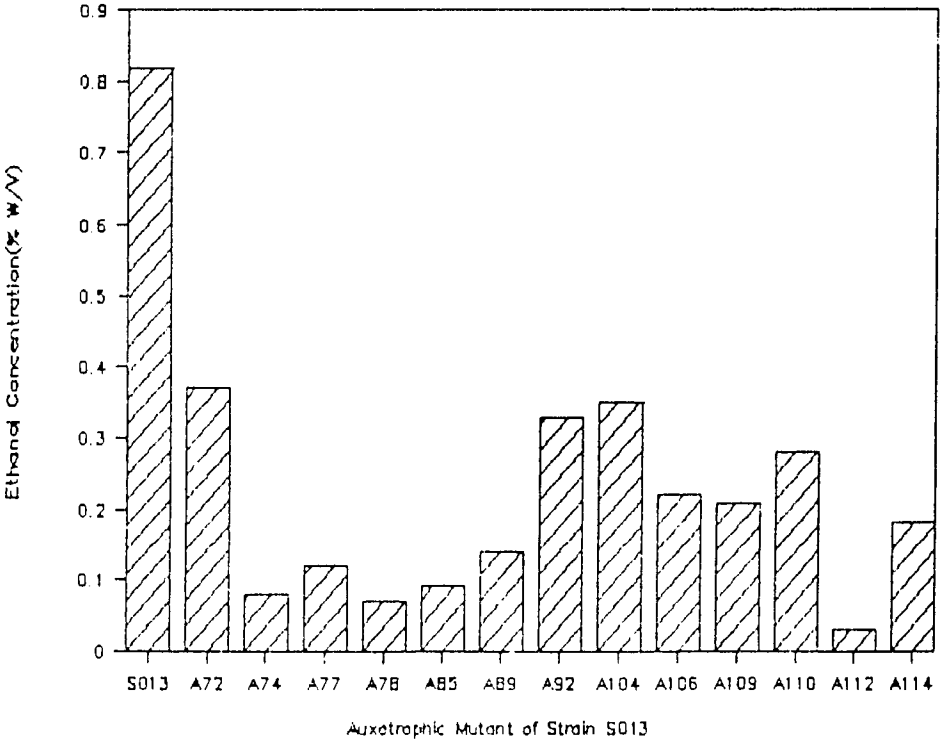


Figure 1 Ethanol fermentation by auxotrophic mutants of strain S013 in 5% NaCl molasses fermentation medium, at 40 C for 4 days.

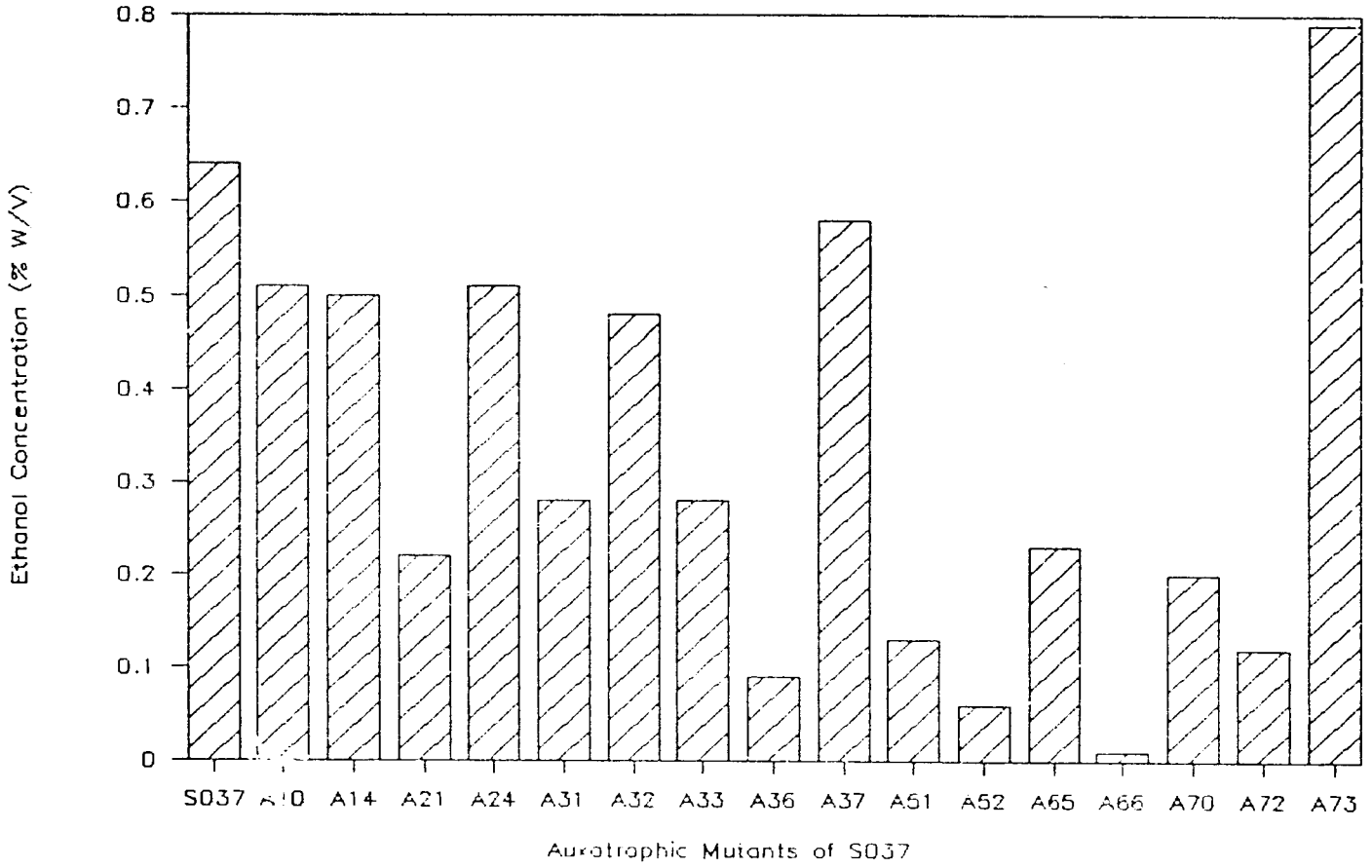


Figure 2 Ethanol fermentation by auxotrophic mutants of strain S037 in 5% NaCl molasses fermentation medium, at 40 C for 4 days.

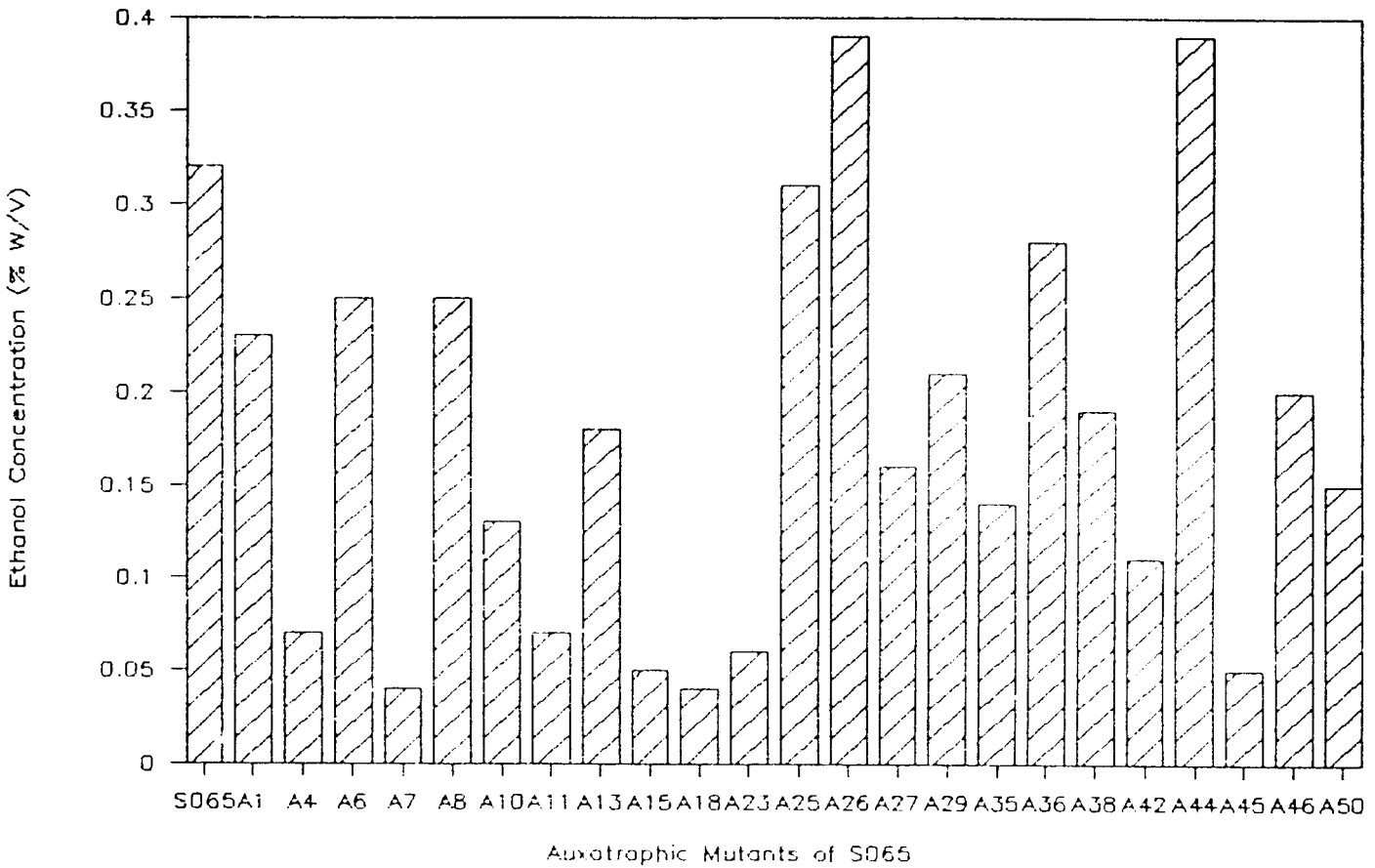


Figure 3 Ethanol fermentation by auxotrophic mutants of strain S065 in 5% NaCl molasses fermentation medium, at 40 C for 4 days.

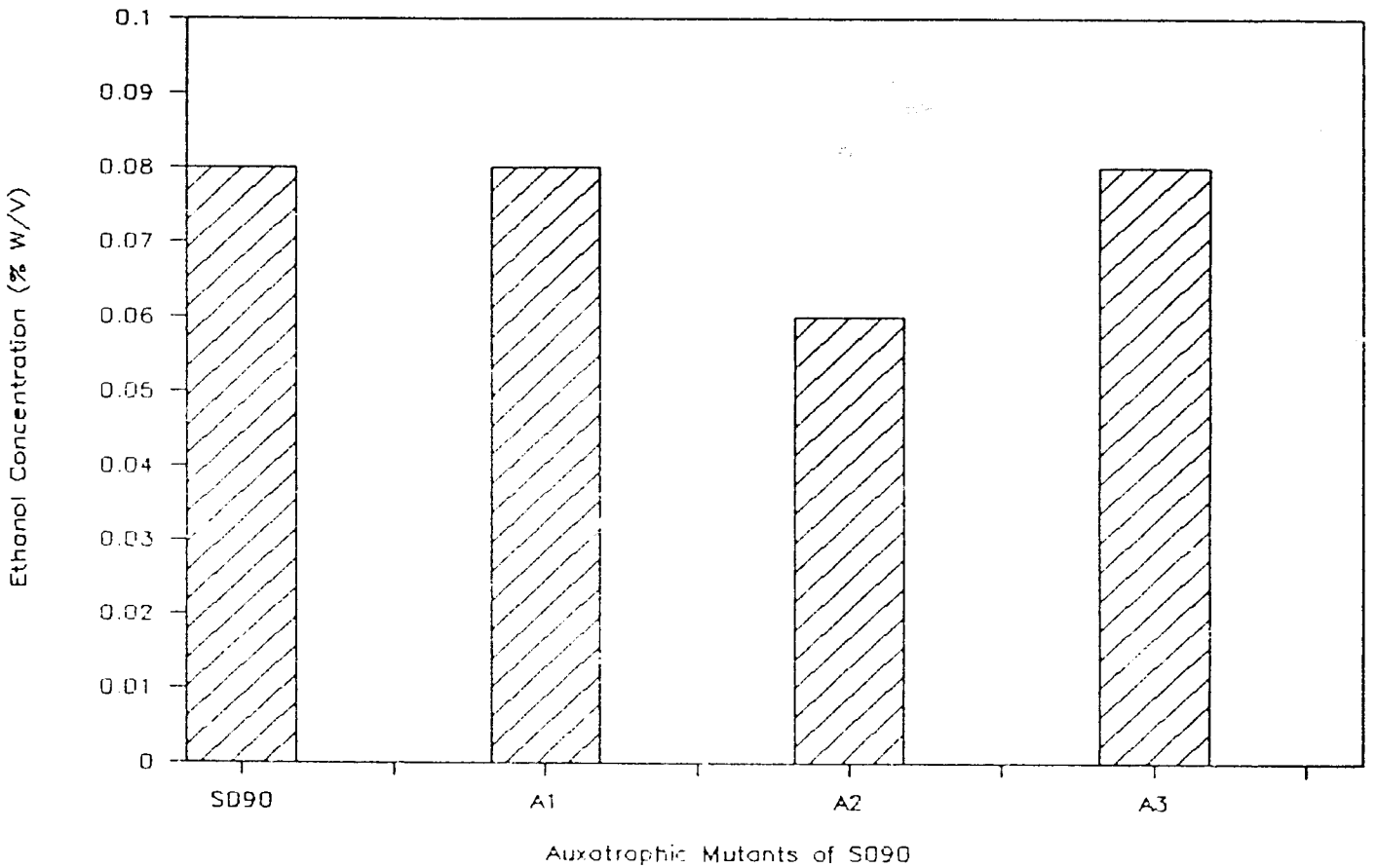


Figure 4 Ethanol fermentation by auxotrophic mutants of strain S090 in 5% NaCl molasses fermentation medium, at 40 C for 4 days.

W/V, and 251-A5 produced 1.84% W/V while wild type produced 2.03% W/V (Figure 5). These two mutants were selected.

Auxotrophic mutants of strain 126 and 356 were investigated for their growth on molasses agar at 40 C. The result showed that both mutants of strain 126 could not grow on molasses agar while a mutant of strani 356 was able tc grow. Therefore the isolation of auxotrophic mutant of strain 126 has to be repeated same as strain 130 and 370. Table 1 summarized the auxotrophic mutants to be used as halotolerant parental strain for protoplast fusion.

Respiratory deficient (RD) mutants of high ethanol producing, strains M30, AM12 and Sc90 obtained in the last reporting period were investigated for ethanol production compared with their wild type strains. The ethanol fermentation was carried out in molasses fermentation broth (molasses 20% sucrose equivalent and 0.05% ammonium sulfate) using standing tube cultivation at 40 C for 2 days. The result showed that all RD mutants of M30 produced very low ethanol concentration when compared with their wild type. Therefore the isolation of RD mutant of M30 was repeated. Totally 218 mutants were picked and tested for ethanol production. Two mutants, M30-RD11 and M30-RD108, which could produce a little lower ethanol concentration were selected to be used for fusion (Figure 6). Ethanol fermentation of 12 respiratory deficient mutants of strain AM12 were carried out and the result was shown in Figure 7. Mutant AM12-RD67 was selected. One RD mutant of Sc90-RD1, obtained was also tested for ethanol production and the result showed that it

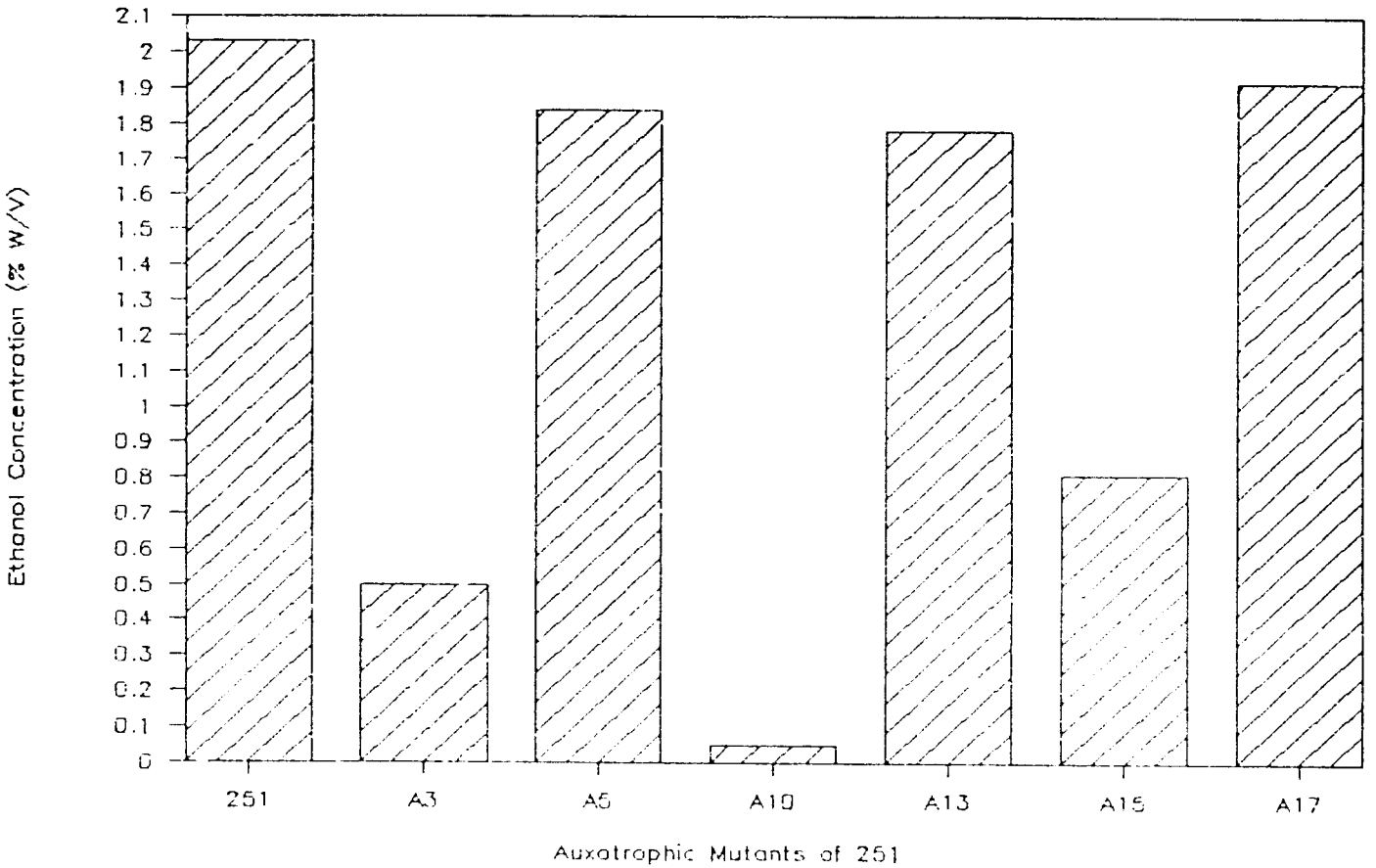


Figure 5 Ethanol fermentation by auxotrophic mutants of strain 251 in 5% NaCl molasses fermentation medium using shaking flask cultivation, at 40 C for 3 days.

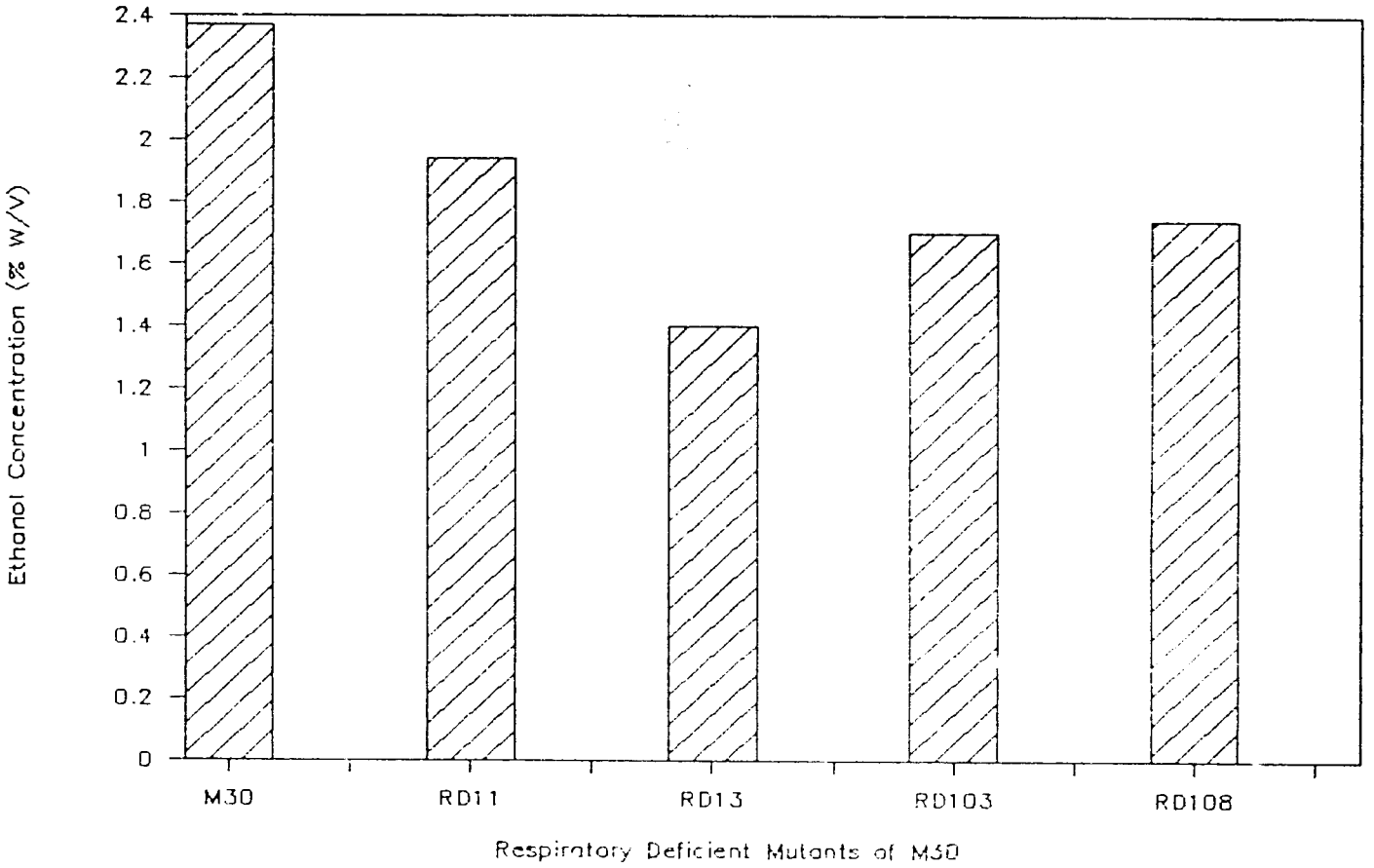


Figure 6 Ethanol fermentation by some respiratory deficient mutants of *S. cerevisiae* M30 in molasses fermentation medium, at 40 C.

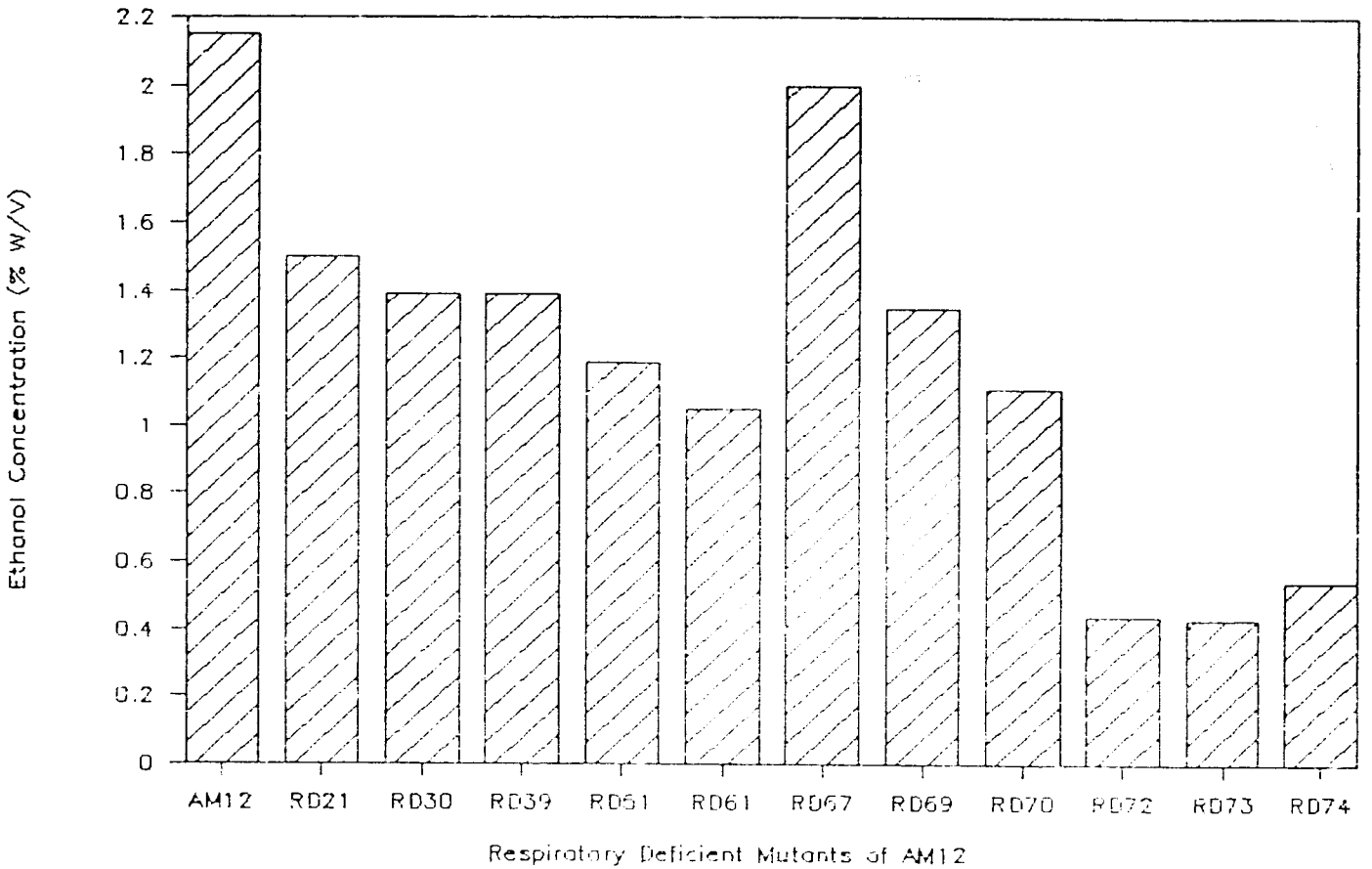


Figure 7 Ethanol fermentation by some respiratory deficient mutants of S. cerevisiae AM12 in molasses fermentation medium, at 40 C.

Table 1 Summarization of the selected auxotrophic mutants to be used as halotolerant parental strain for protoplast fusion.

Wild type	Auxotrophic mutant
Strain selected at 5% NaCl	
S013	S013 - A63
S037	S037 - A79
S065	S065 - A25
	S065 - A44
S090	S090 - A1
	S090 - A3
251	251 - A17
	251 - A5
Strain selected at 17% NaCl	
356	356 - A1

produced ethanol 0.41% W/V while its wild type produced 0.51% W/V. Therefore this mutant was selected to be used in hybridization. Table 2 summarized the respiratory deficient mutants to be used as high ethanol producing parental strain.

The ethanol production of all mutants and their wild types were found to be very low. This is might due to high sugar concentration of the fermentation medium and unflavorable culture conditions such as very high temperature. However, this investigation was performed only to compare the ethanol producing capability of the mutants with their wild type cultures.

2. Protoplast fusion and the selection for potent fusant.

2.1 Protoplast fusion of S.cerevisiae AM12 and strain S013 and the selection for potent fusant.

(1) First batch Protoplast fusion was performed between S. cerevisiae AM12 and S013 and 95 fusants were collected. All fusants obtained were cultivated on molasses agar (molasses 5% sucrose equivalent, 0.05% ammonium sulfate and 1.5% agar) without and with 1.5% NaCl, at 40 C. Growth on salted molasses agar was investigate everyday for 5 days. The result showed that 3 fusants could grow well at 5 days of cultivation (Table 3). These 3 fusants were investigated for growth on 3% sodium chloride molasses agar at 37 and 40C and only one fusant, 6E2 could grow on this medium at both temperatures (Table 4). The ethanol fermentation of fusant

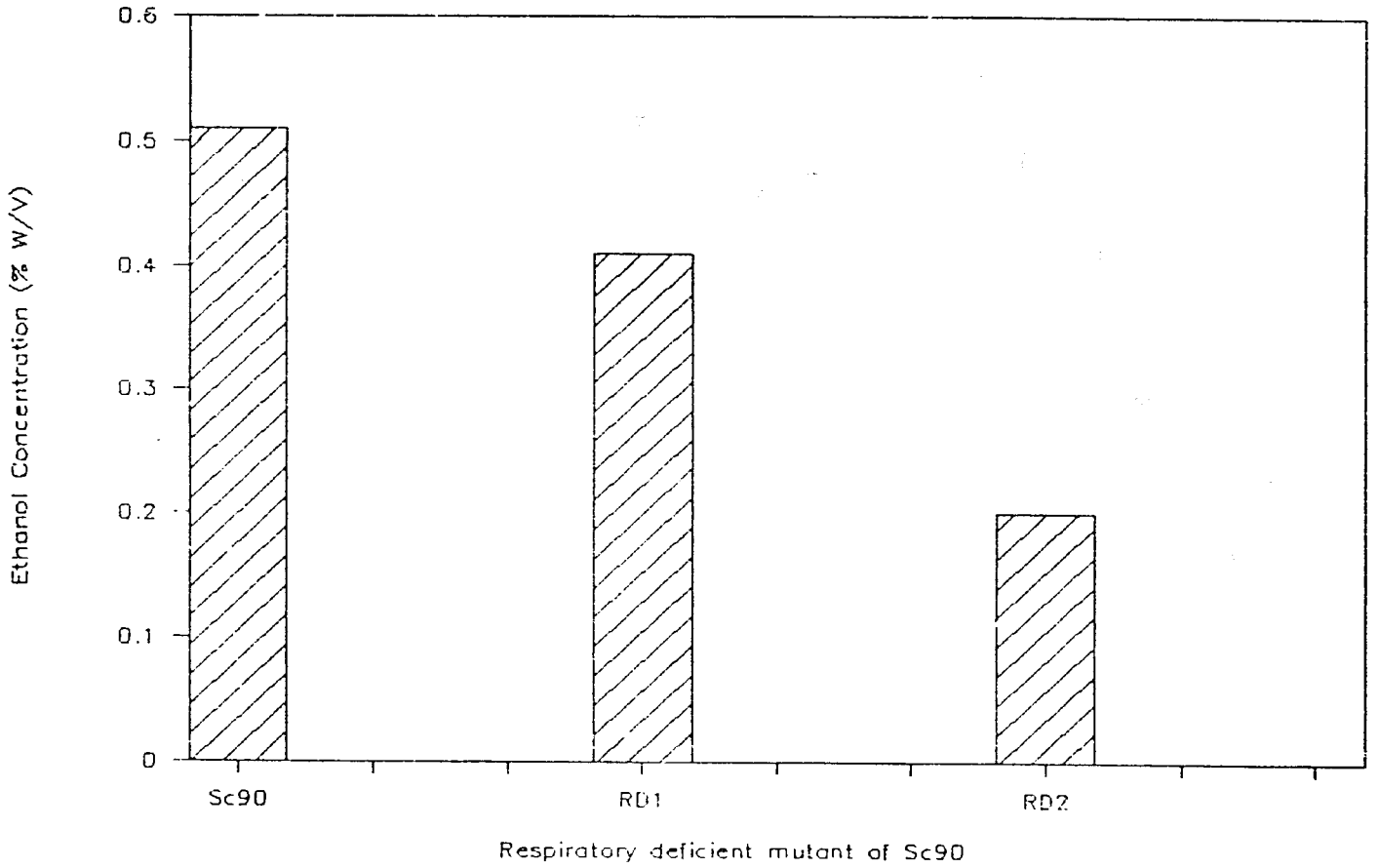


Figure 8 Ethanol fermentation by some respiratory deficient mutants of *S. cerevisiae* Sc90 in molasses fermentation medium, at 40 C.

Table 2 Summarization of the selected respiratory deficient mutants to be used as high ethanol producing strain for protoplast fusion.

Wild type	RD. mutant
S. cerevisiae M30	M30 - RD11
	M30 - RD108
S. cerevisiae AM12	AM12 - RD67
S. cerevisiae Sc90	Sc90 - RD1

Table 3 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* AM12 and S013 on molasses agar containing 0 and 1.5% NaCl at 40 C.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl					
First batch	95	4	4	1	2
Second batch	104	13	9	9	9
1.5% NaCl					
First batch	95	4	4	1	3
Second batch	104	30	31	7	8

Table 4 Growth of fusants obtained from the cross of *S. cerevisiae* AM12 and strain S013 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae AM12	+	+	++	-	+	+
strain S013	+	+	+	-	+	+
fusant 6E2	++	++	+++	++	+++	+++
6E4	-	-	-	-	-	-
6E5	-	-	-	-	-	-

6E2 was tested in 3% sodium chloride molasses fermentation medium (molasses 15% sucrose equivalent, 0.05% ammonium sulfate and 3% sodium chloride) using standing tube cultivation at 40 C for 4 days. The result was shown in Table 5, that fusant 6E2 grew and produced ethanol lower than both parental strains.

(2) Second batch Another batch of protoplast fusion between S. cerevisiae AM12 and S013 was performed and 104 fusants were obtained. Among these fusants only 8 fusants showed good growth on 1.5% sodium chloride molasses agar at 40 C(Table 3).These 8 fusants were selected and investigated for growth on 3% sodium chloride molasses agar at 37 and 40 C. The result indicated that all selected fusant could grow on this medium at both temperatures (Table 6). However, only 4 fusants 7E94, 7E140, 7E164 and 7E217 grew better than their parental strains. These 4 fusants were selected for further investigation.

Ethanol fermentation of these selected fusants were investigated and the result was shown in Table 7. All selected fusants produced very low ethanol concentration when compared with their parental strains.

2.2 Protoplast fusion of S. cerveisiae AM12 and strain S037 and the selection for potent fusant.

S. cerevisiae AM12 was hybridized with S037 using protoplast fusion techniqne and only 3 fusants could be constructed. When they were tested for their growth on

Table 5 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* AM12 and strain S013 in 3% NaCl molasses fermentation medium using standing tube cultivation, at 40 C for 4 days.

Strain	Ethanol % W/V	Growth OD 660 nm
S. cerevisiae AM12	3.09	1.9
strain S013	2.21	1.4
fusant 6E2	1.05	0.9

Table 6 Growth of fusants obtained from the cross of *S. cerevisiae* AM12 and S013 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae AM12	-	+	+	+	++	++
strain S013	-	-	+	+	+	+
fusant 7E94	++	+++	+++	++	++	+++
7E110	++	+++	+++	+	+	++
7E111	++	+++	+++	+	+	++
7E114	++	+++	+++	+	++	++
7E115	++	+++	+++	+	++	++
7E140	++	+++	+++	++	+++	+++
7E164	++	+++	+++	++	+++	+++
7E217	+	++	++	++	+++	+++

Table 7 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* AM12 and strain S013 in 3% NaCl molasses fermentation medium using shaking flask cultivation, at 40 C for 3 days.

Strain	Ethanol % W/V	Growth OD660 nm
S. cerevisiae AM12	4.41	3.3
strain S013	1.76	8.5
fusant 7E94	0.16	0.7
7E140	0.20	0.9
7E164	0.12	0.6
7E217	0.18	0.8

molasses agar without and with 1.5% sodium chloride at 40 C all fusants were able to grow and 2 fusants could grow well (Table 8). Also, on 3% sodium chloride molasses agar both fusants grew better than their parental strains (Table 9).

Ethanol fermentation of both fusants in 3% sodium chloride molasses fermentation medium was investigated. The result showed that fusant B3 produced ethanol (4.69% W/V) a little higher than its parent AM12 (4.41% W/V) while another fusant, B4, produced almost the same amount (4.46% W/V) Table 10. Therefore these two fusant,B3 and B4 were further tested for ethanol production in the same medium but used shaking flask cultivation, at 40 C. From the result fusant B3 and B4 produced maximum ethanol concentration at 60h, 5.63 and 5.69% W/V, respectively. While maximum ethanol concentration produced by AM12 and S037, the parental strains, were obtained at 48h, 5.30 and 3.69% W/V, respectively (Figure 9). However at 48 h both fusants produced ethanol more than their parental strains .

2.3 Protoplast fusion of S. cerevisiae AM12 and strain S065 and the selection for potent fusant.

S. cerevisiae AM12 was fused with S065. A total of 594 fusants were obtained. Their growth were investigated on 3% sodium chloride molasses agar at 37 and 40 C. The result showed that 587 fusants could grow at both 37 and 40 C and 552 fusants grew well (++++ and +++) at 40 C (Table 11). The 552 fusants grew well at 40 C were selected and investigated for their growth on 3% sodium chloride molasses agar

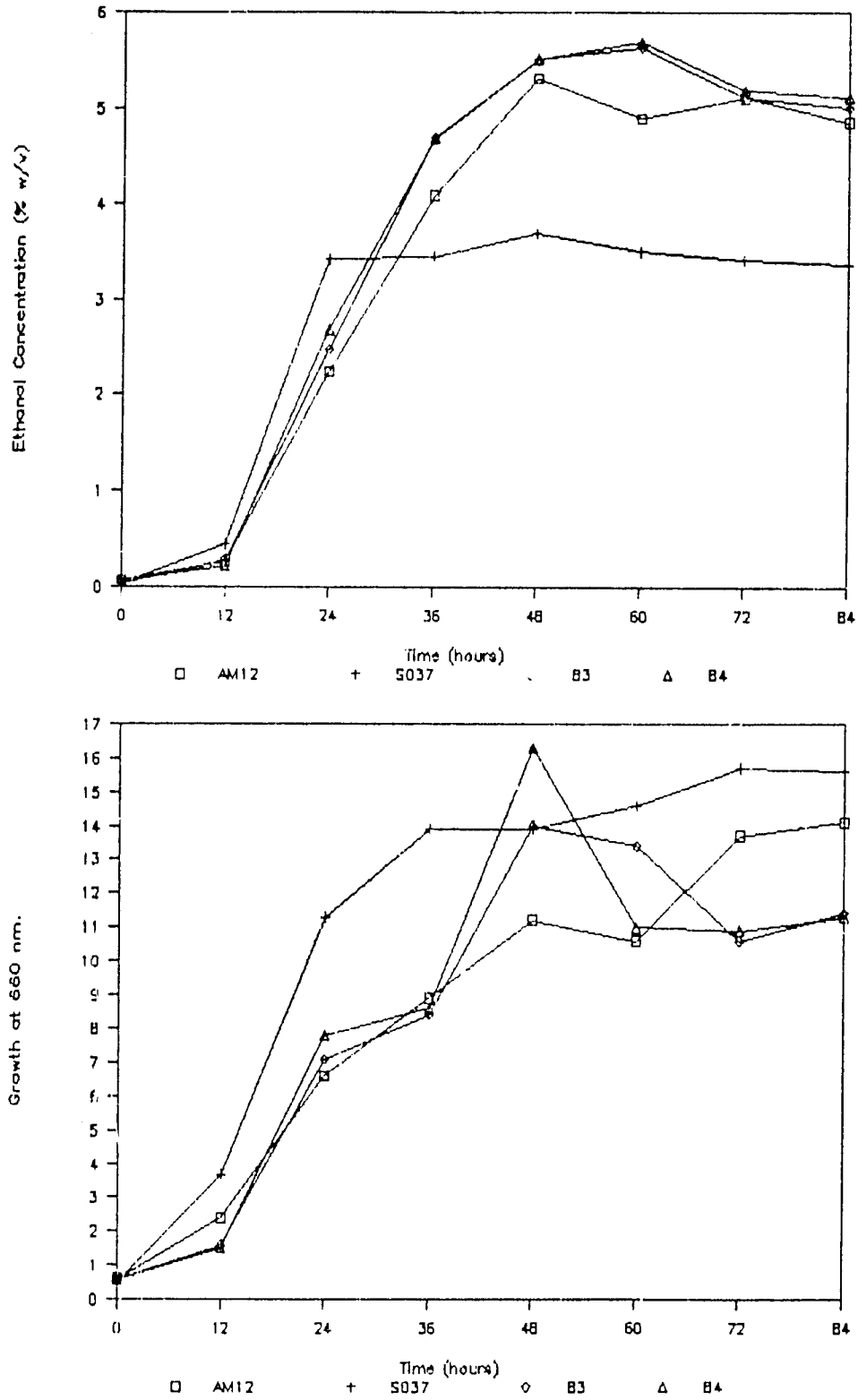


Figure 9 Time-course of ethanol production and growth by fusant B3 and B4 obtained from the cross of S. cerev ae AM12 and strain S037 in 3% NaCl molasses medium using shaking flask cultivation, at 40 C.

Table 8 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* AM12 and S037 on molasses agar containing 0 and 1.5% NaCl at 40 C.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl	3	3	3	0	0
1.5% NaCl	3	3	3	2	2

Table 9 Growth of fusants obtained from the cross of *S. cerevisiae* AM12 and S037 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae AM12	-	+	+	+	++	++
strain S037	-	+	+	+	+	++
fusant B3	++	+++	+++	++	+++	+++
B4	++	+++	+++	++	+++	+++

Table 10 Ethanol fermentation and growth by selected fusants obtained from thhe cross of *S. cerevisiae* AM12 and strain S013 in 3% NaCl molasses fummentation medium using shaking flask cultivation, at 40 C for 3 days.

Strain	Ethanol % W/V	Growth OD660
S. cerevisiae AM12	4.41	3.3
strain S037	1.57	9.5
fusant B3	4.69	2.8
B4	4.46	3.6

Table 11 Growth of fusants obtained from the cross of *S. cerevisiae* AM12 and S065 on 3% NaCl molasses agar.

Growth	Number of fusant	
	At 37 C	At 40 C
Growth ++++	166	105
Growth +++	313	447
Growth ++	95	33
Growth +	13	2
No growth -	7	7
Total	594	594

containing molasses 15% sucrose equivalent, at 40 C. Before this investigation some fusants were segregated while 500 fusants remained stable. Therefore only 500 fusants were tested and the result indicated that 327 fusants grew well (+++) on this medium at 40 C (Table 12). These 327 fusants were further investigated.

Ethanol production by the selected fusants were investigated in 3% sodium chloride molasses fermentation medium using standing tube cultivation, at 40 C. Unfortunately, all fusants produced very low ethanol concentration, less than 0.2% W/V, especially when compared with their parental strains, AM12 and S065 (3.04 and 1.36% W/V, respectively). Therefore no further investigated had been carried out.

2.4 Protoplast fusion of S. cerevisiae AM12 and strain S090 and the selection for potent fusant.

S. cerveisiae AM12 was fused with S090 and 35 fusants were obtained after recomfirmed. Among 35 fusants only 4 fusants showed good growth on 1.5% sodium chloride molasses agar at 40 C (Table 13). Also, all fusants could grow on 3% sodium chloride molasses agar at 37 and 40 C but only one fusant, 7D4, grew better than its parental strains at both temperatures (Table 14).

When fusant 7D4 was investigated for ethanol production at 40 C in 3% sodium chloride molasses fermentation medium the result showed that it produced very

Table 12 Growth of selecte fusants obtained from the cross of *S. cerevisiae* AM12 and S065 on 3% NaCl molasses agar containing molasses as 15% sucrose equivalent.

Growth	Number of fusant	
	At 37 C	At 40 C
Growth ++++	0	0
Growth +++	483	327
Growth ++	9	36
Growth +	8	128
No growth -	0	9
Total	500	500

Table 13 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* AM12 and S090 on molasses agar containing 0 and 1.5% NaCl at 40 C.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl	35	7	7	4	4
1.5% NaCl	35	17	21	4	4

Table 14 Growth of fusants obtained from the cross of *S. cerevisiae* AM12 and S090 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae AM12	-	+	++	+	++	++
strain S090	-	-	+	-	-	+
fusant 7D4	++	++	+++	++	++	+++
7D17	++	+++	+++	+	+	++
7D20	++	+++	+++	+	+	++
7D30	++	+++	+++	+	+	++

low ethanol concentration when compared with its parental strains (Table 15).

2.5 Protoplast fusion of S. cerevisiae AM12 and strain 251.

Protoplast fusion was performed between S. cerevisiae AM12 and 251. A total 12 fusants were obtained. Unfortunately, all fusants could not grow on molasses agar without and with 1.5% sodium chloride at 40 C (Table 16). Therefore they were not be further investigated.

2.6 Protoplast fusion of S. cerevisiae AM12 and 356 and the selection for potent fusant.

Protoplast fusion between S. cerevisiae AM12 and 356 was performed. Totally 38 fusants were obtained. They were tested for growth on 1.5and 3% sodium chloride molasses agar at 37 and 40 C. The result showed that all fusants could grow at both temperatures on salted molasses agar (1.5 and 3% sodium chloride) in Table 17. Therefore all of them were investigated for ethanol production.

Ethanol production of these 38 fusants were investigated in 3% sodium chloride molasses fermentation medium at 40 C. The result showed that no fusant could produced ethanol higher than both parental strains, AM12 and 356, which produced 3.04 and 1.90% W/V, repectively (Table18)

Table 15 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* AM12 and strain S090 in 3% NaCl molasses fermentation medium using shaking flask cultivation, at 40 C for 3 days.

Strain	Ethanol % W/V	Growth OD660 nm
S. cerevsiae AM12	4.41	3.3
strain S090	4.89	7.1
fusant 7D4	0.18	1.0

Table 16 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* AM12 and 251 on molasses agar containing 0 and 1.5% NaCl at 40 C.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl	12	0	0	0	0
1.5% NaCl	12	0	0	0	0

Table 17 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* AM12 and 356 on molasses agar containing 1.5 and 3.0 % NaCl.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
1.5% NaCl 37 C	38	38	38	0	31
40 C	38	34	37	0	32
3.0% NaCl 37 C	38	38	38	0	36
40 C	38	32	37	0	23

Table 18 Ethanol fermentation by selected fusants obtained from the cross of *S. cerevisiae* AM12 and strain 356 in 3% NaCl molasses fermentation medium using standing cultivation, at 40 C for days.

Strain	Ethanol % W/V
S.cerevisiae AM12	3.04
strain 356	1. 9
Fusant 35AM1	1.45
35AM2	1.51
35AM3	1.48
35AM4	1.49
35AM5	1.51
35AM6	NT
35AM7	1.52
35AM8	1.46
35AM9	1.57
35AM10	0.78
35AM11	1.00
35AM12	1.17
35AM13	1.49
35AM14	1.50
35AM15	1.55
35AM16	1.62
35AM17	1.58
35AM18	1.55

Table 18 (continue)

Strain	Ethanol % W/V
35AM19	1.57
35AM20	1.49
35AM21	1.50
35AM22	1.60
35AM23	1.54
35AM24	1.54
35AM25	1.60
35AM26	1.55
35AM27	1.53
35AM28	1.57
35AM29	1.51
35AM30	1.48
35AM31	1.52
35AM32	1.56
35AM33	NT
35AM34	1.46
35AM35	1.48
35AM36	1.52
35AM37	1.51

2.7 Protoplast fusion of S.cerevisiae Sc90 and S013 and the selection for potent fusant.

(1) First batch S. cerevisiae Sc90 was fused with S013 and 183 fusants were collected and isolated into pure cultures. When they were tested for growth on molasses agar without and with 1.5% sodium chloride, at 40 C only 10 fusants grew well at 5 days of cultivation (Table 19). Then their growth were investigated on 3% sodium chloride molasses agar at 37 and 40 C. The result showed in Table 20 that 8 fusants could grow and their growth was better than their parental strains.

Ethanol fermentation by these fusants were investigated in 3% sodium chloride molasses fermentation medium by standing tube cultivation, at 40 C. No fusant could produce ethanol higher than or even the same amount as that produced by the parental strains (Table 21).

(2) Second Batch Another batch of fusion was performed between S. cerevisiae Sc90 and S013, and 2 fusants were obtained (Table 19). Only one fusant, 7G185, grew well on 1.5% sodium chloride molasses agar at 40 C and its growth was much better than the parental strains (Table 22).

This fusant will be further studies for growth and ethanol production in salted molasses medium.

Table 19 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* Sc90 and S013 on molasses agar containing 0 and 1.5% NaCl at 40 C

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl					
First batch	183	57	58	10	10
Second batch	2	1	1	1	1
1.5% NaCl					
First batch	183	79	88	9	10
Second batch	2	1	1	1	1

Table 20 Growth of fusants obtained from the cross of *S. cerevisiae* Sc90 and S013 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae Sc90	+	+	+	-	+	+
strain S013	+	+	+	-	+	+
fusant 6G12	+++	+++	+++	+	++	+++
6G21	++	+++	+++	++	+++	+++
6G22	+++	+++	+++	++	+++	+++
6G24	+++	+++	+++	+++	+++	+++
6G31	-	-	-	-	-	-
6G107	+++	+++	+++	+++	+++	+++
6G108	+++	+++	+++	++	+++	+++
6G109	+++	+++	+++	+++	+++	+++
6G112	-	-	-	-	-	-
6G123	+++	+++	+++	++	+++	+++

Table 21 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* Sc90 and S013 in 3% NaCl molasses fermentation medium using shaking tube cultivation, at 40 C for 4 days.

Strain	Ethanol % W/V	Growth OD660 nm
S. cerevisiae Sc90	2.40	2.0
strain S013	2.21	1.4
fusant 6G12	0.05	0.5
6G21	0.03	0.5
6G22	0.05	0.3
6G24	0.02	0.1
6G107	0.02	0.1
6G108	0.03	0.8
6G109	0.02	0.8
6G123	1.89	4.3

Table 22 Growth of fusants obtained from the cross of S. cerevisiae Sc90 and S013 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae Sc90	-	-	+	[illegible]	-	+
strain S013	-	-	+	+	+	+
fusant 7G185	++	+++	+++	++	++	+++

2.8 Protoplast fusion of S. cerevisiae Sc90 and strain S037 and the selection for polent fusant.

Protoplast fusion which performed between S. cerevisiae Sc90 and strain S037 yielded only 8 fusants. Among these fusants no one showed good growth at 40 C on 1.5% sodium chloride molasses agar (Table 23). Therefore they were not be further selected. .

2.9 Protoplast fusion of S. cerevisiae Sc90 and strain S065 and the selection for potent fusant.

S. cerevisiae Sc90 was hybridized with S065 and 954 fusants were obtained after isolation into pure cultures. The growth of all fusants obtained on 3% sodium chloride molasses agar at 37 and 40 C were observed. The result showed that 2 fusants could not grow at both temperatures while 79 and 48 fesants showed little growth at 40 and 37 C, respectively (Table 24). In this step the fusants that showed good growth at 40 C were selected. Therefore a total of 873 fusants were obtained. However, before further step of selection some fusants died, some segregated and only 668 fusants remained. The growth of these fusants were observed on 3% sodium chloride molasses agar containing molasses 15% sucrose equivalent, at 37 and 40 C. From the result 511 fusants grew well at 40 C (Table 25). These fusants were tested for ethanol production.

The result of ethanol production by 511 fusants showed that no fusant could produce ethanol higher than both

Table 23 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* Sc90 and S037 on molasses agar containing 0 and 1.5% NaCl at 40 C.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl	8	8	8	0	0
1.5% NaCl	8	8	8	0	0

Table 24 Growth of fusants obtained from the cross of *S. cerevisiae* Sc90 and S065 on 3% NaCl molasses agar.

Growth	Number of fusant	
	At 37 C	At 40 C
Growth ++++	82	25
Growth +++	822	848
Growth ++	35	67
Growth +	13	12
No growth -	2	2
Total	954	954

Table 25 Growth of selected fusants obtained from the cross of *S. cerevisiae* Sc90 and S065 on 3% NaCl molasses agar containing molasses as 15% sucrose equivalent.

Growth	Number of fusant	
	At 37 C	At 40 C
Growth ++++	0	0
Growth +++	631	511
Growth ++	28	37
Growth +	9	116
No growth -	0	4
Total	668	668

parental strains. Amount of ethanol produced by these fusants was lower than 0.2% W/V. Therefore they were not be further tested.

2.10 Protoplast fusion of S. cerevisiae Sc90 and strain S090 and the selection for potent fusant.

(1) First batch S. cerevisiae Sc90 was hybridized with S090 and 222 fusants were obtained for the selection. Among 222 fusants only 26 fusants showed good growth on 1.5% sodium chloride molasses agar at 40 C (Table 26). These 25 fusants were investigated for growth on 3% sodium chloride molasses agar at 37 and 40 C. The result was shown in Table 27. From the result 12 fusants could grow on salted molasses medium at both temperatures and they were selected for further studies.

Ethanol production by all selected fusants were investigated in 3% sodium chloride molasses fermentation medium at, 40 C using standing tube cultivation. Table 28 showed the result. It showed that 4 fusants,5F3, 6F41, 6F105 and 6F190, produced higher ethanol concentration than their parental strains, Sc90 and S090, which produced 2.4 and 2.1% W/V, respectively. While other 5 fusants, 5F2, 5F4, 6F119, 6F181 and 6F186, could produce ethanol higher than 2% W/V. Therefore a total of 9 fusants which produced ethanol higher than 2% W/V were selected and investigated for ethanol production in 3% sodium chloride molasses fermentation medium using shaking flask cultivation at 40 C for 4 days. The result showed that all fusants tested could produce

Table 26 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* Sc90 and S090 on molasses agar containing 0 and 1.5% NaCl at 40 C

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl					
First batch	222	42	62	10	21
Second batch	104	27	38	10	10
1.5% NaCl					
First batch	222	76	104	9	26
Second batch	104	48	51	10	10

Table 27 Growth of fusants obtained from the cross of *S. cerevisiae* Sc90 and S090 on 3 % NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae Sc90	++	++	++	+	++	++
Strain S090	+	+	+	-	+	+
Fusant 5F2	+	+	++	+	+	+
5F3	+	+	++	+	+	+
5F4	++	++	++	+	+	++
6F32	-	-	-	-	-	-
6F41	+	+	+	+	+	+
6F89	-	-	-	-	-	-
6F105	+	+	++	+	+	+
6F110	++	+++	+++	++	++	+++
6F111	++	+++	+++	++	++	+++
6F119	++	++	++	+	++	++
6F123	-	-	-	-	-	-
6F126	-	-	-	-	-	-
6F131	-	-	-	-	-	-
6F143	-	-	-	-	-	-
6F144	-	-	-	-	-	-
6F145	-	-	-	-	-	-
6F146	-	-	-	-	-	-

Table 27 (continue)

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
6F147	-	-	-	-	-	-
6F150	-	-	-	-	-	-
6F164	+	++	++	+	+	++
6F170	-	-	-	-	-	-
6F181	+	+	+	+	+	+
6F186	+	+	+	+	+	+
6F190	+	+	+	+	+	+
7F110	-	-	-	-	-	-
7F111	-	-	-	-	-	-

Table 28 Ethanol fermentation and growth by selected fusants obtained from the cross of S. cerevisiae Sc90 and strain S090 in 3% NaCl molasses fermentation medium using standing tube cultivation, at 40 C for 4 days.

Strain	Ethanol % W/V	Growth OD 660 nm
S. serevisiae Sc90	2.40	2.0
strain S090	2.10	2.9
fusant 5F2	2.25	2.4
5F3	2.61	2.9
5F4	2.37	2.4
6F41	3.46	2.5
6F105	2.51	1.57
6F110	0.05	0.6
6F111	0.03	0.9
6F119	2.05	2.9
6F164	1.91	1.7
6F181	2.35	2.7
6F183	2.14	2.0
6F186	2.08	2.0
6F190	2.54	2.5

ethanol higher than that by S090 but only one produced the same amount of ethanol as that by Sc90 (Table 29). These 9 fusants will be further tested.

(2) Second batch Another batch of protoplast fusion was performed between S. cerevisiae Sc90 and S090. In this batch 104 fusants were obtained. When growth on on molasses agar without and with 1.5% sodium chloried at 40 C of these fusants were investigated only 10 fusants showed good growth (Table 26). Also when growth on 3% sodium chloride molasses agar by these 10 fusants were observed the result showed that 6 fusants could grow well at 37 and 40 C (Table 30). These 7 fusants were selected and tested for ethanol production.

The result of ethanol production by the selected fusants showed that no fusant produced ethanol higher or even the same amount as that by their parental strain in salted molasses fermentation medium, at 40 C (Table 31).

2.11 Protoplast fusion of S. cerevisiae Sc90 and strain 251 and the selection for potent fusant.

Only 5 fusants were obtained from the cross of S. cerevisiae Sc90 and 251. Among them only one fusant, 5H4, grew well on 1.5% sodium chloride molasses agar at 37 and 40 C (Table 32). It grew much better than its parental strains at both 37 and 40 C (Table 33).

Ethanol production by fusant 5H4 was determined. The result showed that it produced almost the same amout of

Table 29 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* Sc90 and strain S090 in 3% NaCl molasses fermentation medium using shaking flask cultivation, at 40 C for 3 days.

Strain	Ethanol % W/V	Growth OD660 nm
S. cerevisiae Sc90	3.83	5.1
strain S090	3.47	11.2
5F2	3.64	13.1
5F3	3.69	12.7
5F4	3.69	12.8
6F41	3.77	6.9
6F105	3.55	10.8
6F119	3.70	13.0
6F181	3.72	6.6
6F183	3.78	7.4
6F186	3.83	5.9
6F190	3.79	5.8

Table 30 Growth of fusants obtained from the cross of *S. cerevisiae* Sc90 S090 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. cerevisiae Sc90	-	-	+	-	-	+
strain S090	++	++	+	+	++	++
frsant 7F248	++	++	++	++	+++	+++
7F288	+	++	+++	++	+++	+++
7F289	+	++	++	++	+++	+++
7F290	+	++	++	++	+++	+++
7F291	+	++	++	++	+++	+++
7F292	++	+++	+++	++	+++	+++
7F293	++	+++	+++	++	++	++
7F294	++	+++	+++	++	++	+++
7F295	++	+++	+++	++	++	++
7F296	++	+++	+++	++	++	++

Table 31 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* Sc90 and stain S090 in 3% NaCl molasses fermentation medium using shaking flask cultivation at 40 C for 3 days.

Strain	Ethanol % W/V	Growth OD660 nm
S. cerevisiae Sc90	2.71	3.1
strain S090	4.89	7.1
fusant 7F248	0.25	0.9
7F288	0.22	0.7
7F289	0.14	0.6
7F290	0.14	1.1
7F291	0.17	1.0
7F292	0.12	1.0

Table 32 Summarization of the result of growth by fusants obtained from the cross of *S. cerevisiae* Sc90 and 251 on molasses agar contaning 0 and 1.5% NaCl at 40 C.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar			
		Growth		Good Growth	
		1 day	5 days	1 day	5 days
0% NaCl	5	2	2	0	0
1.5% NaCl	5	2	2	1	1

Table 33 Growth of fusants obtained from the cross of *S. cerevisiae* Sc90 and 251 on 3% NaCl molasses agar.

Strain	37 C			40 C		
	Incubation period(day)			Incubation period(day)		
	1	3	5	1	3	5
S. serevisiae Sc90	+	+	+	+	+	+
strain 251	+	+	+	+	+	+
fusant 5H4	++	++	++	++	++	++

ethanol concentration as the parental strain,Sc90 and higher than another parental strain 251 in 3% sodium chloride molasses fermentation medium using standing tube cultivation at 40 C (Table 34). When shaking flask cultivation was used for ethanol production this fusant produced lower ethanol concentration than both parent strains (Table 35).

As mensioned previously that each of high ethanol producing strain is intented to hybridized using protoplast fusion technique with each of halotolerant strain. Therefore totally 24 crosses will be carried out. At this stage 11 crosses were successully completed (Table 36).

3. Investigation of growth of the selected yeast strains on YPD agar containing various concentrations of sodium chloride

Growth of high ethanol producing strains of S. cerevisiae Sc90, AM12 and M30, were investiged on YPD agar containing various concentrations of sodium chloride 3.0-17.0% at 30 C. Growth of these 3 high ethanol producing strains were reduced when cultivation medium contaied 7% sodium chloride (Table 37). Only little growth was observed at 10% sodium chloride. No growth of these 3 high ethanol producing strains was observed on 11 at 2 days of cultivation but little growth of AM12 and Sc90 were obtained at 5 days of cultivation. On YPD agar containing 12% Sodium chloride and higher growth by these high ethanol producing strains were completely inhibited.

Growth of strain S013 and S037 were reduced when concentration of sodium chloride in the medium was increased

Table 34 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* Sc90 and strain 251 in 3% NaCl molasses fermentation medium using standing tube cultivation at 40 C for 4 days.

Strain	Ethanol % W/V	Growth OD660 nm
S. cerevisiae Sc90	2.40	2.0
strain 251	1.58	5.06
fusant 5H4	2.46	5.8

Table 35 Ethanol fermentation and growth by selected fusants obtained from the cross of *S. cerevisiae* Sc90 and strain 251 in 3% NaCl molasses fermentation medium using shaking flask cultivation at 40 C for 3 days.

Strain	Ethanol % W/V	Growth OD 660 nm
S. cerevisiae Sc90	3.83	5.05
strain 251	2.21	15.8
fusant 5H4	1.85	13.6

Table 36 Summarization of successful protoplast fusion between high ethanol producing strains and halotolerant strains.

No.	cross	No. fusant obtain	No. fusant produce ethanol more than parent*
1	AM12 x S013	95	0
2	AM12 x S037	3	2
3	AM12 x S065	594	0
4	AM12 x S090	35	0
5	AM12 x 251	12	0
6	AM12 x S356	38	0
7	Sc90 x S013(1st batch)	183	0
	Sc90 x S013(2nd batch)	2	NYT**
8	Sc90 x S037	8	0
9	Sc90 x S065	954	0
10	Sc90 x S090(1st batch)	222	4
	Sc90 x S090(2nd batch)	104	0
11	Sc90 x 251	5	1
	Total	2,255	7

* : In standing cultivation, at 40 C

** : Not Yet Test

Table 37 Growth of selected high ethanol producing strains of *S. cerevisiae* on YPD agar containing various concentration of sodium chloride at 40 C.

NaCl concentration (%)	Growth of *S. cerevisiae*					
	AM12 (days)		Sc90 (days)		M30 (days)	
	2	5	2	5	2	5
3	+4	+4	+4	+4	+4	+4
4	+4	+4	+4	+4	+4	+4
5	+4	+4	+4	+4	+4	+4
6	+4	+4	+4	+4	+4	+4
7	+3	+3	+3	+3	+3	+3
8	+3	+3	+3	+3	+3	+3
9	+2	+2	+2	+2	+2	+2
10	+1	+1	+1	+1	+1	+1
11	-	+1	-	+1	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-

to 10% and no growth was observed when concentation of sodium chloride was 13% . Strain S065 and S090 showed reduction in growth when concentration of sodium chloride was increased to 7% and no growth was observed at 13% sodium chloride also. While growth of 251 was reduced at 7% sodium chloride and completely inhibited was observed at 15% (Table 38).

Strain 126, 130, 356 and 370 showed strong growth on medium containg 3-15% sodium chloride. While only little reduction on growth was obtained at 16 and 17% sodium chloride concentration (Table 39).

4. Chromosome separation

Chromosomes of all selected high ethanol producing strain, S. cerevisiae Sc90, AM12 and M30, and selected halotolerant strains, S013, S037, S065, S090, 251, 126, 130, 356 and 370 were separated by orthogonal field alteration electrophoresis (OFAGE) and the results were shown in Figure 10. The results indicated that S. cerevisiae Sc90, AM12 and M30 had the same pattern of chromosomes when observed on gel obtained by OFAGE. The pattern of chromosomes of Sc90, AM12 and M30 seemed to be same as the typical pattern of S. cerevisiae. Also, S090 a selected halotolerant strain seemed to have same pattern of chromosomes as S. cerevisiae. Strain S013,S037, S065 and 251 had same pattern of chromosomes. The strain 126, 130, 356 and 370 which were selected at the same concentration of sodium chloride 17%, showed very similar pattern of chromosome to each other.

Table 38 Growth of halotolerant strains selected at 5% NaCl on YPD agar containing various concentration of sodium chloride at 40 C.

NaCl concentration (%)	Growth of *S. cerevisiae*									
	S013 (days)		S037 (days)		S065 (days)		S090 (days)		251 (days)	
	2	5	2	5	2	5	2	5	2	5
3	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4
4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4
5	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4
6	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4
7	+4	+4	+4	+4	+3	+3	+3	+3	+3	+3
8	+4	+4	+4	+4	+3	+3	+3	+3	+3	+3
9	+4	+4	+4	+4	+3	+3	+3	+3	+3	+3
10	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2
11	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2
12	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
13	-	-	-	-	-	-	-	-	+1	+1
14	-	-	-	-	-	-	-	-	+1	+1
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-

Table 39 Growth of halotolerant strains selected at 17% NaCl on YPD agar containing various concentration of sodium chloride at 40 C.

NaCl concentration (%)	Growth of S. cerevisiae							
	126 (days)		130 (days)		356 (days)		370 (days)	
	2	5	2	5	2	5	2	5
3	+4	+4	+4	+4	+4	+4	+4	+4
4	+4	+4	+4	+4	+4	+4	+4	+4
5	+4	+4	+4	+4	+4	+4	+4	+4
6	+4	+4	+4	+4	+4	+4	+4	+4
7	+4	+4	+4	+4	+4	+4	+4	+4
8	+4	+4	+4	+4	+4	+4	+4	+4
9	+4	+4	+4	+4	+4	+4	+4	+4
10	+4	+4	+4	+4	+4	+4	+4	+4
11	+4	+4	+4	+4	+4	+4	+4	+4
12	+4	+4	+4	+4	+4	+4	+4	+4
13	+4	+4	+4	+4	+4	+4	+4	+4
14	+4	+4	+4	+4	+4	+4	+4	+4
15	+4	+4	+4	+4	+4	+4	+4	+4
16	+3	+3	+3	+3	+3	+3	+3	+3
17	+3	+3	+3	+3	+3	+3	+3	+3

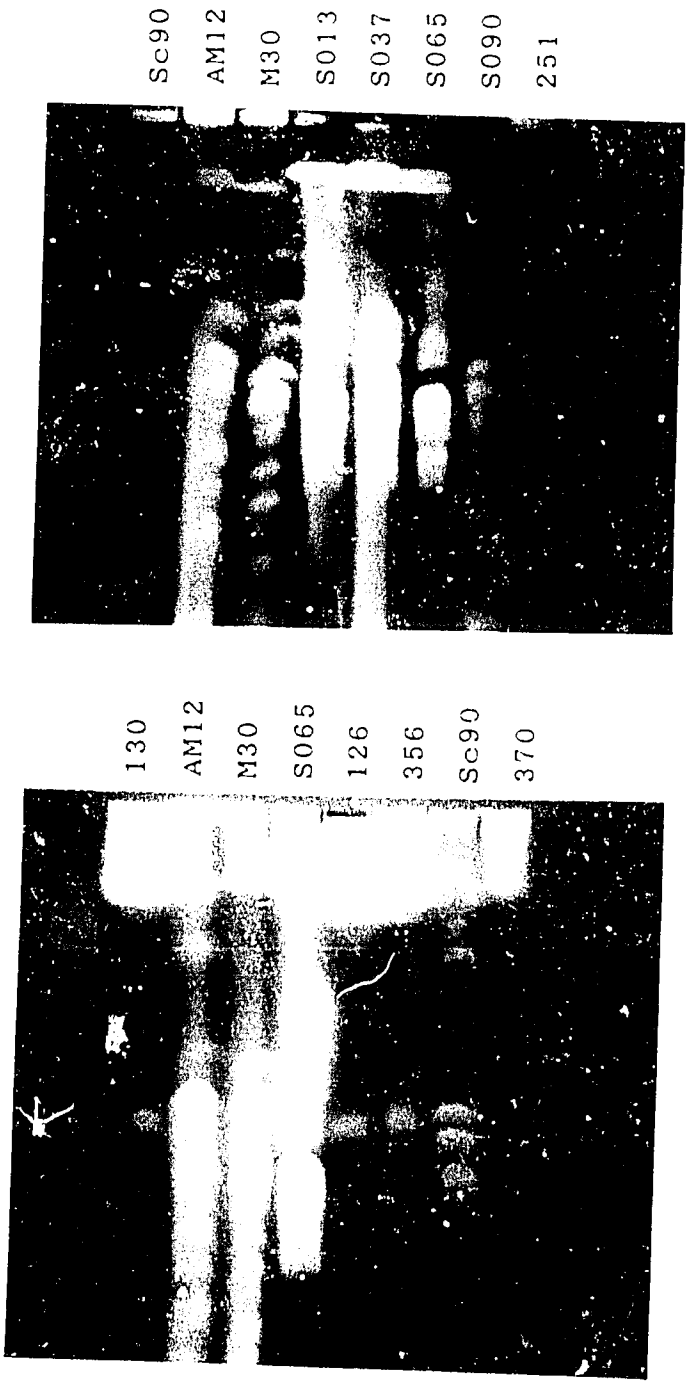


Figure 10 OFAGE of chromosomes of the selected parental strains.

5. DNA preparation

The DNA of selected high ethanol producing strains, Sc90, AM12 and M30, and selected halotobrant strains, S013, S037, S065, S090, 251, 126, 130, 356 and 370 were prepared and investigated by mini-gel electrophoresis. The result was shown in Figure 11.

6. Lithium transformation of plasmid BT131 into high ethanol producing strains

These experiments were carried out because Dr. Hick and Dr. Savitree had been discussed and summarized that if we can not obtain good hybrid by protoplast fusion technique another way to obtain the strain which can produce high ethanol concentration in high salinity molasses mash is gene cloning. Therefore during Dr. Savitree's visit to Dr. Hicks' laboratory we tried to prepare DNA of a selected halotolerant strain and make gene library. But the period was too short so that we were not able to construct gene library of halotolerant strain. Also during Dr. Savitree's visit we tried to establish transformation system for our strains. Dr. Hicks obtained plasmid BT131 from a laboratory in University of California, Davis. This plasmid can be used for yeast transformation and carry sulfometuron methyl (SM) resistance gene. We planned to use this plasmid as vector for carrying halotolerant gene obtained from selected halotolerant strain into selected high ethanol producing strain. Therefore we investigated that whether the plasmid BT131 could be transformed into our high ethanol

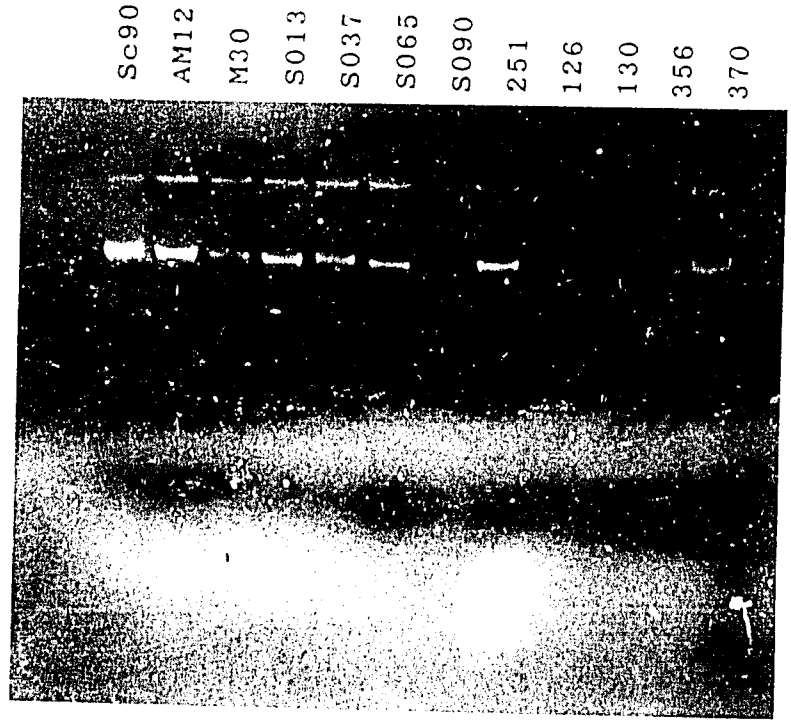


Figure 11 Gel electrophoresis of DNA of the selected parental strains.

producing strains or not. Since this plasmid carry SM resistance gene therefore we want to use SM resistance gene as a marker gene. At first growth by selected high ethanol producing strains were observed on minimal medium containing SM (yeast nitrogen base W/O amino acid 0.67%, glucose 2%,SM 0.3% and agar 1.5%). The result clearly showed that Sc90 could not grow on this medium while AM12 and M30 could grow. However, the growth by AM12 and M30 were lower than normal resistant strain (Figure 12).

Lithium transformations of plasmid BT131 into 3 defferent high ethanol producing strains, Sc90, AM12 and M30 were carried out and the results were summarized in Table 40. Segregation was determined by transferring colonies appeared on selective medium to YPD agar after colonies appeared on YPD agar they were replicated onto selective medium again. If cell carried plasmid BT131 obtained by transformation when it was cultivated on YPD agar during growth of cell plasmid might lose from the cell therefore that cell was not able to grow on selective medium anymore. Stable colony was the colony which still appeared on selective medium after cultivation on rich medium, YPD agar, and transfered onto the selective medium.

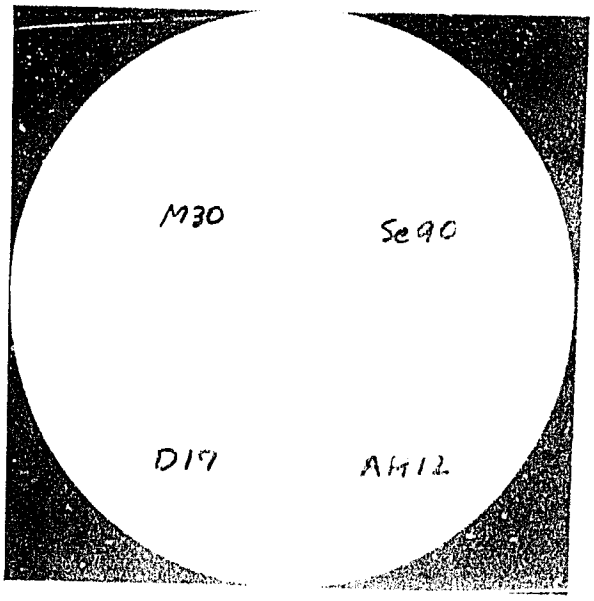


Figure 12 Growth of the selected high ethanol producing strains on medium containg sulfometuron methyl, at 30 C.

Table 40 Transformation of plasmid BT131 into high ethanol producing strains.

Strain	No. of colonies on selective medium (per plate)		Segregation		Frequency of transformation
	without plasmid	with plasmid	without plasmid	with plasmid	
S. cerevisiae Sc90	3	6	6/6 stable	6/6 stable	low
S. cerevisiae AM12	11	151	6/6 stable	6/12 stable	high
S. cerevisiae M30	2	288	3/3 stable	15/18 stable	high

Conclusion

The works had been performed in the last 12 months (August, 1987 to August, 1988) could be summarized as follows:

1. Two auxotrophic mutants of strain 126 and 1 mutant of strain 356 were obtained when NTG 100 mg/ml was used for 20 minutes. However,no auxotrophic mutant of strain 130 and 370 was able to be isolated even mutagenesis was repeated many times.

Respiratory deficient mutants of S. cerevisiae M30 were reconstructed since all mutants obtained in the last reporting period produced very low ethanol concentration.

The selections of all mutants obtained to be used for protoplast fusion were carried out. At this stage auxotrophic mutants of halotolerant strains selected at 5% sodium chloride, S013, S037, S065, S090 and 251, were S013-A63, S037-A73, S065-A25 and S065-A44, S090-A1 and 251-A17, respectively. The auxotrophic mutant of strain 356, a halotolerant strain selected at 17% sodium chloride, was 356-A1.

The selection of respiratory deficient mutants of high ethanol producing strains yielded M30-RD11 and M30-RD108 for S. cerevisiae M30. While AM12-RD67 and Sc90-RD1 were selected for S. cerevisiae AM12 and Sc90, respectively.

2. At this stage 11 crosses between high ethanol producing and halotolerant strains were successfully completed by protoplast fusion technique. The crosses and number of fusants obtained from each cross were as follows:

1) S. cerevisiae AM12 x S013 : 95 fusants
2) S. cerevisiae AM12 x S037 : 3 fusants
3) S. cerevisiae AM12 x S065 : 594 fusants
4) S. cerevisiae AM12 x S090 : 35 fusants
5) S. cerevisiae AM12 x 251 : 12 fusants
6) S. cerevisiae AM12 x 356 : 38 fusants
7) S. cerevisiae Sc90 x S013 : 185 fusants
8) S. cerevisiae Sc90 x S037 : 8 fusants
9) S. cerevisiae Sc90 x S065 : 954 fusants
10) S. cerevisiae Sc90 x S090 : 326 fusants
11) S. cerevisiae Sc90 x 251 : 5 fusants

The results of selections of potent fusants could be summarized that among 11 crosses which were successfully completed only the cross of S. cerevisiae AM12 and S037 yielded two fusants, B3 and B4, which could produce ethanol higher than their parental strains in 3% sodium chloride molasses fermentation medium. Also 9 fusants obtained from the cross of S. cerevisiae Sc90 and S090 could produce ethanol higher than one parental strain, S090 and same as another, Sc90. While the other 9 crosses did not yield any potent fusant.

3. The growth of all selected yeast strains to be used as parental strains for hybridization were investigated on YPD agar containing various concentrations of sodium chloride, 1.0-17.0% at 30 C. The result showed that growth of all selected high ethanol producing strains were reduced when cultivated on 7% NaCl-YPD agar. Growth of S. cerevisiae M30 and Sc90 were completely inhibited by 11% NaCl while 12% was the completely inhibiting concentration for S. cerevisiae AM12.

Growth of S013 and S037, the halotolerant strains selected at 5% NaCl was reduced when cultivated on 10% NaCl-YPD agar while 7% NaCl could reduce growth of strain S065, S090 and 251. Completely inhibiting concentration of sodium chloride for the growth of strain S013, S037, S065 and S090 was 13% and of strain 251 was 15%.

Growth of 4 halotolerant strains selected at 17% sodium chloride, 126, 130, 356 and 370 were very strong on YPD agar containing 3-15% sodium chloride at 30 C. Their growth was reduced when sodium chloride in the medium was increased to 16 and 17% NaCl.

4. Chromosomes of all selected yeasts were separated by OFAGE. The result could be concluded that pattern of chromosomes on gel of the selected strains was similar to each other in the same group but different from the other groups. The pattern of chromosomes on gel of S. cerevisiae M30, AM12 and Sc90 were the same as that of normal S. cerevisiae.

5. The result of lithium transformation of plasmid BT131 into selected high ethanol producing strains indicated that plasmid BT131 could be transformed into our selected high ethanol producing strains, S. cerevisiae M30, AM12 and Sc90. The minimal medium containing sulfometuron methyl could be used as selective medium for transformant.

Work plan for the next period

Work plan for the next period is scheduled as follows :

1. Continue construction of selective marker by mutagenesis for the remaining strains which could not be constructed so far.

2. Continue hybridization of the halotolerant yeast with high ethanol producing yeast by protoplast fusion technique. A total of 13 crosses is planned to be carried out.

3. Ethanol fermention of the selected fusants obtained in this reporting period will be carried out.

4. Selection for the potent fusants from the fusants going to be obtained in the next period.

5. Study on parameters of ethanol fermentation of selected hybrid fusant at laboratory scale.

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