PN-ABM-705

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: July 11, 1989

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. 5.238
(5.0(a2) "Hybridization of Halotolerant Yeast for Alcohol Fermentation

Attachment
1/3

5.238

PN-ABM-705

Progress Report No. 5

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

Kasetsart University

Bangkok Thailand

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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 Tantirungkij
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	: 4 years, August, 1985 to November, 1989
Reporting Period	: September 1, 1988 to February 28, 1989

Bugget Allocation for This Period

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 Prototheca 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 selected halotolerant strain is intended to hybridize with each 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. The auxotrophic mutants of all halotolerant strains selected at 5 % sodium chloride were obtanied by treatment with ethylmethansulfonate (EMS). While the two strains selected at 17 % sodium chloride, strain 126 and356 were isolated by N-methyl-N'-nitro-N-nitrosoguanidine However, no auxotrophic mutant of strain 130 and 370 , 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. Many crosses of protoplast fusion were carried out and a number of hybrid fusants were obtained.

In this reporting period the selected fusants obtained in the last reporting period were investigated for ethanol producing ability in high salinity molasses mash at high temperature using shaking flask culture compaired with the parental strains. Hybridization between halotolerant strain and high ethanol producing strain were also performed in this reporting period and at this stage 11 crosses were successfully completed. The selection of potent fusant from the fusants obtained were carried out.

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

2. 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. 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).

2. Selection for the potent fusant

Selection for the potent hybrid was carried out for many steps at 40 C. At first fusant picked from selective regeneration medium (minimal medium containing glycerol as sole source of carbon) was isotaled into pure culture. The fusant was tested for their ability of growing on molasses agar (molasses 5% sucrose equivalent sugar concentration, 0.05% ammonium sulfate and 1.5% agar) containing 0 and 3 % sodium chloride at 37 and 40 C. The fusant which could grow on these medium was selected and investigated the ethanol fermentation in 3% sodium chloride molasses fermentation medium (molasses 15% sucrose equivalent sugar concentration, 0.05% ammonium sulfate and 3% sodium chloride) at 40 C both by standing tube and shaking flask cultivations.

3. Analysis

Ethanol concentration was determined by using Gas Liquid 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).

Results and Discussion

1. Ethanol production of selected fusants obtained in the last reporting period.

In the last reporting period 9 fusants obtained from protoplast fusion of S. cerevisiae Sc90 and S090 which could produce ethanol higher than S090 in salted molasses medium using standing tube cultivation at 40 c were selected. In this reporting period ethanol production in salted molasses at 40 c was investigated by using shaking flask cultivation. The result revealed that fusant 6F105 produced highest ethanol concentration, 4.71% W/V (Fig. 1). While maximum ethanol produced by the parental strains, S. cerevisiae Sc90 and S090 were 3.86 and 4.75% W/V, respectively. Although the maximum ethanol produced by fusant 6F105 was higher than that produced by S090 but lower than that by S. cerevisiae Sc90.

2. Protopast fusion and the selection for potent fusant

2.1 Protoplast fusion of S. cerevisiae Sc90 and strain 356 and the selection for potent fusant

(1) First batch : Two hundred-forty nine fusants were constructed by protoplast fusion of the high ethanol producing strain, S. cerevisiae Sc90, and the halotolerant strain, 356. When these fusants were tested the ability to grow on molasses agar containing 3% NaCl, at 37 and 40 c, the result showed that among 249 fusants which could grow on

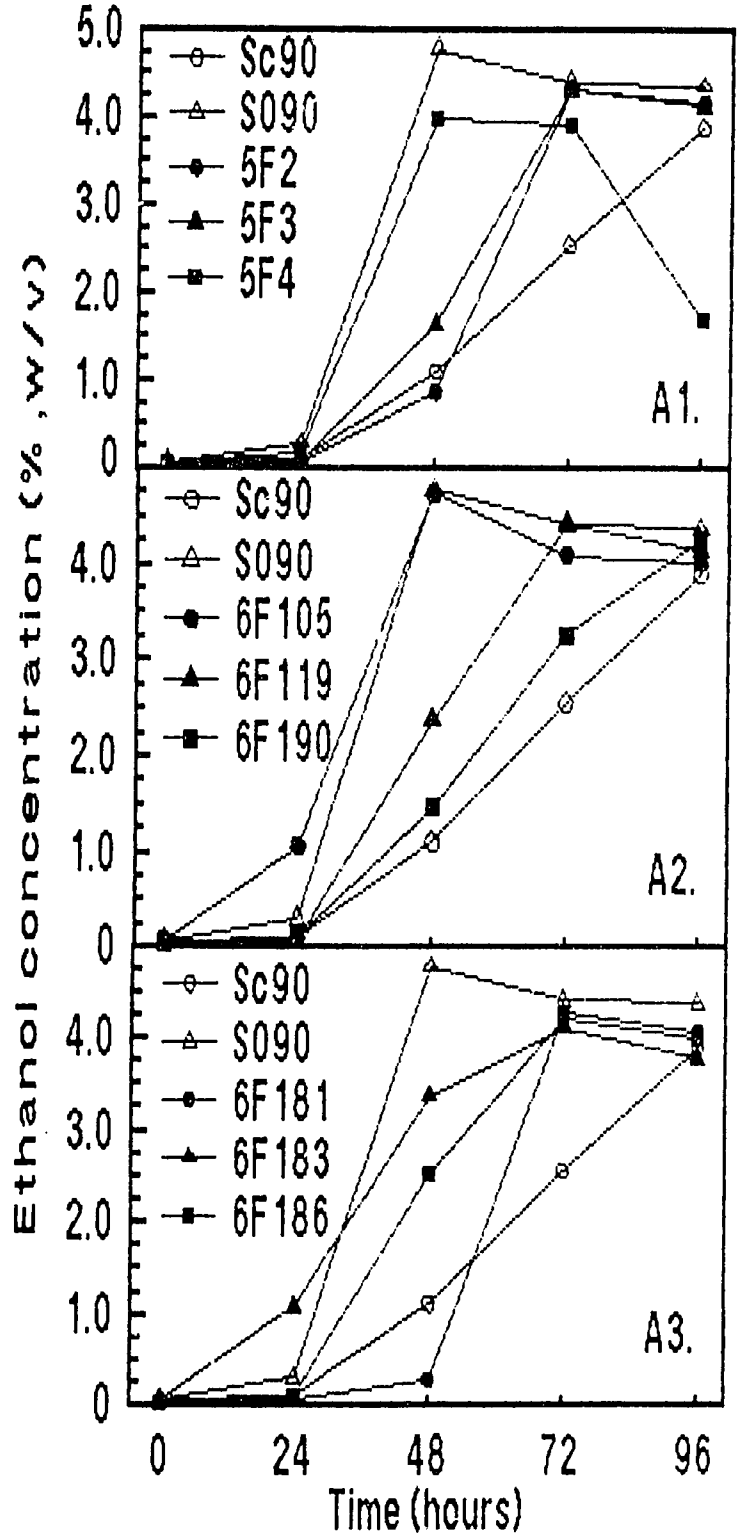

Figure 1A Time-course of ethanol production by fusants, obtained from the cross of S. cerevisiaeSc90 and strain 356 in 3% NaCl molasses medium using shaking flask cultivation, at 40 c.

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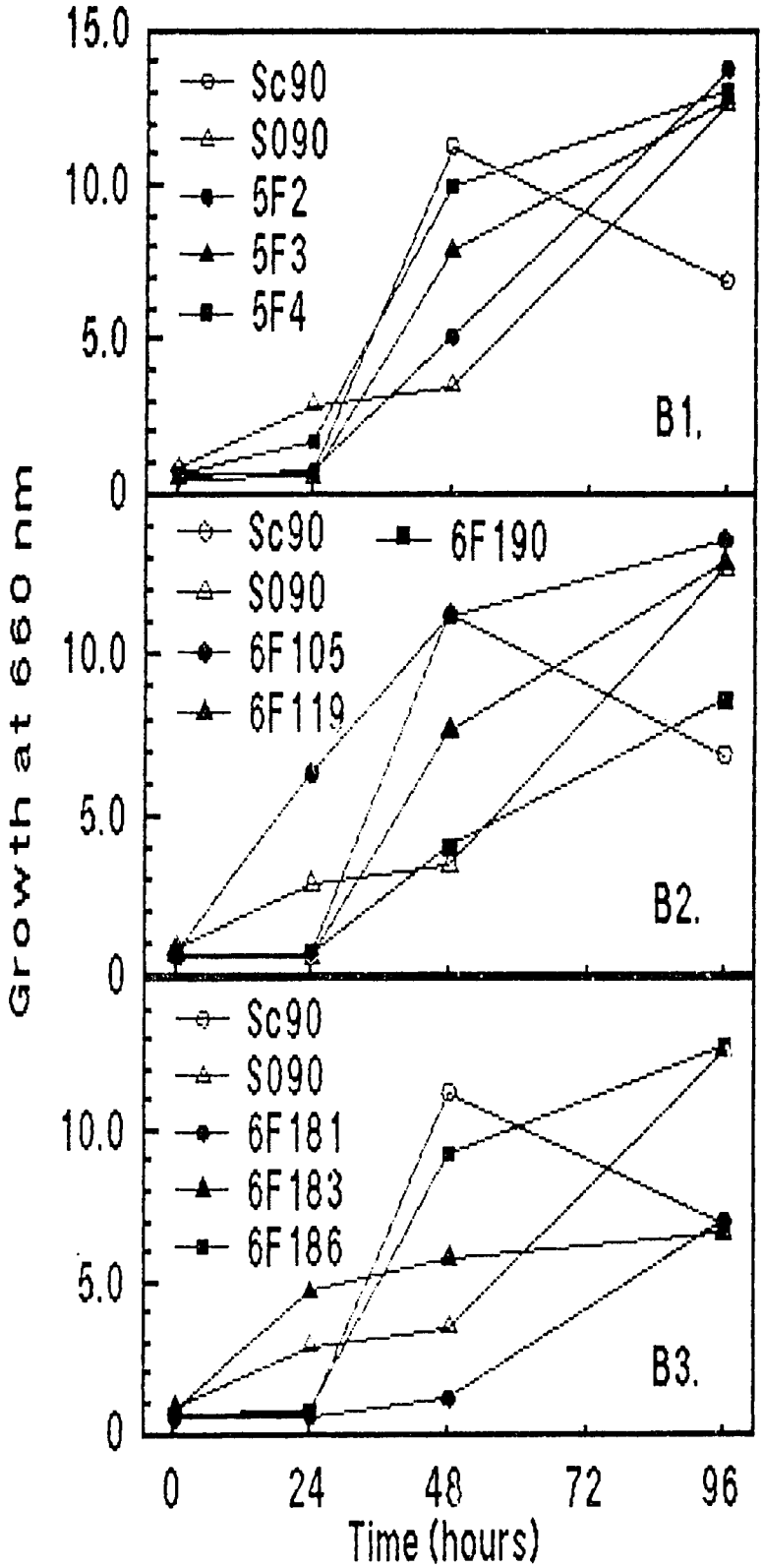

Figure 1B Time-course of growth by fusants, obtained from the cross of S. cerevisiae Sc90 and strain 356 in 3% NaCl molasses medium using shaking flask cultivation, at 40 c.

salted molasses medium after 5 days cultivation at both temperature, only 37 fusants showed good growth at 40 c (Table1). Therefore, these 37 fusants were selected and the ethanol production was investigated . The result of ethanol production showed that all fusants produced ethanol lower than S. cerevisiae Sc90 which produced 1.62% W/V, while most fusants produced ethanol higher than strain 356, which yielded 1.26% W/V (Table 2). There were 2 fusants, 35Sc107 and 35Sc112 produced ethanol 1.54% W/V, which only little lower than that produced by S. cerevisiae Sc90 so that they were selected for further study. Time course of ethanol production by these 2 selected fusants in 3% sodium chloride molasses medium using shaking flask cultivation, at 40 c were determined. Fusant 35Sc107 and 35Sc112 produced maximum ethanol 2.62 and 2.67% W/V, respectively at 24h while S.cerevisiae Sc90 and strain 356 produced 4.52 and 2.16% W/V, respectively at 72h (Fig 2). Since both selected fusants could not produce ethanol higher than S. cerevisiae Sc90 therefore no furtherstudy was did.

(2) Second batch : Another batch of protoplast fusion of S. cerevisiae Sc90 and 356 revealed 111 fusants. Among these fusants only 44 fusants showed good growth after 3 days incubation on 3% sodium chloride molasses agar at 40 c although all flusants tested could grow on this medium (Table 3). Forty-four fusants which showed good growth on 3% sodium chloride molasses agar at 40 c were selected for further study.

Table 1 The result of growth by fusants obtained from the first batch of protoplast fusion between S. cerevisiae Sc 90 and strain 356* on molasses agar containing 3% NaCl.

Incubation temperature	Number of fusant test	Number of fusant grow on molasses agar					
		Growth (day)			Good growth (day)**		
		1	3	5	1	3	5
37 c	249	187	249	249	0	42	107
40 c	249	113	243	249	0	0	37

* Sc 90 and 356 showed good growth since 1 day of incubation at 37 c, while at 40 c good growth was observed at 3 day.

** Good growth = (+++) to (++++)

Table 2 Ethanol production by selected fusants obtained from the cross of *S. cerevisiae* Sc90 and strain 356 in 3% NaCl molasses fermentation medium using standing tube cultivation, at 40 c for 4 days.

Strain	Ethanol, % W/V
S. cerevisiae Sc90	1.62
Strain 356	1.26
fusant 35Sc75	0
35Sc77	1.28
35Sc78	1.44
35Sc79	1.34
35Sc80	1.25
35Sc81	1.39
35Sc82	1.33
35Sc83	1.37
35Sc84	1.33
35Sc85	1.40
35Sc86	1.39
35Sc87	1.42
35Sc88	0
35Sc89	1.47
35Sc90	1.27
35Sc91	1.48
35Sc92	1.46
35Sc93	1.45
35Sc94	1.40
35Sc95	1.44
35Sc96	1.47

Table 2 (continue)

Strain	Ethanol, % W/V
35Sc97	1.40
35Sc98	1.48
35Sc99	1.44
35Sc100	1.37
35Sc101	1.22
35Sc102	1.35
35Sc103	1.48
35Sc104	1.39
35Sc105	1.35
35Sc106	1.42
35Sc107	1.54
35Sc108	1.47
35Sc109	1.45
35Sc110	1.39
35Sc111	1.44
35Sc112	1.54

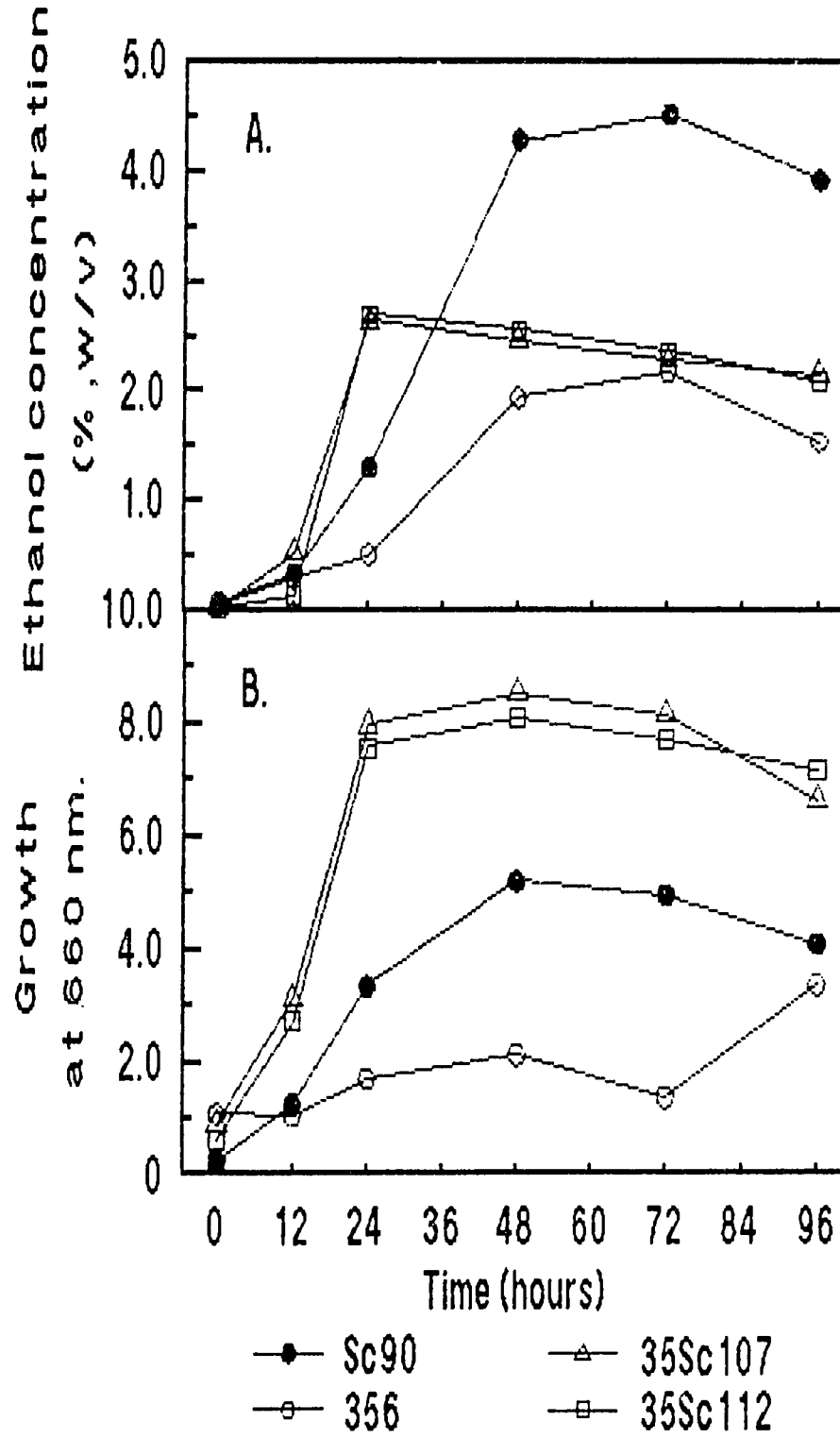

Figure 2 Time-course of ethanol production(A) and growth(B) by fusant 35Sc107 and 35Sc117 obtained from the cross of S. cerevisiae Sc90 and strain 356 in 3% NaCl molasses medium using shaking flask cultivation, at 40 c.

Table 3 The result of growth by fusants obtained form the second batch of protoplast fusion between *S. cerevisiae* Sc90 and strain 356* on molasses agar containing 3% NaCl.

Incubation temperature	Number of fusant test	Number of fusant grow on molasses agar			
		Growth(day)		Good Growth**	
		1	3	1	3
37 c	114	113	114	3	61
40 c	114	101	114	0	44

* Sc90 and 356 showed good growth after 1 day of incubation at 37 c while at 40 c good was obtained at 3 days.

** Good growth = (+++) to (++++)

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

S. cerevisiae AM12 was hybridized with S037 by protoplast fusion technique and 122 fusants were selected from the selective regeneration medium. The result of growth on 3% sodium chloride molasses agar at 40 c revealed that 84 fusants could grew but only 61 fusants grew well (Table 4). These 61 fusants were selected and the ethanol production in salted molasses medium, at 40 c will be determined.

2.3 Protoplast fusion of S. cerevisiae M30 and strain S013 and the selection for potent fusant

Protoplast fusion was carried out between S.cerevisiae M30 and strain S013. Among 217 fusants obtained only 13 fusants showed good growth on 3% sodium chloride molasses agar at 40 c while 17 fusants could grow on molasses agar which no sodium chloride was added (Table 5). All fusants which grew well on 3% sodium chloride molasses agar were selected. The ability to produce ethanol in salted molasses medium at 40 c of these selected fusants will be determined.

2.4 Protoplast fusion of S. cerevisiae M30 and strain S037 and the selection for potent fusant

S. cerevisiae M30 was fused with strain S037 and 148 fusants were selected and tested the ability to grow on 3% sodium chloride molasses agar, at 37 and 40 c. The

Table 4 The result of growth by fusants obtained from protoplast fusion between S. cerevesiae AM12 and strain S037* on molasses agar containing 0 and 3% NaCl, at 40 c.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar	
		Growth	Good Growth**
0% (3 days)	122	109	2
3% (5 days)	122	84	61

* AM12 and S037 showed good growth on molasses agar containing 0 and 3% NaCl.

** Good growth = (+++) to (++++)

Table 5 The result of growth by fusants obtained from protoplast fusion between S. cerevesiae M30 and strain S013* on molasses agar containing 0 and 3% NaCl, at 40 c.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar	
		Growth	Good Growth**
0% (3 days)	217	27	17
3% (5 days)	217	21	13

* M30 and S013 showed good growth on molasses agar containing 0 and 3% NaCl.

** Good growth = (+++) to (++++)

result showed that at 37 c, 123 fusants grew well after only 1 day of cultivation (Table 6). At 40 c, 91 fusants from 123 fusants which could grow after 1 day showed good growth while 117 fusants grew well after 3 days cultivation. Among 117 fusants which showed good growth after 3 days,54 fusants grew better than the others (data did not show), therefore these 54 fusants were selected.

Ethanol production of these selected fusants was investigated in 3% sodium chloride molasses medium using standing tube cultivation, at 40 c.The result showed that no fusant produced ethonol higher than M30 although some fusants produced higher than S037 (Table 7).

2.5 Protoplast fusion of S. cerevisiae M30 and strain S065 and the selection for potent fusant

Protoplast fusion of S. cerevisiae M30 and strain S065 yielded 152 fusants. On 3% sodium chloride molasses agar, at 40 c only 8 fusants could grow and 5 fusants showed good growth (Table 8). All of the fusants that grew well on 3% sodium chloride molasses medium at 40 c were select for further study.

2.6 Protoplast fusion of S. cerevisiae M30 and strain S090 and the selection for potent fusant

(1) First batch : From the cross of S. cerevisiae M30 and strain S090 79 fusants were obtained. When growth on 3% sodium chloride molasses agar was

Table 6 The result of growth by fusants obtained from the first batch of protoplast fusion between S. cerevisiae M30 and strain S037* on molasses agar containing 3% NaCl.

Incubation temperature	Number of fesant test	Number of fusant grow on molasses agar					
		Growth (day)			Good growth (day)**		
		1	3	5	1	3	5
37 c	148	123	123	123	123	123	123
40 c	148	123	128	128	91	117	117

* M30 and S037 showed good growth (+++) after 3 days cultivation

** Good growth = (+++) to (++++)

Table 7 Ethanol production by selected fusants obtained from the cross of S. cerevisiae M30 and strain S037 in 3% NaCl molasses fermentation medium using standing tube cultivation, at 40 c for 4 days.

Strain	Ethanol, % W/V
S. cerevisiae M30	1.00
strain S037	0.47
fusant MS50	0.51
MS51	0.64
MS66	0.48
MS70	0
MS71	0
MS72	0
MS73	0
MS74	0
MS75	0
MS76	0
MS77	0
MS78	0
MS79	0
MS80	0
MS81	0
MS82	0
MS83	0
MS84	0
MS85	0
MS86	0.50

Table 7 (continue)

Strain	Ethanol, % W/V
MS88	0
MS89	0
MS98	0
MS99	0
MS100	0.51
MS101	0
MS102	0
MS103	0
MS104	0
MS105	0
MS106	0.04
MS107	0.53
MS108	0.59
MS109	0.48
MS110	0
MS111	0
MS114	0
MS115	0.65
MS116	0.60
MS117	0.57
MS118	0.56
MS130	0.64
MS131	0.56
MS132	0.49
MS133	0.61

Table 7 (continue)

Strain	Ethanol, % W/V
MS134	0.54
MS135	0.56
MS136	0.49
MS137	0.45
MS138	0.58
MS139	0.45
MS140	0.58
MS141	0.53
MS142	0.58
MS143	0.65

Table 8 The result of growth by fusants obtained from protoplast fusion between S. cerevesiae M30 and strain S065* on molasses agar containing 0 and 3% NaCl, at 40 c.

NaCl in molasses agar	Number of fusant test	Number of fusant grow on molasses agar	
		Growth	Good Growth**
0% (3 days)	152	96	4
3% (5 days)	152	9	5

* M30 and S065 showed good growth on molasses agar containing 0 and 3% NaCl.

** Good growth = (+++) to (++++)

investigated at 37 and 40 c. The result showed that all fusants could grow at both temperature after 3 days cultivation (Table 9). At 37 c, 29 fusants showed good growth after 3 days while 20 fusants grew well at 40 c after the same cultivation period. The fusants which showed good growth at 40 c were selected and tested the ethanol production.

Ethanol produced in 3% sodium chloride molasses medium, at 40 c using shaking flask cultivation by fusant SOM(108)56 was the highest, 2.69 %w/v, among 20 fusants tested (Table10). However, this concentration was lower than that produced by S. cerevisiae M30, 2.8% w/v, although it higher than that produced by S090, 2.27% w/v.

(2) Second batch: Another respiratory deficient mutant of S. cerevisiae M30 was fused with the auxotrophic mutant of strain S090 and only 4 fusants were obtained. These 4 fusants could not grow on 3% sodium chloride molasses agar at 40 c although growth on this medium at lower temperature, 37 c was observed (Table 11).

2.7 Protoplast fusion of S. cerevisiae M30 and strain 251 and the selection for potent fusant

(1) First batch: Protoplast fusion was performed between S. cerevisiae M30 and strain 251. Forty-one fusants was selected to test the ability to grow on 3% sodium chloride molasses agar at 37 and 40 c. The result showed that all fusants could grow on salted medium at both

Table 9 The result of growth by fusants obtained from the first batch of protoplast fusion between S. cerevisiae M30 and strain S090* on molasses agar containing 3% NaCl.

Incubation temperature	Number of fusant test	Number of fusant grow on molasses agar					
		Growth (day)			Good growth (day)**		
		1	3	5	1	3	5
37 c	79	63	79	79	0	29	29
40 c	79	36	79	79	0	20	20

* M30 and S090 showed good growth afater 3 days of incubation at both 37 and 40 c

** Good growth (+++) to (++++)

Table 10 Ethanol production by selected fusante obtained from the cross of *S. cerevisiae* M30 and strain S090 in 3% NaCl molasses fermentation medium using shaking flask cultivation, at 40 c for 3 days.

Strain		Ethanol, % W/V
S. cerevisiae	M30	2.80
strain	S090	2.27
fusant	SOM (108) 41	0.07
	SOM (108) 42	0
	SOM (108) 43	0.17
	SOM (108) 44	0
	SOM (108) 45	0.08
	SOM (108) 46	0
	SOM (108) 47	0
	SOM (108) 52	0
	SOM (108) 53	0
	SOM (108) 54	1.12
	SOM (108) 55	0
	SOM (108) 56	2.69
	SOM (108) 58	0.07
	SOM (108) 59	0
	SOM (108) 60	0
	SOM (108) 69	0
	SOM (108) 74	0
	SOM (108) 75	0
	SOM (108) 76	0
	SOM (108) 77	0

Table 11 The result of growth by fusants obtained from the second batch of protoplast fusion between S. cerevisiae M30 and strain S090* on molasses agar containing 3% NaCl.

Incubation temperature	Number of fusant test	Number of fusant grow on molasses agar					
		Growth (day)			Good growth (day)**		
		1	3	5	1	3	5
37 c	4	4	4	4	0	0	4
40 c	4	4	4	4	0	0	0

* M30 and S090 showed good growth after 3 days of incubation at both 37 and 40 c

** Good growth (+++) to (++++)

temperature after 3 days (Table 12). Good growth was observed by 30 fusants after 3 days, at 37 c. While at 40 c, 6 fusants showed good growth but could be observed only after 5 days of cultivation.

Ethanol production of the fusants which grew well on 3% sodium chloride molasses medium, at 40 c was investigated. Unfortunately, 4 fusants died therefore only 2 fusants were tested. Fusant 251M(108)34 and 251M(108)39 produced ethanol 1.06 and 1.71 %W/V, respectively, in 3% sodium chloride molasses medium, at 40 c, (Table 13). While the parental strain, S. cerevisiae M30 produced 2.8% w/v and strain 251 produced 1.33% w/v. Since both fusants produced ethanol lower than S. cerevisiae M30 therefore no further investigation was performed.

(2) Second batch: Another batch of protoplast fusion between S. cerevisiae M30 and strain 251 was carried out and 338 fusants were constructed. Growth of all fusants were investigated on salted molasses agar at 37 and 40 c.The result showed that 168 fusants grew well after 3 days cultivation at 40 c (Table 14). Among the fusants which showed good growth 85 grew better than the others (data did not showed). Therefore these 85 fusants were selected to test the ethanol production.

Ethanol production in 3% sodium chloride molasses medium at 40 c was investigated. The result was shown in Table 15 that no fusant produced ethanol higher than that produced by both parental strains.

Table 12 The result of growth by fusants obtained from the first batch of protoplast fusion between *S. cerevisiae* M30 and strain S251* on molasses agar containing 3% NaCl.

Incubation temperature	Number of fusant test	Number of fusant grow on molasses agar					
		Growth (day)			Good growth (day)**		
		1	3	5	1	3	5
37 c	41	35	41	41	0	30	30
40 c	41	37	41	41	0	0	6

* M30 and S251 showed good growth after 3 days of incubation.

** Good growth (+++) to (++++)

Table 13 Ethanol production by selected fusants obtained from the cross of *S. cerevisiae* M30 and strain S251 in 3% NaCl molasses fermentation medium using standing tube cultivation, at 40 c for 4 days.

Strain	Ethanol, % W/V
S. cerevisiae M30	2.80
strain 251	1.33
fusant 251M(108)34	1.06
251M(108)39	1.71

Table 14 The result of growth by fusants obtained from the second batch of protoplast fusion between S. cerevisiae M30 and strain 251* on molasses agar containing 3% NaCl.

Incubation temperature	Number of fusant test	Number of fusant grow on molasses agar					
		Growth (day)			Good growth (day)**		
		1	3	5	1	3	5
37 c	338	338	338	338	156	156	156
40 c	338	236	255	255	156	168	168

* M30 and S251 showed good growth after 3 days cultivation.

** Good growth (+++) to (++++)

Table 15 Ethanol production by selected fusants obtained from the cross of S. cerevisiae M30 and strain 251 in 3% NaCl molasses fermentation medium using standing tube cultivation, at 40 c for 4 days.

Strain	Ethanol, % W/V
S. cerevisiae M30	1.24
strain 251	0.59
fusant 25M3	0.50
25M7	0.39
25M8	0.30
25M9	0.48
25M13	0.46
25M16	0.40
25M17	0.38
25M18	0.47
25M28	0.41
25M29	0.41
25M30	0.42
25M31	0.45
25M32	0.43
25M33	0.44
25M34	0.37
25M35	0.47
25M50	0.41
25M51	0.35
25M56	0.45
25M58	0.40

Table 15 (continue)

Strain	Ethanol, % W/V
25M71	0.33
25M76	0.37
25M77	0.37
25M82	0.40
25M85	0.30
25M86	0.39
25M87	0.38
25M88	0
25M106	0.43
25M107	0.45
25M108	0.38
25M109	0.49
25M110	0.31
25M111	0.34
25M113	0.32
25M119	0.34
25M121	0.33
25M122	0.34
25M123	0.43
25M139	0.44
25M140	0.47
25M141	0.42
25M144	0.52
25M145	0.54
25M146	0.68

Table 15 (continue)

Strain	Ethanol, % W/V
25M149	0.37
25M150	0.40
25M159	0.36
25M160	0.4
25M161	0.38
25M162	0.44
25M163	0.48
25M164	0
25M165	0.52
25M202	0.52
25M205	0.48
25M214	0.56
25M219	0.53
25M220	0.47
25M224	0.10
25M229	0.20
25M230	0.12
25M231	0.20
25M232	0.22
25M233	0.07
25M234	0.24
25M235	0.24
25M244	0.12
25M245	0.24
25M251	0.13

Table 15 (continue)

Strain	Ethanol, % W/V
25M255	0.27
25M256	0.26
25M258	0.15
25M263	0.11
25M264	0.21
25M265	0.18
25M266	0.21
25M267	0.26
25M321	0.20
25M322	0.28
25M323	0.40
25M324	0.30
25M325	0.48
25M326	0.29

2.8 Protoplast fusion of S. cerevisiae AM12 and strain S090

Protoplast fusion was carried out between S. cerevisiae AM12, the high ethanol producing strain and S090, the halotolerant strain. From this cross 157 fusants were obtained. They are going to test the growth on salted molasses agar at high temperature, 37 and 40 c.

2.9 Protoplast fusion of S. cerevisiae AM12 and S. cerevisiae M30

Another cross of fusion was performed between two selected high ethanol producing strains of S. cerevisiae, AM12 and M30. Two hundred and eleven fusants obtained from this cross are going to test the growth on salted molasses agar at high temperature.

This project is proposed to construct a new hybrid which can produce high ethanol concentration in high salinity molasses mash therefore we intend to hybridize each of 3 high ethanol producing strains of S. cerevisiae with each of 9 halotolerant strains by protoplast fusion technique. Therefore a total of 24 crosses is planned to carried out. At this stage 18 crosses were successfully completed as showed in Table 16. Unfortunately, very few hybrids showed better capacity to produce ethanol in salted molasses medium at high temperature than their parental strains.

Table 16 Summarization of the successful protoplast fusion between the high ethanol producing strain and halololerant strain

No.	cross	No. of fusant obtained	No.of fusant produced ethanol more than the parent*	Remark
1.	AM12 x S013	95	0	obtained in the last reporting period
2.	AM12 x S037(1)	3	2	obtained in the last reporting period
	AM12 x S037(2)	122	NYT	obtained in this reporting period
3.	AM12 x S065	594	0	obtained in the last reporting period
4.	AM12 x S090(1)	35	0	obtained in the last reporting period
	AM12 x S090(2)	157	NYT	obtained in this reporting period
5.	AM12 x 251	12	0	obtained in the last reporting period
6.	AM12 x 356	38	0	obtained in the last reporting period
7.	Sc90 x S013(1)	183	0	obtained in the last reporting period

Table 16 (continue)

No.	cross	No. of fusant obtained	No.of fusant produced ethanol more than the parent*	Remark
7.	Sc90 x S013(2)	2	NYT	obtained in the last reporting period
8.	Sc90 x S037	8	0	obtained in the last reporting period
9.	Sc90 x S065	954	0	obtained in the last reporting period
10.	Sc90 x S090(1)	222	4	obtained in the last reporting period
	Sc90 x S090(2)	104	0	obtained in the last reporting period
11.	Sc90 x 251	5	1	obtained in the last reporting period
12.	Sc90 x 356(1)	249	0	obtained in this reporting period
	Sc90 x 356(2)	114	NYT	obtained in this reporting period

Table 16 (continue)

No.	cross	No. of fusant obtained	No.of fusant produced ethanol more than the parent*	Remark
13.	M30 x S013	217	NYT	obtained in this reporting period
14.	M30 x S037	148	0	obtained in this reporting period
15.	M30 x S065	152	NYT	obtained in this reporting period
16.	M30 x S090(1)	79	0	obtained in this reporting period
	M30 x S090(2)	4	0	obtained in this reporting period
17.	M30 x 251(1)	41	0	obtained in this reporting period
	M30 x 251(2)	338	0	obtained in this reporting period
18.	AM12 x M30	211	NYT	obtained in this reporting period
	Total	4087	7	

NYT = Not Yet Test

* In molasses medium containing 3 % NaCl using standing cultivation, at 40 c.

Conclusion

The works had been performed in the last 6 months (September, 1988 to February, 1989) could be summarized as follows:

1. Nine fusants obtained by protoplast fusion of S. cerevisiae Sc90 and S090 were tested for ethanol production in 3% sodium chloride molasses medium at 40 c using shaking flask cultivation. The result revealed that no fusant produced ethanol higher than that produced by both parental strains.

2. In this reporting period 7 crosses between high ethanol producing strains 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 Sc90 x 356 : 363 fusants
2) S. cerevisiae AM12 x S037 : 122 fusants
3) S. cerevisiae M30 x S013 : 217 fusants
4) S. cerevisiae M30 x S037 : 148 fusants
5) S. cerevisiae M30 x S065 : 152 fusants
6) S. cerevisiae M30 x S090 : 83 fusants
7) S. cerevisiae M30 x 251 : 379 fusants

Some fusants obtained were already screened for the potent fusant. So far no fusant showed better ability of ethanol production in high salinity molasses mash at high temperature.

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.

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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