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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 10/22/87

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
PR#3 rec'd 10/22/87

Attachment

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Progress Report No. 3

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 Tantirungkit
Project Advisor	: Dr. Jaroon Kumnuanta
Project Consultants	: Prof. Dr. G.R. Fink Prof. Dr. J. 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, 1986 to August 31, 1987

Budget Allocation for This Period

: 835,470.14 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 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 concentration of brackstrap molasses itself, the combined effects of which are detrimental to fermentation activities. The inhibiting effect on yeast growth enables other microorganism 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, especially for yeasts. 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 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, a 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 flocculated hybrids from a flocculated 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 salt concentrations are 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% ethanol in the presence of 18% sodium chloride and 3.2% without salt (Onishi, 1963). Yamagata and Fujita (1970) isolated and identified several strains of halotolerant yeasts 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 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. In the last reporting period 2 steps of screening were already performed. Seven strains; 016, S013, S019, S037, S065, S090 and 251 were selected at 5% sodium chloride. And at 17% sodium chloride 11 strains; strain 065, 090, 092, 126, 130, 229, 246, 346, 356, 366, 370 and 390 were selected.

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

In this reporting period final screening of halotolerant strains at both level of sodium chloride were carried out. The selective marker of some selected high ethanol producing strains and halotolerant strains were already constructed. Hybridization between high ethanol producing strains and halotolerant strains through protoplast fusion were carried out with successful between some pairs.

Objectives

Overall objectives

The project is aimed at constructing a new hybrid from two different foundation strains, namely halotolerant strain and an industrial ethanol production 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 production 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 finally select potent halotolerant yeast to be used as parental strain for protoplast fusion.

2. To construct the selective (genetic) marker of both parental strains for protoplast fusion.

3. To construct the new hybrid by hybridization of high ethanol producing strain and halotolerant strain through protoplast fusion technique.

Materials and Methods

1. Final screening for potent halotolerant yeast.

1.1 At 5% sodium chloride.

The composition of medium used was molasses of 20% sucrose equivalent sugar concentration, 5% sodium chloride and 0.05% ammonium sulfate.

The strains obtained from secondary screening were cultured in 5-liter fermentor using 3 liters working volume. Inoculum was cells cultivated in 5% glucose-YPD medium on rotary shaker 150 rpm, at 37° C for 17-18 hours. Initial pH of fermentation medium was 4.5 . During cultivation in fermentor temperature was controlled at 40° C and agitation was 200 rpm. Culture broth was analysed for concentration of ethanol and cell every 4-6 h.

1.2 At 17% sodium chloride

At 17% sodium chloride, medium consisted of molasses of 5% sucrose equivalent sugar concentration, 17% sodium chloride and 0.05% ammonium sulfate was used.

Selected strains obtained from secondary screening were cultivated in 500 ml. Erlenmeyer flask using 200 ml.

of medium. The incubation was performed at 40° C on rotary shaker at 70-80 rpm. Cell and ethanol concentration were analysed every 12 hours.

2. Identification of selected halotolerant strains

The taxonomic characteristics of selected halotolerant strains were studied according to the methods described in "The Yeast, a taxonomic study", second revised edition by Lodder (1970).

3. Construction selective (genetic) marker of selected parental strains

3.1 Isolation of auxotrophic mutant

Cells were mutagenized by treatment with ethyl methanesulfonate(EMS) or N-methyl,N'-nitro-N-nitrosoguanidine(NTG) following the method of Fink (1986) with some modification. And treatment of spores by EMS was done following the method of Oshima and Takano(1980) with some modification.

3.2 Isolation of respiratory deficient mutant

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

4. 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).

5. Screening of hybrid fusant

Screening for high ethanol producing fusants are being carried out using salted molasses medium at 40° C.

6. 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. Final screening for potent halotolerant yeast

1.1 At 5% sodium chloride.

At this sodium chloride concentration, good ethanol producing strains were expected to obtain. The 7 strains, i.e. S013, S019, S037, S065, S090, 016 and 251 obtained from secondary screening were final screened. The result of ethanol production by these selected strains was shown in Fig.1,a. The results revealed that strain S013, S037 and 251 produced almost the same amount of ethanol but at different incubation period. Strain S013 produced 3.2% by weight at 60 hours while strain S037 and 251 produced 3.1% at 72 hours and 3.0% at 48 hours, respectively. Strain S065, S090 S019 and 016 produced ethanol 2.9% at 72 hours, 2.7% at 72 hours, 2.2% at 72 hours and 1.4% at 60 hours, respectively.

The result of growth by these 7 strains was presented in Fig.1,b. Strain 251 showed highest growth OD 66o nm was 10.6 . While growth of strain S037, S013, S065 and S090 gave OD as 7.9, 5.7, 4.8 and 4.1, respectively. Very low growth was obtained by strain S019 and 016 which OD were 1.8 and 0.9, respectively.

By consideration the result of ethanol production the strain S013, S037, 251, S065 and S090 were selected to be used as halotolerant parent.

1.2 At 17% sodium chloride.

To obtain halotolerant yeast which could grow at high concentration of sodium chloride 17% sodium chloride was used. Twelve strains, i.e. 065, 090, 092, 126, 130, 229, 246, 346, 356, 366, 370 and 394 obtained from secondary screening were investigated for growth and ethanol production by shaking flask cultivation. The result of growth by these 12 strains were shown in Fig.2,a. The result presented that strain 370 gave highest cell concentration, OD 660 nm was 4.8 at 72 hours and by strain 356 was 4.6. While the other 10 strains did not show much different in optical density. Therefore, strain 370 and 356 were selected based on this good growth. However, the ethanol produced by these 12 strains were compared with the result that only 4 strains produced ethanol more than 1% by weight (Fig.2,b). Two of them were strain 370 and 356 each produced ethanol 1.6% by weight. The other two were strain 126 and 130 which produced ethanol 1.5 and 1.4% by weight, respectively. Hence, a total of 4 strains, i.e. 370, 356, 126, and 130 were selected for hybridization which high ethanol producing strain.

2. Identification of selected halotolerant strains

From the characteristics described in Table 1 and 2, the strains selected at 5% sodium chloride, i.e. S013, S037, 251, S065 and S090 were identified according to the key of general and species in "The Yeast, a taxonomic study", second edition as follows;

S013 was identified as Torulopsis grabrata (Fig.3,a)
S037 was identified as Torulopsis candida (Fig. 3,b)
251 was identified as Torulopsis bovina (Fig. 3,c)
S065 was identified as Saccharomyces rouxii (Fig. 3,d)
S090 was identified as Torulopsis bovina (Fig. 3,e)

When considered the characteristics described in Table 3 and Table 4, the 4 strains obtained by selection at 17% sodium chloride, i.e. 370, 356, 126 and 130 were difficult to identify. However, by their ability to assimilate nitrate and formation of ascospore with warty wall (Fig. 4), these 4 strains could be identified as Citromyces spp. But ascus wall of these 4 strains was different from the ascus wall described in the standard description for genus Citromyces. Since the ascus wall described was thick but of these 4 strains was easy to rupture. And from the other characteristics excepted the ability to assimilate potassium nitrate these 4 strains were close to Torulaspora group, especially Saccharomyces fermentati. However, their

characteristics were closer to *Citromyces spp.* than to *S. fermentati*.

3. Construction of selective (genetic) marker by mutation

To facilitate the selection of hybrid fusants, auxotrophic mutant is being used as one parental strain while another parent is respiratory deficient mutant.

At first auxotrophic mutants of selected halotolerant strains were isolated by ethylmethane sulfonate(EMS) and the result was shown in Table 5. The result presented that by using EMS auxotrophic mutant could be isolated only from the strains screened at 5% sodium chloride. We tried to isolate auxotrophic mutant by using UV and EMS with UV. However, no mutant could be isolated. So far, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) are being used. The 3 mutants of strain 126 and 2 of strain 356 were successfully isolated. And the other 2 strains are being mutagenized using NTG.

Respiratory deficient mutant of high ethanol producing strains, *S. cerevisiae* Sc90, M30 and AM12 were isolated and the result was shown in Table 6.

4. Protoplast fusion

Each of selected halotolerant strain is intended to hybridize with each of high ethanol producing strain using protoplast fusion technique. So far, successful protoplast fusion was obtained from some crosses as shown in Table 7. The result presented that although many colonies were picked from selective regeneration medium (minimal medium containing glycerol), on which both parental strains could not grow but fusion product could regenerate and further grow, only lower than half of fusion hybrids were obtained from each cross. This might due to segregation of fusion products when they were transferred to new selective medium.

The hybrids fusant obtained are being investigated for ethanol production in salted molasses medium at 40° C.

Conclusion

The work that has been performed in last 12 months (August, 1986 to August, 1987) could be summarized as followed:

1. Final screen for potent halotolerant yeast for hybridization of two levels of sodium chloride. At 5% sodium chloride, strains S013, S037, 251, S065 and S090 were selected. While at 17% sodium chloride strain 370, 356, 126 and 130 were selected.

The selected strains were identified according to the method described in "The yeast, a taxonomic study" second revision edition. Strain S013 was identified as Torulopsis grabata. Strain S037 was Torulopsis candida. Strain 251 and S090 were identified as Torulopsis bovina. And strain S065 was Saccharomyces rouxii. The other 4 strains obtained by selection at 17% sodium chloride, i.e. 370, 356, 126 and 130 were roughly identified as Citromyces spp.

2. To facilitate the selection of hybrid fusant selective (genetic) markers of selected halotolerant and high ethanol producing strains were constructed by mutation. The auxotrophic mutants of all strains selected at 5% sodium chloride were able to isolate by EMS treatment. But no auxotrophic mutant of the strains

selected at 17% sodium chloride were obtained by treatment with EMS, UV or EMS with UV. However, auxotrophic mutants of strain 126 and 356 were able to isolate using NTG, so far. While halotolerant parental strains are auxotrophic mutant high ethanol producing strain should be respiratory deficient mutant. Therefore, respiratory deficient mutant of selected high ethanol producing strains, M30, AM12 and Sc90 were successfully isolated to be used as another parent.

3. So far, successful protoplast fusion was obtained from some crosses, i.e. AM12 with S013, AM12 with S037, AM12 with S090, AM12 with 251, Sc90 with S013, Sc90 with S037, Sc90 with S090 and Sc90 with 251. Totally 1,078 hybrid fusants were selected on selective regeneration medium.

Screening of potent hybrid fusants are being carried out by investigation of ethanol production in salted molasses medium at 40° C.

Work plan for the next period

Work plan for the next period is scheduled as follows:

1. Continue construction of selective marker by mutation of some selected strains that could not construct in the last reporting period.
2. Continue protoplast fusion of selected strains.
3. Screening of the potent hybrid fusant by investigation of ethanol production in salted molasses medium at high temperature.
4. Study on parameters of ethanol fermentation of selected hybrid fusant at laboratory scale.

References

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Table 1 Taxonomic characteristics of halotolerant strains selected at 5% sodium chloride.

		Strain no.				
		S013	S037	251	S065	S090
Cell shape		Ovoid	Spherical, ovoid	Spherical, ovoid	Spherical, ellipsoidal amoeboid	Ovoid
Cell size	wide	3-5 u	4-7 u	2.5-6 μ	2.5-4 μ	4-6 μ
	long	4-7 u	5-8 μ	4-8 μ	4-6 μ	5-8 μ
Cell arrangement		singly	singly	singly pair, group	singly, pair small cluster	singly
Pseudomycelium		form	not form	not form	not form	not form
Vegetative reproduction		Multilateral budding				
Sexual spore		not found				
KNO3 assimilation		-	-	-	-	-
Ethylamine HCl assimilation		-	-	-	+	+
Growth in vitamin free medium		-	+	-	-	-
Growth on glucose yeast extract agar						
1. containing 50% glucose		-	-	+w	+	+
2. containing 60% glucose		-	-	+w	+	+

+w = Weak

Table 2 Fermentation of various sugars and assimilation of carbon compounds by the strains selected at 5% NaCl.

Carbon compounds	Strain no.				
	S013	S037	251	S065	S090
Fermentation					
glucose	+	+	+	+	+
sucrose	-	+	-	-	-
galactose	-	+	-	-	-
maltose	-	+	-	-	-
lactose	-	-	-	-	-
raffinose	-	+	-	-	-
mellibiose	-	-	-	-	-
Assimilation					
glucose	+	+	+	+	+
sucrose	-	+	-	-	-
galactose	-	+	-	+	-
maltose	-	+	-	-	-
lactose	-	-	-	-	-
raffinose	-	+	-	-	-
trehalose	+	+	-	-	-
mellibiose	-	+	-	-	-
mannitol	-	+	-	+	-
soluble starch	-	-	-	+	-
L-arabinose	-	-	-	-	-
glycerol	+	-	+w	+	-

+w = Weak

Table 3 Taxonomic characteristics of halotolerant yeast strains selected at 17% Sodium chloride.

		Strain no.			
		126	130	356	370
Cell shape		Spherical, subglobose	Spherical, subglobose	Spherical, subglobose	Spherical, subglobose
Cell size	wide	4-10 μ	3.5-8 μ	3.5-8 μ	3.5-8 μ
	long	4-10 μ	3.5-8 μ	3.5-8 μ	3.5-8 μ
Cell arrangement		singly	singly	singly	singly
Pseudomycelium		form	form	form	form
Vegetative reproduction		Multilateral budding			
Ascospore formation		form	form	form	form
Number of ascospore per ascus(Fig. 4)		1	1	1	1
Ascospore shape		Spheroidal	Spheroidal	Spheroidal	Spheroidal
Ascospore wall		warty	warty	warty	warty

Table 4 Fermentation of various sugars and assimilation of carbon compounds by the strain selected at 17% NaCl.

Carbon compounds	Strain no.			
	126	130	356	370
Fermentation				
glucose	+	+	+	+
sucrose	+	+	+	+
galactose	-	-	-	-
maltose	+	+	+	+
lactose	-	-	-	-
raffinose	+	+	+	+
mellibiose	-	-	-	-
Assimilation				
glucose	+	+	+	+
sucrose	+	+	+	+
galactose	-	-	-	-
maltose	+	+	+	+
lactose	-	-	-	-
raffinose	+	+	+	+
trehalose	-	-	-	-
mellibiose	-	-	-	-
mannitol	+	+	+	+
soluble starch	-	-	-	-
L-arabinose	-	-	-	-
glycerol	+w	+w	+w	+w

+w = Weak

Table 5 Number of auxotrophic mutants of selected halotolerant yeasts obtained by treatment with EMS.

Strain	No. of auxotrophic mutant obtained
Selected at 5% NaCl	
S013	42
S037	27
S065	31
S090	3
S251	7
Selected at 17% NaCl	
126	0
130	0
356	0
370	0

Table 6 Number of respiratory deficient mutants of selected high ethanol producing strains of *Saccharomyces cerevisiae* obtained.

Strain	No. of respiratory deficient mutant obtained
M30	16
AM12	11
Sc90	8

Table 7 Successful protoplast fusion.

Cross	No. of fusion performed	No. of colony picked from selective regeneration medium	No. of fusant obtained
AM12(RD) X S013(56A)	4	1170	298
AM12(RD) X S037(73A)	2	1322	4
AM12(RD) X S090(A)	1	580	2
AM12(RD) X 251(A)	1	1148	12
Sc90(RD) X S013(56A)	3	990	177
Sc90(RD) X S037(73A)	1	1152	8
Sc90(RD) X S090(A)	4	1890	587
Sc90(RD) X 251(A)	2	1080	5

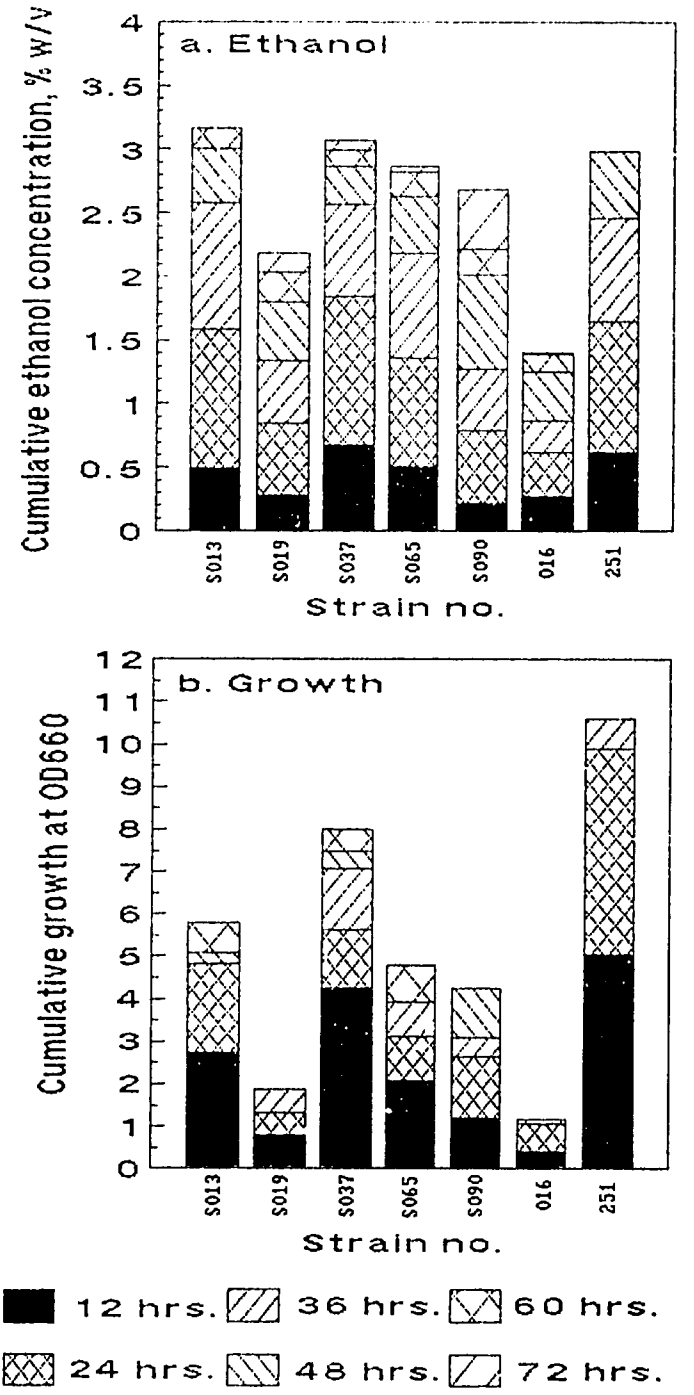

Figure 1 Ethanol production(a) and growth(b) by halotolerant strains in molasses medium containing 5% NaCl, at 40° C.

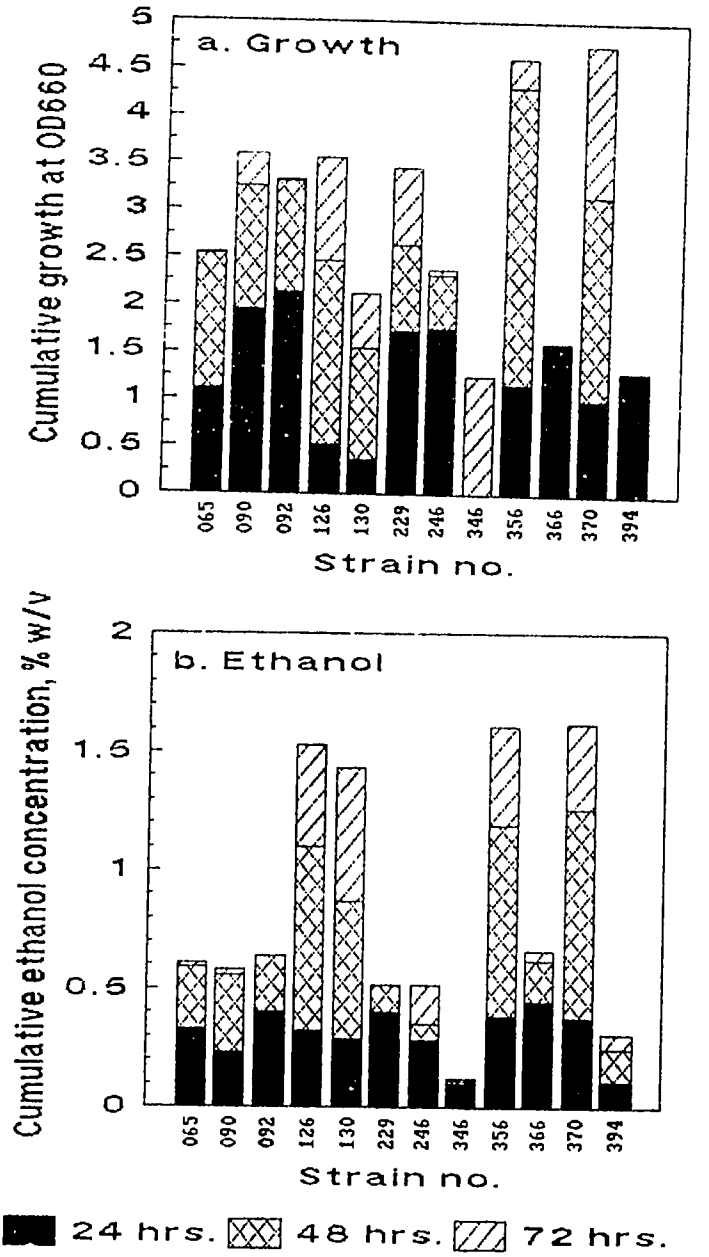

Figure 2 Growth(a) and ethanol production(b) by halotolerant strains in molasses medium containing 17% NaCl, at 40° C.

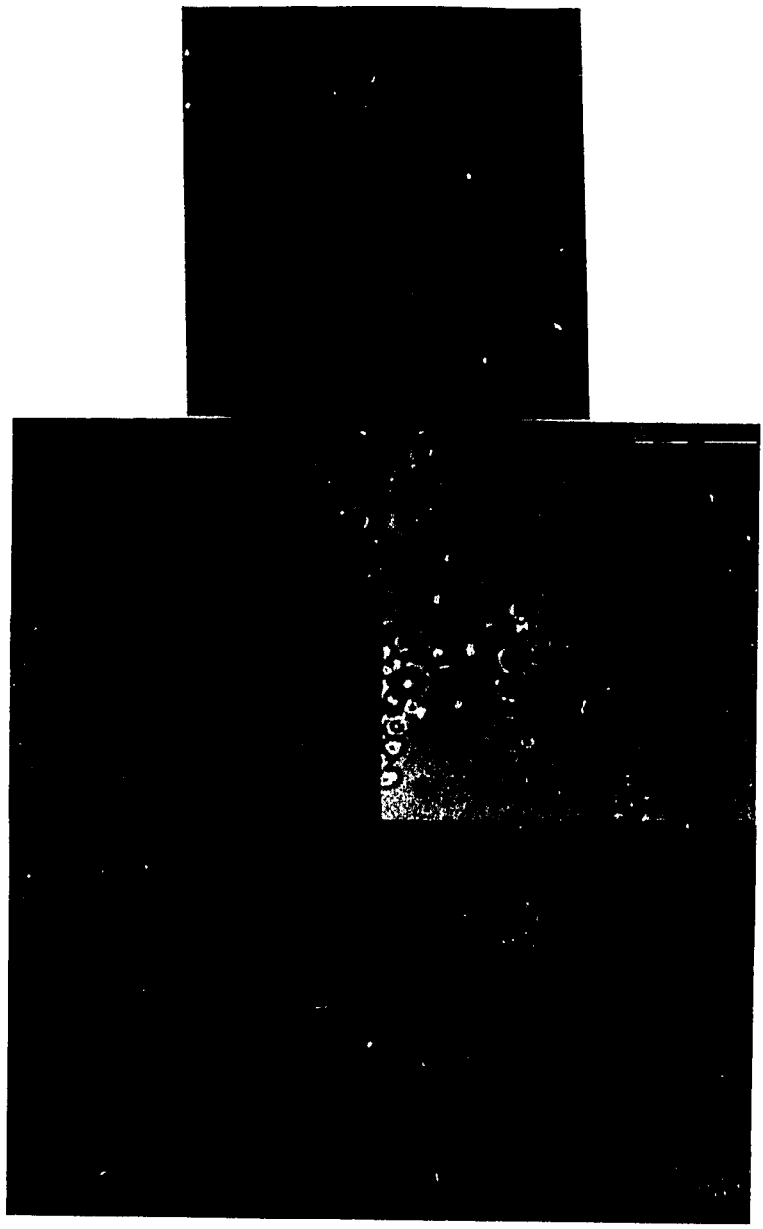

Figure 3 Photograph showed vegetative cells of halotolerant strains selected at 5% NaCl (a) strain S013, (b) strain S037, (c) strain 251, (d) strain S065 and (e) strain S090 (1000X).

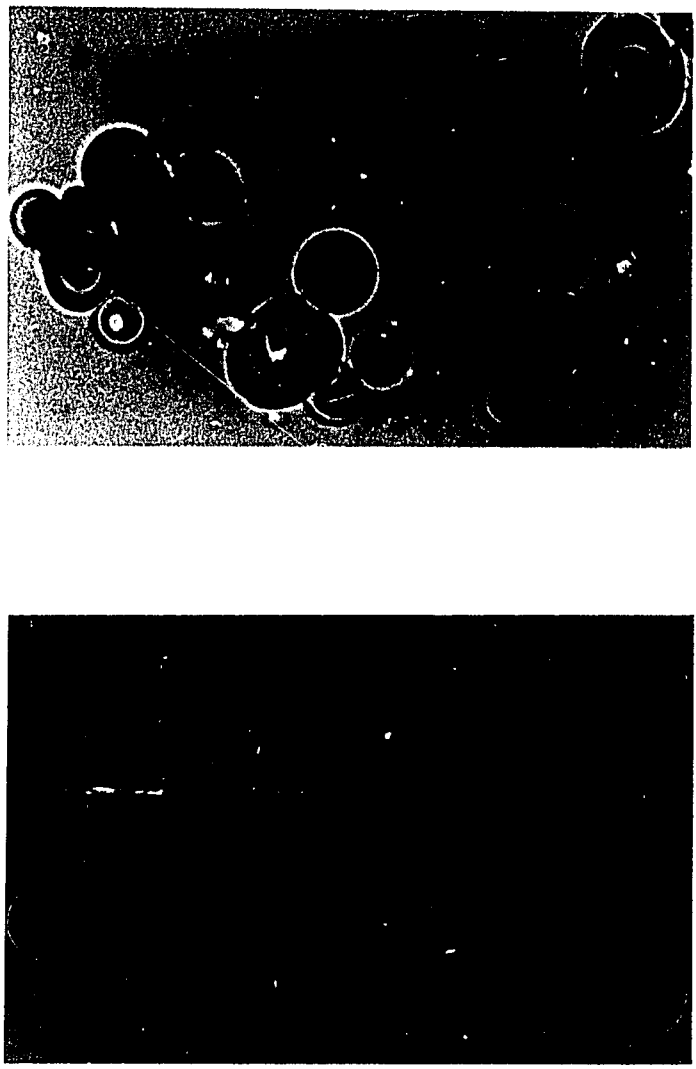

Figure 4 Photograph showed one ascospore in an ascus of (a) strain 126 and (b) strain 356 (1000X).