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AGENCY FOR INTERNATIONAL DEVELOPMENT
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DATE: 8/9/88

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. 238
PR Aug 85 - Jul 86

Attachment

PROGRESS REPORT

(August 1985 - February 1986)

THAILAND-USAID PROJECT

ON

Hybridization of Halotolerant Yeast for the Production of Alcohol Fuels

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Backgroud Information

This progress report covers the work period from initiation of the project August 1985 to February 1986. Therefore the first activity was procurement of equipments necessary for research work of this project and recruitment of research personnels. The financial plan and activity plan are attached herewith. The second activity was concerning with the research work of the project which will be discussed later on. Since this is the first progress report on the research work, the following paragraph is describing the back ground of the project.

Overall objectives of this project is to construct a new yeast hybrid from two different foundation strains, namely, halotolerant strain and industrial ethanol production strain, through protoplast fusion technique. The halotolerant yeast which tolerates to high salt concentration will be used as a parent strain. The industrial ethanol production strain, *Saccharomyces cerevisiae* , which can ferment cane molasses at high rate and yield high ethanol concentration (over 12 % by volume) will be used as another parent strain. The hybrid obtained from fusion will incorporate the characteristics of producing high ethanol concentration in high salinity mash which derived from the recombination of desirable characteristics from the parent strains. This project aims to use protoplast fusion technique for hybridization of

yeast strains mentioned earlier for construction of hybrid strain which is difficult to be achieved by the conventional mating technique. Consequently, the new hybrid strains obtained will be tested under laboratory condition and pilot scale trial for their performances of fermentation.

It is anticipated that the selected hybrid will be used for industrial ethanol production, especially for distilleries where brackish water or high salinity well water is the only available resources.

The technical work plan of this three years project is devided into 3 phases as follow:

Year 1; Isolation, collection and selection of halotolerant yeast to be used as a parent strain for protoplast fusion.

Yesr 2; Hybridization of selected halotolerant strains with industrial ethanol production strains and test for their performances.

Year 3; Study on ethanol fermentation characteristics and kinetic studies of selected hybrids in salinity mash at laboratory and pilot scale.

The target goal of research in this period is to isolate and collect halotolerant yeast to be used as a parent for protoplast fusion. This report describes the progress of activities dealing with collection of samples from brine fermented products and isolation of fermentative halotolerant yeast from those samples. Selection of the haloto-

lerant yeast strains from stock cultures also included. The performance of ethanol fermentation by industrial alcohol production strains to be used as one parent will be investigated.

Introduction

Halotolerant or salt-tolerant yeast, a kind of osmophilic yeast, is usually present in various kinds of fermented food where high salt concentration are used in the processes such as miso, soysauce, fermented vegetables etc. (Onishi, 1963; Yong and Wood, 1974) or in products of high sugar content such as honey, molasses, etc. (Onishi, 1963). Predominant strain usually found in these products is Saccharomyces rouxii which can tolerate to 20-22% of sodium chloride (Lee and Lee, 1970). Mori and Windisch (1982) found that most of the salt-tolerant strains isolated from shoyu mashes and miso pastes can tolerate to concentration of salt over 3.5 molar of sodium chloride. Although S. rouxii can produce ethanol (Lee et al, 1971), however the efficiency of alcohol fermentation by these strains are very low, for instance, S. rouxii var. halomembranis produced only 1.9 % of 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 yeast from

miso and soya mash and found that the tolerant range of yeast varied widely between 0-22 % sodium chloride. Dhevahuti (1979) investigated S. rouxii in fermenting soy mash and found that this species involued in soysauce fermentation in Thailand as well and the two mating types were found in different plans.

Meterials and Methods

I. Collection of samples for isolation of halotolerant yeast

1. Type of samples The samples suitable for isolation of yeast are various kinds of fermented food, food and products of high sugar content, such as fermented fish, soysauce, miso, pickle, palm sugar, molasses, etc.

2. Sampling area Samples were collected from many provinces in every parts of Thailand in order to obtain different strains from various locations to cover the broad base of distribution.

3. Sampling method Both solid and liquid samples about 20-50 grams were collected in plastic sampling bag. The samples were collected at the active fermenting stage wherever possible.

II. Isolation of halotolerant yeast

Isolation procedure was divided into 2 steps. First the samples were enriched in enrichment medium A and B . Medium consisted of molasses of 10 % sucrose equivalent sugar concentration, 0.05 % ammonium sulfate and 5 % sodium

chloride. Medium B consisted of molasses of 5 % sucrose equivalent sugar, 0.05 % ammonium sulfate and 17 % sodium chloride. Cultivation was performed at 37 and 40 °c for 3 days. Second step, the fermented mash of both A and B medium was streak on molasses agar A and B, respectively, and incubated at 37 and 40 °c. After colonies appeared on molasses agar they were restreaked on the same medium to purify the cultures and kept in molasses medium A as stock cultures. The purpose of using medium A is to isolate the good alcohol fermenting yeasts while medium B is to isolate the salt-tolerant yeasts. The 5 % salt in medium A is relatively high for ethanol fermentation while in the real practice the salt in the mash will be less than 2 %. Also fermentation at 37 and 40 °c is required for the selection of yeast strains for ethanol fermentation in the tropics.

III. Selection of yeasts which can grow in the medium containing 5 % sodium chloride from stock yeast culture.

The stock cultures were obtained from the collection of 3 laboratories in Kasetsart University. The selection was performed by cultivation of all cultures on molasses medium containing molasses of 10 % sucrose equivalent sugar concentration, 0.05 % ammonium sulfate and 5 % sodium chloride (A), at 37 and 40 °c for 3 days then growth and amount of carbondioxide produced was investigated. Those culture having good growth with various fermentation were selected for further investigations.

IV. Preliminary investigation on ethonol fermentation ability of industrial yeast strains.

Ethanol production in molasses medium was carried out using flask culture. The fermentation medium consisted of molasses of 20 % sucrose equivalent sugar concentration and 0.05 % of ammonium sulfate and pH was adjusted to 4.5 The fermentation was done in 500 ml Erlenmeyer flask with 200 ml medium and incubated at 37 °c on reciprocal shaker, 80 rpm. Inoculum was prepared by cultivation of yeast in YPD medium (2 % glucose, 2 % polypeptone and 1 % yeast extract) at 37 °c on rotary shaker, 150 rpm for 18-20 h. Initial cell concentration of fermentation medium was adjusted to the optical density at 660 nm approximately 1.5. The sample was taken at appropriate cultivation time and analysed for concentration of ethanol and cell mass.

Ethanol was analysed using Liquid Gas chromatography under the following conditions: packing material polyethylene-glycol 20 M in glass column, 1 m length and 3 mm inner diameter, carrier gas nitrogen at 40 ml/min, temperature of column and injector, 70 and 125 °c, respectively, propanol was used as internal standard. Cell concentration was measured as optical density of the cell suspension at 660 nm.

Results

I. Collection of samples for isolation of halotolerant yeasts

A total of 216 samples were collected from many provinces in Thailand. Figure 1 indicated the locations which samples were collected. The 216 samples consisted of 65 samples of molasses, 71 samples of fermented fishes, 17 samples of different kinds of pickle, 23 samples of fermented soybean (miso), 17 samples of shrimp paste, 6 samples of fish sauce, 3 samples of fermented shells, 2 samples each of fermented mushrooms, palm sugar, soil from alcohol factory and distillery waste, 1 sample each of fermented pork, fermented shrimp, tamarind, dry salted squid, salted crab and salted lettuce.

II. Isolation of halotolerant yeast

Three hundred and thirty three strains of halotolerant yeasts were isolated from this 216 samples as shown in table 1. It was summarized in table 2 that 92 strains were isolated from molasses, 124 strains from fermented fishes, 44 strains from pickles, 31 strains from fermented soybean (miso), 10 strains from fermented mushroom, 8 strains from fermented shells, 2 strains from fermented pork and salted crab, 6 strains from palm sugar, 5 strains from soil in alcohol factory, 3 strains from distillery waste and salted lettuce and 1 strain from fermented shrimp,tamarind and dry salted squid. There was no halotolerant yeast isolated from shrimp paste and fish sauce.

Table 2 described the number of halotolerant yeast isolated from the molasses medium contained 5 and 17 % sodium chloride and at 37 and 40 °c. The result indicated that from 333 strains, 250 strains were isolated on 5 % sodium chloride containing molasses medium at 37 °c and 42 strains at 40 °c. Another 34 strains were isolated on 17 % sodium chloride containing molasses medium at 37 °c and 7 strains at 40 °c.

III. Selection of yeasts which can grow in the medium containing 5 % sodium chloride from stock yeast culture

A total of 693 strains obtained from stock yeast cultures were tested for the ability of growth on the molasses medium containing 5 % sodium chloride. The results are summarized in the table 3. Only 261 strains could grow on salted molasses medium and considered as halotolerant yeast.

IV. Summary of ethanol fermentation ability of industrial yeast strain

The industrial strains being used in this project were obtained from many sources as described in table 4.

V. Preliminary investigations of ethanol fermentation ability of industrial yeast strains.

Figure 2 and 3 represented time-course of ethanol production and growth of cells, at 35 °c from *S. cerevisiae* strains TJ 1, TJ 3, Sc 90, M 30, N 1, B 120, 191 and 281. The result indicated that *S. cerevisiae* Sc 90 which currently

used in most factories produced highest ethanol concentration, 9.15 % by weight after 48 h, while strain AM 12, B 120, M 30, 281 and 191 produced 8.89, 8.81, 8.68, 8.29, and 8.13 % by weight of ethanol, respectively. The other 3 strains TJ 1, TJ 3 and N 1 produced only 7.16, 4.63 and 6.55 % by weight of ethanol after 48 h of cultivation. Considering from cell mass strain AM 12 given highest cell concentration as measured by optical density at 660 nm was 22.92. The optical density of strain Sc 90, 191, 281, B 120 and N 1 were 20.85, 20.19, 19.95, 19.14 and 19.11, respectively, while TJ 3 and TJ 1 were 9.78 and 6.93, respectively.

Conclusion

The work that has been carried out in the first 6 months (August 1985 to February 1986) could be summarized as follows :

1. Halotolerant strains have been isolated from various sources of brine fermented products and molasses collected from different parts of the country.

2. Isolation of halotolerant strains were carried out by enrichment of the medium selective for fermentative yeasts that can proliferate and ferment at 5 and 17 % salt concentrations. The strains also selected for their ability to grow at 37 and 40 °c.

3. A total of 333 strains of halotolerant yeasts were isolated for further screening. Another 693 strains from culture collections also included in the test.

4. Further screening program will be the selection of good fermenting yeast at 5 % salt concentration. Another group to be selected is the 17 % salt tolerant yeasts with moderate fermenting activity. In addition a few strains of Saccharomyces rouxii will be obtained from other culture collections. After selection they will be used as halotolerant parental strain for hybridization.

5. Nine strains of yeast for industrial ethanol fermentation were tested for their ability to ferment ethanol at high rate and it was found that the strain currently used by most of the factory is the best one. So this strain will be used as ethanol producing parental strain for hybridization with the halotolerant strains.

6. Selection of the halotolerant strains will be continued in the next period.

Plan of work in the next period

In the next period the strains to be used as both parents will be selected and work plan is scheduled as the following :

1. Screening for potent halotolerant yeast to be used as a parent.

In this experiment all 594 strains of halotolerant yeasts will be screened for potent halotolerant yeast to be used for protoplast fusion. The screening will be divided into 2 systems. The first is to obtained the halotolerant yeast which can grow at high salt concentration, although ethanol production is relatively low. The second is to obtain the strains which can ferment ethanol at high rate and yield in 5 % sodium chloride containing molasses medium. Flow diagram of screening is shown in figure 4.

2. Screening for highest ethanol production strains from industrial yeasts to be used as another parent. The screening will be carried out in molasses medium with and without sodium chloride as shown in figure 5.

References

Boonyaratanakornkit, M., P. Karuwanna, L. Kraidej, S. Phoopat and P. Tammarate. 1981. Alcohol production from locally available raw materials. Final report presented to National research council of Thailand.

Dhevahuti, P. 1978. Study on yeast in soysauce and miso. M.S. Thesis, Kasetsart University. Bangkok, Thailand.

Kumnuanta, J., W. Yongmanitchai, P. Tammarate, P Leelasulitham. 1983. New yeast strain for alcohol production. Proceeding of the conference on Science and Technology. 27-29 October 1983.

Lee T.S. and S.K. Lee. 1970. Studies on yeast for brewing soysauce. IV. Culture condition of the osmophilic yeasts in high concentration of NaCl. J. of Korean Agr. Chem. Soc. 13(3): 193-195. Food Science and Technology Abstract. Vol.5.

Lee, T.S., S.K. Lee, Y.M. Chu and O.K. Shin .1971. Studies on yeasts for brewing soy sauce VI. Industrial utilization of *Saccharomyces rouxii* T9 in the brewing of soy sauce. J. of Korean Agr.Chem. Soc. 14(2): 121-129. Food Science and Technology Abstract. Vol.6, 3T168.

Mori H. and S. Windisch. 1982. Homothallism in sugar-tolerant *Saccharomyces rouxii* J. Ferment. Tech. 60(2).

Onishi, H. 1963. Osmophilic yeasts. Advances in Food Res. 12: 53-94.

Yamamoto, M. and S. Fukui. 1977. Fusion of yeast protoplasts. Agr. Biol. Chem. 41: 1829-1830.

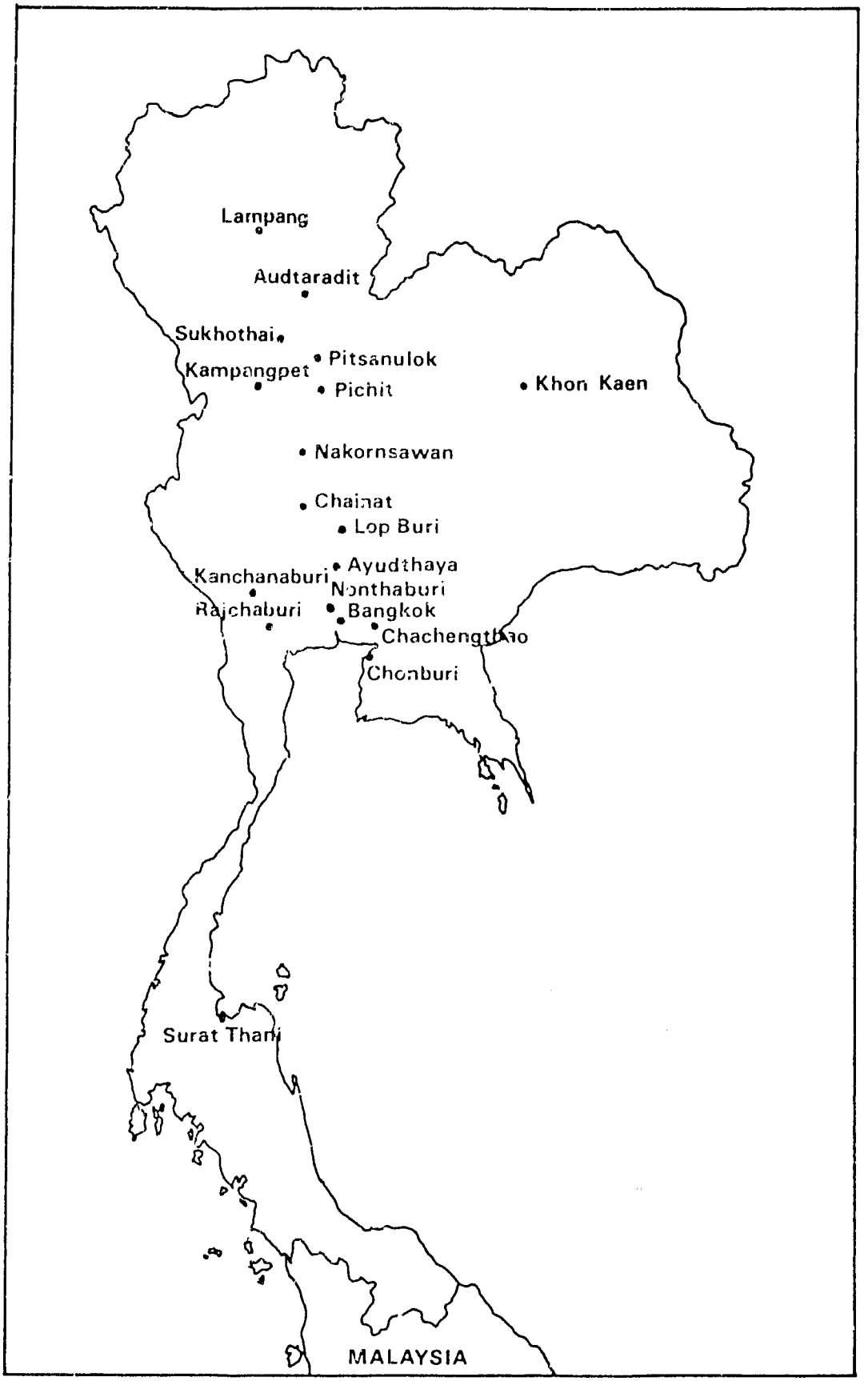

Fig. 1 Map of Thailand showing sites of sample collection.

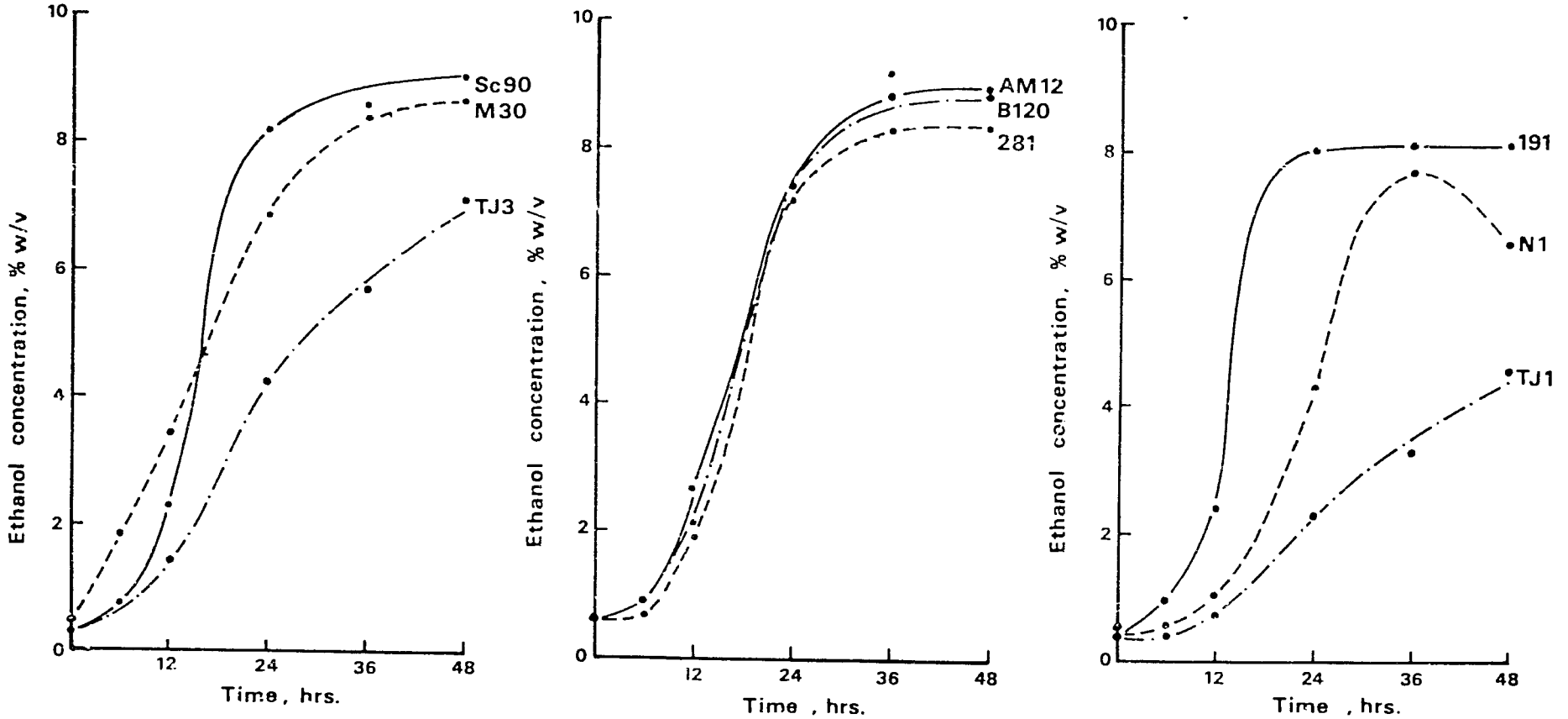

Fig.2 Ethanol production by collected industrial yeast strains in molasses medium.

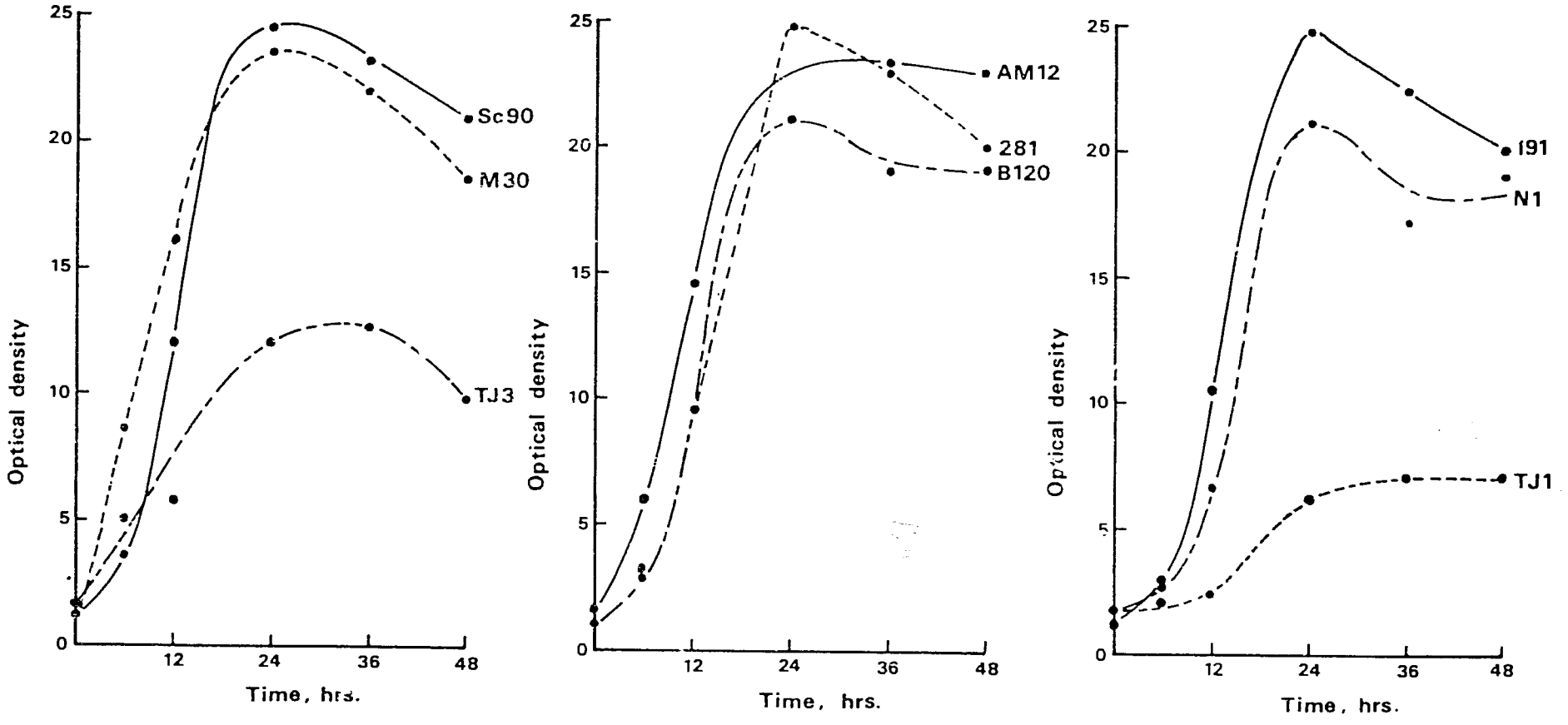

Fig.3 Growth of collected industrial yeast strains in molasses medium.

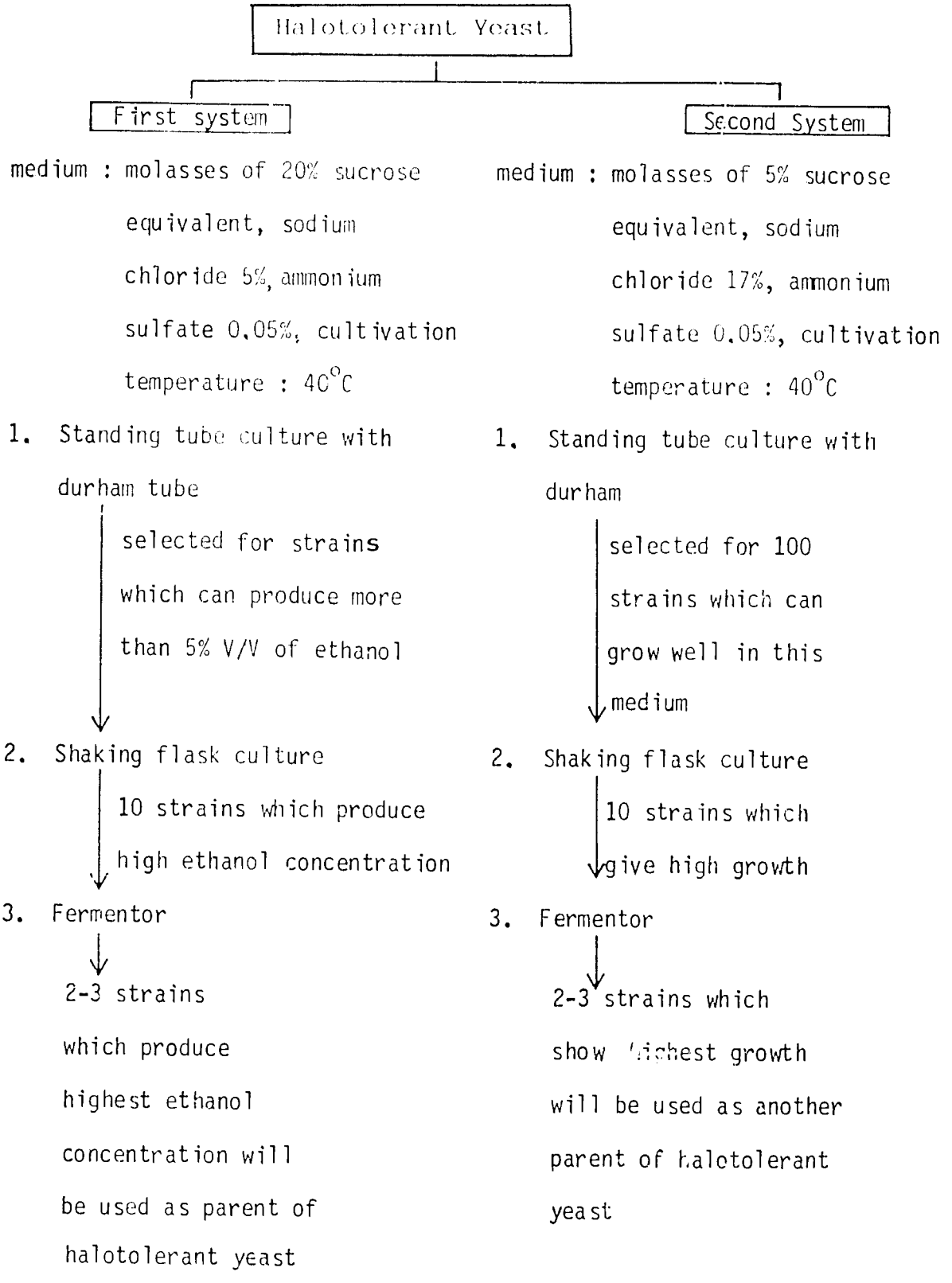

Figure 4 Flow diagram of screening for potent halotolerant yeast.

Industrial ethanol production strains

↓

1. Shaking flask culture using molasses medium (molasses of 20% sucrose equivalent, 0.05% ammonium sulfate) at 35°C

↓ select the strains which can produce more than 8% by weight of ethanol

2. Fermentation carry out in fermentor with and without sodium chloride at 40°C

↓

2-3 strains which can produce highest ethanol concentration in molasses medium without sodium chloride

Figure 5 Flow diagram of screening for highest ethanol production strains from industrial yeast

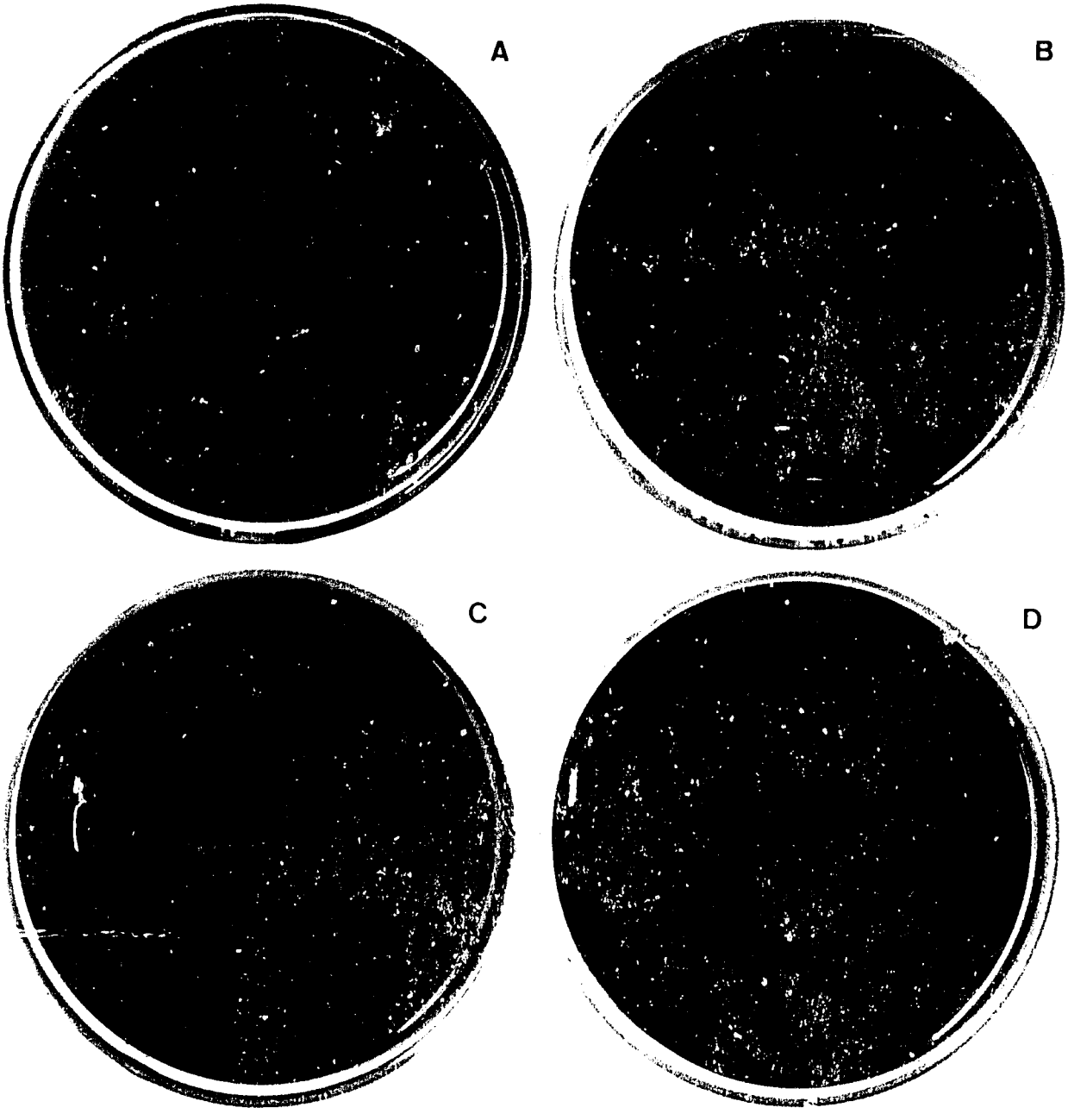

Fig.6 Photograph of colonies of some halotolerant yeasts cultivated on molasses medium contained 5% sodium chloride. A : strain no.157, B : strain no.227 C : strain no.228, D : strain no. 237

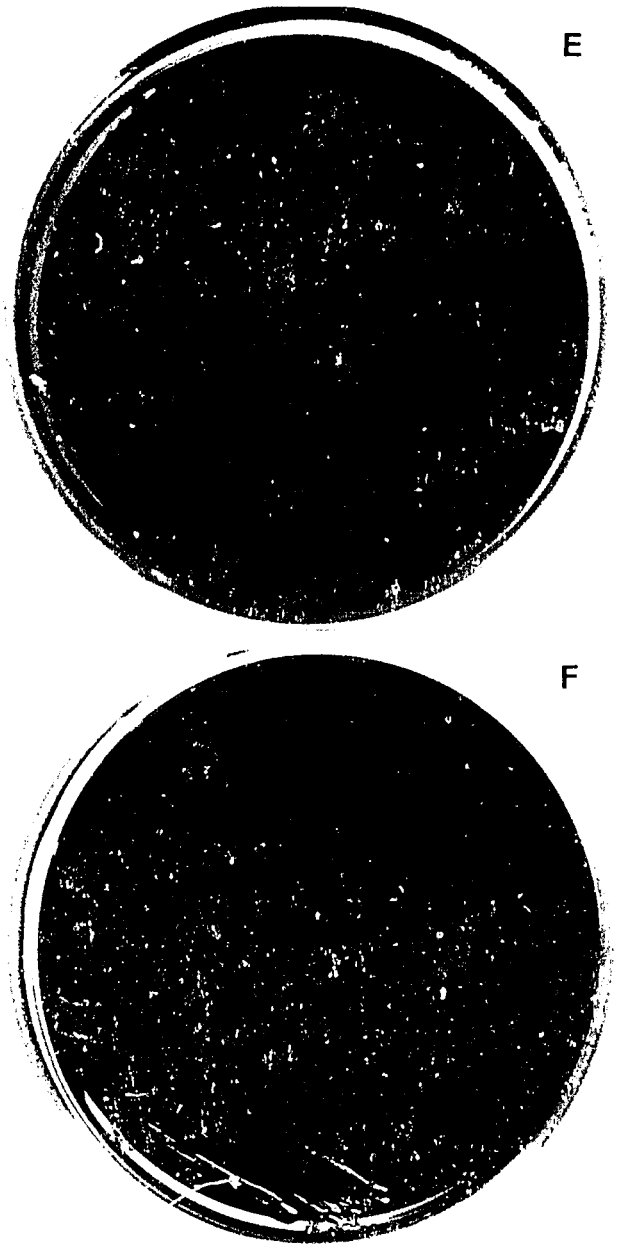

FIg.6 (Cont.)

E : strain no.248 , F : strain no. 252

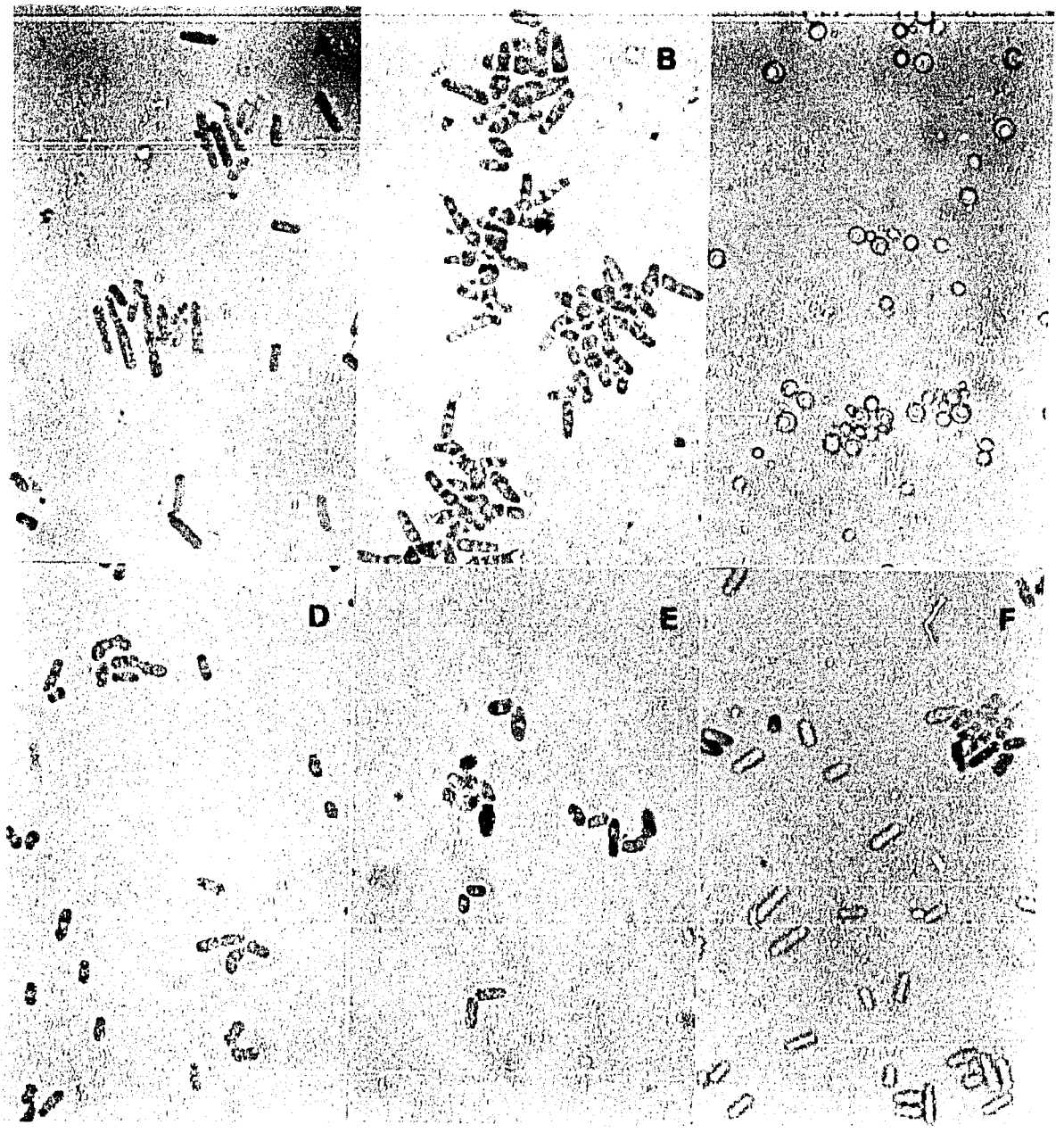

Fig.7 Micrograph of cells of some halotolerant yesats cultivated on molasses medium contained 5% sodium chloride. A : strain no.157, B : strain no. 227, C : strain no.228, D : strain no. 237, E : strain no.248, and F : strain no. 252.

Table 1 Lists of halotolerant yeasts isolated from various location in Thailand

sample No.	Product	Location	Isolation method 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
1	Bamboo shoot pickle	Nakornsawan	+				001
1	" ---- "	"		+			002
1	" ---- "	"		+			003
2	Lettuce pickle	Kanchanaburi	+				004
2	" ---- "	"	+				005
2	" ---- "	"		+			006
2	" ---- "	"		+			007
3	Lettuce pickle	Sukhothai	+				008
3	" ---- "	"		+			009
4	Fermented mushroom	Kampangpet	+				010
4	" ---- "	"	+				011
4	" ---- "	"			+		012
4	" ---- "	"		+			013
4	" ---- "	"		+			014
4	" ---- "	"				+	015
5	Onion pickle	Kanchanaburi	+				016
5	" ---- "	"	+				017
5	" ---- "	"		+			018
6	Fermented mushroom	Sukhothai	+				019
6	" ---- "	"			+		020
6	" ---- "	"			+		021
6	" ---- "	"		+			022
7	Bamboo shoot pickle	Kampangpet	+				023
8	Fermented shell	Pitsanulok	+				024
8	" ---- "	"			+		025
8	" ---- "	"		+			026
8	" ---- "	"				+	027

Table 1 (Cont.)

Sample No.	Product	Location	Isolation method 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain
9	Lettuce pickle	Pitsanulok	+				028
10	Lettuce pickle	Kampangpet	+				029
10	" ---- "	"	+				030
10	" ---- "	"	+				031
10	" ---- "	"		+			032
11	Pla-chom (Fermented fish)	Pitsanulok	+				033
11	" ---- "	"	+				034
12	Lettuce Pickle	Kampangpet	+				035
12	" ---- "	"		+			036
13	Bamboo shoot pickle	Pichit	+				037
13	" ---- "	"	+				038
13	" ---- "	"	+				039
13	" ---- "	"		+			040
13	" ---- "	"				+	041
14	Bamboo shoot pickle	Pichit	+				042
14	" ---- "	"	+				043
14	" ---- "	"		+			044
14	" ---- "	"		+			045
15	Lettuce pickle	Pichit	+				046
15	" ---- "	"	+				047
15	" ---- "	"		+			048
15	" ---- "	"		+			049

Table 1 (Cont.)

Sample No.	Product	Location	Isolation Method 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
16	Salted crab	Pitsanulok	+				050
16	" ---- "	"		+			051
17	" ---- "	"	+				052
17	" ---- "	"	+				053
17	" ---- "	"		+			054
17	" ---- "	"		+			055
18	Fermented Shell	Chachengthao	+				056
18	" ---- "	"		+			057
19	Miso (Fermented soy bean)	Chachengthao			+		058
19	" ---- "	"			+		059
20	Bamboo shoot pickle	Pitsanulok	+				060
20	" ---- "	"		+			061
21	Palm sugar	Chachengthao	+				062
21	" ---- "	"			+		063
21	" ---- "	"		+			064
21	" ---- "	"				+	065
22	Ginger pickle	Chachengthao	+				066
22	" ---- "	"		+			067
23	Tamarind	Chachengthao		+			068
24	Pla-ra (Fermented fish)	Kampangpet	+				069

Table 1 (Cont.)

Sample No.	Product	Location	Isolation Method 5%NaCl 37℃	5%NaCl 40℃	17%NaCl 37℃	17%NaCl 40℃	Strain No.
25	Miso (Fermented soy bean)	Kampangpet	+				070
25	" ---- "	"		+			071
26	Plar-ra (Fermented fish)	Kampangpet	+				072
27	Plar-ra (Fermented fish)	Pitsanulok	+				073
28	" ---- "	"	+				074
28	" ---- "	"	+				075
29	" ---- "	"	+				076
29	" ---- "	"			+		077
30	" ---- "	"	+				078
30	" ---- "	"			+		079
31	" ---- "	"	+				080
32	Pla-ra (Fermented fish)	Sukhothai	+				081
32	" ---- "	"			+		082
33	" ---- "	"			+		083
34	Soil (Distillery waste treatment plant)	Audtaradit	+				084
35	Soil (Molasses storage tank)	"	+				085
35	" ---- "	"			+		086
35	" ---- "	"			+		087
35	" ---- "	"	+				088

Table 1 (Cont.)

Sample No.	Product	Location	Isolation method 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
42	Pla-ra (Fermented fish)	Audtaradit	+				110
42	" ---- "	"	+				111
42	" ---- "	"		+			112
43	Nam (Fermented pork)	Kanchanaburi		+			113
43	" ---- "	"		+			114
44	Molasses	Audtaradit	+				115
44	" ---- "	"		+			116
45	Distillery waste	Audtaradit		+			117
46	Palm Sugar	Kanchanaburi	+				118
46	" ---- "	"		+			119
47	Pla-chao (Fermented fish)	Pichit	+				120
47	" --- "	"			+		121
48	Pla-chom (Fermented fish)	Pichit	+				122
49	Pla-som (Fermented fish)	Pitsanulok	+				123
49	" ---- "	"	+				124
50	Pla-chom (Fermented fish)	Pichit	+				125
50	" ---- "	"			+		126
50	" ---- "	"			+		127
51	Pla-chom (Fermented fish)	Chainat	+				128
51	" ---- "	"			+		129

Table 1 (Cont.)

Sample No.	Product	Location	Isolation method 5%NaCl 37°C	5%NaCl 40°C	17%Nacl 37°C	17%Nacl 40°C	Strain No.
36	Molasses	Audtaradit	+				089
36	" ---- "	"			+		090
36	" ---- "	"		+			091
36	" ---- "	"				+	092
37	Molasses	Pitsanulok	+				093
37	" ---- "	"		+			094
38	Distillery waste	Audtaradit	+				095
38	" ---- "	"		+			096
39	Molasses	Audtaradit	+				097
39	" ---- "	"			+		098
39	" ---- "	"		+			099
39	" ---- "	"		+			100
39	" ---- "	"				+	101
40	Molasses	Audtaradit		+			102
40	" ---- "	"			+		103
40	" ---- "	"		+			104
40	" ---- "	"		+			105
40	" ---- "	"				+	106
41	Molasses	Audtaradit	+				107
41	" ---- "	"			+		108
41	" ---- "	"		+			109

Table 1 (Cont.)

Sample No.	Product	Location	Isolation method 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
52	Dry salted squid	Pichit			+		130
53	Pla-som (Fermented fish)	Nakornsawan	+				131
	" ---- "	"	+				132
53	" ---- "	"			+		133
54	Pla-ra (Fermented fish	Nakornsawan	+				134
55	Pla-ra (Fermented fish)	Pitsanulok			+		135
56	Pla-ra (Fermented fish)	Pichit	+				136
56	" ---- "	"			+		137
57	Pla-som (Fermented fish)	Pichit	+				138
57	" ---- "	"			+		139
58	Miso (Fermented soy bean)	Nakornsawan			+		140
59	Pla-ra (Fermented fish)	Nakornsawan	+				141
59	" ---- "	"			+		142
59	Pla-ra (Fermented fish)	Nakornsawan			+		143
59	" ---- "	"			+		144
60	Miso (Fermented soy bean)	Nakornsawan			+		145
61	Pla-ra (Fermented fish)	Supanburi	+				146
61	" ---- "	"			+		147

Table 1 (Cont.)

Sample No.	Product	Location	Isolation methods 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
62	Miso (Fermented soy bean)	Nakornsawan			+		148
63	Miso (Fermented say bean)	Nakornsawan	+				149
63	" ---- "	"			+		150
64	Pla-som (Fermented fish)	Nakornsawan	+				151
64	" ---- "	"	+				152
64	" ---- "	"	+				153
64	" ---- "	"	+				154
64	" ---- "	"	+				155
65	Onion pickle	Bangkok	+				156
66	Pla-chao (Fermented fish)	Bangkok	+				157
66							
66	" ---- "	"	+				158
66	" ---- "	"	+				159
67	Fermented shell	Bangkok	+				160
67	" ---- "	"	+				161
68	Miso (Fermented soy bean)	Bangkok	+				162
68	" ---- "	"	+				163
69	Pla-som	Bangkok	+				164
70	Pla-ra (Fermented fish)	Bangkok	+				165

Table 1 (Cont.)

Sample No.	Product	Location	Isolation methods 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
70	Pla-ra (Fermented fish)	Bangkok	+				166
70	" ---- "	"	+				167
71	Fermented Shrimp	Bangkok	+				168
72	Pla-ra (Fermented fish)	Rajchaburi	+				169
72	" ---- "	"	+				170
72	" ---- "	"	+				171
73	Pla-ra (Fermented fish)	Bangkok	+				172
73	" ---- "	"	+				173
73	" ---- "	"	+				174
74	Pla-ra (Fermented fish)	Bangkok	+				175
74	" ---- "	"	+				176
75	Pla-som (Fermented fish)	Bangkok	+				177
75	" ---- "	"	+				178
76	Pla-ra (Fermented fish)	Bangkok)	+				179
76	" ---- "	"	+				180
76	" ---- "	"	+				181
77	Pla-chom (Fermented fish)	Bangkok	+				182
78	Pla-chao (Fermented	Bangkok	+				183

Table 1 (Cont.)

Sample No.	Product	Location	Isolation methods 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
78	Pla-chao (Fermented fish)	Bangkok	+				184
78	" ---- "	"	+				185
79	Pla-ra (Fermented fish)	Bangkok	+				186
80	Pla-ra (Fermented fish)	Bangkok	+				187
81	Tai-pla (fish intestine)	Bangkok	+				188
82	Pla-ra (Fermented fish)	Bangkok	+				189
83	Salted Lettuce	Bangkok	+				190
83	" ---- "	"	+				191
83	" ---- "	"	+				192
84	Garlic pickle	Bangkok	+				193
85	Pla-ra (Fermented fish)	Bangkok	+				194
86	Miso (Fermented soy bean)	Bangkok	+				195
87	Pla-ra (Fermented fish)	Bangkok	+				196
87	" ---- "	"	+				197
88	Molasses (5-6/9/84)*	Nonthaburi (Thai Tam Distillery)	+				198
89	Molasses (29-30/12/84)	"	+				199

* Lot No. of molasses samples, sampling date

Table 1 (Cont.)

Sample No.	Product	Location	Isolation Methods 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Stain No.
90	Molasses (27/9/84)	Nonthaburi (Thai Tam Distillery)	+				200
91	Molasses (5/10/83)	"	+				201
92	Molasses (13/11/84)	"	+				202
93	Molasses (11-12/9/84)	"	+				203
94	Molasses (21/9/84)	"	+				204
95	Molasses (2/11/84)	"	+				205
96	Molasses (14/8/84)	"	+				206
97	Molasses (2/1/84)	"	+				207
98	Molasses (25/7/84)	"	+				208
99	Molasses (20/8/84)	"	+				209
100	Molasses (10/10/83)	"	+				210
101	Molasses (5/12/84)	"	+				211
102	Molasses (29-30/8/84)	"	+				212
103	Molasses (17/9/84)	"	+				213
104	Molasses (14/1/84)	"	+				214
105	Molasses (7/12/84)	"	+				215
106	Molasses (21/1/84)	"	+				216
107	Molasses (16/8/84)	"	+				217
108	Molasses (22/12/85)	"	+				218
109	Molasses (6/9/84)	"	+				219
110	Molasses (4/9/84)	"	+				220

Table 1 (Cont.)

Sample No.	Product	Location	Isolation methods 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
111	Molasses (21/11/84)	Nonthaburi (Thai Tam Distillery)	+				221
112	Molasses (25/8)	"	+				222
113	Molasses (7/11/84)	"	+				223
114	Molasses (27/9/83)	"	+				224
115	Molasses (6/12)	"	+				225
116	Molasses (12/84)	"	+				226
117	Molasses	Ayudthaya (Sampling from ship)	+				227
117	Molasses	"	+				228
118	Molasses	"	+				229
119	Molasses	"	+				230
119	Molasses	"	+				231
120	Molasses	"	+				232
121	Molasses	"	+				233
121	Molasses	"	+				234
122	Molasses (mix tank)	Ayudthaya Distillery	+				235
122	" ---- "	"	+				236
123	Molasses (1985)	"	+				237
124	" ---- "	"	+				238
125	" ---- "	"	+				239
125	" ---- "	"	+				240

Table 1 (Cont.)

Sample No.	Product	Location	Isolation Methods 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
125	Molasses (1985)	Ayudthaya Distillery	+				241
125	" ---- "	"	+				242
126	" ---- "	"	+				243
127	" ---- "	"	+				244
127	" ---- "	"	+				245
128	Molasses (1983)	"	+				246
129	Molasses (1985)	"	+				247
130	" ---- "	"	+				248
131	" ---- "	"	+				249
132	" ---- "	"	+				250
133	Onion pickle	Bangkok	+				251
134	Pla-chao (Fermented fish)	Bangkok	+				252
134	" ---- "	"	+				253
134	" ---- "	"	+				254
135	Pla-ra (Fermented fish)	Bangkok	+				255
135	" ---- "	"	+				256
136	Pla-chom (Fermented fish)	Bangkok	+				257
137	Pla-ra (Fermented fish)	Bangkok	+				258
138	" ---- "	"	+				259
139	Pla-chom (Fermented fish)	"	+				260

Table 1 (Cont.)

Sample No.	Sample Name	Sampling Place	Isolation method 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
140	Molasses	Nonthaburi (Thai Tam Distillery)	+				261
141	Som-Fug (Fermented fish)	Lop Buri	+				262
142	" ---- "	"	+				263
143	" ---- "	"	+				264
144	" ---- "	"	+				-
145	" ---- "	"	+				265
146	Pla-ra (Fermented fish)	Bangkok	+				266
147	" ---- "	"	+				267
147	" ---- "	"	+				268
147	" ---- "	"	+				269
148	" ---- "	"	+				270
148	" ---- "	"	+				271
149	" ---- "	"	+				272
149	" ---- "	"	+				273
150	Molasses	Ayudthaya	+				274
150	"	"	+				275
150	"	"	+				276
150	"	"	+				277
151	"	Khon Kaen	+				278
152	"	Surat Thani	+				279

Table 1 (Cont.)

Sample No.	Sample Name	Sampling Place	Isolation method 5%NaCl 37℃	5%NaCl 40℃	17%NaCl 37℃	17%NaCl 40℃	Strain No.
153	Molasses	Ayudthaya	+				280
154	"	Chonburi	+				281
155	"	Surat Thani	+				282
156	"	Lampang	+				183
157	"	Kanchanaburi	+				284
158	"	Ayudthaya	+				285
159	"	Khon Kaen	+				286
160	"	"	+				287
161	"	"	+				288
162	"	Ayudthaya	+				289
162	"	"	+				290
163	Pla-chao (Fermented fish)	Bangkok	+				291
163	"	"	+				292
164	"	"	+				293
165	"	"	+				294
166	"	Chonburi	+				295
167	"	"	+				296
168	"	"	+				297
169	"	"	+				298
170	"	Khon Kaen	+				299
171	"	"	+				300
172	"	"	+				301

Table 1 (Cont.)

Sample No.	Sample Name	Sampling Place	Isolation method 5%NaCl 37°C	5%NaCl 40°C	17%NaCl 37°C	17%NaCl 40°C	Strain No.
173	Pla-chao (Fermented fish)	Bangkok	+				302
173	"	"	+				303
174	"	"	+				304
174	"	"	+				305
175	"	"	+				306
175	"	"	+				307
175	"	"	+				308
176	"	"	+				309
176	"	"	+				310
177	"	"	+				311
177	"	"	+				312
178	"	"	+				313
178	"	"	+				314
179	Miso (Fermented soybean)	"	+				315
180	"	"	+				316
180	"	"	+				317
181	"	"	+				318
182	"	"	+				319
183	"	"	+				320
184	"	"	+				321
184	"	"	+				322
185	"	"	+				323

Table 1 (Cont.)

Sample No.	Sample Name	Sampling Place	Isolation method 5%NaCl 37 °C	5%NaCl 40 °C	17%NaCl 37 °C	17%NaCl 40 °C	Strain No.
186	Miso (Fermented fish)	Bangkok	+				324
187	"	"	+				325
187	"	"	+				326
188	"	"	+				327
189	"	"	+				328
190	"	"	+				329
191	"	"	+				330
192	"	"	+				331
193	"	"	+				332
193	"	"	+				333
194	Shrimp paste (Kapi)	Chachengthao	-				-
195	"	"	-				-
196	"	"	-				-
197	"	"	-				-
198	"	"	-				-
199	"	"	-				-
200	"	"	-				-
201	"	"	-				-
202	"	"	-				-
203	"	"	-				-
204	"	"	-				-

Table 1 (Cont.)

Sample No.	Sample Name	Sampling Place	Isolation method				Strain No.
			5%NaCl		17%NaCl		
			37°C	40°C	37°C	40°C	
205	Shrimp paste (Kapi)	Chachengthao					-
206	"	Kanchanabu[illegible]					-
207	"	"					-
208	"	Rajchaburi					-
209	"	Bangkok					-
210	"	"					-
211	Fish sauce	Bangkok					-
212	"	"					-
213	"	"					-
214	"	"					-
215	"	Rajchaburi					-
216	"	"					-

Table 2 Summarized on number of halotolerant yeast isolated in the medium containing 5 and 17% of sodium chloride and cultivated at 37 and 40°C

Product	Number of Sample	Number of isolates				Total
		5% NaCl		17% NaCl		
		37°C	40°C	37°C	40°C	
Molasses	65	76	9	4	3	92
Fermented fish	71	107	1	16	-	124
Pla-ra	(32)	(47)	(1)	(10)	(-)	
Pla-chom	(7)	(8)	(-)	(3)	(-)	
Pla-som	(6)	(13)	(-)	(2)	(-)	
Pla-chao	(20)	(34)	(-)	(1)	(-)	
Som-fug	(5)	(4)	(-)	(-)	(-)	
Tai-pla	(1)	(1)	(-)	(-)	(-)	
Pickles	17	26	17	-	1	44
Bamboo shoot	(6)	(10)	(8)	(-)	(1)	
Lettuce	(6)	(10)	(7)	(-)	(-)	
Onion	(3)	(4)	(1)	(-)	(-)	
Ginger	(1)	(1)	(-)	(-)	(-)	
Garlic	(1)	(1)	(-)	(-)	(-)	
Fermented soy bean (Miso)	23	24	1	6	-	31
Fermented mushroom	2	3	3	3	1	10
Fermented shell	3	4	2	1	1	8
Feemented pork (nam)	1	-	2	-	-	2
Fermented shrimp (Kung-chom)	1	1	-	-	-	1

Table 2 (Cont.)

Product	Number of sample	Number of isolates				Total
		5% NaCl		17% NaCl		
		37°C	40°C	37°C	40°C	
Palm sugar	2	2	2	1	1	6
Soil (alcohol factory)	2	2	1	2	-	5
Distillery waste	2	1	2	-	-	3
Tamarind	1	-	1	-	-	1
Dry salted squid	1	-	-	1	-	1
Salted crab	1	1	1	-	-	2
Salted lettuce	1	3	-	-	-	3
Shrimp paste (Kapi)	17	-	-	-	-	-
Fish sauce	6	-	-	-	-	-
Total	216	250	42	34	7	333

Table 3 Number of halotolerant yeasts from stock culture isolated from foods and alcoholic beverages in Thailand.

No. of Strains Tested	History of strain isolation		No. of Strains grow in 5%NaCl molasses medium	Strain No.
	Sample Name	Location		
10	Ragum, fruit	Surat Thani	1	S001
18	Pineapple juice	"	3	S002-S004
17	Orange juice	"	2	S005-S006
12	Tamarind juice	"	1	S007
12	Sator (Rice Wine)	"	2	S008-S009
16	Bitter berry in syrup	"	2	S010-S011
16	Lettuce pickle	"	1	S012
17	Garlic pickle	"	1	S013
17	Green tamarind in syrup	"	2	S014-S015
11	Ragum in syrup	"	2	S016-S017
15	Green mango in syrup	"	2	S018-S019
18	Palm fruit	"	2	S020-S021
13	Mixture (Sugar, Salt, Chilli)	"	-	-
10	Cucumber pickle	"	3	S022-S024
17	Sauce (Sugar syrup, Vinigar, Chilli)	"	2	S025-S026
15	Kao mark (Saccharified red rice)	"	1	S027
17	" ---- "	"	1	S028
17	Kao mark (Saccharified rice)	"	2	S029-S030
11	" ---- "	"	9	S031-S039
10	" ---- "	"	10	S040-S049

Table 3 (Cont.)

No.of Strains Tested	History of strain isolation		No.of Strains grow in 5%NaCl molasses medium	strain No.
	Sample Name	Location		
17	Kao mark (Saccharified rice)	Surat Thani	7	S050-S056
14	" ------ "	"	-	-
15	Kao mark (Saccharified red rice)	"	3	S057-S059
15	" ------ "	"	4	S060-S063
12	Shrimp paste	"	-	-
19	Mark, fruit	"	1	S064
11	Jack fruit	"	1	S065
16	Cane sugar	"	-	-
11	Look pang (Chinese yeast cake)	"	9	S066-S074
16	Rice wine	Chiang Mai	16	S075-S090
14	Mango	Surat Thani	14	S091-S104
13	Crunrun	"	13	S105-S117
14	Honey	"	14	S118-S131
10	Mango	"	10	S132-S141
12	Strawberry	Chiang Mai	6	S142-S148
17	Rose apple	Surat Thani	-	-
11	Maprang, fruit	"	11	S149-S159
12	Grape	"	12	S160-S171
2	Grape	"	2	S172-S173
2	Pineapple	Bangkok	2	S174-S175

Table 3 (Cont.)

No.of Strains Tested	History of strain isolation		No.of Strains grow in 5%NaCl molasses medium	Strain No.
	Sample Name	Location		
4	Banana	Bangkok	1	S176
2	Banana	"	1	S177
1	Orange	"	-	-
4	Sugar apple	"	4	S178-S181
1	Care flower	"	1	S182
5	Makok, fruit	"	4	S183-S186
2	Tamato	"	2	S187-S188
2	Jujube, fruit	"	1	S189
4	Mango	"	4	S190-S193
4	Onion pickle	"	2	S194-S195
4	Palm fruit	"	3	S196-S198
5	Rambutan	"	2	S199-S200
1	Lotus seed	"	-	-
1	Star goose berry	Chiang Mai	-	-
5	Orange	"	5	S201-S206
1	Lunfon flower	"	1	S206
3	Tor (Peach)	"	2	S207-S208
3	Papaya	Chiang Rai	3	S209-S211
2	Chak flower	"	1	S212
3	Banana	"	3	S213-S215
2	Nam (Fermented pork)	"	-	-
2	Tomato	"	1	S216

Table 3 (Cont.)

No. of Strains Tested	History of strain isolation		No. of Strains grow in 5%NaCl molasses medium	Strain No.
	Sample Name	Location		
1	Nam (Fermented pork)	Chiang Rai	1	S217
5	Makok, fruit	"	3	S218-S220
1	Tao nao (Fermented soy bean, Bacillus)	"	1	S221
3	Grape	"	2	S222-S223
6	Putcha, Fruit	"	4	S224-S227
4	Lamut, fruit	"	3	S228-S230
1	Orange	"	1	S231
5	Palm sugar	"	3	S232-S234
1	Shrimp paste	Lumpun	1	S235
1	Steamed glutinuous rice	"	1	S236
1	Makok, fruit	"	-	-
3	Mieng (Fermented tea leaf)	"	2	S237-S238
1	Miso (Fermented soy bean)	"	1	S239
2	Banana	"	2	S240-S241
4	Pineapple	Lumpang	3	S242-S244
3	Sugar apple	"	3	S245-S247
3	Sugarcane	"	2	S248-S249
2	Rose apple	"	1	S250
3	Tup tim, fruit	"	2	S251-S252
4	Makok, fruit	"	3	S253-S255
1	Nam (Fermented pork)	Chiang Mai	-	S
2	Flower	"	1	S256

Table 3 (Cont.)

No.of Strains Tested	History of strain isolation		No.of Strains grow in 5%NaCl molasses medium	Strain No.
	Sample Name	Location		
1	Tor (Peach)	Chiang Mai	1	S257
1	Brown sugar	"	1	S258
2	Fermented plum	"	-	-
2	Cassia sap	Chiang Rai	-	-
3	Teak sap	"	3	S259-S261
		Total	61	261

Table 4 Summary of ethanol fermentation ability of industrial yeast strains.

Strain Name	Source	Ethanol Fermentation Performance	Reference
S.cerevisiae Sc 90	Ayudthaya Alcohol Factory Thailand	produced 12.04% by weight of ethanol at 37°C in molasses medium with 20% inverted sugar in 200 l fermentor	Boonyaratana-kornkit et al. (1980-1981)
S.cerevisiae M 30	Surat Thani Alcohol Factory Thailand	produced 9.8, 9 and 6% by volume of ethanol at 35, 37 and 40°C respectively in molasses medium with 20% inverted sugar	Boonyaratana-kornkit et al. (1980-1981)
S.cerevisiae 191	isolated from mold bran of vinegar factory in China (stock culture of Institute of Food Research and Product Development)	produced 7.2% by volume of ethanol after 70 h of cultivation in cassava presaccharified by mold bran in 10L fermentor	Boonyaratana-kornkit et al. (1980-1981)

Table 4 (Cont.)

Strain Name	Source	Ethanol Fermentation Performance	Reference
S.cerevisiae 281	stock culture of Institute of Food Research and Product Development	produced 9.18% by Volume of ethanol in 25% glutinous rice slurry after enzyme analyse hydrolysis.	Boonyaratana-kornkit et al. (1980-1981)
S.cerevisiae TJ 1	isolated from Thai soil, stock culture of Department of Microbiology Kasetsart University Thailand	produced 10.35, 8.75 and 6.25% by volume of ethanol in glucose medium at 35,40 and 42°C respectively.	Seki et al. (1983)
S.cerevisiae TJ3	isolated from molasses mash stock culture of Department of Microbiology Kasetsart University Thailand	produced 14.6 and 13.9% by volume of ethanol in glucose medium at 30 and 35°C respectively	Seki et al. (1983)

Table 4 (Cont.)

Strain Name	Source	Ethanol fermentation Performance	Reference
S.cerevisiae N1	a strain isolated from mash of Awamari (Okinawa split) brewing, stock culture of Department of Fermentation Technology, Osaka University, Japan	produced 14.0 12.5 and 9.6% by weight of ethanol in glucose medium at 30, 35 and 40°C respectively	Seki et al. (1983)
S.cerevisiae AM12	a hybrid obtained by protoplast fusion of TJ1 and N1	produced 20.38 17.5 and 10% by volume of ethanol in glucose medium at 35, 40 and 42°C respectively	Seki et al. (1983)
S.cerevisiae B 120	non identified	produced 14% by volume of ethanol in industrial scale using molasses as raw material at 48 h of cultivation	Kumnuanta et al. (1983)

Activity Plan

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

Hybridization of Halotolerant Yeast for the Production of Alcohol Fuels.

Activities	1985					1986												1987												1988											
	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N
1. Procurement of Equipments																																									
2. Collection of samples																																									
3. Isolation of halotolerant yeast																																									
4. Screening of potent halotolerant strains for hybridization																																									
5. Screening of high ethanol producing strain																																									
6. Study the characteristics of selected halotolelant and high ethanol producing strain to be used as parent for hybridization																																									
7. Mutation of potent strains inorder to construct the selective marker																																									
8. Selection of mutant																																									
9. Protoplast fusion of selected strain																																									
10. Screening of the potent fusant																																									
11. Study on parameters of alcohol fermentation of fusants under laboratory and pilot scale trial																																									
12. Study in detail for characteristics of fusant strains																																									
13. Two researcher go for training in U.S.																																									
14. One researcher go for training in U.S.																																									
15. U.S. expert come to Thailand for consultancy																																									
16. Reporting 16.1 Progress																																									
16.2 Final																																									
Projected costs $	36,821		5,300			4,300		4,300		19,3[illegible]			14,[illegible]					1,300			11,800			14,[illegible]			[illegible]			4,[illegible]			4,[illegible]							
Baht	994,167		143,100			116,100		116,100		521,1[illegible]			3[illegible],2[illegible]					11[illegible],100			318,[illegible]			394,200			116,1[illegible]			116,1[illegible]			11[illegible],1[illegible]			—				

Financial Plan

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

Hybridization of Halotolerant Yeast for the production of Alcohol Fuels.

Cost Element	1985		1986				1987				1988			total
	Aug-Oct	Nov-Jan	Feb-Apr	May-Jul	Aug-Oct	Nov-Jan	Feb-Apr	May-Jul	Aug-Oct	Nov-Jan	Feb-Apr	May-Jul		Baht
1. Salary	20,817	36,450	36,450	36,450	36,450	36,450	36,450	36,450	36,450	36,450	36,450	19,683		495,000
2. Equipment	866,700													866,700
3. Material and Supply	47,250	47,250	47,250	47,250	47,250	47,250	47,250	47,250	47,250	47,250	47,250	47,250		567,000
4. Travel and Transportation, local	16,200	16,200												32,400
5. Micellaneous Journal, Report and Publication	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700	13,500		43,200
6. Training in U.S.					405,000			202,500						607,500
7. Central Research Facility Cost	40,500	40,500	29,700	29,700	29,700	29,700	29,700	29,700	29,700	29,700	29,700	29,700		378,000
8. Consulation						278,100			278,100					556,200
= Total (Baht)	994,167	143,100	116,100	116,100	521,100	394,200	116,100	318,600	394,200	116,100	116,100	110,133		3,456,000

1 U.S. $ = 27 Baht