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Construction of High Ethanol Fermenting Halotolerant Hybrid by Intergeneric Protoplast Fusion of *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii*

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ABSTRACT

Saccharomyces cerevisiae M30, a high ethanol producing flocculent strain, was hybridized with *Zygosaccharomyces rouxii* TISTR1750, a halotolerant non-flocculent strain, by polyethylene glycol induced protoplast fusion. The protoplast fusion of these two yeast genera occurred with frequency of $5.89X10^{-5}$. Three fusants, namely RM11, RM37 and RM58, were selected based on their high ethanol fermentation efficiency in glucose broth containing 1.5 and 3% sodium chloride. Ethanol fermentation performance in salted medium of the three fusants revealed that in glucose broth containing 1.5, 3, 5 and 7% sodium chloride RM11 produced the highest ethanol concentrations and yields when compared to the other two fusants and both parental strains. Ethanol production in semisynthetic medium consisted of 18% glucose and 3% sodium chloride in 5 l fermentor with controlling of pH not to be lower than pH 4.5, temperature at 30 C and 300 rpm agitation speed also showed that the efficiency of ethanol production of the fusant RM11 was the highest comparing with both parental strains. It revealed maximal ethanol as 6.85% by weight with 87% of theoretical yield at 60 h of cultivation. *S. cerevisiae* M30 produced ethanol 6.5% by weight with 83% of theoretical yield, while *Zygosac. rouxii* TISTR1750 produced 6.36% by weight with 79% of theoretical yield at the same cultivation period. The fusant RM11 exhibited flocculation the same as that of *S. cerevisiae* M30.

Key words : ethanol fermentation, intergeneric protoplast fusion, *S. cerevisiae*, *Zygosac. rouxii*, halotolerant

INTRODUCTION

Ethanol production in certain area is encountered with a problem of high salinity water in the fermentation process which markedly affected to fermentation ability of yeast. Generally, *Saccharomyces cerevisiae* has the ability to ferment ethanol with high efficiency in medium without sodium chloride. In contrast, in high concentration of sodium chloride its ethanol fermentation is decreased while production of the other products such as glycerol, 2,3-butanediol, acetoin, acetaldehyde and volatile acids are increased (Umemoto *et al.*, 1967; Tajima and Yashizumi, 1975; Limtong *et al.*, 1986). High concentration of sodium chloride was found to inhibit the activity of various enzymes in glycolytic pathway (hexokinase, aldolase and thriose phosphate isomerase)

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consequently to decreasing of ATP level which then stimulates glycerophosphate dehydrogenase activity that converts dihydroxyacetone phosphate to glyceraldehyde 3-phosphate and requires coenzyme NADH. Lacking of NADH causes the reduction of alcohol dehydrogenase activity because it also requires NADH, so that lower ethanol concentration is produced (Tajima and Yashizumi, 1975). Moreover sodium chloride was reported to decrease maximum specific growth rate of *S. cerevisiae* (Watson, 1970).

To solve the problem of low efficiency of ethanol production in high concentration of sodium chloride, a better strain that can tolerate high sodium chloride concentration, a salt tolerant strain, is required. The salt tolerant or halotolerant yeast is a kind of osmophilic yeast usually find in the presence of high concentration of salt and sugar (Onishi, 1963; Lee and Lee, 1970; Tokuoka, 1992). Fermented foods where high salt concentrations are used in the process, such as soy sauce, miso etc. are good sources for halotolerant yeast. Usually predominant strains find in these products is *Zygosaccharomyces rouxii* (formerly *S. rouxii*) which can tolerate to 20-22% sodium chloride concentration (Lee and Lee, 1978). This yeast accumulates glycerol intracellularly in response to increase the concentration of sodium chloride in growth medium (Yagi, 1988). However, the efficiency of ethanol fermentation by halotolerant strains are relatively low (Onishi, 1963). So better approach to obtain a strain which ferments ethanol with high efficiency and tolerats to high sodium chloride concentration is to hybridize the ethanol fermenting strain to the halotolerant strain. Of hybridization techniques, protoplast fusion is superior to the others since it is disregard for mating type and ploidy of parental strains. Also it can be used to hybrid the parental strains of different genera (Pina *et al*., 1986). This paper reports the construction of a high ethanol producing halotolerant hybrid by hybridization of *S. cerevisiae* and *Zygosac. rouxii* using protoplast fusion technique.

MATERIALS AND METHODS

Microorganisms

The yeast strains used in this study were *S. cerevisiae* M30, a high ethanol producing and flocculent strain of the Department of Microbiology Faculty of Science, Kasetsart University, Thailand and *Zygosac. rouxii* TISTR1750, a halotolerant and non-flocculent strain of Thailand Institute of Scientific and Technological Research. The strains were cultivated and maintained on yeast extract peptone dextrose (YPD) agar (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar).

Mutation

Ascospores of *S. cerevisiae* M30 were mutated using ethyl methane sulfonate (EMS) as the method of Oshima and Takano (1980). Respiratory deficient mutants of *Zygosac. rouxii* TISTR1750 were isolated following the method described by Seki *et al*. (1983).

Protoplast preparation

Eight ml of over night yeast culture in YPD broth cultivated at 30°C was centrifuged at 3,000 rpm for 5 min. Cells were washed twice and resuspended in sterile distilled water to make concentration of 10^7-10^8 cells/ml. Four ml of cell suspension was centrifuged and then 4 ml of lytic enzyme solution (0.075 mg/ml Zymolyase 20T, 0.6 M KCl or 2.0 M KCl, 10 mM β-mercaptoethanol and 50 mM phosphate buffer, pH 7.5) was added to cells pellet. The mixture was incubated at 30°C for 3-4 h. Protoplasts were collected by centrifugation at 3,000 rpm for 10 min. The protoplasts of *Zygosac. rouxii* were washed and resuspended in 4 ml of 2 M KCl solution, while 0.6 M KCl was used for

protoplasts of *S. cerevisiae*. The protoplasts and remaining cells were counted under microscope by using high power objective and calculated as percentage of protoplast formation.

Protoplast fusion and regeneration

Protoplast fusion was induced by 30% polyethyleneglycol (PEG)-6000 containing 50 mM $CaCl_2$ as the method used by Seki *et al*. (1983) except that the incubation was performed at 20°C for 15 min. Fused protoplasts were collected, diluted with 2 M KCl and then pipetted on to surface of selective regeneration medium (SRM: 0.67% Difco-yeast nitrogen base without amino acid, 2% glycerol, 2% Difco-agar and 2M KCl) and overlaied with SRM containing 3% of Difco-agar and incubated at 30°C. Fusants grew selectively on the SRM were collected. Regeneration of protoplast of parental strains was carried out by the same method as mentioned previously except complete regeneration medium (CRM: 1% yeast extract, 2% peptone, 2% glucose and 2% or 3% Difco-agar) was used.

Ethanol fermentation

Fermentation medium was YPD broth consisted of 18% glucose (YPD_{18} broth) and various amount of sodium chloride. For primary screening of the fusants, fermentation was performed in test tube with standing incubation at 30°C for 48 h For secondary screening, fermentation was performed in 250 ml Erlenmeyer flask contained 50 ml of fermentation medium and incubated by standing with periodical shaking every day at 30°C for 96 h Ethanol fermentation by shaking flask culture was investigated using 100 ml of YPD_{18} broth contained various concentrations of sodium chloride in 250 ml Erlenmeyer flask and incubated on rotary shaker, 80 rpm at 30°C. Fermentation in fermentor was carried out using 3 l of medium consisted 18% glucose, 0.6% yeast extract, 0.4% ammonium sulfate, 0.2% magnesium sulfate, 0.8% potassium dihydrogen sulfate and 3% sodium chloride in 5 l jar fermentor. (Microferm fermentor, New Brunchwick Scientific, USA). The culture was agitated at 300 rpm, pH was controlled not lower than 4.5 by 5 N sodium hydroxide and temperature was controlled at 30°C.

Analysis

Ethanol concentration was analyzed by gas chromatography (Shimadzu GC-9A and CR-3A recorder, Shimadzu Co. Ltd., Japan) using column (3.2 mm × 3 m) packed with 20 M polyethylene glycol on 60-80 mesh shimalite support. Flame ionized detector was used. The temperature of column was 100°C and of injection port was 200° C. Nitrogen was used as carrier gas with flow rate of 40 ml/min. N-propanol, 4% w/v, was an internal standard. Cell concentration was determined by spectrophotometer as optical density at 660 nm (Shimadzu UV-visible recording spectrophotometer UV-240). Cell dry weight was obtained from conversion of optical density value by using calibration cure. Glucose concentration was analyzed by Nelson-Somogyi Method (Nelson, 1944). Glycerol concentration was analyzed by using glycerol test kit of Boehringer Mannheim Gmbh Co. Ltd. (Germany). Flocculation was determined as sedimentation efficiency (S.E.) following the method described by Limtong (1986).

RESULTS AND DISCUSSION

Isolation of mutants

To facilitate the selection of hybrid fusant, selective markers were constructed for both parental strains by mutation. From 97 auxotrophic mutants obtained by EMS treatment on ascospores of *S. cerevisiae* M30 that produced within 3 days on McClary's acetate agar, mutant A13 was selected for subsequent protoplast fusion based on its similar ethanol fermentation ability as *S. cerevisiae* M30.

Respiratory deficient mutant RD14 of *Zygosac. rouxii* TISTR1750 was selected from 165 mutants obtained since it produced ethanol same as its wild type (data not showed).

Protoplast fusion

By protoplasing cells (10^8 cells/ml) of auxotrophic mutant A13 of *S. cerevisiae* M30 with lytic enzyme solution contained 0.075 mg/ml Zymolyase 20T for 4 h, no cell was remained in the suspension, this means 100% of protoplasts was obtained (Table 1). However, only 10^7 protoplasts/ml was detected. The number of protoplasts obtained was lower than the number of initial cells used, even no cell was remained, which may resulted from lysis of protoplasts during protoplasing. To isolate protoplast RD14 of *Zygosac. rouxii* TISTR1750 cells had to be pretreated by suspending in β-mercaptoethanol or dithioerythritol at 30° C for 15 min before protoplasing. Pretreatment by dithioerythritol yielded higher protoplast (data not showed). This result supported the result of Arnold and Garrison (1979) who reported that in protoplasing of *Zygosac. rouxii* using snail enzyme with dithioerythritol pretreatment 85% of cells were converted to protoplasts. Though dithioerythritol pretreatment on cells of RD14 of *Zygosac. rouxii* TISTR1750 resulted in better protoplasing than without and with β-mercaptoethanol pretreatment, only 70.23% of protoplasts in the suspension was obtained (Table 1). Protoplasts of auxotrophic mutant A13 of *S. cerevisiae* M30 regenerated on YPD medium contained 0.6 M KCl with 20% regeneration rate, that calculated from number of colonies on complete regeneration medium divided by number of protoplasts obtained from direct count. While regeneration rate of RD14 of *Zygosac. rouxii* TISTR1750 was 14.57% (Table 1). The low regeneration rate of both protoplasts may revealed that the conditions for protoplasing or/and regeneration were not optimized.

Protoplast suspension of *S. cerevisiae* M30 (A13), 7.65×10^7 protoplasts/ml, was mixed with protoplast suspension of *Zygosac. rouxii* TISTR1750 (RD14), 5.46×10^7 protoplasts/ml, in the ratio of 1:1. Only PEG induced fusion products which exhibited prototrophic and respiratory sufficient characteristics obtained from crossing of both parental strains were able to regenerate and yield colonies on selective regeneration medium. One hundred fifty five colonies appeared on selective

Table 1 Protoplasts isolation of the auxotrophic mutant of *S. cerevisiae* M30 (A13) and respiratory deficient mutant of *Zygosac. rouxii* TISTR1750 (RD14) and their regeneration rate.

Number of cells and protoplasts (cells and protoplasts/ml)	Number of remaining cells (cells/ml)	Protoplast formation (%)	Number of colonies on CRM (CFUs/ml)	Regeneration rate (%)
S. cerevisiae M30 (A13) 7.65×10^7	0	100	1.53×10^7	20.00
Zygosac. rouxii TISTR1750 (RD14) 5.46×10^7	1.13×10^2	70.23	7.96×10^6	14.57

Remark: Initial cell concentration used for protoplast isolation was 10^8 cells/ml .

regeneration medium were collected. The protoplast fusion frequency which defined as the ratio of the number of colonies on selective regeneration medium to the number of colonies on complete regeneration medium was 5.89×10^{-5}. The protoplast fusion frequency of this partner revealed higher value than that reported by Pina *et al.* (1986) on intergeneric protoplast fusion of *S. cerevisiae* and *Zygosac. fermentati*, 2×10^{-7}.

Screening for the fusants produced high ethanol in salted medium

One hundred fifty-five fusants were primary screened by determination of ethanol fermentation in YPD_{18} broth contained 1.5 and 3% sodium chloride using standing tube cultivation at 30°C. Twelve fusants produced ethanol higher than both parental strains in medium contained both concentration of sodium chloride were selected for secondary screening (data not showed). Secondary screening which was performed in the same medium by standing flask cultivation at 30°C for 96 h showed that 6 fusants, RM24, RM39, RM68, RM11, RM37 and RM58, produced higher ethanol concentration than both parental strains in medium contained 1.5% sodium chloride. They produced ethanol in the range of 6.50-6.70% by weight (Table 2). Fusant RM11, RM37 and RM58, showed slightly higher ethanol fermentation than the other 3 fusants, therefore they were selected for further study.

Ethanol production in salted medium

To investigate ethanol fermentation, growth and glucose utilization in salted medium, the fusants were cultivated in YPD_{18} broth contained 1.5, 3 and 7% sodium chloride and incubated by shaking on rotary shaker, 80 rpm, at 30°C. Higher sodium chloride concentration in medium resulted in lower ethanol fermentation in all fusants and parental strains tested. Maximal ethanol concentrations produced by the fusant RM11 in all salted media were the highest when compared with the other fusants and both parental strains (Figures 1a, 2a, 3a and 4a). In medium contained 1.5, 3, 5 and 7% sodium chloride, RM11 produced maximal ethanol 7.10, 6.59, 5.74 and 4.46% by weight at 48, 60, 84, and 84 h, respectively. In the above mentioned 4 salted media, RM37 produced ethanol 6.85, 6.55, 5.57 and 3.9% by weigh at 48, 72, 84 and 96 h,

Table 2 Ethanol production of secondary selected fusants in YPD_{18} broth contained 1.5 and 3% sodium chloride by standing cultivation at 30°C for 96 h.

Strains	Ethanol concentration (% w/v)	
	1.5% NaCl	3% NaCl
S. *cerevisiae* M30	6.46	6.23
Zygosac. rouxii TISTR1750	6.31	6.21
RM11	6.70	6.47
RM24	6.50	6.20
RM37	6.67	6.43
RM39	6.51	6.19
RM58	6.65	6.41
RM68	6.48	6.22

respectively and RM58 produced 6.88, 6.58, 5.60 and 4.09% by weight at 48,72, 84 and 84 h, respectively. While *S. cerevisiae* M30 produced maximum ethanol 6.48, 5.96, 3.59 and 2.64% by weight at 48, 72, 96 and 120 h, respectively, *Zygosac. rouxii* TISTR1750 produced 6.42, 6.32, 5.34 and 3.71% by weigh at 48, 60, 120 and 96 h, respectively. The lowest ethanol production by *S. cerevisiae* M30 in medium contained various concentrations of sodium chloride except in 1.5% sodium chloride suggested the low tolerant to sodium chloride of this strain.

At higher concentration of sodium chloride lower growth was attained in all strains, especially in *S. cerevisiae* M30, the high ethanol producing and non-halotolerant strain. Growth of the three fusants were not much different from *Zygosac. rouxii* TISTR1750, the halotolerant parental strain in all concentrations of sodium chloride. *S. cerevisiae* M30 revealed clearly lower growth than the others, except in 1.5% sodium chloride that showed only slightly lower (Figures 1b, 2b, 3b and 4b). Glucose utilization by the fusants and *Zygosac rouxii* TISTR1750 were not different in all concentrations of sodium chloride (Figures 1c, 2c, 3c and 4c), while the glucose utilization by *S. cerevisiae* M30 was the lowest, especially in medium contained 5 and 7% sodium chloride.

Ethanol yields in medium contained 1.5 and 3% sodium chloride of *S. cerevisiae* M30 were higher than that of *Zygosac. rouxii* TISTR1750, in contrast in medium contained 5 and 7% sodium chloride were lower (Figure 5). Among the fusants ethanol yield of RM11 was the highest. The results suggested that high ethanol fermentation ability in low concentration of sodium chloride of this fusant attained from *S. cerevisiae* M30 while its halotolerance attained from *Zygosac. rouxii* TISTR1750. The ethanol yields of the other 2 fusants, also suggested that they attained both characteristics from the parental strains.

The results of ethanol fermentation in various salted media indicated that the increase of sodium chloride concentration consequently to the increase of osmotic pressure caused the decrease of ethanol fermentation and growth of both halotolerant and non-halotolerant yeasts. This finding was similar to the others (Watson, 1970; Tajima and Yashizumi, 1975).

Ethanol fermentation in salted medium in fermentor

Since RM11 produced ethanol with the highest concentration and yield, therefore its ethanol fermentation in semisynthetic medium consisted of 18% glucose and 3% sodium chloride by using fermentor was investigated. RM11 produced maximal ethanol concentration 6.85% by weight at 60 h of cultivation (Figure 6). While *S cerevisiae* M30 produced 6.50% by weight at 72 h, *Zygosac. rouxii* TISTR1750 produced 6.35% by weight at 60 h (Figures 7 and 8). Maximal growth of RM11, 3.35 g/l, was attained at 48 h while 3.27 and 3.25 g/l were produced by *S. cerevisiae* M30 and *Zygosac rouxii* TISTR1750 at the same period. Based on sugar consumption ethanol yield of RM11 was 87% of the theoretical yield, while those of *S. cerevisiae* M30 and *Zygosac. rouxii* TISTR1750 were 83 and 79% of theoretical yield, respectively (Table 3).

Glycerol was an osmoregulator produced by most yeasts when they were transferred to salted medium (Van Zyl *et al*., 1993), therefore glycerol in culture broth was determined. The amount of glycerol released to culture broth by halototerant yeast, *Zygosac. rouxii* TISTR1750, was the highest. It produced 0.35 g/l of glycerol at 12 h of cultivation, though the concentration was slightly decreased after that for a short period it still maintained higher concentration than in the other strains (Figure 9). RM11 also showed the same pattern of glycerol releasing as *Zygosac. rouxii* TISTR1750, though

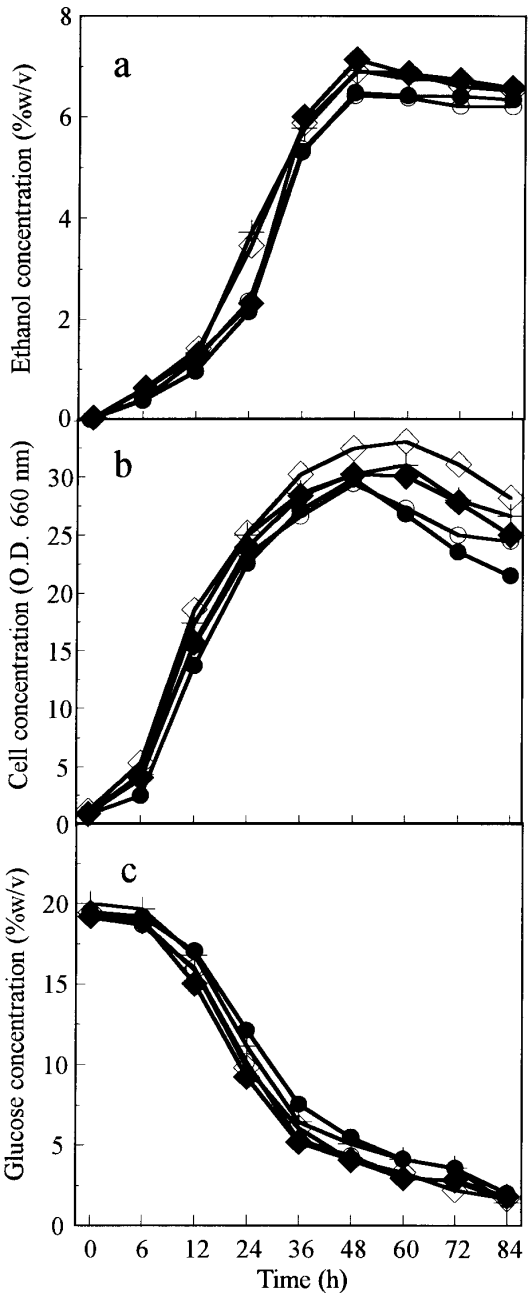

Figure 1 Ethanol fermentation (a), growth (b) and glucose consumption (c) by fusant RM11 (◆), RM37 (◇), RM58 (+) and parental strains *S. cerevisiae* M30 (●) ans *Zygosac. rouxii* TISTR 1750 (○) in YPD_{18} broth contained 1.5% NaCl.

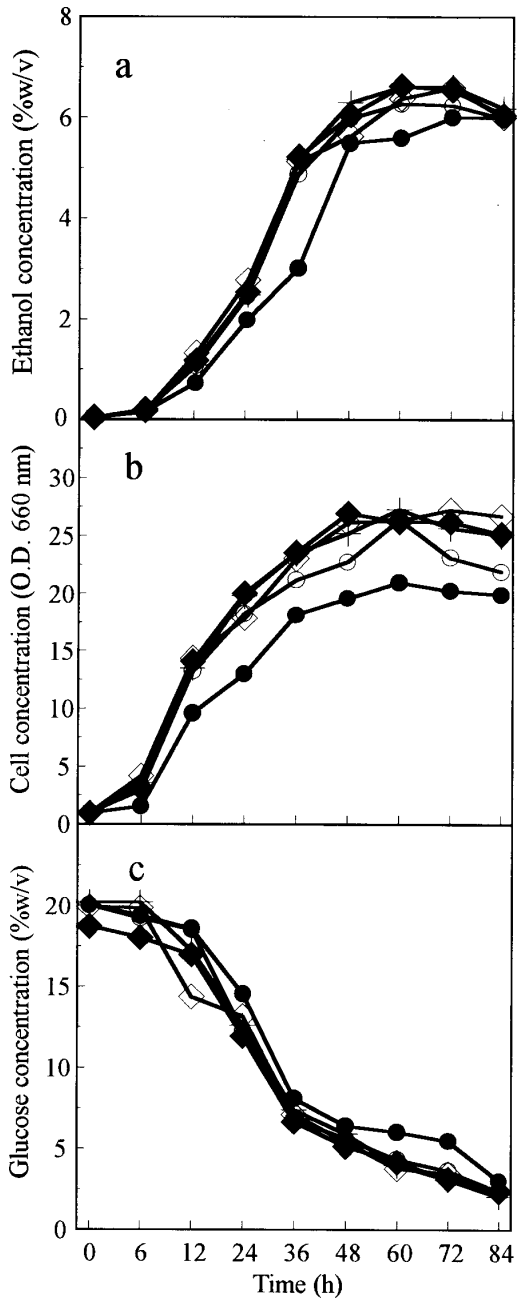

Figure 2 Ethanol fermentation (a), growth (b) and glucose consumption (c) by fusant RM11 (◆), RM37 (◇), RM58 (+) and parental strains *S. cerevisiae* M30 (●) ans *Zygosac. rouxii* TISTR 1750 (○) in YPD_{18} broth contained 3.0% NaCl.

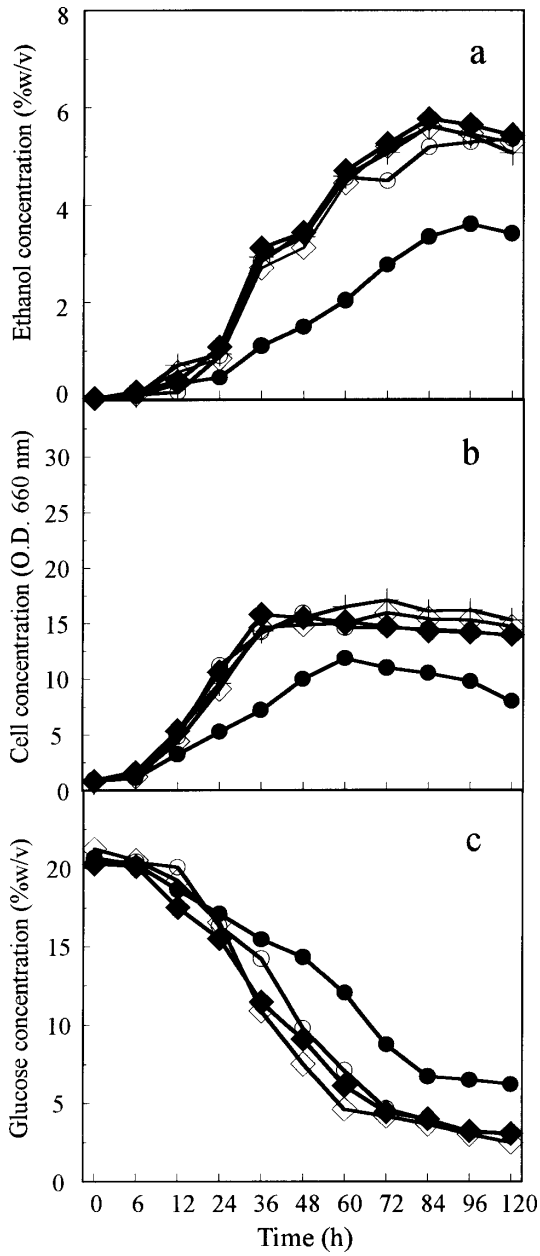

Figure 3 Ethanol fermentation (a), growth (b) and glucose consumption (c) by fusant RM11 (◆), RM37 (◇), RM58 (+) and parental strains *S. cerevisiae* M30 (●) ans *Zygosac. rouxii* TISTR 1750 (○) in YPD_{18} broth contained 5.0% NaCl.

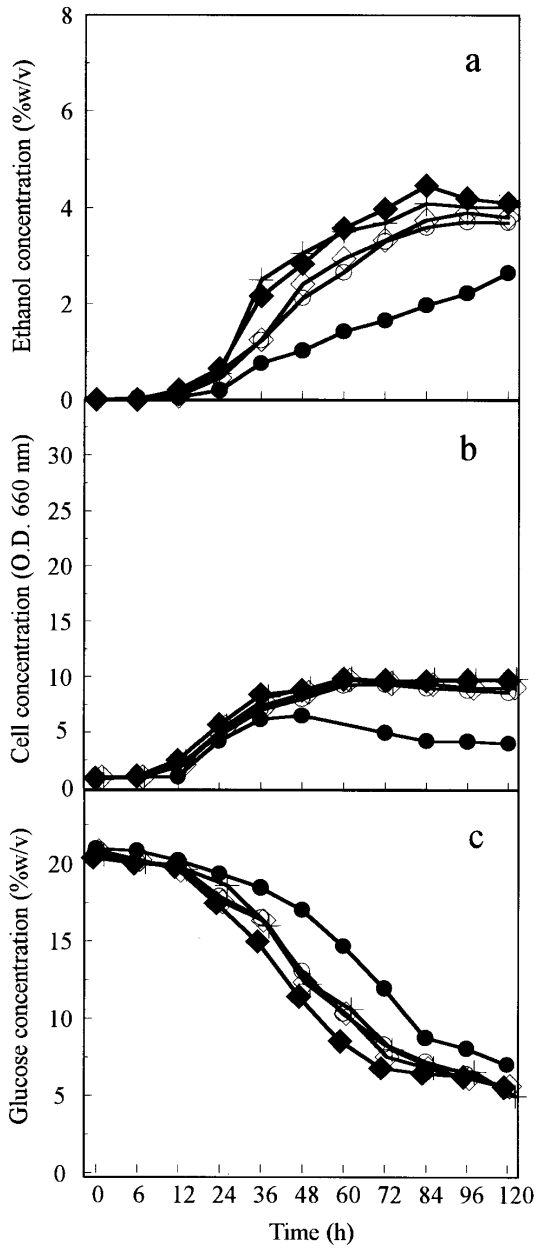

Figure 4 Ethanol fermentation (a), growth (b) and glucose consumption (c) by fusant RM11 (◆), RM37 (◇), RM58 (+) and parental strains *S. cerevisiae* M30 (●) ans *Zygosac. rouxii* TISTR 1750 (○) in YPD_{18} broth contained 7.0% NaCl.

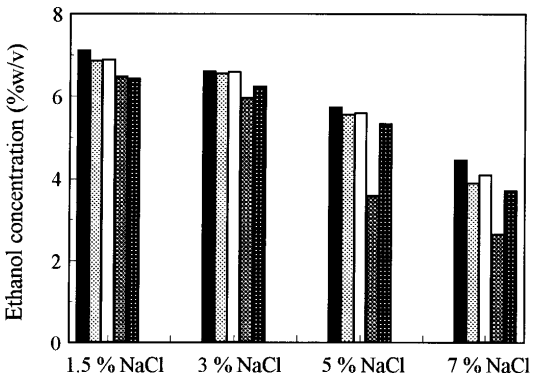

Figure 5 Comparison of maximal ethanol concentration produced in YPD_{18} contained 1.5, 3, 5 and 7% NaCl by fusants RM11 (■), RM37 (▩), RM58 (□) and parental strains *S. cerevisiae* M30 (▩) and *Zygosac. rouxii* TISTR 1750 (■).

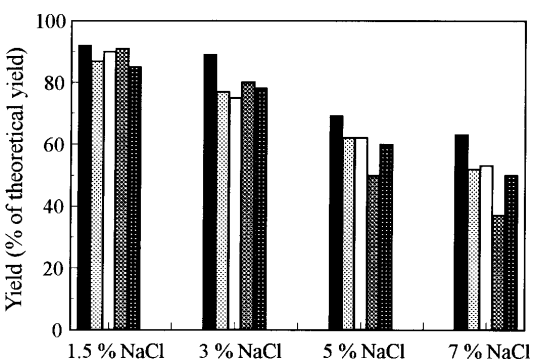

Figure 6 Comparison of ethanol yield in YPD_{18} broth contained 1.5, 3, 5 and 7% NaCl compared with theoretical yield by fusants RM11 (■), RM37 (▩), RM58 (□) and parental strains S. cerevisiae M30 (▩) and *Zygosac. rouxii* TISTR 1750 (■).

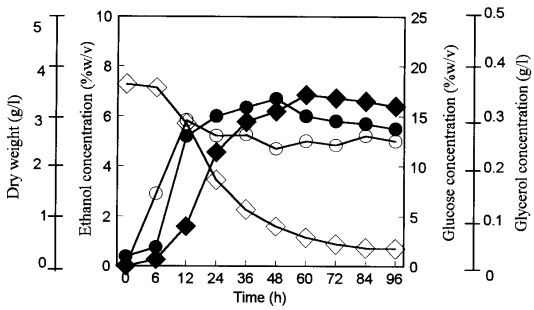

Figure 7 Ethanol fermentation (◆), growth (●), glucose consumption (◇) and glycerol production (○) by the fusant RM111 in medium contained 3% NaCl in fermentor.

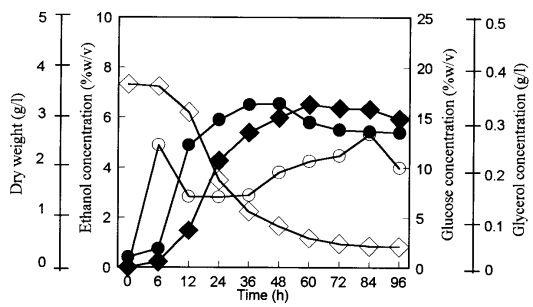

Figure 8 Ethanol fermentation (◆), growth (●), glucose consumption (◇) and glycerol production (○) by *S. cerevisiae* M30 in medium contained 3% NaCl in fermentor.

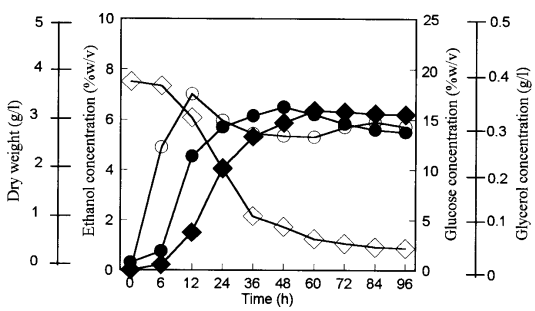

Figure 9 Ethanol fermentation (◆), growth (●), glucose consumption (◇) and glycerol production (○) by *Zygosac. rouxii* TISTR1750 in medium contained 3% NaCl in fermentor.

Table 3 Comparison of ethanol fermentation, growth and glucose consumption of the selected fusant RM11 and its parental strains in medium contained 3% sodium chloride in jar fermentor.

	S. cerevisiae M30	*Zygosac. rouxii* TISTR1750	RM11
Initial cell concentration (O.D.660 nm)	1.26	0.93	1.18
Maximum dry weight (g/l)	3.27	3.25	3.35
Maximum ethanol concentration (%w/v)	6.50	6.36	6.85
Sugar concentration (%w/v)	18.30	18.80	18.21
Residual sugar (%w/v)	2.92	3.11	2.86
Maximum fermentation rate (g/l/h)	0.23	0.21	0.24
Yield of ethanol production (% of theoretical yield)	83	79	87
Cell mass production (g/l/h)	0.35	0.31	0.37
Sedimentation efficiency (S.E.)	0.90	0	0.75

lower maximal glycerol was attained, 0.29 g/l, at the same cultivation period (Figure 7). The non-halotolerant strain, *S. cerevisiae* M30, released maximum glycerol 0.25 g/l at 6 h which then drastically decreased to the concentration lower than 0.2 g/l though after 36 h slightly increased was reattained (Figure 8).

The fusant RM11 revealed the ability of flocculation same as the flocculent parental strain, *S. cerevisiae* M30 (S.E.0.9), though its S.E. (0.75) was lower (Table 3).

CONCLUSION

Intraspecific protoplast fusion of *S. cerevisiae* M30 and *Zygosac. rouxii* TISTR1750 in this study succeeded with relatively high protoplast fusion frequency. Though the fusants obtained were not confirmed by DNA content and genetic analysis but their fermentation, growth and glucose utilization profiles in salted medium supported that they were hybrids obtained from genetic recombination. The selected hybrid fusant RM11 revealed higher ethanol fermenting efficiency in medium contained various concentrations of sodium chloride, 1.5-7%, than its parental strains. Ethanol fermentation of the selected fusant RM11 in medium contained 3% sodium chloride in jar fermentor not only revealed higher ethanol concentration than both parental strains but its ethanol yield and fermentation rate were also higher. The selected fusant also attained the flocculation characteristic from the flocculent parental strain.

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