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Original Article

Isolation of high ethanol resistant strains of *Saccharomyces cerevisiae*

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Abstract

Several strains of *Saccharomyces cerevisiae* were isolated from different sources. These strains were under taken for ethanol tolerance analysis and growth under stress condition. Exponential phase to various concentrations of ethanol (2–26% v/v) for 1 h was used for isolation of resistant strains. Viable cells were isolated and purified by inoculation of diluted samples on yeast extract peptone agar medium at 30 ºC for 24 h. All resistant strains were grown on solid aerobic low peptone medium supplemented with 24% (v/v) ethanol but had variations at growth intensity in this condition. Invertase enzyme activity in yeast isolates was measured and comparing with control samples resistant strains did not show significant difference in the enzyme activity. As resistant strains to ethanol have different content of fatty acids in cell membrane and resistance mechanisms, they can be used in various fields of biotechnology such as ethanol production, recombinant-protein expression, trehalose production and novel drug development.

Keywords: Ethanol; Tolerance; *Saccharomyces cerevisiae*

INTRODUCTION

Saccharomyces cerevisiae is an important microorganism in bio-industry and its tolerance to ethanol is one of the main characteristics to decide whether it can be used as a biofermentation resource (1). Thus, in the industrial ethanol production, there are many important factors which should be considered such as ethanol or sugar tolerance of strains, and enzymatic activities of their invertase for good operation in this regard (2).

It is widely accepted that the sake and distilling yeasts have an inherently higher tolerance towards ethanol than do brewing yeasts. This phenomenon is correlated with ethanol concentration, which is produced in these yeasts (3). Excess amount of ethanol has been reported to cause mitochondrial DNA damage and degrades bio-membranes (4) in yeast cells and inactivate some enzymes such as hexokinase and dehydrogenase (3). Thus it is considered as a growth inhibitor for microorganisms (2). Moreover, ethanol can dissolve fatty acid constituents of the cell membranes, disrupt cytoplasmic membrane rigidity (1), stop mitochondrial bio-molecules translocation and proton motive force (5) and finally cause cell death (2). According to these phenomena, resistant strains to ethanol have many mechanisms to overcome ethanol perils. For example, those cells of *S. cerevisiae* which have been grown under conditions of the presence of ethanol appear to increase the amount of monounsaturated fatty acids in cellular lipids (6). Many reports have suggested a relationship between the fatty acid compositions of lipid membranes and ethanol stress

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tolerance (7). More over, specific genes have been indicated to be expressed under stress conditions and their products exert an important role in the cell maintenance and more life span such as *BEM2, PAT1, ROM2, VPS34* and *ADA2* (8). BEM2 is a GTPase activating protein, while ROM2 is a GDP-GTP exchanging factor and VPS34 is a phosphatidylinositol kinase and ADA2 is a component of the nucleosomal histone acetyltransferase complex (7).

Approaches to measure ethanol tolerance have involved determinations of ethanol effects on cell growth, fermentative ability, viability and batch culture performance (5). It is important to note that yeast strains have proper invertase enzyme activity (8). Invertase is one of the important extracellular enzymes in *Saccharomyces* that is responsible for converting sucrose to its subunits, glucose and fructose (8).

As resistance to ethanol and sugar are very important in ethanol production at large scale, resistant strains to ethanol stress are valuable for biotechnological fermentation (1). These strains are able to extend the process of fermentation for longer time and produce distinct (recombinant) products in the presence of ethanol (4,9). In addition, resistant strains to ethanol stress have shown to have other abilities like resistance to other stresses such as osmotic pressure and oxidative and heat (4). In this paper, we have examined ethanol affects on the viability and growth parameters of several yeast strains.

MATERIALS AND METHODS

Microorganisms and Media

Two commercial bioindustrial yeasts were collected from their producers originating from Frings (Germany) and S.I.Lesaffre (France) companies (Yeast types No. 1 and 2, respectively). One type of yeast was gifted from Persian Type Culture Collection (PTCC) No. 1038 (Yeast type No. 3) and the fourth strain of yeast was obtained from microbiology research laboratory of the University of Isfahan (Yeast type No. 4). These yeasts were collected and cultured on solid yeast extract peptone glucose medium (YEPG) (1% yeast extract, 1% peptone, 2% glucose and 1.5% agar) for 48 h at 30 ºC for purity identification and on solid slant yeast extract glucose plus chloramphenicol (0.5% yeast extract, 2% glucose, 1.49% agar-agar and 0.01% chloramphenicol) for maintenance in 4 ºC (10). All chemical, media and reagents that were used in this research were purchased from Merck (Germany).

Exogenous ethanol effect

Samples of exponential growth phase of yeast cells in liquid media were subjected to ethanol stress. To do the experiment, first the optical density of yeast cultures in broth YEPG were measured at 620 nm by Zeiss spectrophotometer (Specord S 10). Then 1×10^7 yeast cells corresponding to the observed OD were treated with 1 ml of 2-26% (v/v) ethanol and incubated for 1 h at room temperature. After the stress treatment, cells were centrifuged and suspended in 1 ml of 50 mM phosphate buffer, pH 6.8. Then cell suspensions were diluted with the same buffer and plated on YEPG agar. Yeast cells survival was determined by colony counting after incubation at 30 ºC for 24 h as described earlier (3,5).

Treatment of yeast cells on solid medium

All type of yeasts were transferred on aerobic low peptone (ALP) media containing 1.5% agar, 0.1% $(NH_4)_2SO_4$, 0.05% pancreatic digest of casein, 0.05% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.02% KCl, 0.02% phenol red and 5% ethanol (2-26% v/v) on surface culture. After inoculation, sealed solid medium were incubated at 30 ºC for 24, 48 and 72 h. Growth intensity of different cells and medium color changes for each set of samples were compared with positive

control. Positive control was solid ALP medium without ethanol that contains surface culture of the used yeast strains.

Assay of invertase activity

Estimation of invertase activity in yeast cells was done according to Osho method (5). Sucrose, as an appropriate substrate was used in acetate buffer (0.1 M, pH 5.0) and was converted to hexoses by the enzymatic activity of invertase. This reaction was performed at 30 ºC for 5 min and free hexoses in solution were bound to dinitrosalicylic acid reagent (7). Then, absorbance of colored mixture was measured at 540 nm by spectrophotometer. According to standard curve of glucose, enzyme activity was calculated based on free hexsose content which was produced after adding the substrate (1,5). The amount of enzyme which released 1 µmole of the reduced sugar per minute was defined as one unit of invertase activity.

Statistical methods

Data from each experiment was statistically analyzed using SPSS 9.0.0 software. For analyzing data, t-test and analysis of variance (ANOVA) methods in 1% confidence level was applied (6). t-test indicates differences between the samples in each treatment while, ANOVA analysis shows, variations between several treatments in each test.

RESULTS

Effect of exogenous ethanol on growth of yeast cells

The yeast types were exposed to ethanol concentrations 2 to 16% v/v as described in material and method section. Cell growth was evaluated by colony counting. Results indicate growth changes in different yeast cells at 1% confidence level which were significant, also showed significant differences between treatments (Fig. 1). According to Fig. 1, it is obvious that two yeast strains No. 3 and 4 were more resistant than others. Thus, these two strains were subsequently exposed to 18-26% (v/v) ethanol for resistance estimation (Fig. 2). Few cells of these two types of yeast tolerated 24% (v/v) ethanol concentration (cell growth was 22% and 11% for strains 3 and 4, respectively). In order to isolate resistant strains, samples were transferred to the solid YPG medium and were exposed to 24% (v/v) ethanol stress again. Cell growth assessment indicated that variation of resistance was inter-strains dependent.

Inoculation of yeast cells to solid ALP + ethanol medium

All yeast strains and all isolated samples from yeast types No. 3 and 4 were transferred to solid ALP medium containing 2-16% (v/v) and 16-26% (v/v) ethanol, respectively. In these media the only available carbon source to produce energy for yeast strains was ethanol. After inoculation, we observed various modes of growth in yeasts presumably relating to the type of the cells (Table 1 and 2). Yeast cells that consume ethanol immediately produce acid/sour odor and cause medium color change from yellow to red. However yeasts which consume ethanol slowly don't cause sour odor and color change. By this differential experiment, yeast strains No. 3 and 4 were classified as group 1, while the rest were considered as group 2.

Invertase enzyme activity

To understand whether higher resistance to ethanol is due to the greater activity of invertase in yeast strains, the activity of this enzyme was measured. According to Table 3, enzyme activity in yeast strains No. 1 and 2 was higher than yeast strains No. 3 and 4. More interestingly, resistant strains to 24% (v/v) ethanol showed an invertase activity less than parental yeasts in the same condition.

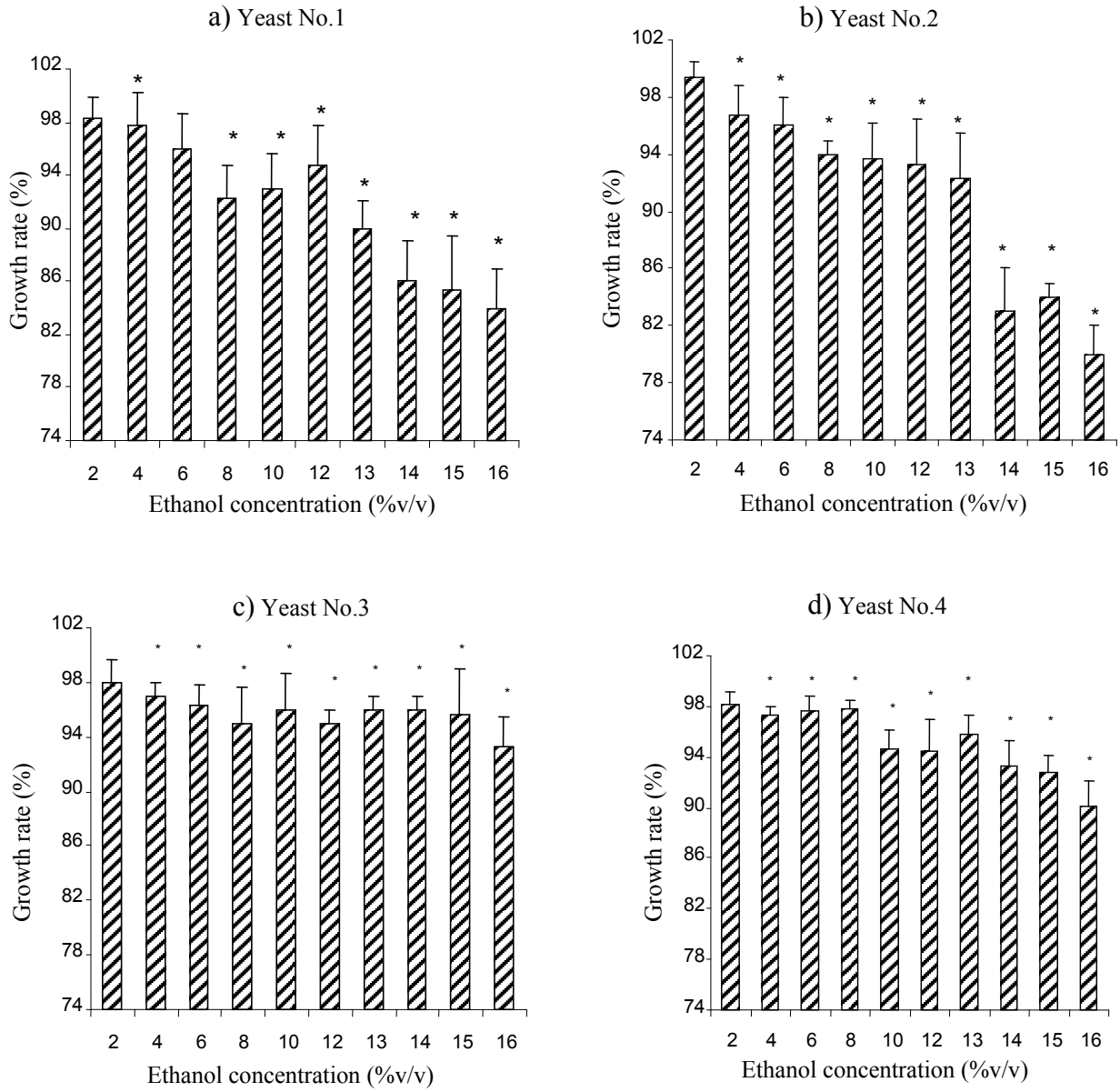

Fig. 1. Effects of 2-16% (v/v) ethanol increment on the viability of different yeast cells. Cells were treated by various concentrations of ethanol at 25 ºC for 1 h in three repeats and growth rate was calculated by plate counting. Statistical evaluations showed that significant differences between level of treatments versus 2% ethanol (**P*<0.05).

Table 1. Growth intensity of different yeast cells (main types) in two groups on solid ALP medium supplemented with different concentration of ethanol.

Yeast strain	Growth intensity							
	2%[a]	4%	6%	8%	10%	12%	14%	16%
Yeast 1[b]	4+[c]	4+	4+	4+	4+	3+	3+	3+
Yeast 2	4+	4+	3+	3+	3+	3+	2+	2+
Yeast 3	3+	2+	2+	2+	2+	2+	1+	1+
Yeast 4	4+	4+	4+	3+	3+	3+	2+	2+

a) Ethanol concentration on solid medium (%, V/V).
b) Name of main commercially type of yeasts.
c) Growth intensity of main yeasts on solid medium that compare with control.

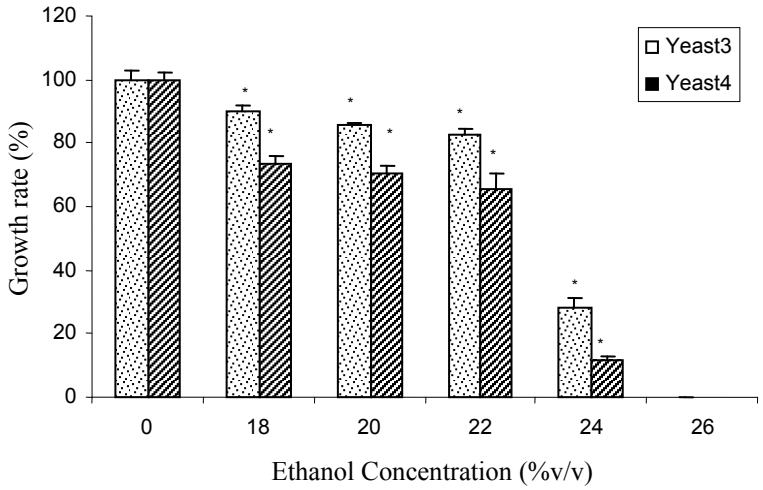

Fig. 2. Effect of 18-26% (v/v) ethanol on the viability of yeast types 3 and 4 cells. Viability was calculated after standard plate counting. Resistant strains were isolated from 24% (v/v) ethanol. **P*<0.05, significantly different from 0% ethanol (control).

Table 2. Growth intensity of different yeast cells (isolates from strains No. 3 and 4 subtypes) in two groups on solid ALP medium supplemented with different concentration of ethanol.

Yeast strain	Growth intensity				
	16%	18%	20%	22%	24%
Yeast No. 3-1[a]	3+	2+	2+	2+	1+
Yeast No. 3-2	3+	2+	1+	1+	1+
Yeast No. 3-3	3+	2+	2+	2+	1+
Yeast No. 3-4	3+	2+	2+	2+	1+
Yeast No. 3-5	3+	2+	2+	1+	1+
Yeast No. 4-1	3+	2+	2+	2+	1+
Yeast No. 4-2	3+	2+	1+	1+	1+
Yeast No. 4-3	3+	2+	2+	2+	1+
Yeast No. 4-4	3+	2+	2+	1+	1+
Yeast No. 4-5	3+	2+	2+	2+	1+

a) Name of resistant strains that were isolated from two main yeasts (No. 3 and No. 4).

Table 3. Invertase activity of commercial yeast strains (Yeast 1-4) and ten resistant colonies isolated from yeast strains No. 3 and No. 4 (Subtypes).

Yeast strain	Invert sugar release (g/L)
Yeast 1	37.5
Yeast 2	33.6
Yeast 3	29.6
Yeast 4	30.9
Yeast 3-1	1.5
Yeast 3-2	3.6
Yeast 3-3	2.8
Yeast 3-4	7.8
Yeast 3-5	2.1
Yeast 4-1	3.4
Yeast 4-2	2.1
Yeast 4-3	11.8
Yeast 4-4	2.6
Yeast 4-5	2.2

DISCUSSION

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