

*Full Length Research Paper*

# Screening of *Saccharomyces cerevisiae* for high tolerance of ethanol concentration and temperature

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The fermentation process of sugar feedstock materials at industrial scale requires the utilization of microorganisms capable of working at high ethanol concentration and high temperatures. The selection of *Saccharomyces cerevisiae* strains, able to ferment sugars obtained from different material at temperatures above 35°C with high ethanol yield, has become a necessity. Three yeast strains were irradiated with gamma ray and screened for their ability to grow and ferment molasses in a temperature range of 35-45°C. The yeasts were placed in a liquid medium, and irradiated at different doses (0.1, 1, 2, 3, 4, 5 and 10 KGy/h). Although all the isolated strains had growth (in agar plates) at 35 and 40°C, but just two strains showed growth at 42°C, and there was no growth at 45°C. Two pure yeast strains were isolated (PTCC<sup>5269</sup> M<sub>3</sub> and Areni M<sub>7</sub>). The efficiency of temperature and high concentrations of ethanol tolerant strains were more than double of ethanol production compared with using the initial strains of yeast. All resistant strains were tested on liquid medium of molasses, and nutrients with 30% (v/v) ethanol had significant difference (P>0.01) for growth intensity at same condition with initial strains.

**Key words:** Bioethanol, gamma radiation, *Saccharomyces cerevisiae*, thermotolerant.

## INTRODUCTION

*Saccharomyces cerevisiae* is an important micro-organism in bio-industry and its tolerance to temperature and ethanol concentration is one of the main characteristics used for deciding whether it can be used as a bio-fermentation resource (Osho, 2005). 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 for good operation (Furukava et al., 2004). One of the problems associated with fermentation of sugar is the high temperatures (35-45°C) and high ethanol concentration (over 20%). Tolerance to high temperatures and ethanol concentrations are important factors of microorganisms for increasing efficiency at industrial scale. The fermentation efficiency of *S. cerevisiae* at high temperatures is very low due to increased fluidity in membranes to which the yeast responds by changing its

fatty acids composition (Mager and Siderius, 2002; Schuller et al., 2004).

Stress or environmental stimuli can cause structural changes and/or metabolic changes in an organism acting as expression activator for genes involved in the synthesis of specific compounds that protect the organism (Lieckefeldt et al., 1993). The factors triggering the expression of this type of genes can be biotic or abiotic. Biotic factors induce changes in the gene expression of the guest, giving rise to the synthesis of specific compounds that generate resistance to the strange organism. Abiotic stresses can be temperature, osmotic stress, anaerobic conditions, heavy metals, growth regulators, ultraviolet or gamma radiation, metabolic repressors, and pH (Brosnan et al., 2000). Stress due to temperature has been the most studied abiotic factor, where both heat and cold induce the synthesis or storing of a group of proteins that increase stress resistance (Steensma and Linde, 2001). Some successful attempts to adapt yeasts to high temperatures have been described. *S. cerevisiae* yeasts, capable of

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fermenting at 40 and 45°C have been obtained using progressive cultures (De Barros et al., 1998). Additionally, thermo-tolerant yeasts have been obtained by selecting survivors after a shock process at relatively high temperatures. El Sheikh and Berry (1980) demonstrated that 15 min of incubation at 55°C resulted in yeasts tolerant to higher temperature than non-incubated controls. The objective of this work was to select *S. cerevisiae* strains capable of fermenting glucose at temperatures above 35°C with an ethanol yield of at least 70% of theoretical value in Fermentation process.

Excess amount of ethanol has been reported to cause mitochondrial DNA damage and degrades bio membranes in yeast cells (Swiecilo et al., 2000). Ethanol can dissolve fatty acid constituents of the cell membranes; disrupt cytoplasmic membrane rigidity (Osho, 2005). Many reports have accentuated a relationship between the fatty acid compositions of lipid membranes and ethanol stress tolerance (You et al., 2003) which stop mitochondrial bio molecules translocation and proton motive force (Ekunsanmi and Odunfa, 1990) and finally cause cell death. According to these phenomena, resistant strains to ethanol have many mechanisms to overcome ethanol perils.

Invertase enzyme activity propriety in the yeast strain is very important. Invertase is one of the important extra-cellular enzymes in *Saccharomyces* that is responsible for converting sucrose to its subunits, glucose and fructose (Sengupta et al., 2000).

Approaches to measure ethanol tolerance involve determination of ethanol effects on cell growth, fermentation ability, viability and batch culture performance (Ekunsanmi and Odunfa, 1990). High ethanol tolerant strains are able to extend the process of fermentation for longer time and produce distinct products in the presence of ethanol (Swiecilo et al., 2000). Resistant strains to ethanol stress have other abilities like resistance to other stresses such as osmotic pressure and oxidative and heat (Swiecilo et al., 2000). Mc Kee and Lawrence (1979) found that partially different sets of gene functions are required for the production of different kinds of mutations induced by <sup>60</sup>Co gamma rays in *S. cerevisiae*. This observation was very similar to others made previously with respect to UV mutagenesis (Lawrence and Christensen, 1976, 1978, 1979).

Induction of mutation in *S. bouldarii* was carried out by 1, 2, 3, 4 and 5 KGy exposure of  $\gamma$  irradiation. Results revealed that the survival percentages were decreased by increasing the doses of  $\gamma$  rays whereas the survival percentage was 2.67% at exposure dose of 5 KGy. On the other hand, the mutant percentages were increased by increasing the radiation intensities, that is, doses. The highest numbers of mutants were induced as a result of 4 KGy dose of  $\gamma$  rays applications, which gave the highest mutants percentage (14.29%) (Abosereh et al., 2006).

To-on et al. (2007), with the yeast fermentation performance reviews collected from various sources, were able to isolate a particular strain capable of producing

15% ethanol at temperature of 40°C in 48 h. It was called thermo-tolerant.

Benton et al. (2006), with DNA analysis in different strains of yeast exposed to different doses of gamma rays (1, 10 and 100 Gy), observed significant differences between their DNA. Akachaa et al. (2007) suggested that low doses of  $\gamma$ -ray (10 and 20 Gy) significantly increased the enzyme activity on *S. cerevisiae*. They also described the impact of irradiation on immobilization efficiency of biocatalyst entrapped on to alginate gel beads. When yeast irradiated to a dose of 20 Gy was immobilized, alcohol-dehydrogenase stability improved up to 1.4 times at 45°C compared to the immobilized non-irradiated cells (Ben Akachaa et al., 2007).

This study attempted to screen *S. cerevisiae* isolates for high tolerance of ethanol concentration and high temperature ranges.

## MATERIALS AND METHODS

### *S. cerevisiae* strains

One of the original types of *S. cerevisiae*, PTCC<sup>5269</sup> (Persian type culture collection) was obtained from the Persian Type Culture Collection of Yeast Cultures which is used in the alcohol industry building, Tehran, Iran (Type NO. 1). Another strain, TTCC<sup>1323</sup> (Turkish type culture collection) was kindly provided by Turkish Type Culture Collection, Ankara, Turkey (Type NO. 2) and third strain was isolated from commercial indigenous wine yeast strains in Areni, Armenia (Type NO. 3). All these were used in the study.

### Media

The yeast strains were kept in solution of 10% molasses, 1 g urea, 0.3 g magnesium sulphate, 0.3 g ammonium phosphate and 0.3 g potassium sulphate, all resolved in 1 L of distilled water, with pH solution set at 5.6. Then the medium was autoclaved at 121°C for 15 min (El Sheikh and Berry, 1980). When required, the yeast culture was activated by transferring to yeast extract peptone agar (YEPA) medium as a complete medium plates (Santangelo, 2006). Inoculum was prepared by putting 0.1 ml of yeast with sampler and added to 100 ml of the above medium. After 24 h incubation at 35°C the culture contained approximately  $7.2 \times 10^2$  cells per ml determined by the method of Camacho-Ruiz et al. (2003). The cells were grown before irradiation up to the stationary phase of growth on a solid growth medium.

### Radiation

*S. cerevisiae* culture was radiated with <sup>60</sup>Co as a source of Gamma ray at different doses (0.1, 1, 2, 3, 4, 5 and 10 KGy/h) such that about minimum 10% of the cells defunct (Abosereh et al., 2006). The dried sample of *S. cerevisiae* (10 g) was milled with the blender for 30 s to yield a powder about 50%. Cultures were irradiated in air at ambient temperature and atmosphere pressure. *S. cerevisiae* cells were grown in YEPA broth medium with shaking at 15°C. For irradiation, the dried samples were packed in polyethylene bags and irradiated (Justin et al., 2001). Mutagenesis of yeast strain was carried out at the Centre of Biotechnology Research, Tabriz, Iran. Cultures were irradiated at ambient temperature and atmosphere

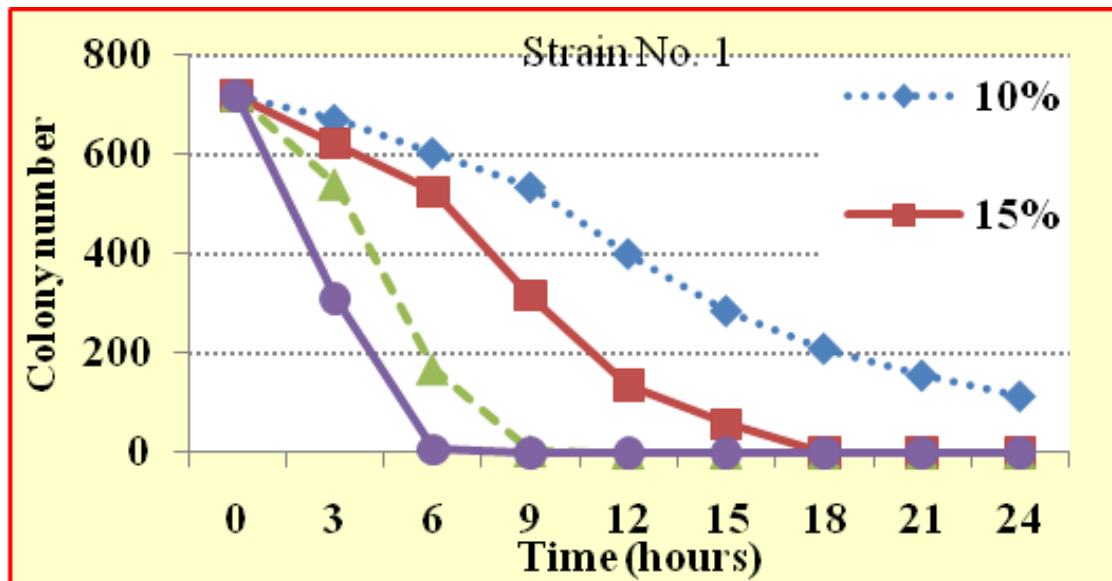


Figure 1. Grown chart of strain No.1 in different concentrations of ethanol.

pressure by shaking with gentle agitation.

#### Mutant cells selection

Serial dilution was prepared from each strain and 1 ml of solution containing  $7.2 \times 10^2$  cells of yeast suspension was added to medium (10% molasses and 2 g/L; urea, 1.00 g;  $MgSO_4$ , 0.3 g;  $NH_4PO_4$ , 0.3 ml;  $K_2SO_4$  sterilized at 121°C for 15 min) with different concentrations of ethanol- 15, 20, 25 and 30% (Sherman et al., 1986). Test tubes containing mentioned medium were incubated at different temperatures (35, 38 and 42°C). Survival cell density was evaluated by plating 0.1 ml of each treatment onto the surface of malt agar medium once every 3 to 72 h. Plates were then incubated for 48 h at 35°C and colonies were counted. Survival yeast percentages were estimated for each treatment.

Survival yeast colonies in medium containing 20% alcohol incubated at 38°C were transferred to medium containing 25% alcohol and incubated at 38 and 42°C for 48 h. Survival colonies in 25% alcohol incubated at 38°C were cultured at complete medium for 48 h. Then 0.1 ml of this isolates inoculated to 30% alcohol in medium mentioned above was incubated at 38 and 42°C for 48 h.

Colonies from irradiated strains which had growth at 42°C (from each treatment of three tested strains) were isolated in a maintenance medium, and evaluated by measuring the  $CO_2$  displacement in a growth medium in a standard tube saccharimeter at 42°C for 24 h.

Irradiated solution of yeast with the gamma-ray was transferred in medium (CM) and yeast survival was compared in different dose of gamma rays with three different initial populations. Also their tolerance to temperatures and different concentrations of alcohol was investigated.

Few colonies grown in 17% alcohol concentration on 40°C were incubated after mixing for 48 h and then their tolerance to alcohol concentrations sundries (15, 17, 20, 25 and 30%) and temperature (35, 40, 45, 50 and 55°C) was investigated. Then the colonies grown in 20% alcohol concentration and temperature of 45 degrees, and also grown in CM for 48 h were assessed again at high concentrations of alcohol and high temperatures. It is done in order to get a particular strain of yeast that has a maximum

temperature tolerance and alcohol.

## RESULTS AND DISCUSSION

### Evaluation of ethanol production

A sample of each strain (0.1 mol) with approximately  $7.2 \times 10^4$  cells per ml was placed in test tubes containing different ethanol concentrations (10, 15, 17.5 and 20%) and maintenance medium for incubating for 24 h (Camacho-Riz et al., 2003). Once in every two hours the samples were cultured in Petri dishes and incubated at 35°C for 48 h. Number of survival colony was significant between strains. Renaults showed that:

1. In 10% ethanol concentration, all the strains had growth but survival number for strain No.3 was more than the other two strains. Cell density decrease in strain No. 2 was more than the others. Statistically, no significant differences were observed in number of colonies between three strains (Figures 1, 2 and 3).
2. Growth in 15% ethanol concentration showed that strains No. 1 after 6 h was decreased and after 18 h no colonies were formed. In strain No. 2 reducing of population was faster than the others, and after 15 h the population vanished. In strain No. 3 after 24 h only a few colonies were formed; therefore it showed that this strain had more ethanol tolerance than the others.
3. In 17.5% ethanol, decreasing of population density in all three strains was faster. Strains No. 1 and 2 after 9 h and strains No. 3 after 12 h of growth and biomass production ceased.
4. Growth in 20% ethanol and evaluation of survival cell

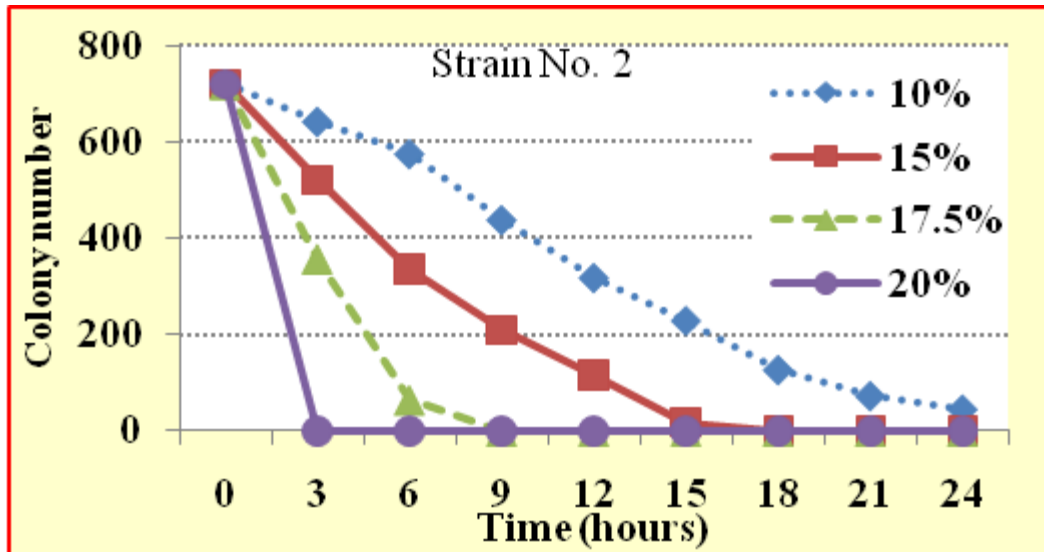


Figure 2. Grown chart of strain No.2 in different concentrations of ethanol.

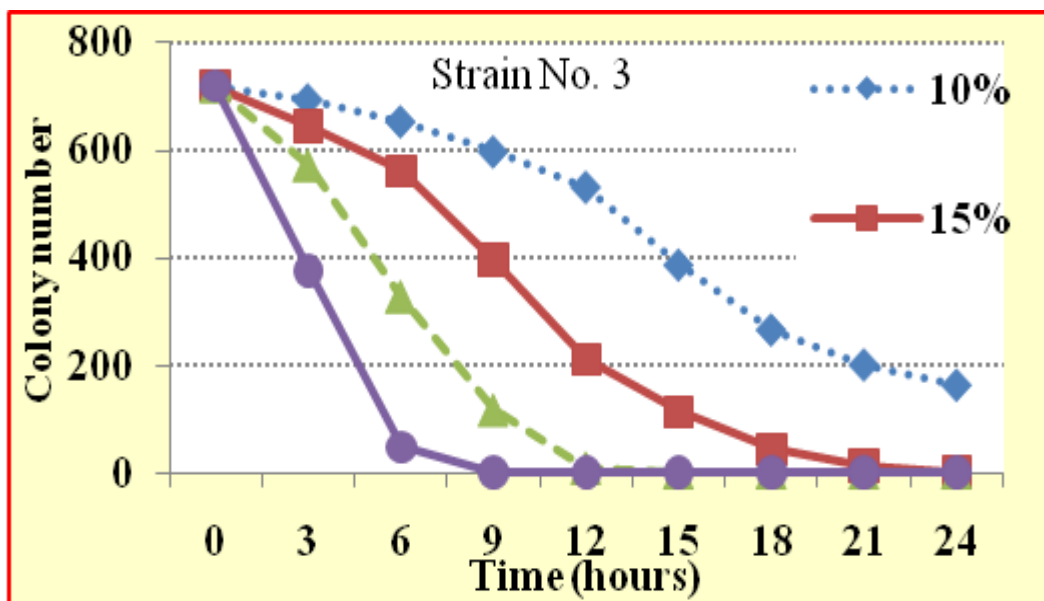


Figure 3. Grown chart of strain No.3 in different concentrations of ethanol.

showed that yeast No. 1 and 3 after six and yeast No. 2 after three hours had no growth.

#### Evaluation of ethanol production at different temperature conditions

All yeast strains exhibit growth and ethanol production at 25 and 42°C. As shown in Figure 4, yeasts No.1 and 3 had the highest activity with 110.1 and 98.6 g/L respectively at 35°C. But yeast No. 2 had the lowest

activity and produced only 85.3 g/L at 30°C.

The yeasts (No. 1 and 3) which had growth at 35°C in the maintenance medium were incubated with shaker at 50°C for 6 h (Cakar et al., 2004) and then the growth medium and ethanol production were evaluated at 35, 38, 42 and 45°C. This thermal shock was repeated several times with different samples so it helped to reach the yeast with higher temperature tolerance. After thermal shock, tolerance range in yeasts No.1 and 3 increased to 42 and 38°C respectively (Figure 4).

The highest increase in ethanol production was

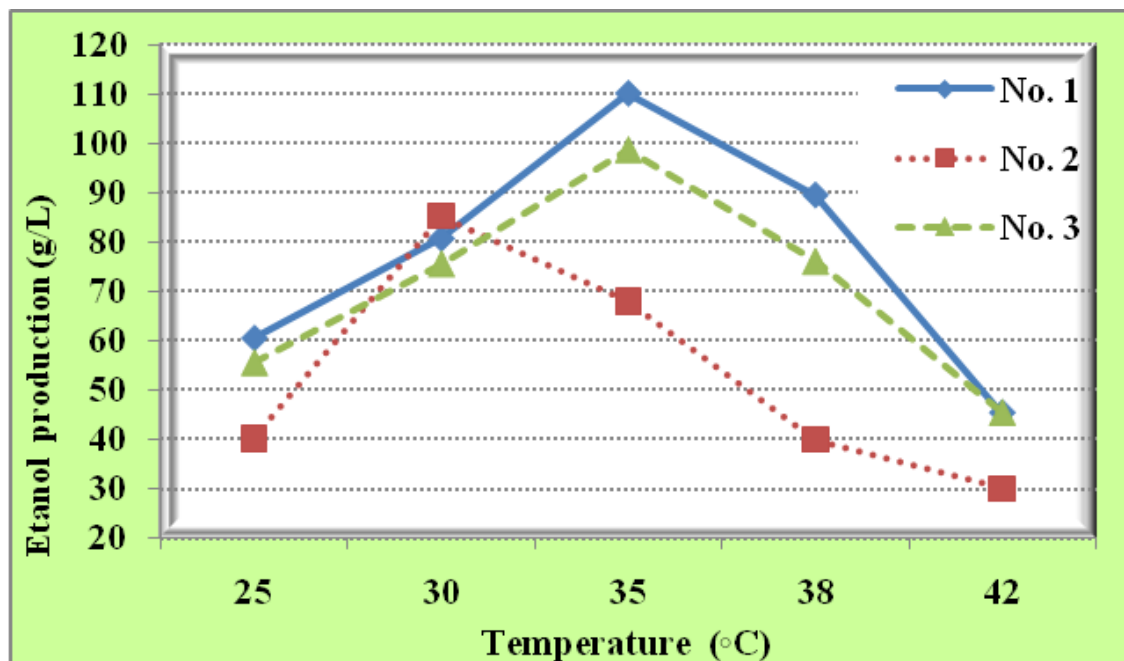


Figure 4. Ethanol production potential for three strains in different temperatures.

obtained by yeast No. 1 TS strain (Been exposed to thermal shock). After thermal shock treatment maximum 110.1 g/L ethanol production was observed at 35°C; the same amount at 38°C within 56 h produced by the same yeast. The yeast No. 3 TH ethanol production was 98.6 g/L during 60 h incubation at 38°C. Considering the range of tolerance to ethanol in strains No. 1 and 3 these two yeast strains can be selected for more study.

#### Effect of gamma ray irradiation on yeast

Solution of selected yeast strains was irradiated by different doses of gamma ( $\gamma$ ) ray. Gamma irradiation, as a physical method, is known to cause injury to microorganisms and has been used widely for creating mutagenesis (Abosereh et al., 2006). Mutation as a result of gamma radiation was achieved by doses of 0.1, 1, 2, 3, 4, 5 and 10 KGy. The results showed that the gamma radiation higher than 1 KGy caused the death of a large number of yeasts in all yeast strains (Table 1). There was no growth at 10 KGy radiation dose. This result confirmed the work of Wang et al. (2001) on the characterization of *S. cerevisiae* mutant.

Evaluating the effect of gamma radiation on survival yeast enzyme activity, it was observed that the enzyme activity of *S. bouldarii* was increased to 30-35% by radiation with low doses (0.1, 1 and 2 KGy) (Abosereh et al., 2006).

240 survived yeasts were obtained after  $\gamma$  irradiation treatments. Strains No. 1 and 2 were evaluated in terms of survival ability in 25 and 30% ethanol concentration

and ethanol production capability in temperature of 38 and 42°C.

Strains tolerance to high concentrations of ethanol (25 and 30%) was incubated with shaker in sugar beet molasses of 20% (Cazetta et al., 2007) for 72 h at 38 and 42°C. Once in every three hours, 0.1 ml of each treatment was transferred to plates containing malt agar medium and colony formation represented survival (Reagan et al., 1995).

There was significant differences ( $p < 0.01$ ) between irradiated yeasts with the initial strains for traits of ethanol production yield and abilities to survive in high ethanol concentration at high temperatures. By Duncan mean comparison method two isolated strains were selected for maximum ethanol production in the high temperatures (Table 2). The isolated strains had two time efficiency of ethanol production more than the strain used in the alcohol industry. Also, they had the ability to survive and grow in 25-30% ethanol concentration.

Means comparison of ethanol production of the thermo-tolerant and ethanol tolerant yeasts with untreated strains of *S. cerevisiae* PTCC<sup>5269</sup> (No. 1) and *S. cerevisiae* Areni (No. 2) are shown in Table 2. Yeast fermentation medium contains molasses of 20% (w/v); yeast extract of 0.5% (w/v), pH 5.5 and starts with a 10% inoculum on a shaking incubator at 38 and 42°C with 80 rpm for 72 h average value of four replicates.

Of the 12 isolated yeast evaluated in the experiment at 38 and 42°C, only PTCC<sup>5269</sup> M<sub>3</sub> and Areni M<sub>7</sub> had growth and produced highest bio-ethanol. Ethanol yield obtained 23.50% (v/v) and 22.60% (v/v) at 72 h. These two isolated strains had significant difference ( $p < 0.01$ ) with

**Table 1.** Effect of different doses of Gamma irradiation on survival of yeast strains.

Dose of $\gamma$ ray (KGY)	Survival colonies					
	No. 1		No. 2		No. 3	
	N/ml	%	N/ml	%	N/ml	%
0	720	100	720	100	720	100
0.1	719	99.9	720	100	712	98.9
1	685	95.1	546	75.8	653	90.7
2	556	77.2	371	51.5	387	53.8
3	349	48.5	223	31.0	165	22.9
4	190	26.4	98	13.6	59	8.2
5	47	6.5	13	1.8	3	0.4
10	0	0.0	0	0.0	0	0.0

**Table 2.** Mean comparison of ethanol production for isolated tolerant yeast with initial strain of *S. cerevisiae* ( $p < 0.01$ ).

Isolated yeast	Ethanol % (v/v)	
	38°C	42°C
	PTCC <sup>5269</sup> M <sub>1</sub>	18.50 E
PTCC <sup>5269</sup> M <sub>2</sub>	21.97 B	19.12 B
PTCC <sup>5269</sup> M <sub>3</sub>	23.90 A	23.50 A
Areni M <sub>1</sub>	20.30 CD	19.50 B
Areni M <sub>2</sub>	21.37 BC	19.60 B
Areni M <sub>3</sub>	20.10 CD	19.20 B
Areni M <sub>4</sub>	21.20 BC	19.50 B
Areni M <sub>5</sub>	20.30 CD	17.80 B
Areni M <sub>6</sub>	18.80 E	17.10 B
Areni M <sub>7</sub>	23.80 A	22.60 A
Areni M <sub>8</sub>	20.30 CD	19.20 B
Areni M <sub>9</sub>	19.37 DE	17.10 B
PTCC <sup>5269</sup> (No. 1) Check	14.85 F	11.54 C
Areni (No. 3) Check	16.61 G	10.55 C

other treated and untreated strains of *S. cerevisiae* PTCC<sup>5269</sup> (No. 1) and Areni (No. 2). There was no growth and fermentation at 45°C by treated and untreated yeasts. Some of them were capable of growing and producing ethanol in stress conditions. The differences could be attributed to genotypic qualities of each microorganism. Similar effects were reported previously by Abdel-Fattah et al. (2000).

Edgardo et al. (2008) reported that thermo-tolerant strain of organosolv-pretreated *P. radiata* could produce 22 g/L ethanol (73% of the theoretical ethanol yield).

While the untreated strain can produce only 3.5 g ethanol/L (12% of the theoretical ethanol yield).

The stability of the acquired stress resistance phenotype was also tested for all mutant clones by analyzing their relative level of stress resistance after five batch growth cycles (about 10 generations). All clones exhibited unaltered stress resistance (data not shown).

## Conclusion

The gamma irradiation treatments made it possible to find two strains, PTCC<sup>5269</sup> M<sub>3</sub> and Areni M<sub>7</sub>, with high ethanol production yield at high temperature statement than the control strains. Employing these two gamma ray and temperature treated strains can cause increased ethanol concentration up to 25%. Thus fermentor evacuation and loading time can reduce three times and residual sugar well remain at minimum amount at any time of fermentation. In current study, the stress resistances of various individual clones selected from final mutant populations were determined. The results generally revealed heterogeneous populations. To understand the genetic background of these highly double-stress resistant mutant clones, which is the second step in inverse metabolic engineering (Bailey et al., 1996), their transcriptome and/or proteomic analysis would be necessary.

Achievement of thermal and high ethanol tolerant strains led greater ethanol yield in fermentation process than the control strains. By using these strains it is possible to convert pre-treated sugar material into ethanol at industrial scale more efficiently.

Kiransree et al. (2000) produced with molasses containing 14% sugar, maximum 53.2 and 45 (g/l) at 30 and 40°C respectively by thermo-tolerant strain. That strain showed 12% W/V ethanol tolerance. Isolated strain was also characterised for its ethanol producing ability using various starchy substrates in solid state and submerged fermentation.

The isolated strains (PTCC<sup>5269</sup> M<sub>3</sub> and Areni M<sub>7</sub>) are now ready for being commercialised as a new fermentative strain for bio-ethanol production. So these strains have been deposited at the Department of Biotechnology, Urmia University, Urmia, Iran, and can be released for research and commercial purpose upon request. These cultures have economical importance for use in alcohol production during hot seasons.

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