International Scholars Journals

African Journal of Virology Research ISSN 2756-3413 Vol. 18 (1), pp. 001-006, January, 2024. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Advancing Biofuel Technology: Mutation of Saccharomyces cerevisiae for Improved Furfural Resilience and Bioethanol Output

Rahimian Zarif Bahareh[1]* and Azin Mehrdad[2]

[1]Department of biology, Science and Research Branch, Islamic Azad University, Ayatollah Ashrafi Esfahani Highway, Simon Bolivar Blvd., Tehran 1477893855, Iran.

[2]Department of Biotechnology, Institute of Advanced Technology, Iranian Research Organization for Science and Technology, 27, Shahid Mousavi St., Enghelab Ave., Tehran 15815-3538, Iran.

Accepted 18 November, 2023

High ethanol yield is a desired property of industrial yeast strains. A significant problem in fermentative conversion of lignocellulosic materials to ethanol is the formation of furfural and hydroxymethylfurfural (HMF), which are formed during acid hydrolysis of lignocellulose. Furfural has been known to create strong inhibition in metabolism of the yeast *Saccharomyces cerevisiae*. In this article, the result of random mutagenesis of a native strain of *S. cerevisiae* by ultra violet (UV) light and nitrous acid is presented. By screening the cells in the presence of furfural, a potent mutant was selected which produced 36.7% more bioethanol than the parent strain, in the presence of 0.2% (v/v) furfural.

Key words: Bioethanol, furfural, mutation, nitrous acid, *Saccharomyces cerevisiae* PTCC 5315, UV radiation.

INTRODUCTION

Fuel ethanol has been regarded as a favorable alternative energy source, which is both renewable and environmental (Ogawa et al., 2000). Nearly, 73% of the whole ethanol produced globally, corresponds to fuel ethanol (Cardona and Sanchez, 2007; Balat et al., 2008; Zhang et al., 2010). Ethanol produced from lignocellulosic materials is called second generation bioethanol and regarded as a carbon neutral fuel. The market for bioethanol has been growing in many countries. To promote bioethanol utilization, it is necessary to reduce the production cost. Wood is one of the most adequate and great resources of the lignocellulosic materials used for bioethanol production (Okuda et al., 2007).

Saccharomyces cerevisiae is utilized mostly in batch fermentations to convert sugars to ethanol for the production of potation and biofuels. Despite the distinct importance of this process, the physiological routes which limit the rate of glycolysis and ethanol production are not fully understood (Casey and Ingledew, 1986; Ingram and Buttke, 1984; Moulin et al., 1984).

Realizing these routes, contributes to pave important steps toward the development of improved organisms and process conditions for more and faster ethanol production. As a well known fact, ethanol is remarkable in displaying strong inhibition on the growth and metabolism of yeast cells (Xue et al., 2008; Cot et al., 2007).

Therefore, strain improvement could result in increasing the ethanol production capacity of current fermentation plants and decreasing in the cost of production (Dombek and Ingram, 1987). The study of ethanol fermentation has gained significance because of expanding demand for it in recent years as a motor fuel supplement to gasoline. Fast fermentation and high ethanol levels are desirable to minimize capital costs and distillation energy, where excellent yields are essential for process economics. The substrate is the principle cost component for industrial ethanol production and it is necessary that, ethanol production could be carried out with cheap substrates (Ratnam et al., 2005).

A significant problem in fermentative conversion of lignocellulosic materials to ethanol is the severe inhibitory

*Corresponding author. E-mail: bahareh_r_z@yahoo.com. Tel: +98 21 44453952 or +98 21 44869458. Fax: +9821 77929868.

effects often created by lignocellulosic hydrolysates (Cardona and Sanchez, 2007; Fang et al., 2010).

Furfural has been known to create strong inhibition in metabolism of *S. cerevisiae* under anaerobic conditions both during batch cultivation (Palmqvist et al., 1999) and in glucose-limited chemostats (Fireoved and Mutharasan, 1986; Sarvari et al., 2001).

Furfural has distinct inhibitory effects on the growth rate, as well as on the fermentation rate of yeasts (Banerjee et al., 1981; Palmqvist and Hahn-Hägerdal, 2000).

However, only few studies have reported the effects of furfural under aerobic conditions. These effects are significant, since inhibition caused by furfural during respiratory growth has an immense impact on yeast multiplication in an ethanol production plant based on a lignocellulosic feedstock (Horváth et al., 2003).

Induced mutagenesis by application of physical and chemical mutagens is an uncomplicated and straightforward method for yeast strain improvement. Selection process, after treatment of cells by mutagens, has been used to a considerable extent in improvement of yeasts (Sridhar et al., 2002).

In this study, UV radiation (Calam, 1970; Kiran, 2000a, b) and nitrous acid (Azin and Noroozi, 2001) were used to mutagenize *S. cerevisiae*, which was then, cultured in the presence of furfural to select for furfural resistant and strong ethanol producers. Since furfural was used for selection of mutants, most of the isolates showed either increased or at least unaltered levels of bioethanol production as compared with the native strain.

MATERIALS AND METHODS

Microorganism

S. cerevisiae T12 (PTCC 5315), a native strain that showed good ethanol production and furfural resistance, which was isolated from an industrial waste water in Ghazvin Province (North-west of Iran), was used in this study as native type strain. The yeast was kept on potato dextrose agar (PDA) and subcultured every two weeks. Twenty four hours aged colonies were used to inoculate yeast extract peptone dextrose broth containing (g/l): yeast extract, 10; peptone, 20; dextrose, 20. The cultures were incubated for 20 h on a shaker-incubator at 150 rev. min^{-1}, 26°C. The growth was checked by counting the cell number by Neubauer counting chamber and also by measuring the culture absorbance at 600 nm, spectrophotometerically (Sridhar et al., 2002).

UV treatment and selection of mutants: 0.1 ml of a suspension of about 1.3×10^8 cells/ml were plated on YEPD agar medium, containing (g/l): yeast extract,10; peptone, 20; dextrose, 20; agar, 15; pH 5.5. The plates were exposed to short wavelength UV light (280 nm) from a distance of 20 cm by using a Philips™ 30 W germicidal UV lamp (Baltz, 1986; Sinha and Chakrabarty, 1977) from 0 to 350 s, till a survival of about 0.01% was obtained. Percentage of survived cells after UV light irradiation was measured by culturing the untreated cells on furfural containing YEPD medium. To screen for furfural resistant mutants, freshly distilled furfural (0.15% (v/v) was added to the medium. Appropriate UV dose was determined as that dose in which, the survived cell fraction was 0.01%.

Nitrous acid treatment and selection of mutants

Concentrations of 0.02 to 0.2 M of $NaNO_2$ in acetate buffer (0.2 M, pH 4.5) were added to the washed and centrifuged cells of *S. cerevisiae* PTCC 5315 in a manner that a cell density of about 1.3×10^8 cells/ml was prepared (Carlton and Brown, 1981). The solution was thoroughly shaken and after 10 min, 1 ml of cell suspension was drawn off and diluted in phosphate buffer (0.2 M, pH 7.1) to stop the reaction (Azin and Noroozi, 2001). Percentage of survived cells after nitrous acid treatment was measured by culturing the treated and untreated cells on YEPD medium containing 0.2% (v/v) freshly distilled furfural and comparing the number of appeared colonies. In that concentration of nitrous acid that 99.99% of yeast cell were killed, colonies were screened for resistance against furfural. Appropriate nitrous acid dose was determined as that dose in which, the survived cell fraction was 0.01%.

Fermentation experiments

After selecting the most resistant mutants, they were kept on YEPD agar, containing 0.2% (v/v) furfural. For preparation of precultures, the mutants were inoculated to 25 ml flasks, containing 5 ml YEPD broth and incubated on rotary-shaker at 150 rev. min^{-1}, 30°C, for 20 h. Then, 2 ml of preculture was transferred to 18 ml of a synthetic medium containing (g/l): glucose, 190; yeast extract, 10; $(NH_4)_2SO_4$, 0.6; $(NH_4)_3PO_4$, 1.2; pH 5.3 and placed on rotary-shaker at 150 rev. min^{-1}, 30°C, for 8 h to complete the aerobic growth phase. After this phase, the cultures were aseptically transferred to 25 ml Bijou bottles for 40 h to complete the anaerobic, ethanolic fermentation period. Both the preculture and production medium contained 0.2% (v/v) furfural.

Analytical methods

The concentration of biomass was determined by centrifugation of culture for 15 min at 6000×*g*, washing the cells with distilled water and drying the precipitated mass at 103°C for 24 h and weighing the residues, in three replicates (Sarvari et al., 2001). Cell concentration was also determined by measuring its absorbance specrophotometrically at 620 nm and microscopic cell counting by Neubaur counting chamber.

For determination of glucose concentration enzymatic glucose kit (Shimenzyme Co., Tehran, Iran) was used. The ethanol concentration was measured by gas chromatography (GC-14A, OV17 column, Shimadzu Corp., Japan) equipped with a UV detector, after distillation of the culture media and separation of ethanol. Chromatography was performed in initial oven temperature of 50°C, final temperature at 90 and 230°C injecting temperature, using nitrogen as carrier gas at a flow rate of 30 ml/min (Grob and Barry, 2004). All the tests were done in three replicates.

RESULTS

During two stages of mutagenesis, lethal doses and frequency of selected mutants were calculated as presented in Figures 1 and 2. As shown in Figure 1, the best UV dose (time of irradiation), which have been calculated as ratio of surviving cells to the total number of cells at the beginning of treatment, has been observed after 290 s of exposure to U.V. light. This was the point at which, 99.99% of the initial cells were killed. For nitrous

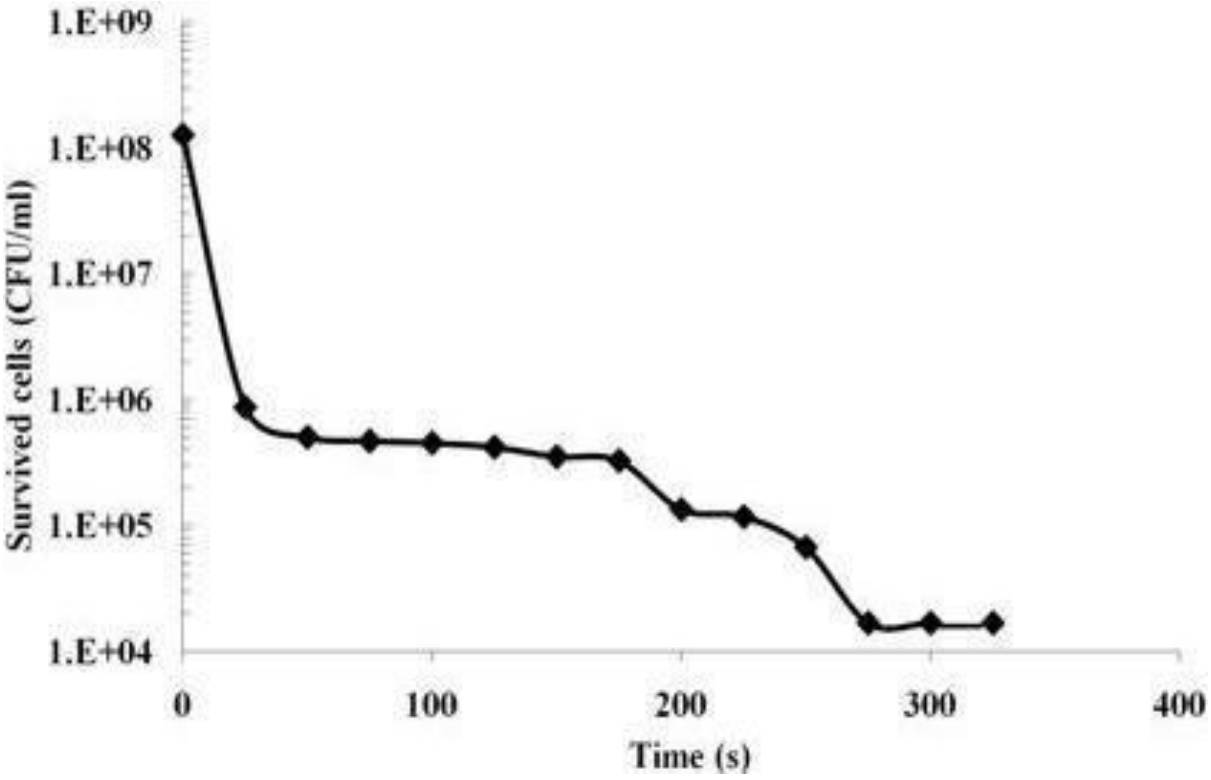

Figure 1. Effect of UV light on survival of *S. cerevisiae* PTCC 5315 cells.

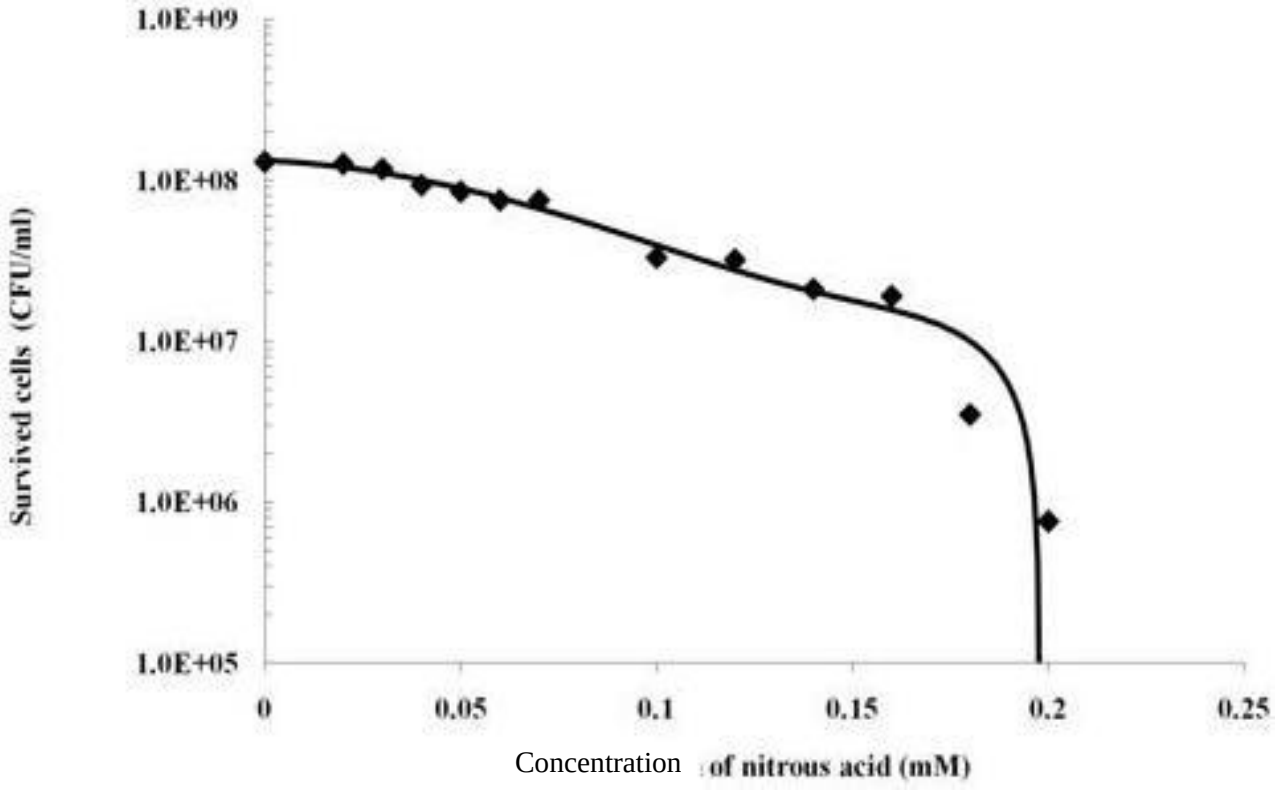

Figure 2. Effect of nitrous acid on survival of *S. cerevisiae* PTCC 5315 cells.

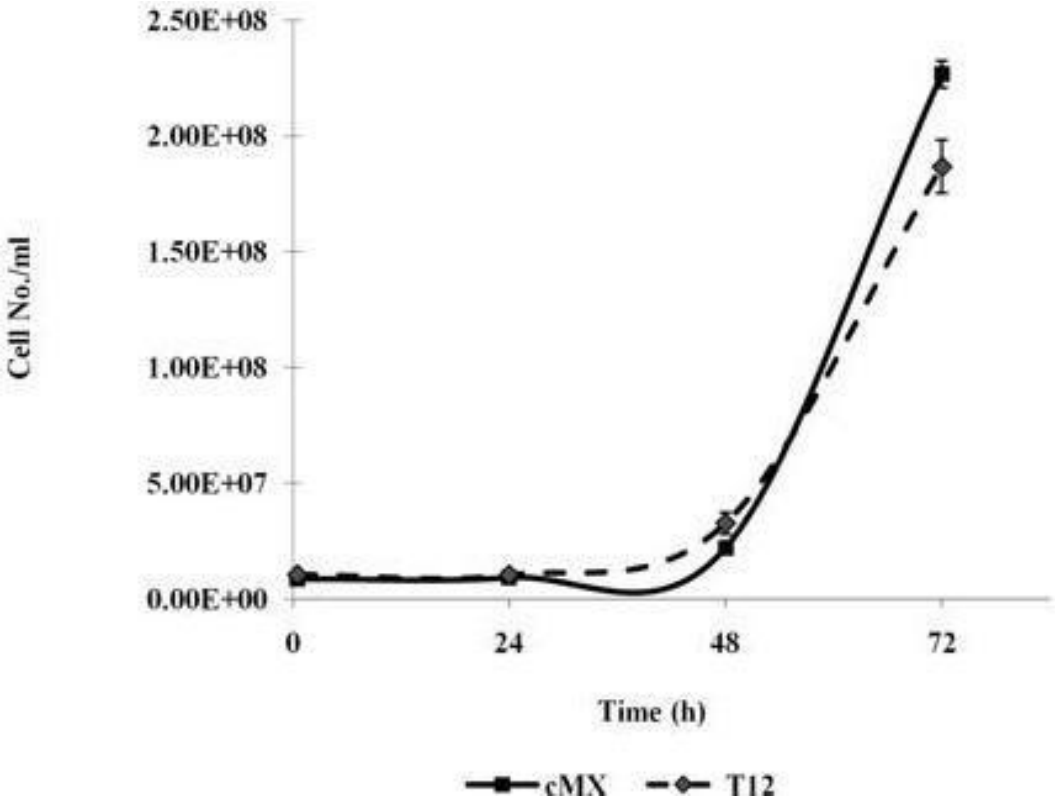

Figure 3. Growth kinetics of native type and mutant strains. Error bars represent the standard deviation of 3 replicates.

acid, 0.2 M concentration was found to be the appropriate dose for the same killing rate (Figure 2). Furthermore, it was found that, at 0.2% (v/v) concentration of furfural, no growth was observed in native type yeast.

After sequential treatment of native *S. cerevisiae* PTCC5315 by UV radiation and nitrous acid, the best mutants obtained at each step, were determined based on resistance to furfural (0.2% (v/v)) in YEPD agar medium. After selection of the resistant mutants, they were compared with native type strain regarding the production of ethanol. Finally, it was found that, the ethanol production in a mutant strain, designated cMX, was 36.7% higher than the native strain.

Growth kinetic

Figure 3 compares the growth kinetics of mutant strain, cMX, with that of the native type, PTCC5315. As it is shown, although, at the early phases of growth, there was no significant difference between the trend of growth, however, after 72 h, there was a significant difference ($P = 0.012$, $\alpha = 0.05$), as tested by statistical t-test. While the specific growth coefficient (μ) of native type strain (between 0 to 72 h) was measured to be 0.24 h^{-1}, the same figure for the mutant was 0.36 h^{-1}. This difference in growth is in accordance with the recorded differences between sugar consumption and ethanol production, between two strains (Figure 4).

The mutant strain, cMX, comparing with the native type, has been able to consume more sugar and produce more ethanol. Comparing the yield of the ethanol by the following formula:

$$Y_{P/S} = \frac{\text{[produced ethanol] g/l}}{\text{[consumed substrate] g/l}}$$

show that, while *Yp/s* in strain PTCC 5315 was 0.38, the mutant strain had *Yp/s* of 0.4, which shows a better conversion of sugar to ethanol in the presence of furfural.

DISCUSSION

Hydrolysis of lignocellulosic substances by acids produces hydrolyzates that contain toxic compounds that inhibit fermentation. They are classified into three groups: (1) weak acids from hemicellulose or sugar degradation (for example, acetic acid and formic acid); (2) furan derivatives from sugar degradation (for example, furfural and 5-hydroxymethy furfural [HMF]); (3) phenolic

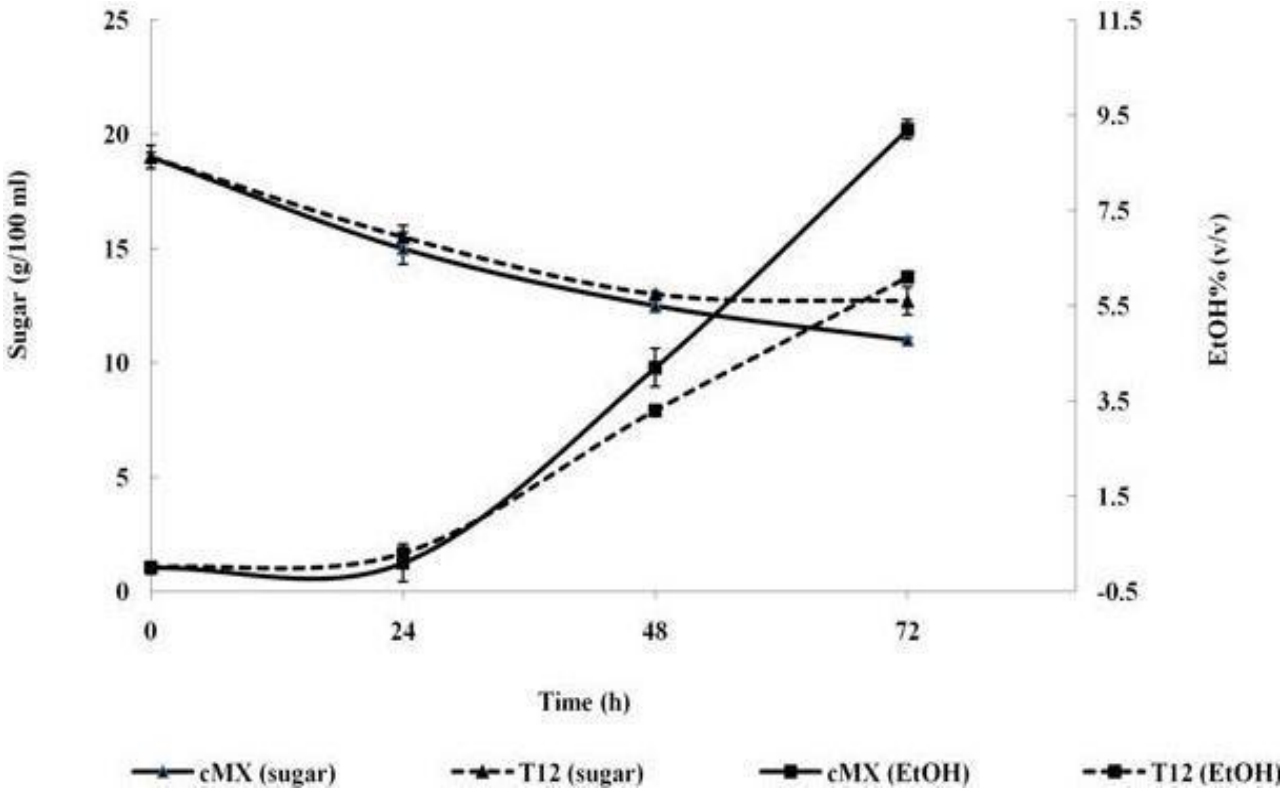

Figure 4. Rate of sugar consumption and ethanol production of native type and mutant strains. Error bars represent the standard deviation of 3 replicates.

compounds from lignin degradation (for example, 4-hydroxybenzoic acid, vanillin and catechol). Of these compounds, furans inhibit the growth and fermentation of ethanol-producing microorganisms most strongly (Cardona and Sanchez, 2007; Balat et al., 2008).

Effects of ethanol fermentation by furans *in vitro* measurements showed that, furfural and HMF directly inhibited alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH) (Modig et al., 2002). Directed evolution or adaptive strategies are paths to mimic the common selection procedures (Sauer, 2001). Directed evolution is based on the tentative improvement of cellular properties through iterative genetic diversification (induced or not, by mutagens) and selection procedures. Several mechanisms may explain the inhibition.

Liu et al. (2005) reported that, after at least 100 transfers, *S. cerevisiae* 307-12H60 and 307- 12H120 showed enhanced ability to reduce HMF at concentrations as high as 30 and 60 mmol l^{-1}, respectively. Moreover, both strains grew and metabolized glucose faster than the control strain Y-12632.

Induction of mutation by UV and MNNG followed by selection was affluently used for improvement of baking yeast strains in Poland and USSR (Johnston and Oberman, 1979). Uma and Polasa (1990) have reported the effect of UV in improving the biomass and ethanol yield using mesophilic *S. cerevisiae* at 03°C (Uma and Polasa, 1990).

In this article, the procedures of creating a novel mutant of *S. cerevisiae* which was screened from a mutated population of the yeast, sequentially treated by UV light and nitrous acid and screened in increasing concentration of furfural is described. As a result, a mutant was isolated which was able to produce 36.6% more ethanol, than the parent strain, in the presence of 0.2% (v/v) furfural. It showed better glucose consumption rate in this condition, too.

Further studies are underway to find the differences of the mutant and parent strains at the proteomics level, the result of which will be presented.

ACKNOWLEDGEMENTS

This work was financed by Iranian Research Organization for Science and Technology by grant no. 10121857. The technical assistance of Miss Sepideh Shokri and Farzaneh Ahadzade from Department of Biotechnology, Iranian Research Organization for Science and Technology is gratefully appreciated.

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