Advances in Bioresearch
Adv. Biores., Vol 5 (4) December 2014: 66-72
©2014 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV 7.20 [Poland]

Advances in Bioresearch

ORIGINAL ARTICLE

Enhancement of Ethanol Production by Inducing Ethanol-Tolerance in *Saccharomyces cerevisiae* U1

Yuvraj C[2], Priyanka P[2] and Aranganathan V[1*]
[1]Department of Biochemistry, Jain University, Bangalore -560 011, Karnataka, India
[2]Department of Biotechnology, Jain University, Bangalore -560 011, Karnataka, India
E-mail: bioarang@yahoo.co.in

ABSTRACT
Ethanol tolerance is an important characteristic of ethanol producing yeast. Present study has focused on the development of high ethanol tolerant yeast strain by random mutation using UV radiation and EtBr. UV mutant U1 displayed significantly improved ethanol tolerance upto 20% ethanol and shown a higher viability during incubation. FTIR spectra analysis of wild and mutant strain in the region corresponding to cell wall polysaccharides shows significant modifications. Mutated strain exhibits a higher IR absorption intensity at 1082 cm^{-1} stretching and contribution of glucan band as major cell wall content. The amount of ethanol produced from sugarcane and tomato juice using potential U1 mutant strain was about 40 %, 36 % respectively higher when compared with wild strain.
Keywords: *Ethanol tolerance, Mutation, FTIR, Saccharomyces cerevisiae, ADH gene*

Received 02/10/2014 Accepted 21/11/2014

How to cite this article:
Yuvraj C, Priyanka P and Aranganathan V.Enhancement of Ethanol Production by Inducing Ethanol-Tolerance in *Saccharomyces cerevisiae* U1. Adv. Biores., Vol 5 [4] December 2014: 67-72: DOI: 10.15515/abr.0976-4585.5.4.6772

INTRODUCTION

A sustainable energy in future depends on an increased share of renewable energy, especially in developing countries. Renewable energy is one of the most efficient ways to achieve sustainable development. Increasing its share in the world matrix will help to prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply on a global scale

Fuel ethanol, which has a higher octane rating than gasoline, accounts for approximately two-thirds of the world's total annual ethanol production of more than 21,812 million gallons. Yeasts from the ancient time have been used by mankind for the production of alcoholic beverages and leavened bread [1]. Ethanol production was the foremost biotechnological, yeast derived commodity from many years [2]. Cost-effective ethanol production depends on, among other factors, rapid and high yielding conversion of carbohydrate to ethanol, which in itself depends on improvements in the survival and performance of yeast cells under industrial conditions [3].

Among all the factors, ethanol is considered to be the major stress responsible for decreased ethanol production and stuck fermentation [4]. Excess concentration of ethanol nearby 8% (v/v) causes phospholipids of lipid bilayer of cell membrane and organelles to become hyperpolarized thereby increasing membrane fluidity and consequently decreasing membrane integrity [5, 6]. Ethanol is an inhibitor of yeast cells as the concentration of ethanol increases in the medium it increases the cell vitality and death rate [7]. High ethanol production capability of ethanologenic yeasts under the presence of high ethanol is one of the most important factors for ethanol production.

The development of such strains is of great economic value to industries involved in fermenting, distilling and refining ethanol. Several studies to date have focused interest on ethanol tolerance of ethanol-producing yeasts based on the presumption that ethanol-tolerant yeast strains would have enhanced ethanol productivities and yields [8, 9, 10].

The aim of present study is (i) Induce mutation and characterization of *Saccharomyces cerevisiae* (ii)

analysis of ethanol tolerant capability (iii) Application on ethanol production from agro products.

MATERIALS AND METHODS

Chemicals and Equipments

Yeast extract Peptone Dextrose agar (YEPDA) media, Potassium dichromate, Sulfuric acid, EtBr, Agarose and Tris Base where purchased from Himedia, India. Fungal genomic DNA isolation kit (RKN 19, Chromous Biotech, Bangalore), PCR analyzer (Corbett Research, Australia), FT-IR spectrophotometer (Perkin Elmer GX)

Cultivation of Yeast strain

Yeast strain (Saccharomyces cerevisiae) MTCC 170, procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The lyophilized strain was activated according to Sambrook and Russell, [11] using YEPD media. All components of media were autoclaved together for 15 mins at 121°C. For liquid culture cells were inoculated into a 250ml Erlenmeyer flask containing 50ml of YPD and the flask were incubated 33°C for 24hrs. YEPDA agar plates containing 4%, 8%, 12% and 16 % ethanol were 1st selection media for ethanol-tolerance.

Mutation on MTCC 170 strain

Mutation studies were carried out on plates containing pure culture. Plate exposure was preferred due to better exposure and static condition over broth culture. Physical random mutation was induced using Ultraviolet (UV) rays on pure cultivated strains according to Unaldi et al., [12] with minor modifications. Plates were exposed to UV light at a distance of 20 cm with interval of 10, 15, 20 and 30 mins using UV transilluminator. Strains were designated as U1, U2, U3 and U4 respectively. All the plates were incubated at 33°C for 24hrs.

Simultaneously chemical mutation of pure cultivated strain was carried out using EtBr at a concentration range of 0.25, 0.50, 0.75 and 1 mg/ml in YEPDA media plates. Chemically treated strains were designated as E1, E2, E3 and E4 respectively and plates were incubated at same conditions.

Strain Selection

Saccharomyces cerevisiae mutants were screened for 2nd level selection using YEPDA broth containing high ethanol concentration for ethanol-tolerance ability. The ethanol tolerance capability of the mutants in YEPDA medium was examined in comparison to the wild type.

Molecular Characterization

Yeast DNA was isolated by using standard protocol provided in fungal genomic DNA isolation kit. DNA amplification has been done by using primers specific to ADH (Alcohol dehydrogenase) gene which were designed using Oligoanalyser and sequence of ADH gene was retrieved from NCBI. Both the isolated genomic DNA and PCR amplified products had been resolved by using agarose gel electrophoresis.

Fourier transform infrared (FTIR) spectroscopy analysis

FTIR spectroscopy analysis was carried out to examine the changes in cell wall content of yeast strain before and after mutation. Spectra were recorded for 72h old yeast cultures at room temperature using Perkin Elmer GX FT-IR spectrophotometer. All measurements on two independent cultures normalized in the range of 860 cm^{-1} to 1360 cm^{-1} and analyzed using Labspec 6 Raman software.

Fermentation of Agro products

The presence of more sucrose content makes sugarcane and tomatoes to be selected for the ethanol production. Fresh sugarcane juice was collected by crushing the sugarcane stem for the fermentation process. Fresh tomatoes were brought from the local market just before the use. The tomatoes were washed and dry weight was measured. Then tomatoes were crushed into juice in a grinder without using any water. 1 liter of each juice was autoclaved and used for fermentation process separately.

Submerged fermentation was carried out separately with 10 ml of wild and U1 strain inoculum (1×10^6 cells/ml) using modular baby fermenter of 3 liter capacity. Fermentation conditions of pH 4.0, at 37°C have been maintained for 72 hrs for maximum activity [13]. The fermented products have been subjected to simple distillation for the separation of ethanol from aqueous mixture at70°C; due to the boiling point of ethanol is approximately 78.37°C. The amount of ethanol produced was measured using Potassium Dichromate method developed by William and Darwin [14].

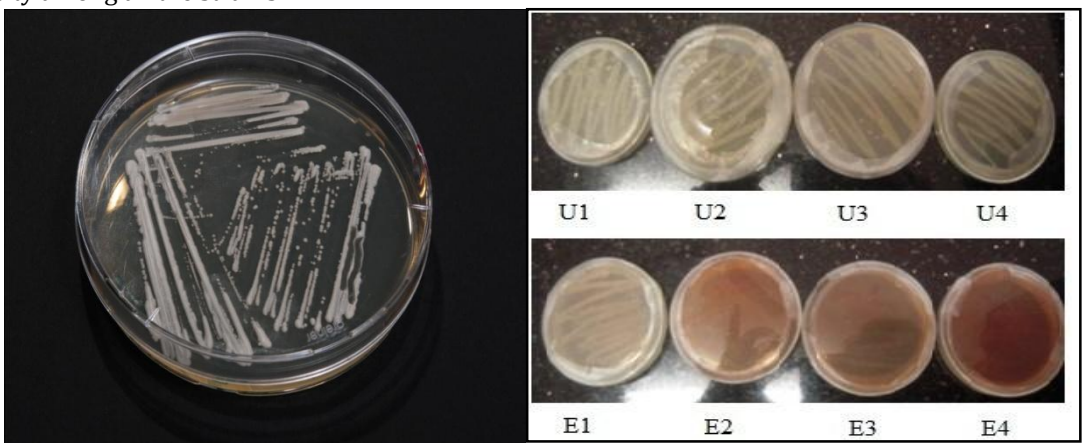
RESULTS AND DISCUSSION

Cultivation and selection

Figure 1a shows the growth of wild yeast strain on YEPDA media. Microscopic observations like large, unicellular, globose to ellipsoidal budding cells or blastoconidia without flagella reveals as *Saccharomyces cerevisiae* which was validated according to Kurtzman et al., [15].

Figure 1b shows the growth pattern of mutant strains on high ethanol strength. The UV mutant strains

(U1 to U4) were shown growth in all the four plates, whereas EtBr mutated strain shown growth only at 0.25mg/ml EtBr concentration (E1). The other mutants (E2 to E4) have shown nearly zero growth may be due to excess concentration of EtBr which leads to the loss of mitochondrial DNA [16].
The growth pattern of 2nd level selection was shown in figure 2. Strain U1 showed best ethanol tolerant capacity among all the strains.



A B

Fig. 1 Colonies of Yeast on YEPDA plates (a) Wild strain (b) UV and EtBr mutant strain

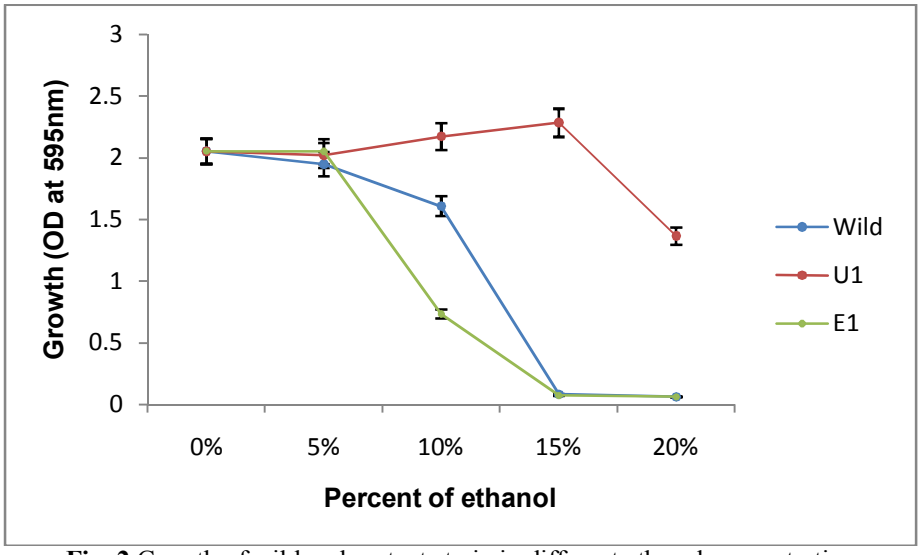


Fig. 2 Growth of wild and mutant strain in different ethanol concentration

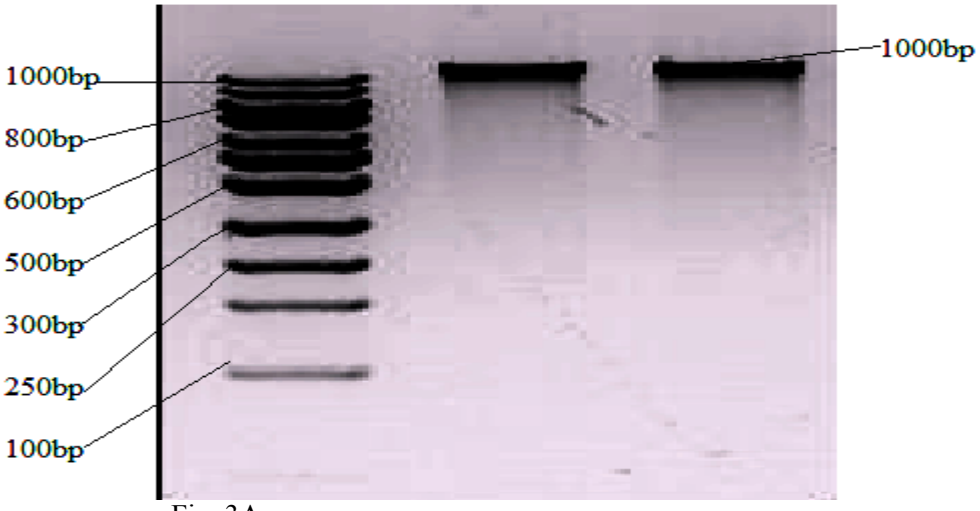


Fig. 3A

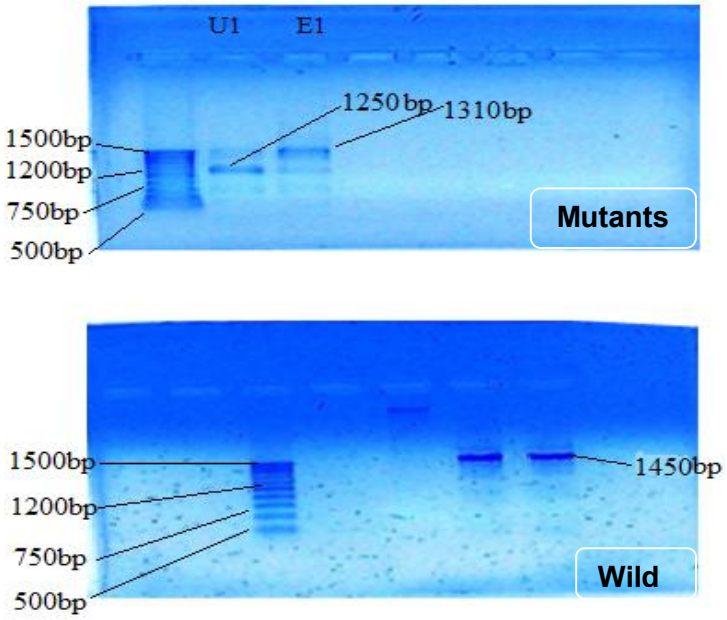
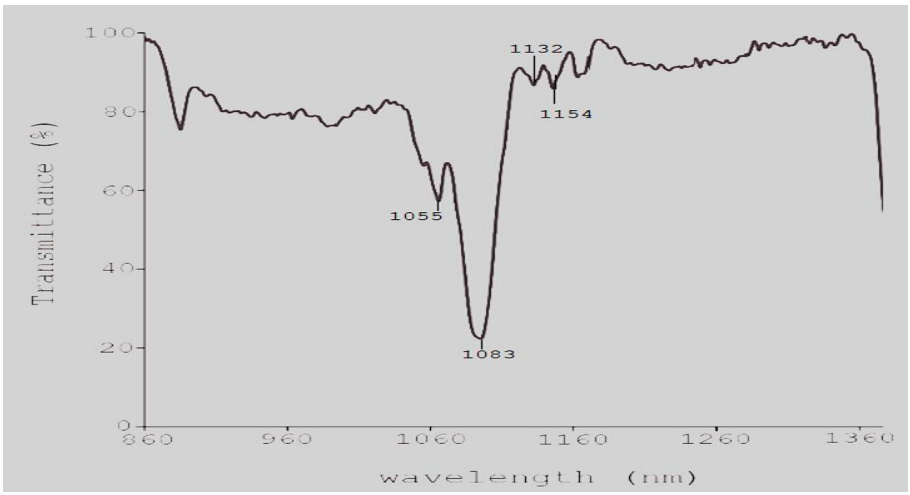
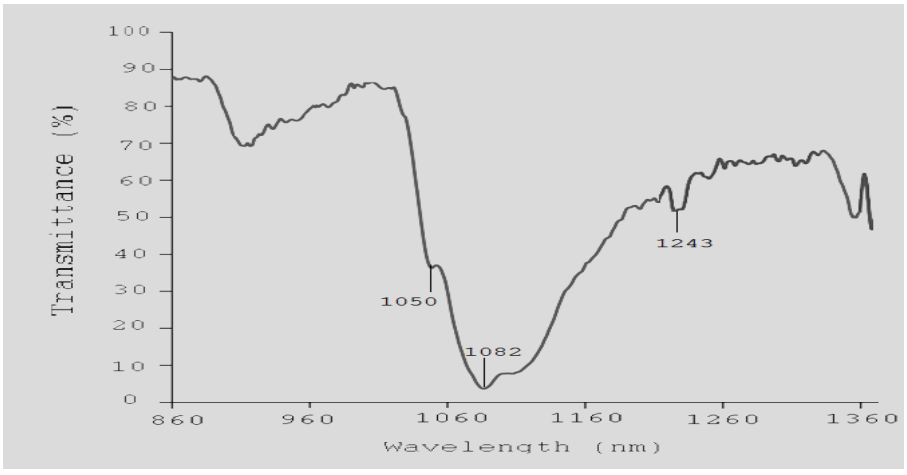


Fig. 3B

Fig. 3 (a) yeast gDNA on agarose gel (b) PCR amplified product of wild and mutants using ADH1 primers



A



B

Fig. 4 FTIR spectra of Yeast cell wall (a) Wild (b) U1 mutant

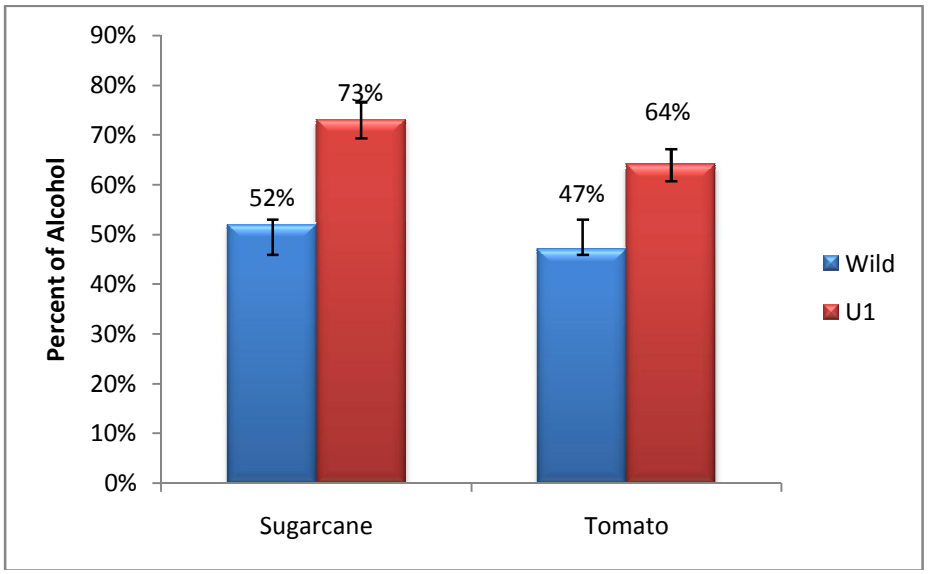


Fig. 5 Ethanol production percentage from sugarcane and Tomato juice

Molecular Characterization

Figure 3a shows the isolated yeast genomic DNA on agarose gel under UV-light. Similarly the 35 cycles of PCR amplified ADH gene in both wild and mutant strain was depicted in the figure 3b.

PCR results shown difference in amplification pattern in wild and mutant strains. Wild strain shows single amplified product of 1450bp size whereas mutant strains exhibit multiple band amplification ranging from 1250bp to 1310bp which may be due to base change in the DNA sequence after mutagenesis which in turn resulting in altered activity of mutated strains in ethanol stressed environment.

There is a correlation between the presence of an ADH gene, the amount of lipid fatty acid unsaturation of these strains and its ability to tolerate externally added ethanol. The main target for the toxicity of alcohol is the membranes [17]. The more tolerant strains generally shows higher unsaturation index (UI) value, which indicates that in these strains induced the rigidity of the membrane other than the fatty acid composition [18]. Chatterjee et al [19] also found the correlation between the amount of unsaturated fatty acids and the heat shock response induced by ethanol on yeast cells.

According to Heipieper et al [20] it has been postulated that KIADH2 of *Kluyveromyces lactis* may be involved in adaptation to high ethanol concentrations, which was verified by the kinetic parameters of the ADH isozymes of *K. lactis* reported by Bozzi *et al* [21].

FTIR spectroscopic analysis

The FT-IR spectroscopic analysis of wild and mutants U1 yeast strain is shown in figure 4a and 4b. Each spectrum presented is an average of three independent measurements; from this comparison it is clear that spectral modifications are present in the mutated sample. The mutated U1 strain exhibits a higher IR absorption intensity 1082 cm^{-1}stretching and contribution of C-O-C, C-C and C-OH of β (1→3) glucan band [22]. The characteristics bands demonstrate the content of carbohydrates increased in UV mutant strains.

The major yeast cell wall carbohydrate components glucans and mannans are presented in the IR range of 860 cm^{-1} to 1360 cm^{-1} [23]. Bands in these regions are assigned to C-O-C, C-C and C-OH glycosidic stretching vibrations of the pyranose rings of the carbohydrates [24]. Vibrations of C–OH, C–O–C and C–C groups are in a very crowded spectral region between 1050 and 1160 cm^{-1}. Symmetric PO_2 stretching was also appeared in the same region, since the glucan and mannan content are higher than phosphate in yeast cells suggests that the vibrations in this spectral region characterize carbohydrates [25].

The results of the present study are in agreement with the data of Galichet et al [26] who showed that the polysaccharide region is presented in the spectral region of 950 and 1190 cm^{-1} and the spectral profile reflects the presence of sugars in the yeast cell wall. Increased total carbohydrate content in the mutant strain determines cell wall rigidness and stability was validated with the previous study done by Zimkus et al [27].

Fermentation and Ethanol production

The amount of ethanol produced by the fermentation process was shown in figure 5. The results of the fermentation by UV-mutants from wild type produced 40.3 and 36.1 % more ethanol from sugar cane and tomato juice respectively. The results suggested that the enhancement of ethanol production by the

mutant U1 was mainly due to the improvement of ethanol-tolerance. Ethanol tolerance is complex phenotypic behavior controlled at DNA level, genetic studies of responses to ethanol and other environmental stresses will leads to the development of more tolerant and fermentation efficient strains [28, 29].

Yeast cells with high viability due to the higher unsaturation index (UI) are the important factor to increase ethanol production [8]. Previous studies have shown the correlation between increasing ethanol tolerance and higher ethanol productivity by fermentation process [9, 30, 31]. Bai et al [32] reported around 14 % more ethanol production using commercial *S. cerevisiae* strain between 30 to 32 °C. The present study demonstrated the high ethanol yield around 40% at 37°C proves the stability and potential of the UV-mutant strain.

CONCLUSION

Results of present studies indicate the importance of ethanol tolerant capacity of yeast for the high ethanol productivity. The developed ethanol tolerant strain will be the promising candidate for ethanol production at commercial level. Further researches on this strain under scale-up fermentation conditions are planned for high ethanol production.

REFERENCES

1. Chambers, P. (2007). Yeasts: products and discovery (YPD) in Australia. Microbiol. Aust. 28: 43.
2. Demain, A.L., (2009). Biosolutions to the energy problem. J Ind. Microbiol. Biotechnol. 36: 319– 332.
3. Snowden, C., Schierholtz, R., Poliszczuk, P., Hughes, S., Van Der Merwe, G. (2009). *ETP1/YHL010c* is a novel gene needed for the adaptation of *Saccharomyces cerevisiae* to ethanol. FEMS Yeast Res. 9: 372–380.
4. Gibson, B.R., Lawrence, S.J., Leclaire, J.P., Powell, C.D., Smart, K.A. (2007). Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol. Rev. 31: 535– 569.
5. Lloyd, D., Morrell, S., Carlsen, H.N., Degn, H., James, P.E., Rowlands, C.C. (1993). Effects of growth with ethanol on fermentation and membrane fluidity of *Saccharomyces cerevisiae*. Yeast 9: 825–833.
6. Ly, H.V., Block, D.E., Longo, M.L. (2002). Interfacial tension effect of ethanol on lipid bilayer rigidity, stability, and area/molecule: a micropipet aspiration approach. Langmuir 18: 8988–8995.
7. Birch, R.M. and Walker, G.M. (2000). Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 26: 678–687.
8. Thammasittirong, N.R.S., Chamduang, T., Phonrod, U., Sriroth, K. (2012). Ethanol production potential of ethanol-tolerant Saccharomyces and non- Saccharomyces yeasts. Pol. J Microbiol. 61:219–221.
9. Fiedurek, J., Skowronek, M., Gromada, A. (2011). Selection and adaptation of *Saccharomyces cerevisiae* to increased ethanol tolerance and production. Pol. J Microbiol. 60: 51–58.
10. Shi, D.J., Wang, C.L., Wang, K.M. (2009). Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. J Ind. Microbiol. Biotechnol. 36:139–147.
11. Sambrook, J., Russell, D.W.: Molecular cloning a laboratory manual. 1(3), 6.31-6.32 (2001).
12. Unaldi, M.N., Arikan, B., Coral, G. (2002). Isolation of alcohol tolerant, osmotolerant and thermotolerant yeast strains and improvement of their alcohol tolerance by UV mutagenesis. Acta. Microbiol. Pol. 51(2): 115-120.
13. Lin. Y, Zhang, W., Chunjie, Li., Sakakibara, K., Tanaka, S., Kong, H. (2012). Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. Biomass Bioenerg. 1-7.
14. Williams, M. B., Darwin, R. (1950). Colorimetric determination of ethyl alcohol, Anal. Chem. 22: 1556.
15. Kurtzman, C.P., Robnett, C.J. (1998). Identification and phylogeny of ascomycetous yeast from analysis of nuclear large subunit 26S ribosomal DNA partial sequences. A. Van Leeuw. 73: 331-371.
16. Slonimski, P. P., Perrodin, G., Croft, J.H. (1968). Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient non-chromosomal petites. Biochem. Biophys. Res. Commun. 30: 232-239.
17. Jones R.P., Greenfield P.F. (1987) Ethanol and the fluidity of the yeast plasma membrane, Yeast 3: 223–232.
18. Isken S., de Bont J.A.M. (1998). Bacteria tolerant to organic solvents, Extremophiles 2, 229–238.
19. Chatterjee, M.T., Khalawan S.A., Curran B.P.G. (1997). Alterations in cellular lipids may be responsible for the transient nature of the yeast heat shock response, Microbiology 143, 3063–3068.
20. Heipieper, J.H, Isken, S., Saliola, M. (2000). Ethanol tolerance and membrane fatty acid adaptation in adh multiple and null mutants of *Kluyveromyces lactis.* Res. Microbiol. 151: 777–784.
21. Bozzi, A., Saliola, M., Falcone, C., Bossa, F., Martini, F. (1997). Structural and biochemical studies of alcohol dehydrogenase isozymes from *Kluyveromyces lactis*, Biochim. Biophys. Acta 1339: 133–142.
22. Mitchell, A.J., Scurfield, G. (1970). An assessment of infrared spectra as indicators of fungal cell wall composition. Aust. J. Biol. Sci. 23: 345-360.
23. Bahmed, K., Quiles, F., Bonaly, R., Coulon, J. (2003). Fluorescence and infrared spectrometric study of cell walls from *Candida, kluyveromyces, Rhodotorula* and *schizosaccharomyces* yeasts in relation with their chemical composition. Biomacromolecules. 4(6): 1763-1772.
24. Jesus, J., Ojeda, M., Romero-Gonzalez, E., Robert, T., Bachmann, R., Dyvean, G. E., Steven, A., Banwart, A. (2008). Characterization of the cell surface and cell wall chemistry of drinking water bacteria by combining XPS, FTIR spectroscopy, modeling and potentiometric titrations. Langmuir. 24(8): 4032-4040.

25. Keith, E., Laidig, K., Speers, P., Streitwieser, A.(2000). Complexation of Li+, Na+ and K+ with water and ammonia. Coord. Chem. Rev. 125: 197-204.
26. Galichet, A., Sockalingum, G. D., Belarbi, A., Manfait, M. (2001). FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: study of an anomalous strain exhibiting a pink-colored cell phenotype FEMS Microbiology Letters 197: 179-186.
27. Zimkus, A., Misiunas, A., Chaustova, L. (2011) Spectroscopic study of yeast *Saccharomyces cerevisiae* cell wall structure. Chemija. 22(2): 80–84.
28. Ding, M.Z., Wang, X., Yang, Y., Yuan, Y.J. (2012) Comparative metabolic profiling of parental and inhibitors-tolerant yeasts during lignocellulosic ethanol fermentation. Metabolomics 8: 232–243.
29. Li, H., ML, M., Luo, S., Zhang, R.M., Han, P., Hu, W. (2012). Metabolic responses to ethanol in *Saccharomyces cerevisiae* using a gas chromatography tandem mass spectrometry-based metabolomics approach. Int. J Biochem. Cell Biol. 44: 1087–1096
30. Hou, L.: Novel methods of genome shuffling in *Saccharomyces cerevisiae*. (2009). Biotechnol. Lett. 31:671–677.
31. Basso, L.C., De Amorim, H.V., de Oliveira, A.J., Lopes, M.L. (2008). Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res. 8: 1155–1163.
32. Bai, F.W., Anderson, W.A., Moo-Young, M. (2008). Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol. Adv. 26: 89–105.