Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2620

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **5 (1):** 631-639 (2017)

International Journal of Pure & Applied Bioscience

Review Article

OPEN ACCESS

Yeast: Main Source of Ethanol and Biofuel

Ningaraj Dalawai*, Krupa, K. N., Subhash Bharani, Vinayak Turaidar, Harinikumar, K. M. and Vijayakumar Swamy H. V.

Department of Plant Biotechnology, UAS, GKVK, Bengaluru

*Corresponding Author E-mail: subash4586@gmail.com

Received: 16.02.2017 | Revised: 25.02.2017 | Accepted: 26.02.2017

ABSTRACT

Biofuels are liquid or gaseous fuels produced from biomass resources and used in place of, or in addition to diesel, petrol or other fossil fuels for transport, stationary, portable and other applications. An efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economical as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases. Yeast cells are facultative anaerobes and under anaerobic conditions they can ferment glucose to ethanol. S. cerevisiae is ideal for ethanol production due to several properties comprising fast growth rates, efficient glucose repression, efficient ethanol production and tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels. In this review, the potential use of yeast as sources of energy, bioactive compounds, high-value chemicals, tools for ethanol production and recent progress in applications are reported.

Key words: *Yeast, Biofuel, Ethanol Production, Ethanol Tolerance, Environment.*

INTRODUCTION

Yeasts are unicellular fungi that reproduce by budding (asexual) and also fusion (sexual). *Saccharomyces cerevisiae* is budding yeast mainly used for ethanol production. A well-known property of yeast is that they are responsible for the conversion of fermentable sugars into alcohol and other by-products. Consistent yeast performance during the fermentation process requires both accurate cell counts plus assessment of cellular viability.

A number of yeasts were isolated from various natural sources such as slime fluxes of trees, insects and insect frass, flowers and similar substrata. Several of the isolates, from represented species have been described in recent years. Members of the high ethanol tolerant species of *S. cerevisiae* were unique to natural fruit surface. The additional isolations have contributed additional knowledge regarding the natural habitat of some of the species[23].

Recently, the petroleum products are running out of race due to unbalanced relation between supply and demand besides air pollution of sources. The hike in petrol cost is mainly due to shortage of resources which leads to search for alternate fuel to replace fossil fuels.

Cite this article: Dalawai, N., Krupa, K.N., Bharani, S., Turaidar, V., Harinikumar, K.M. and Swamy, H.V., Yeast: Main Source of Ethanol and Biofuel, *Int. J. Pure App. Biosci.* **5(1):** 631-639 (2017). doi: http://dx.doi.org/10.18782/2320-7051.2620

An eco-friendly bio-ethanol is one such alternate fuel that can be used in unmodified petrol engines with current fuelling infrastructure and it is easily applicable in present day combustion engine, as mixing with gasoline[13].

Yeast

Yeasts do not form a specific taxonomic or phylogenetic grouping. At present it is estimated that only 1% of all yeast species have been described[19]. The term "yeast" is often taken as a synonym for *S. cerevisiae*. However the Phylogenetic diversity of yeasts is shown by their placement in both divisions Ascomycota and Basidiomycota. The budding yeasts (true yeasts) are classified in the order Saccharomycetales[3].

Saccharomyces cerevisiae (baker's yeast) is currently used in research to increase the yield of the production of bio-ethanol from sugars through Zymase enzyme. Yeasts do not require sunlight to grow, but do use sugars as a source of energy. S. *cerevisiae* cells use three major pathways for growth on glucose[21].

Among the yeasts, *S. cerevisiae* still remains the prime species for ethanol production. Previous published reports showed that the ethanol tolerance and sugar utilization efficiency of yeast may be improved by altering the nitrogen sources in fermentation medium[34,36].

Taxonomy of yeast (*S. cerevisiae*)

Kingdom : Fungi	Phylum : Ascomycota
Subphylum : Saccharomycotina	Class : Saccharomycetes
Order : Saccharomycetales	Family : Saccharomycetaceae
Genus : *Saccharomyces*	

Fermentation

First, the fermentation of glucose: $C_6H_{12}O_6(s) \rightarrow 2CH_3CH_2OH\ (l) + 2CO_2\ (g)$

Second, the oxidation of glucose: $C_6H_{12}O_6(s) + 6O_2\ (g) \rightarrow 6CO_2\ (g) + 6H_2O\ (l)$

Third, the oxidation of ethanol: $CH_3CH_2OH\ (l) + 3O_2\ (g) \rightarrow 2CO_2\ (g) + 3H_2O\ (l)$

These pathways show that *S. cerevisiae* cells can grow in both an oxygen free environment and an oxygen rich setting. It shows that growth can occur when glucose becomes very limited or absent and oxygen is present. The first pathway is interesting for research, because it involves the production of ethanol[27].

Historically the relationship between mankind and yeast developed that led to the discovery of fermented beverages. Numerous inventions have led to improved technologies and capabilities to optimize fermentation technology on an industrial scale. The role of brewing yeast in the beer-making process is reviewed by Elizabeth *et al*[8]., and its importance as the main character is highlighted. On considering the various outcomes of functions in a brewery, it has been found that these functions are focused on supporting the supply of yeast requirements for fermentation and ultimately to maintain the integrity of the product.

Saccharomyces cerevisiae is one of the most important fungi used by humans throughout recorded history. This yeast represents the prototype for fermentative yeast responsible for fermentation of foods, such as wine, beer and bread. In recent years they have made a significant impact on both the scientific community and industry. The yeast *S. cerevisiae* gained particular attention from 1990's as it has served as scientific tool and was first eukaryotic organism to have its genome sequenced. They are playing a major role in production of biofuel[2].

Ancient brewers domesticated yeast due to selection of the best fermentation agent, although the full biochemical process was not

understood. This intimate association between mankind and *Saccharomyces cerevisiae* was further demonstrated during a study of the genetic diversity among 651 wine yeast strains from 56 geographical origins worldwide[20].

Therefore strong economic incentives can be revealed by improving production processes resulting in a substantial growth for the ethanol industry in the near future[5].

Industrial production of ethanol

In assessing a yeast strain for industrial use, specific physiological properties are required[7]. Ethanol tolerance, sugar tolerance and invertase activity are some of the important properties for use in industrial ethanol production[17].

Saccharomyces have been genetically engineered to ferment xylose, one of the major fermentable sugars present in cellulosic biomasses, such as agricultural residues, paper wastes, and wood chips. Such a development means that ethanol can be efficiently produced from more inexpensive feed stocks, making cellulosic ethanol fuel a more competitively priced alternative to gasoline fuels.

The fermentation of wine is initiated by naturally occurring yeasts present in the vineyards. Many wineries still use nature strains, however many use modern methods of strain maintenance and isolation. The bubbles in sparkling wines are trapped carbon dioxide, the result of yeast fermenting sugars in the grape juice. One yeast cell can ferment approximately its own weight of glucose per hour. Under optimal conditions *S. cerevisiae* can produce up to 18 per cent, by volume, ethanol with 15 to 16 per cent being the norm. The sulphur dioxide present in commercially produced wine is actually added just after the grapes are crushed to kill the naturally present bacteria, moulds, and yeasts.

A succession of yeasts was observed during fermentation of a guardant with *S. cerevisiae* being the predominant species. Candida sake, *Kluyveromyces marxianus* var. drosophila rum and apiculate yeasts were also frequent. Transient yeast species were found in variable numbers, probably due to the daily addition of sugar-cane juice. Killer yeasts were isolated and may have a role in the exclusion of some transient and contaminant species.

The ability of yeast to convert sugar into ethanol has been harnessed by the biotechnology industry, which has various uses including ethanol fuel. The process starts by milling a feed stock, such as sugar cane, sweet corn, or cheap cereal grains, and then adding dilute sulphuric acid, or fungal amylase enzymes, to break down the starches in to fermentable sugars. Yeasts are then added to convert fermentable sugars to ethanol, which is then distilled off to obtain ethanol upto 96 % concentration[3].

Molecular characterization of yeast

The applicability of different PCR-based techniques, random amplified polymorphic DNA, cleaved amplified polymorphic sequence and SSRs (simple sequence repeats) to genetically identify, typify and discriminate among *S. cerevisiae* strains, was investigated by Perez *et al*[25]. The analysis and comparison of the genetic profiles obtained for each of the techniques allowed determination of the degree of polymorphism, genetic diversity and relationships among the strains. Consequently, the discriminatory power and usefulness of the molecular techniques assayed was established. SSR analysis was the most effective method due to its high level of polymorphism, the ease of interpretation of the genetic profiles obtained, and the speed of use.

Techera *et al*[32]., differentiated nine industrial wine strains of *S. cerevisiae* using microsatellite (Simple Sequence Repeats, SSR) markers. Six of the strains were indigenous yeasts currently used as high-density starter monocultures by the Uruguayan wine industry. Unequivocal differentiation of these six native strains and three commercial *S. cerevisiae* wine strains was achieved by PCR amplification and polymorphism analysis of loci containing microsatellite markers. They recommend the use of this reproducible and simple molecular method to routinely discriminate wine yeast strains. Microsatellites are superior to other methods for typing yeasts because the results can be exchanged as quantitative data. Knowledge of the

frequencies of the alleles for different SSR markers will eventually lead to an accurate typing method to identify industrial wine yeast strains.

Gallego *et al*[11]., evaluated the usefulness of different molecular techniques for the genetic analysis of *S. cerevisiae* strains. Three commonly used PCR-derived genetic methods, random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs) and simple sequence repeats (SSRs; microsatellites), were used to characterize 27 wine yeast strains of *S. cerevisiae* from the "Denominacion de Origen Vinos de Madrid" (Spain). Using these methods, they were able to overcome certain limitations associated with classical taxonomic methods. Based on the presence or absence of amplified fragments for each genotype, AFLPs and SSRs showed a similar discriminatory power superior to that of the RAPDs. Genetic relationships between strains were also estimated using the three methods. In general, very poor correlations were found, reflecting the different genomic regions for which the methods are screened. Results are discussed in terms of which molecular technique is most appropriate for use with a particular aspect of genetic evaluation.

Perez *et al*[25]., selected six polymorphic microsatellite loci for their use as molecular markers in the identification, typification and genetic differentiation of *S. cerevisiae* strains. The selection was undertaken following a search of the genomic DNA database of *S. cerevisiae* for simple tandem repeat sequences (microsatellites) of di-and trinucleotides. The genetic variability generated by these markers was evaluated in 51 isolates. The discriminatory power produced by combining the information obtained by the six microsatellites was very high. A total of 57 alleles, which generated 44 genotypes, were found. The multiple analyses of microsatellites proved to be a powerful and agile tool for analysing the genome of *S. cerevisiae* populations.

Molecular markers

Microsatellite or SSRs (Simple Sequence Repeats) markers are tandem repetitive DNA sequences of short units (1-10 nt) dispersed throughout the genome and co-dominantly inherited. Microsatellite loci of yeast have a high degree of variability and could have a very important application as sequence tagged site (STS) markers[4], giving rise to an innovative development in the genetic analysis of these eukaryotic organisms.

The genome of *S. cerevisiae* has an abundance of microsatellites that are distributed throughout its 16 chromosomes, giving rise to numerous polymorphic alleles and also present in the mitochondrial DNA. Some microsatellites have been used successfully to characterize and discrimination among *S. cerevisiae* strains.

This molecular approach is based on the PCR amplification of fragments using oligonucleotides complementary to single repetitive sequences present in the target DNA. These repetitive sequences are called microsatellites. Some of the most frequently used are (GTC) 5, (GTG) 5, (GACA) 4, M13 phage (GAGGGTGGCGGTTCT). This technique differs of RAPD in the use of a higher annealing temperature (55 ºC) in microsatellite analysis instead of 37 ºC in RAPD. The application of a higher annealing temperature drives more specific primer hybridization what consequently ensures a higher reproducibility.

Mechanism of ethanol tolerance

Ethanol is inhibitor of growth of microorganisms. It has been reported to the damage mitochondrial DNA in yeast cells[16] and to cause inactivation of some enzymes, such as hexokinase and dehydrogenase. Some strains of the yeast *S.cerevisiae* show tolerance and can adapt to high concentrations of ethanol[12]. Many studies have documented the alteration of cellular lipid composition in response to ethanol exposure. It has been found that *S. cerevisiae* cells grown in the presence of ethanol appear to increase the amount of monounsaturated fatty acids in cellular lipids. Since cell membranes have

received extensive consideration as primary targets of ethanol stress, many reports have suggested a relationship between the fatty acid compositions of lipid membranes and ethanol stress tolerance. Although the correlation between ethanol tolerance and increased degree of fatty acid unsaturation of membrane lipids of *S. cerevisiae* is well documented, a causal relationship is not yet established.

Ethanol is clearly inhibitory for yeasts. Cell growth stops at relatively low ethanol concentrations, and fermentation stops at relatively higher ones[28]. Decreases in the rate of ethanol production are related to decreases in viable cell count[1]. Cell growth inhibition by ethanol is non-competitive and has been described as either a linear or an exponential function of ethanol concentration[1].

Damore *et al*[6]., found out that a number of factors such as plasma membrane composition, media composition, mode of substrate feeding, osmotic pressure, temperature, intracellular ethanol accumulation, and by product formation have been shown to influence the ethanol tolerance of yeast. Media composition was found to have a profound effect upon the ability of a yeast strain to ferment concentrated substrates (high osmotic pressure) and to ferment at higher temperatures. Supplementation with peptone-yeast extract, magnesium, or potassium salts has a significant and positive effect upon overall fermentation rates. An intracellular accumulation of ethanol was observed during the early stages of fermentation. As fermentation proceeds, the intracellular and extracellular ethanol concentrations become similar. In addition, increases in osmotic pressure are associated with increased intracellular accumulation of ethanol. However, it was observed that nutrient limitation, not increased intracellular accumulation of ethanol, is responsible to some extent for the decreases in growth and fermentation activity of yeast cells at higher osmotic pressure and temperature.

A positive correlation was observed between cell viability and trehalose concentration. When leakage of electrolytes from the cells was recorded by observing changes in conductivity of the medium, we found that ethanol increases leakage, but the presence of trehalose reverses that effect[22].

The effect of salt stress on ethanol endurance of yeast cells was studied. Cells grown under increased NaCl concentrations were more ethanol tolerant than controls. The increase in trehalose content under hyper-saline conditions has been suggested to allow cells to withstand higher ethanolic conditions. There seems to be an overlap between osmotolerance and ethanol endurance in *Saccharomyces cerevisiae*[29].

Excess amount of ethanol has been reported to cause mitochondrial DNA damage and degrades bio membranes in yeast cells[30].

Takahashi *et al*[31]., reported that five ethanol-sensitive mutants isolated from approximately 7000 mutants created by transforming yeast cells with a transposon (mTn-lacZ/LEU2)-mutagenized genomic library could grow normally in a rich medium but they could not grow in the same medium containing 6% ethanol. Sequence analysis of the mutants revealed that the transposon was inserted in the coding regions of BEM2, PAT1, ROM2, VPS34 and ADA2. These five genes are required for growth under ethanol stress.

To enhance the ethanol tolerance of *S. cerevisiae*, the *Arabidopsis thaliana* FAD2 gene and/or the *S. cerevisiae* OLE1 gene were over expressed in this yeast. The transformation over expressing these two genes could not only synthesize dienoic acids but also increased the unsaturated fatty acid content of membrane lipid and then showed the highest viability in the presence of 15 % (v/v) ethanol[18].

You *et al*[37]., examined the effects of different unsaturated fatty acid compositions of *Saccharomyces cerevisiae* on the growth-inhibiting effects of ethanol. The unsaturated fatty acid (UFA) composition of *S. cerevisiae* is relatively simple, consisting almost exclusively of the mono-UFAs palmitoleic acid (_9Z-C16:1) andoleic acid (_9Z-C18:1), with the former predominating. Both UFAs are

formed in *S. cerevisiae* by the oxygen- and NADH-dependent desaturation of palmitic acid (C16:0) and stearic acid (C18:0), respectively, catalysed by a single integral membrane desaturase encoded by the *OLE1* gene. They systematically altered the UFA composition of yeast cells in a uniform genetic background (i) by genetic complementation of a desaturase-deficient *ole1* knockout strain with cDNA expression constructs encoding insect desaturases with distinct regioselectivities (i.e.,_9 and _11) and substrate chain-length preferences (i.e., C16:0 and C18:0); and, (ii) by supplementation of the same strain with synthetic mono-UFAs. Both experimental approaches demonstrated thatoleic acid is the most efficacious UFA in overcoming the toxic effects of ethanol in growing yeast cells. Furthermore, the only other UFA tested that conferred a nominal degree of ethanol tolerance is *cis*-vaccenic acid (_11Z-C18:1), whereas neither _11Z-C16:1 nor palmitoleic acid (_9Z-C16:1) conferred any ethanol tolerance. They also showed that the most ethanol-tolerant transformant, which expresses the insect desaturase *Tni*NPVE, produces twice as much oleic acid as palmitoleic acid in the absence of ethanol and undergoes a fourfold increase in the ratio of oleic acid to palmitoleic acid in response to exposure to 5 % ethanol. These findings are consistent with the hypothesis that ethanol tolerance in yeast results from incorporation of oleic acid into lipid membranes, affecting a compensatory decrease in membrane fluidity that counteracts the fluidizing effects of ethanol.

Many reports have accented a relationship between the fatty acid compositions of lipid membranes and ethanol stress tolerance[37] which stop mitochondrial bio molecules translocation and proton motive force[7] and finally cause cell death. According to these phenomena, resistant strains to ethanol have many mechanisms to overcome ethanol perils.

Ca^{2+} ions in the medium decrease the plasma membrane permeability of yeast cells subjected to ethanol stress and enhance the ethanol tolerance[14].

Osho[24] isolated seventeen wine yeasts from fermenting cashew apple juice were screened for ethanol and sugar tolerance. Two species of *Saccharomyces* comprising of three strains of *S. cerevisiae* and one *S. uvarum* showed measurable growth in medium containing 9 % (v/v) ethanol. They were equally sugar tolerant having good growth in medium containing 25 % (w/v) glucose. Two of the strains (*S. cerevisiae*) were found to possess higher invertase activities than the remaining two. Further search for industrially useful yeasts in tropical fruits is suggested.

Ergosterol is an essential component of yeast cells that maintains the integrity of the membrane. It was investigated as an important factor in the ethanol tolerance of yeast cells[26].

Ethanol can dissolve fatty acid constituents of the cell membranes; disrupt cytoplasmic membrane rigidity[24]. During fermentation, sugars lead to the production of ethanol and carbon dioxide. Increasing the concentration of ethanol delays the growth of the yeast, which eventually stops the fermentation[17]. It is important that the yeast strain used be able to survive the highest ethanol concentration produced. For beer, these concentrations range in 3–9 %, for grape wine 11–15 % and for honey wine up to 17 %[24,33]. Alcohol tolerance is particularly important for alcoholic fermentation while, producing ethanol for fuel. We need to have yeast with a tolerance more than 25 %.

Fujita *et al*[10]., screened a set of homozygous diploid deletion mutants of the yeast *Saccharomyces cerevisiae* genes required for tolerance to aliphatic alcohols. The screen identified 137, 122 and 48 deletion mutants sensitive to ethanol, 1-propanol and 1-pentanol, respectively. A number of the genes required for ethanol tolerance were those also required for tolerance to other alcohols.

The concentrations of Mg^{2+}, glycine, yeast extract, biotin, acetaldehyde and peptone were optimized by a uniform design process for ethanol production by *S. cerevisiae*. Using non-linear step-wise regression analysis, a predictive mathematical model was established. Concentrations of Mg^{2+} and

peptone were identified as the critical factors: 50 mM Mg^{2+} and 1.5 % (w/v) peptone in the medium increased the final ethanol titre from 14.2 % (v/v) to 17 % (v/v) in 48 hr[9].

Ethanol tolerance has yet to be clearly defined, although it has been reported to be producible under defined conditions and appears to be under complex genetic control. Ethanol has three major effects on yeast, it decreases the rate of growth and of fermentation and it reduces overall level of cell viability. In this experiment the attempt will be made to check the viability of yeast cells under different conditions of ethanol[35].

Hu *et al*[15]., reported that till now as many as 251 genes have been predicted to be involved in influencing ethanol tolerance in yeast. Candidacy of these genes was determined from a tested phenotypic effect following gene knockout, from an induced change in gene function under an ethanol stress condition, or by mutagenesis.

A brief procedure is as follows, the yeast strains which are confirmed through the PCR for the ADHase (Alcohol dehydrogenase) production will be used for this study. Those strains which are positive for PCR will be grown in the tubes containing different concentrations of ethanol in YEPDA broth. The optical density will be measured immediately after inoculation of the strains and then incubation will be done at 24-48 hr. @ 30 °C. Growth expressed as generation time will be determined by measuring the optical density of cultures at 595 nM. The concentration of alcohol at which the yeast just inhibited will be accessed as the ethanol tolerance of yeast.

REFERENCES

1. Aiba, S., Shoda, M. and Nagatani, M., Kinetics of product inhibition in alcohol fermentation. *Biotechnol. Bioeng*., **10:** 845-865 (1968).
2. Angeles, M., Perez, F., Javiergallego, and Hidalgo, P., Evaluation of molecular techniques for the genetic characterization of *Saccharomyces cerevisiae* strains. *FEMS Microbiol Letters.*, **205:** 375-378 (2001).
3. Anonymous., Fuel Ethanol Production. *Genomics: GTL*. Systems Biology for Energy and Environment., *p*. 234-245 (2006).
4. Beckmann, J.S. and Soller, M., Towards a unified approach to the genetic mapping of eukaryotes based on sequence tagged microsatellite sites. *J. Biotechnol*., **8:** 930-932 (1990).
5. Carlos, R. and Roberto, De, L., Ethanol fermentation from sugarcane at different maturities. *Ind. Crops Products,* **33(2):** 333-337 (2011).
6. D'amore, T., Panchal, C.J., Russell, I. and Stewart, G.G., A Study of ethanol tolerance in yeast. *Crit. Rev. Biotechnol.,* **9(4):** 287-304 (1990).
7. Ekunsanmi, T.J. and Odunfa, S.A., Ethanol tolerance, sugar tolerance and invertase activities of some yeast strains isolated from steep water of fermenting Cassavas tubers. *J. Appl. Bacteriol.,* **69:** 672-675 (1990).
8. Elizabeth, J. Lodolo., Johan, L.F., Kock, Barry, C. Axcell, and Martin Brooks., The yeast *Saccharomyces cerevisiae* the main character in beer brewing. *FEMS Yeast Res.*, **8:** 1018–1036 (2008).
9. Fan-Qiang Wang, Cui-Juan Gao, Chun-Yu Yang and Ping XU., Optimization of an ethanol production medium in very high gravity fermentation. *Biotechnol Lett.*, **29:** 233–236 (2007).
10. Fujita, K., Matsuyama, A., Kobayashi, Y. and Iwahashi, H., The genome-wide screening of yeast deletion mutants to identify the genes required for tolerance to ethanol and other alcohols. *FEMS Yeast Res.*, **6(5):** 744 -750 (2006).
11. Gallego, F.J., Perez, M.A., Martinez, I. and Hidalgo, P., Microsatellites obtained from database sequences are useful to characterize *Saccharomyces cerevisiae* strains. *American. J. Enol. vitic.*, **49:** 350-352 (2008).
12. Ghareib, M., Youssef, K.A. and Khalil, A.A., Ethanol tolerance of *Saccharomyces cerevisiae* and its relationship to lipid content and composition. *Folia. Microbiol*., **33:** 447–452 (1988).

13. Hansen, Alan, C., Qin Zhang, Peter, W.L. and Lyne., Ethanol diesel fuel blends – a review. *Bioresource Technol.*, **96:** 277-285 (2005).
14. Hu, C.K., Bai, F.W. and An, L.J., Enhancement in ethanol tolerance of a self- flocculating yeast by calcium ion through decrease in plasmalemma permeability *Sheng Wu Gong Cheng Xue Bao.*, **19(6):** 715-19 (2003).
15. Hu, X.H., Wang, M.H., Tan, T., Li, J.R., Yang, H., Leach, L., Zhang, R.M. and Luo, Z. W., Genetic dissection of ethanol tolerance in the budding yeast *Saccharomyces cerevisiae*. *Genetics.*, **175(3):** 1479-1487 (2007).
16. Ibeas, J.I. and Jimenez, J., Mitochodrial DNA loss caused by ethanol in *Saccharomyces* flor yeasts. *Appl. Environ. Microbiol*., **63:** 7–12 (1997).
17. Jimenez, J. and Benitez, T., Characterization of yeasts for ethanol production. *Appl. Microbiol. Biotechnol.*, **25:** 150-154 (1986).
18. Kajiwara, S., Suga, K., Sone, H. and Nakamura, K., Improved ethanol tolerance of *Saccharomyces cerevisiae* strains by increase in fatty acid unsaturation via metabolic engineering. *Foods Food Ingredients J. Japan.*, **208(4):** 261-269 (2003).
19. Kurtzman, C.P. and Piskur, J., Taxonomy and phylogenetic diversity among the yeasts Comparative genomics: using fungi as models., **15:** 29-46 (2006).
20. Legras, J.L., Merdinoglu, D., Cornuet, J.M. and Karst, F., Bread beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. *Mol Ecol.*, **16:** 2091–2102 (2007).
21. Loureiro, V. and Malfeito-Ferreira, M., Spoilage yeasts in the wine industry. *Int. J. Food Microbiol.*, **86:** 23-50 (2003).
22. Mansure, J.J., Panek, A.D., Crowe, L.M. and Crowe, J.H., Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochem. Biophys. Acta.*, **191(2):** 309-16 (1994).
23. Miller, M.W., Phaff, H.J and Snyder, H.E., On the occurrence of various species of yeast in nature. *Mycopathologia.*, **16(1):** 1-18 (1962).
24. Osho, A., Ethanol and sugar tolerance of wine yeasts isolated from fermenting cashew apple juice. *J. Biotechnol.*, **4(7):** 160–163 (2005).
25. Perez, M.A., Gallego, F.J., Martinez, I. and Hidalgo, P., Detection, distribution and selection of microsatellites (SSRs) in the genome of the yeast *Saccharomyces* spp. as molecular markers. *Letters Appl Microbiol*., **33:** 461-466 (2001).
26. Shobayashi, M., Mitsueda, S., Ago, M., Fuji, T., Iwashita, K. and Lefuji, H., Effect of cultural conditions on ergosterol biosynthesis by *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.*, **69(12):** 2381-2388 (2005).
27. Slaa, J., Gnode, M. and Else, H., Yeast and fermentation: the optimal temperature, *Journal of Organic Chemistry*: *Chem. Dut. Aspects*., p.134 (2009).
28. Stokes, S.L., Influence of temperature on the growth and metabolism of yeasts, *The yeasts.*, **2:** 119-134 (1970).
29. Sukesh Chander, S., A possible role of trehalose in osmotolerance and ethanol tolerance in *Saccharomyces cerevisiae. FEMS Yeast Microbiol. Lett*., **152(1):** 11 (1997).
30. Swiecilo, A., Krawiec, Z., Wawryn, J and Bartosz, G., Effect of stress on the life span of the yeast S. cerevisiae. *Acta Biochim Polonica*., **47:** 355-364 (2000).
31. Takahashi, T., Shimoi, H and Ito, K., Identification of genes required for growth under ethanol stress using transposon mutagenesis in *Saccharomyces cerevisiae*. *Molecular Genetics and Genomics*., **265(6):** 1112-1119 (2001).
32. Techera. G.A., Jubany, S., Carrau. F.M. and Gaggero. C., Differentiation of industrial wine yeast strains using microsatellite markers. *Letters in Appl Microbiol.*, **33(1):** 71-75 (2001).
33. Teramoto, Y., Sato, R. and Ueda, S., Characteristics of fermentation yeast isolated from traditional Ethiopian honey wine. *Afr. J. Biotechnol.*, **4(2):** 660–662 (2005).
34. Thomas, G., Bernhardt and Christopher R. Morse., Fatty Acid Ethyl Esters in the Blood as Markers for Ethanol Intake. *Jama*., **276(14):** 1152-1156 (1996).

35. Xue, Y.M. and Jiang, N., Study on ethanol tolerance of *S. cerevisiae* X330 under very high gravity medium. *Sheng Wu Gong Cheng Xue Bao.*, **22(3):** 508-513 (2006).
36. Yalcin, S.K. and Ozbas, Z.Y., Effect of ammonium sulphate concentration on growth and glycerol production kinetics of two endogenic wine yeast strains. *J. Biotechnol.*, 7: 89-93 (2008).
37. You, K.M., Rosenfeild, C.L. and Knipple, D.C., Ethanol tolerance in yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. *Appl. Environ. Microbiol.*, **63(3):** 1499-503 (2003).