KAVAKA 42: 87-100(2014)

Yeast diversity, adaptation and thermotolerance

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(Submitted in November, 2013 ; Accepted on June 15, 2014)

ABSTRACT

Yeasts are a group of microbes that are distributed among *Basidiomycota* and *Ascomycota* phyla of fungal kingdom. The yeasts are having considerable importance in the field of agriculture, economics and medicine. It is estimated that till today about 99% of the potential diversity of this group of eukaryotic microorganisms is yet to be studied. Fast growth of global economics due to industrialization and also global warming has changed the present scenario of the industry and switching them towards eco-friendly processes. There is thus an increasing interest globally to discover new microbial species for economical exploitation that necessitates understanding their biodiversity, adaptation, ecological role and importance. Biodiversity of yeast not only provides the catalogue of life on earth, but their characterization and identification help in understanding the potential use of these eukaryotic organisms in the production of novel biochemicals and enzymes useful in the pharmaceutical, agriculture, food, cosmetics and chemical industries. This review focuses on the diversity of yeast and their adaptation with special emphasis on thermo tolerance, mechanism and its applications.

Keywords: Yeast, biodiversity, adaptation, ecology, thermotolerance, bioethanol.

INTRODUCTION

Yeasts are unicellular eukaryotic microbes and are probably one of the oldest and earliest life forms domesticated by mankind, for fermentation and baking (Walker *et al.*, 2002). These microbes belong to the fungal kingdom with 1,500 species under 100 genera and recognized as new production vehicles in modern biotechnological processes (Kurtzman and Fell, 2006). Ecologically most often yeasts are inhabitants of sugar rich environment like naturally occurring skin of fruits and berries, plant exudates, soil and insects (Suh *et al.*, 2005). They were also found as part of skin flora, gut flora of mammals and insects and deep sea environments (Kutty and Philip, 2008). Very little is known about the ecological functions and biodiversity of yeast, as 99% of the potential diversity of this group of eukaryotic microorganisms is yet to be studied (Jack *et al.*, 2000). Till now only few dedicated studies on yeast biodiversity have been reported from tropical zones of the planet and southern hemisphere when compared to northern hemisphere where majority of the yeast catalogued are discovered in countries from this region (Enrique *et al.*, 2011). Understanding the ecological functions and biodiversity of microbes helps in the utilization of their potential applications in the industry and helpful for human welfare. Hence, the current review is focused on the diversity, thermotolerance and applications of yeasts.

BIODIVERSITY AND ECOLOGY OF YEAST

The essence of biology is the variation in life forms and thus comes the biodiversity which has become the intrinsic feature of not only to living organism but also to diversity of strains and varieties. Global industrialization, conversion of tropical rainforests into irrigated agricultural landforms and natural events are the major concerns of changes or loss in the biodiversity of microorganisms (Hoekstra *et al.*, 2005). Mutations provide the ability in microorganisms to adapt to different environments and the speed of adaptation depends on the rate of beneficial mutations (Gregory and Andrew, 2007). Dormancy in yeast is one trait that allows species to contend with changes in the environment (Caceres and Tessier, 2003). Therefore, studies on ancient yeast species or strains provides a platform and opportunity to help us to understand the yeast origin and biodiversity over the time (Gomes *et al.*, 2009). Moreover several studies described that naturally fermenting *Saccharomyces* populations can vary from place to place and from time to time (Lopandic *et al.*, 2008). To understand the origin of few strains, extensive sampling combined with strong population genetic structure is required. At present, studies on phenotypic diversity of *S. cerevisiae* have been dominated by genetically similar laboratory or domesticated yeast strains (Warringer *et al.*, 2011). When compared with yeast strains from non-arboreal environments, domesticated strains are already known to have evolved into a number of phenotypes such as resistance to copper, sulphites and osmotic stress and their enological properties (Hyma *et al.*, 2011). But studies on diversity of phenotypes present in arboreal populations are yet to be uncovered; their discovery will greatly help to understand the power of yeast genetics, ecology and evolutionary aspects of wild *S. cerevisiae.*

In order to understand the structure of wild yeast, Wang *et al.* (2012) collected numerous samples from diverse arboreal habitats like fruit, bark, soil and rotten wood of primeval forests undisturbed by humans, secondary forests, planted orchards and urban trees in both tropical and temperate regions across China. Wang and his colleagues discovered a number of surprisingly diverse lineages of *S. cerevisiae* from both primeval and secondary forests of China. Genetic analysis of 99 *S. cerevisiae* isolates revealed eight genetically distinct groups, five of which are basal to all previously defined groups, including that from North American oak trees. Interestingly, the three

groups that fell within previously described populations were all isolated from secondary forests and orchards. While some amount of recombination can be inferred from genealogical in-congruence among the 13 loci used, the three basal groups of strains obtained from primeval forests were mostly diverse and remained distinct. *S. arboricolus* is the most recently discovered species which was isolated from oak and evergreen trees in China by Wang and Bai (2008). *S. bayanus* a brewing strain derived from wild stock and *S. eubayanus* was also recently found in forests in Patagonia (Libkind *et al.*, 2011). However, *S. cerevisiae* is prevalent species in human-associated fermentations. The identification of wild *S. cerevisiae* from oak trees in Siberia and North America (Naumov *et al.*, 2000) suggested that *S. cerevisiae* is not entirely a human commensal species. Subsequent studies on population genetics showed that wild oak tree populations are differentiated from those associated with humans (Hyma *et al.*, 2011). The present understanding of yeast population has however came from strains associated with human fermentations with the exception of yeast isolated from immune compromised patients (Muller *et al.*, 2011).

Several wine growing regions in the world described the ecosystem of yeast community present in fresh grape must and in alcoholic fermentation, analyzed on the basis of growth of cultivable yeast microflora on nutrient media (Lopandic *et al.*, 2008). The most widely used techniques in oenology is the PCR-DGGE method, which has reported detection limits between 10^2 CFU/mL in pure cultures and 10^4 CFU/mL in wine or must samples (Andorra *et al.*, 2008). In recent years, scientists have used real-time quantitative PCR (QPCR) to detect and quantify microbes in different alimentary environments (Blackstone *et al.*, 2003). The QPCR technique can detect as low as one cell per mL. Zott *et al.*, (2010) used specific oligonucleotide primers designed for real-time quantitative PCR (QPCR) to analyze several important non-*Saccharomyces* yeasts and *Saccharomyces* spp. in the fresh wine must, finished wine and those present during fermentation. This study confirms the usefulness and the relevance of QPCR for studying non-*Saccharomyces* yeasts in the complex yeast ecosystem of grape must and wine. The specificity of all primer couples for their target yeast species were validated and the QPCR methods developed were compared with a classic approach of colony identification by RFLP-ITS-PCR on cultured samples. Independent of location and grape variety, non-*Saccharomyces* (NS) species like *Candida, Hanseniaspora, Torulaspora, Pichia, Issatchenkia* and *Metschnikowia* genera were found. *Candida stellata* has also been mentioned (Di Maro *et al.*, 2007) but *Candida zemplinina* has been recently described as a separate species, which is very closely related to *C. stellata* and it is the most abundant species of the *Candida* genus in wine and must samples (Csoma and Sipiczki, 2008).

Grape berry surface is an unstable habitat that changes greatly according to the stage of grape ripening; this itself is dependent on several environmental factors, such as rapid changes in temperature, humidity and UV radiation, nutritive limitations, and the application of agrochemical treatments (Cordero-Bueso *et al.*, 2011). Milanovic *et al.* (2013) evaluated influence of fungicide treatments on the abundance and diversity of yeast communities colonizing grape berry surfaces in an organic vineyard (copper/ sulphur-based products) and a conventional vineyard (commonly used fungicides). Studies have been carried out on grape berries and in juice during fermentation, using culture-dependent and independent approaches **(Fig. 1)**. Mature grapes generally have abundant yeast and were slightly higher than grapes treated with conventional fungicides. Compared with the conventional one, organic vineyard showed less yeast species diversity in the initial grape samples. *Metschnikowia pulcherrima* was widely found in the conventional samples but occasionally found in organic ones. *Candida zemplinina* and *Hanseniaspora uvarum* (>50%) are the typical yeasts species found dominant in both vineyards and colonise surfaces of mature grape berries.

Soka and Susanto (2010) isolated a total of 24 yeasts from three different kinds of fermented orange juices and subjected to diversity analysis using restriction fragment length polymorphism (RFLP) on the internal transcribed spacer (ITS) region. This analysis revealed different amplified PCR restriction profiles and fragment sizes for each type of orange juice (**Table 1**). Yeasts isolated from the same type of orange juice showed identical restriction patterns. Sequencing of ITS regions infer that specific yeast species was detected from each type of orange, i.e., *Pichia veronae* from Indonesian Pontianak orange, *Cryptococcus albidosimilis* from Sunkist orange and *Issatchenkia orientalis* from Indonesian Medan orange.

The role of yeast in the fermented foods is not understood but occurrence of mixed mycobiota comprising the genera *Candida, Debaryomyces, Dekkera, Eotrichum, Hanseniaspora, Kodamaea, Kluyveromyces, Meyerozyma, Pichia, Rhodotorula, Saccharomyces, Trichosporon* and *Zygosaccharomyces* have been reported. Anna *et al.* (2013) compared yeast populations in marketed ogi, mawe, gowe and tchoukoutou using a combination of culture-dependent and independent molecular methods. The study was carried out to understand the specific yeast characteristics and their significance for fermentation and product quality which helps to create a new and better-suited platform for their selection. Anna *et al.* (2013) isolated 236 yeasts and identified based on different migration profiles on denaturing gradient gel electrophoresis (DGGE) and 26S rRNA gene sequencing (**Table 2**). *Candida krusei* was the yeast most frequently isolated and has strongest predominance in maize based products. Other predominant yeasts present at equal or lower incidence were *Clavispora lusitaniae* and *Saccharomyces cerevisiae* in ogi and mawe, *Clavispora lusitaniae*, *Candida tropicalis* and *Kluyveromyces marxianus* in gowe and *Clavispora lusitaniae*,

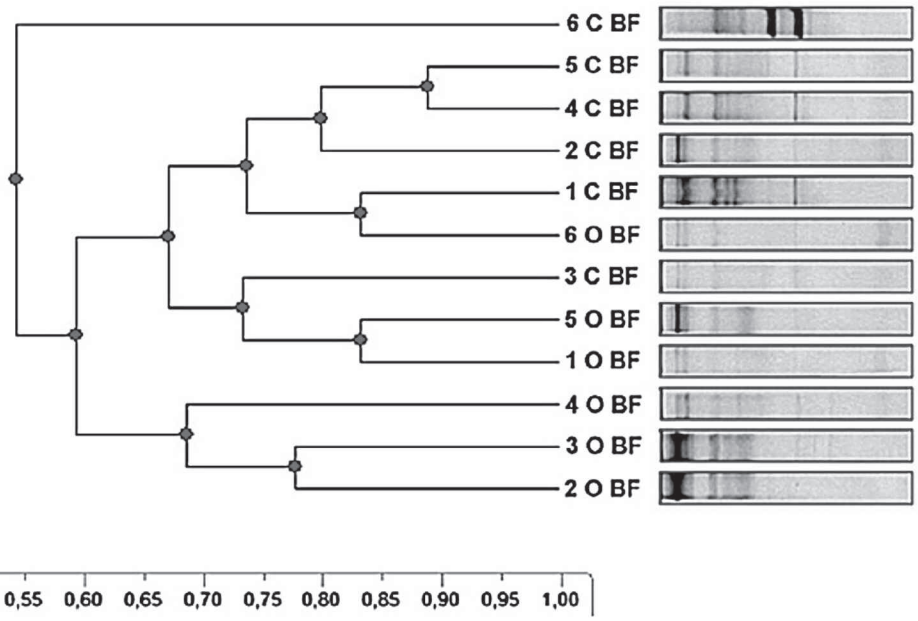

Fig. 1. UPGMA cluster analysis of the DGGE profiles of the samples collected from the organic (1O–6O) and conventional (1C–6C) vineyards at the beginning of the fermentation, based on the bands revealed. Scale: the average distances between the samples. BF = beginning of fermentation. (Milanovic *et al.,* 2013).

Table 1: PCR amplification products and restriction fragments of yeasts isolated from three types of orange using Hha 1, Hin f1 and Hae III. (Soka *et al.,* 2010)

Types of Oranges	PCR Fragments (bp)	Restriction Fragments (bp)		
		HhaI	*HinfI*	*HaeIII*
Indonesian Pontianak Orange	600	300+300	300+300	480+70
Sunkist Orange	650	300+300	350+380	500+70+60
Indonesian Medan Orange	500	200+200+50	250+180	400+80+20

Saccharomyces cerevisiae and *Candida rugosa* in tchoukoutou. Grouping of 164 *Candida krusei* isolates by rep-PCR analysis indicated that several biotypes were involved in fermentation of the four products. The DGGE analysis on the DNA directly extracted from the food matrices demonstrated the presence of *Dekkera bruxellensis* and *Debaryomyces hansenii*, which were not detected by the culture-based approach.

Yeasts are mainly responsible for the alcoholic fermentation and characteristic of this process is that different yeast species are active sequentially during the fermentation, each being substituted by the next (Lopandic *et al.*, 2008). Thus an analysis of the population dynamics of the *Saccharomyces bayanus* var. *uvaru*m yeast in wine fermentations carried out under industrial-scale vinification in the Ribera del Duero region, which has a relatively warm climate, this yeast was found dominating during the middle stage of the fermentation process, when the temperature range was between 20 and 25 ºC. The low frequency of detection of *S. bayanus* var. *uvarum* at the end of fermentation could be indicative of its lower ethanol tolerance compared to *S. cerevisiae*. *Uvarum* yeast species has a typical fermentation profile in grape must that is

Table 2: Identification of yeasts from marketed samples of ogi, mawè, gowé and thoukoutou by PCR-DGGE. For each product, three different sample sites in Benin were chosen, indicated in roman numbers. The number of isolates for each species and the total number (in % in brackets) are shown for each product. (Anna *et al.,* 2013)

Yeast Species	Ogi				Mawe				Gowe				Tchoukoutou				All products
	I	II	III	Tot	I	II	III	Tot	I	II	III	Tot	I	II	III	Tot	Tot
Candida Krusei	19	19	19	57(96)	20	16	18	54(92)	19			19(32)	10	18	6	34(59)	164(69)
Clavispora lusitaniae			1	1(2)			1	1(2)	1		3	4(6)		1	11	12(20)	18(8)
Saccharomyces cerevisiae	1			1(2)		4		4(6)					8	1		9(15)	14(6)
Candida tropicalis										3	16	19(32)					19(8)
Kluyveromyces marxianus										17	1	18(30)					18(8)
Candida rugosa															3	3(5)	3(1)
Total	**20**	**19**	**20**	**59**	**20**	**0**	**19**	**59**	**20**	**20**	**20**	**60**	**18**	**20**	**20**	**58**	**236**

significantly different from *S. cerevisiae*. It produces lower and higher amounts of acetic acid and glycerol, respectively compared with *S. cerevisiae* and produces relatively large amounts of alcohols such as 2-phenylethanol in wine (Tosi *et al.*, 2009). In other studies, significant differences have been reported in the aroma profile of Malvasia delle Lipari wines fermented by *S. cerevisiae* and others by *S. bayanus* var. *uvarum*, a higher score was attributed to the latter in tasting (Muratore *et al.*, 2007).

Saccharomyces cerevisiae that is usually associated with fermented beverages and a wide diversity of phylogenetically unrelated yeasts may form a part of the soil microbial community (Sniegowski *et al.*, 2002). Some of these yeasts that are, however, originated from other habitats such as vegetative debris or animals are transcended or fortuitously present in the soil were found to be autochthonous soil yeast that includes both ascomycetous and basidiomycetous species (Lachance and Starmer, 1998). Yeast are usually found in soil close to fruit bearing plants because the spoiled fruit deposited in the soil will act as nutrient which enrich the soil yeast (Lachance and Starmer, 1998). The simple organic carbon compounds associated with root exudates are readily assimilated by yeasts and as a result larger yeast populations usually occur in the rhizosphere than further away in the bulk soil (Cloete *et al.*, 2009).

The largest unexplored and extreme biospheres of earth are glacier habitats (cryosphere). In recent years the thrust area of research for biotechnology is related to studies on psychrophilic yeasts from glacier habitats (Buzzini *et al.*, 2012). Arctic habitats harbour around 46 species of yeasts isolated from various habitats like Siberian sediments, sands and permafrost layers from Iceland soils, from ancient Greenland ice cores, from Svalbard glacier associated habitats and from glaciers in Alaska (Uetake *et al.*, 2012). Singh *et al.* (2013) studied ten strains of cryophilic yeasts from glacier ice cores of Svalbard, Arctic. Five species of yeasts [*Cryptococcus adeliensis* (MLB-18), *C. albidosimilis* (MLB-19), *C. saitoi* (MLB-22), *Rhodosporidium lusitaniae* (MLB-20) and *Rhodotorula mucilaginosa* (MLB-27)] were identified by performing the sequence analysis of these isolates using D1/D2 domain. Studies on the effect of temperature on the growth of these isolates showed decrease in number of viable yeast cells with the increase in the depth of ice core indicating that the yeast cells in the porous surface layer of ice survive better than those in the deeper layers. Cryophilic yeasts play an important role in nutrient cycling in the ice and glacial habitats by secreting wide range of extracellular enzymes to utilize traces of available nutrients which help them in their survival and adaptation in the cryosphere (Welander, 2005). To survive at lower temperatures, psychrophilic yeast accumulates high concentration of polyunsaturated fatty acids (PUFAs) in their membranes which regulate the organisms' membrane fluidity (Pathan *et al.*, 2010). Increased concentrations of unsaturated fatty acids are also reported from psychrophilic yeasts such as *Candida, Leucosporidium* and *Torulopsis* and the fungus *Microdochium nivale* (Istokovics *et al.*, 1998). Adaptation strategies in microbes vary due to enzyme secretion, PUFA accumulation and antifreeze protein secretion, but the isolated psychrophilic microorganisms adapt to the low temperature by enzyme and PUFA secretion rather than antifreeze proteins (Fiedurek *et al.*, 2003).

ADAPTATION STRATEGIES OF YEAST

a) Sexual selection

The processes that lead to differential mating success among individuals may influence the evolutionary trajectory and biodiversity of populations. To test the effect of the strength of sexual selection on the fate of populations, Reding *et al.* (2013) allowed *Saccharomyces cerevisiae* to evolve for approximately 250 generations with altered sex ratios and found a reduction in the rate of adaptation in yeast exposed to strong sexual selection as compared to those exposed to weak or no sexual selection. Specifically, weak sexual selection populations adapted more efficiently to a harsh environment than strong sexual selection population, and asexual populations were in between these extremes and that strong sexual selection erases the benefits of sexual reproduction. This pattern was maintained during an environmental change (i.e. moving from glucose to sucrose media). These observations highlight the importance of sexual selection in shaping macro-evolutionary patterns and biodiversity. Furthermore, the asexual populations could only adapt via new mutations where as the sexual populations had the advantage of additional genetic variation from new alleles suggesting that the effect of strong sexual selection is powerful.

The study of Reding *et al.* (2013) suggests that it is possible that mutations which increase pheromonal signalling are separate from those that increase growth. In a well adapted population, pheromone signaling may be an indicator of genetic fitness, but in a new environment, in the presence of strong sexual selection, it is not. Further it was also found that crosses between populations experiencing strong sexual selection were less likely to mate than crosses between populations that experienced little or no sexual selection. This trend of pre-zygotic isolation, though not statistically significant, is in the predicted direction and given fewer numbers of generations. Finally, Reding *et al.* (2013) concluded that strong sexual selection pressures hinder adaptation, possibly influence population decline and extinction, and may increase the potential of pre-zygotic isolation.

b) Acquisition of new genes

The acquisition of new genes via horizontal transfer or gene duplication has been the dominant mechanism thus far implicated in the evolution of microbial pathogenicity. In

contrast, the role of many other modes of evolution such as changes in gene expression regulation remains unknown. A transition to a pathogenic lifestyle has recently taken place in some lineages of *Saccharomyces cerevisiae*. Hunter *et al.* (2012) identified involvement of physically interacting proteins in endocytosis that has experienced multiple cis-regulatory mutations that down-regulate gene expression levels in a pathogenic yeast. To test whether these adaptations affect virulence, a panel of single-allele knockout was created in strains whose hemizygous state is similar to the genes of adaptive down-regulations and their virulence was measured in a mammalian host. It was noticed that there is no growth in standard laboratory conditions, and all the strains were more virulent than their wild-type progenitor, suggesting that these adaptations played a role in the evolution of pathogenicity. Furthermore, genetic variants at these loci were associated with clinical origin across 88 diverse yeast strains, suggesting the adaptations may have contributed to the virulence of a wide range of clinical isolates. Pleiotropic effects of these adaptations on a wide range of morphological traits which have been reduced by compensatory mutations at other loci were also detected. These results suggest that cis-regulatory adaptation can occur at the level of physically interacting modules and one such polygenic adaptation led to increased virulence during the evolution of pathogenic yeast.

For most known cis-regulatory adaptations, pleiotropy is thought to be minimized as a result of their tissue specificity (Manceau *et al.*, 2011). Since unicellular species such as yeast cannot reduce pleiotropy in this way, they may be dependent on compensatory mutations to mitigate any unavoidable deleterious pleiotropic effects. This was observed for the endocytosis complex adaptations, whose pleiotropic effects on morphology have apparently been compensated by other loci.

c) Weak organic acids (WOAs)

WOAs are most widely used for preservation, to prevent fungal spoilage of foods and beverages. Exposure of *S. cerevisiae* to WOA leads to cellular acidification and anion accumulation. Pre-adaptation of cultures reduced the rate of acidification caused by weak acid exposure, due to changes in plasma membrane or cell wall composition. In order to adapt to sub lethal concentrations of the acids and grow, yeast cells activate ATP consuming membrane transporters to remove protons and anions. To study the extent of ATP depletion on growth inhibition in sorbic or acetic acid treated cells, Azmat Ullah *et al.* (2013) analyzed the effect of the reduction of proton and anion pumping activity on intracellular pH (pHi), growth, and energy status upon exposure to the hydrophilic acetic acid (HA) and the lipophilic sorbic acid (HS). Lipophilic WOA stress induces the plasma membrane ATP-binding cassette (ABC) transporter Pdr12p (Hatzixanthis *et al.*, 2003), which play a role in the adaptation of yeast to these weak acids by pumping out anions by utilizing the energy, either ATP or the membrane potential or the proton gradient (Henriques *et al.*, 1997). Further a stronger reduction of ATP with growth reduction rather than with growth inhibitory concentrations of both acids was observed and it was concluded that growth inhibition is not due to the ATP reduction caused by proton pumping, but rather it was due to the sorbate anion pumping. A reduction of proton pumping activity may reduce ATP consumption, but the resulting decrease of pH affects growth more. Intracellular pH (pHi) affects many cellular processes and even a slight deviation of pHi affects intracellular metabolic reactions, as it influences the ionization states of acidic and basic side chains of amino acids and thereby protein activity. Cellular activities counteracting acidification and anion accumulation consume ATP (Piper *et al.*, 1998). In yeast, intracellular acidification is partly counteracted by the activity of PMA1 plasma membrane H+-ATPase pump. PMA1 is an essential gene that encodes the major pHi regulator in baker's yeast (Serrano *et al.*, 1986). It pumps H+ ions out of the cell using ATP hydrolysis at 1:1 ratio and consumes about 20% of the ATP during normal conditions and up to 60% during weak acid stress (de Kok *et al.*, 2012). ATP utilization was differentially regulated during moderate and severe stress conditions. The energy depletion alone is not the cause of growth inhibition during HA or HS stress. Rather, the cells appear to reduce ATP consumption in high stress conditions that prevent futile cycling and maintain energy reserves for growth resumption in more favorable conditions.

DETERMINATION OF DIVERSITY

The classification of yeasts was primarily based on morphology and physiology, in particular the capacity to assimilate particular carbon source. The limitations of these methods lead to the application of molecular approaches, but different techniques often generated inconclusive findings. Kurtzman and Fell (1998) and colleagues established variation in the D1/D2 region of the large subunit (25S) rDNA as the benchmark for categorizing yeast and understanding the relatedness between strains (Kurtzman and Robnett, 1998). Application of this sequence-based taxonomy resulted in major re-organization and reclassification within the *Saccharomyces* complex. The highly polyphyletic nature of the original *Kluyveromyces* genus required renaming of most of its species and the genus now retains just six species (Lachance, 2007). Some well known '*Kluyveromyces*' species such as *K. thermotolerans* and the original type species, *K. polysporus,* are no longer part of the *Kluyveromyces* genus and the type species is now *K. marxianus. K. dobzhanskii* is the species most closely related to *K. marxianus*. An appreciation of the phylogenetic relationship between the *Kluyveromyces* and *Saccharomyces* genera is important while considering the genetics and metabolism of yeast within these genera.

The differentiation of species and strains of yeast *S. cerevisiae* and *S. bayanus* var. *uvarum* has been performed by analysis of restriction patterns of the MET2 gene and electrophoretic karyotyping as a suitable technique for discriminating the yeast clones (Le Jeune *et al.*, 2007). The MET2 gene was amplified using the primers described by Hansen and Kielland-Brandt (1994). The Non-*Saccharomyces* (NS) yeasts were identified to genus or species level by amplification of the 5.8S-ITS region of ribosomal genes as described by Esteve-Zarzoso *et al.* (1999). NS strains showed patterns with the absence of bands running below the region of 500 kb, which are specific to *S. cerevisiae* strains (De Jonge *et al.*, 1986). The strains of *S. bayanus* var. *uvarum* were differentiated from *S. cerevisiae* by the presence of two small chromosomes in the region of 245–370 kb, instead of three as in *S. cerevisiae* as reported by Naumov *et al.* (2000; 2002). *S. bayanus* var. *uvarum* yeasts were present mainly mid-way through the fermentation but were in competition with the Non-*Saccharomyces* (NS) and *S. cerevisiae*. This yeast was detected as the predominant species when the temperature of fermentation was in the range between 20 and 25 ºC. Within each yeast population, the presence of several different strains were detected and differentiated by their karyotypes. The diversity of the *S. cerevisiae* yeast was higher than S. *bayanus* var. *uvarum.*

ELECTROPHORETIC KARYOTYPING AND RFLP ANALYSIS

To detect the diversity among wine-fermenting yeast populations, single-cell colonies are analyzed by using molecular methods such as RFLP of genomic DNA or mitochondrial DNA, microsatellite analysis and delta sequencing or karyotyping. To characterize the composition of the *Saccharomyces* population, Csoma *et al.* (2010) examined 10 physiological properties, measuring the production of 7 secondary metabolites during micro-fermentation of the grape must. Csoma *et al.* (2010) analyzed *S. cerevisiae* and *S. uvarum* strains isolated during spontaneous fermentations of musts from four white grapevine varieties and one red grapevine variety in four traditional wine growing regions located in Southern and Central Hungary that differ from Tokaj in climate, soil and wine making technology. They focused on *Saccharomyces* yeasts of Tokaj, a wine region shared by Northeast Hungary and East Slovakia, where *S. uvarum* is a regular and significant component of the fermenting yeast populations (Antunovics *et al.*, 2005). Electrophoretic karyotype analysis was carried out using the BIORAD CHEF-II system as described by Antunovics *et al.* (2005) or the CHEF-III system as described by Sipiczki (2004) for comparative characterization of 86 isolates. CHE-III gave a better resolution in the region of large chromosomes. Further the relatedness of the karyotype was determined by analyzing the banding patterns with the UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm available at (http://www.tinet.cat/~debb/UPGMA/) [Garcia-Vallve *et al.*, 1999]. To differentiate between *S. cerevisiae* and *S. uvarum*, the method of NTS PCR-RFLP was used as described by Nguyen and Gaillardin (1997). The D1/D2 domains of the 26S rDNA were amplified with the primers NL-1 and NL-4 (O'Donnell, 1993) and sequenced with an ABI PRISM 3700 (Applied Biosystems) sequencer using the PCR primers (**Table 3**).To perform sequence similarity searches and pair wise sequence comparison studies, blast studies were carried out. This study revealed that the *S. cerevisiae* populations of certain wines are quite homogeneous in certain traits but highly diverse in other properties. The findings indicated that alcoholic fermentation in grape wines is performed by highly diverse yeast consortia rather than by one or two dominating strains.

RAPID QPCR METHOD

Presence of inhibitory compounds in wine such as tannins, polysaccharides, and pigments, may reduce the efficiency of DNA isolation and/or amplification, producing false negative results (Tessonniere *et al.*, 2009). Methods like

Table 3: RFLP analysis of the 5.8S-ITS region and sequence information for the D1/D2 domains of 26S rDNA gene of yeasts isolated from fermentation starters for Hong Qu glutinous rice wine. (Xu-Cong *et al.,* 2013).

Restriction profile	No of Isolates	Amplified product (bp)	Restriction fragments (size in bp)[a] *Cfo I*	*Hinf I*	*Hae III*	Closet relative species	Matching nucleotides (identity %)[b]	Accession number [c]
I	14	620	300+265+60	320+300	400+115+90	*Pichia guilliermondii*	542/542(100%)	JF439367
II	41	880	385+365+150	365+155	320+230+150	*Saccharomyces cerevisiae*	546/546(100%)	JN083825
III	233	680	320	358+325	600+80	*Saccharomycopsis fibuligera*	543/543(100%)	HM107786
IV	35	650	290+200	310+310	550	*Pichia fabianii*	541/541 (100%)	EF550321
V	5	890	383+162+144	524+366+268	636+221	*Candida glabrata*	551/551 (100%)	HM591682
VI	5	595	278	268	358+97	*Cryptococcus heveanensis*	573/575 (99%)	AF075467
VII	10	630	330+300	350+160+120	500+70+60	*Cryptococcus albidus*	564/566(99%)	AY296054
VIII	30	880	385+365+150	365+155	320+230+180+150	*Saccharomyces cerevisiae*	544/544(100%)	EF116915
IX	47	810	750+110	410+340	640	*Saccharomyces malanga*	537/540(99%)	EU057553
X	28	640	316+242+114	340+230+75	425+215	*Rhodotorula mucilaginosa*	558/559(99%)	GU373744
XI	17	684	592	180+150+110	587	*Sporobolomyces nylandii*	555/560(99%)	AB279629
XII	22	640	560	315+315	580	*Wickerhamomyces anomalus*	558/560 (99%)	FJ972217
XIII	13	684	300 + 260	365 + 330	380+110 + 80	*Rhodosporidium toruloides*	547/548 (99%)	FJ515241

[a] Restriction fragments smaller than 60 bp could not be visualized, [b] Sequence identity in the D1/D2 region of isolates of the 26S ribosomal gene and the closest relative species in the NCBI GenBank database. [c] Accession number of the sequence of the closest relative in the NCBI GenBank database found by BLAST search.

RFLP-ITS-PCR on colonies and QPCR methods made it possible to analyze the dynamics of all 6 target NS yeasts, revealing that the minor NS yeasts, like *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* species, were present throughout the fermentation process. Zott *et al.* (2010) reported *Hanseniaspora uvarum* was the major NS yeast, even after 25 days of vinification and similar results showing a high population of *Hanseniaspora* spp. at the end of a mixed culture with *Saccharomyces cerevisiae* was observed by Hierro *et al.* (2007) using QPCR method. The presence of *Hanseniaspora uvarum* at the end of alcoholic fermentation was also reported by Mills using direct molecular methods (Mills *et al.*, 2002). The methods developed in this research have been useful for studying NS yeasts and *Saccharomyces cerevisiae* dynamics in co-fermentation experiments. QPCR is also useful for evaluating the implications of NS yeast under real production conditions in future ecological studies like the impact of addition of *S. cerevisiae* in wine must in industries.

THERMOTOLERANCE

Generally yeast grows relatively at low temperatures in comparison with bacteria. During fermentation, the metabolic activity of yeasts results in exothermic production of heat and the quantity of heat released varies with different substrates and increases with increase in substrate concentration (Jones *et al.*, 1981; Lyons, 1981). The fermentation of pentoses results in the release of larger quantities of heat than hexoses (Holderby and Moggio, 1960). The control of temperature during fermentation is required to avoid the rise in temperature of medium and prevent growth inhibition; otherwise it will lead to incomplete fermentation, thereby decreasing the fermentation efficiency (Roxas and Anguila, 1971). But the thermotolerant yeasts grow with upper temperature limits of 42 - 45 ° C (Arthur and Watson, 1976), which helps in overcoming the limitations associated with mesophilic yeast, and hence, thermotolerant yeast has more practical applications than other types.

a) Factors responsible for Thermotolerance

1) Trehalose as a stress protectant

Trehalose, a sugar produced by a wide variety of organisms, is important in providing protection from desiccation (Singer and Lindquist 1998). Its concentrations increase in the presence of elevated temperatures, ethanol, desiccation and hydrogen peroxide as part of the cellular response (Gadd *et al.*, 1987). In most of the fungi, prokaryotes and nematodes, increased tolerance to adverse environmental conditions are found to possess high levels of trehalose (Van Laere, 1989).

Recent work done on yeast indicate that trehalose also promotes survival under conditions of extreme heat, by enabling proteins to retain their native conformation at elevated temperatures and by suppressing the aggregation of denatured proteins (Hottiger *et al.*, 1994) and cooperate with HSPs to promote protein refolding. Trehalose is also believed to be associated with alterations in the viscosity and water activity of the medium, leading to a decrease in the solvation layer of proteins and thus help in protein stabilization (Sola-Penna and Meyer-Fernandes, 1998). The recovery of cells from heat shock, however, gets impaired if they fail to degrade trehalose after the stress has passed. The finding that the disaccharide is localized exclusively to the nuclear cytosolic compartment, inferring the function of trehalose in *S. cerevisiae* (Kellner *et al.*, 1982). The restriction of trehalose to this region results in approximately 0.5M cytosolic trehalose concentrations (Hottiger *et al.*, 1994), which would drastically affect the constituents of the cytoplasm (Kellner *et al.*, 1982). With high concentrations of trehalose in *in-vitro*, the temperature range over which proteins retain their native state was found to be extended (Hottiger *et al.*, 1994). The role of trehalose in tolerance of various *S. cerevisiae* strains was determined by the analysis of mutations affecting trehalose levels. Cells carrying the bcy1-1 mutation in the regulatory subunit of cAMP dependent protein kinase are unable to synthesize high levels of trehalose and are sensitive to heat. In contrast, cells with the cyr1-2 mutation in the adenylate-cyclase structural gene produce trehalose constitutively and are thermotolerant (Hottiger *et al.*, 1989).

2) ATPase and transcriptional factors for stress tolerance of the yeast

Heat-driven oxidative stress has been reported to be correlated with the loss of cell viability in yeasts (Davidson and Schiestl, 2001). Calcium is an essential micronutrient for yeast growth and $CaCl_2$ was found to increase the tolerance of *S. cerevisiae* to lethal heat treatments (Fedoseeva *et al.*, 2010). Microarray analysis of *S. cerevisiae* incubated with calcium found to induce the expression of more than 160 genes, including the Ca^{2+}-dependent kinase, ATPase and transcriptional factors, which contribute to the stress tolerance of the yeast (Yoshimoto *et al,.* 2002). Liu *et al.* (2012) reported that heat shock could induce significant accumulation of ROS in yeast cells, resulting in the decline of its biocontrol ability. ROS also play an important role in the signal transduction of senescence and apoptosis in yeast (Madeo *et al.*, 2002). Further, it was found that *Debaryomyces hansenii* show a significant higher level of ROS accumulation after heat shock treatment at 40 °C for 20 min, and lower levels of ROS by adding 1 or 5 mM exogenous calcium in the medium. High amounts of ROS increase levels of carbonylated proteins and cause oxidative damage to cell components like proteins, lipids and nucleic acids and finally loss of cell viability. The addition of exogenous calcium, however, could markedly reduce the protein carbonylation of the yeasts (Reverter-Branchat *et al.*, 2004). The detoxification of ROS is dependent on the antioxidant enzymes like CAT, SOD,

and POD. When treated with exogenous Ca^{2+}, SOD and POD levels in *D. hansenii* increased (**Table 4**). Similar observations have been recorded by Navarrete *et al.* (2009), who observed that NaCl exerted a protective effect against oxidative stress and exogenous Ca^{2+} can improve the tolerance of yeasts to heat stress, particularly in *D. hansenii*. The exogenous Ca^{2+} could enhance the antioxidant enzyme activities, which in turn could reduce ROS accumulation and inhibit protein oxidative damage.

3) Thermotolerance by adaptation

The heat shock response is among the most fundamentally important and ubiquitous stress responses in nature. Heat shock proteins (HSPs), which are induced in response to various stress conditions were also considered to play an important role in conferring thermotolerance (Lindquist and Kim, 1996). Many HSPs are constitutively expressed at moderate temperatures and play a role in increasing the folding and assembly of proteins (Piper, 1993). Furthermore, two distinct types of regulatory factors are involved in the induction of heat responsive genes, a heat shock factor (Hsf1) and general stress transcription factors (Msn2 and Msn4). The genes activated by Hsf1 mostly encode chaperones and components associated with ubiquitin-related protein degradation systems; whereas the genes activated by the Msn2 and Msn4 transcriptional activators encode enzymes involved in carbohydrate metabolism and antioxidant functions (Boy-Marcotte *et al.,* 1999). Even in the absence of stress, Hsf1 binds as a trimer to canonical heat shock elements (HSEs) in the promoter of target heat shock protein (HSP) genes (Giardina and Lis, 1995). When *Saccharomyces cerevisiae* or *Candida albicans* cells are exposed to an acute heat shock, Hsf1 becomes hyper-phosphorylated and activated, leading to the transcriptional induction of these target HSP genes, thereby promoting cellular adaptation to the elevated temperature (Gallo *et al.*, 1993).

Atsushi *et al.* (2013) obtained a thermotolerant *Saccharomyces cerevisiae* yeast strain YK60 1 by stepwise adaptation of parental strain MT8 1. YK60 1 exhibited faster growth than MT8 1 at 30 °C and even grew at 40 °C, at which MT8 1 could not grow. To investigate the mechanisms as to how MT8 1 acquired thermotolerance, DNA microarray analysis was performed. The analysis revealed the induction of stress responsive genes encoding heat shock proteins and trehalose biosynthetic enzymes in YK60 1. Furthermore, non targeting metabolome analysis of YK60 1 showed that it accumulated more trehalose that contributes to stress tolerance than MT8-1.

4) MAPK kinase signaling for Thermotolerance

For a cell, the cell wall and cell membrane which are essential for the maintenance of cellular integrity and shape need to be changed and repaired to respond to environmental stresses such as heat stress as well as morphological changes during the cell cycle (Lesage and Bussey, 2006). This cell wall remodeling is controlled by a Pkc1-activated mitogen-activated protein (MAP) kinase cascade known as the cell wall integrity pathway. The MAPK cascade leads to transcription activation through the MAPK Slt2 transcriptional activator, leading to the expression of genes required for cell wall remodeling (Levin, 2005). These MAPkinase pathways contribute to thermotolerance in *S. cerevisiae,* and are also important for virulence, as MAP kinase defective *Candida albicans* mutant's display attenuated virulence in infection models (Guhad *et al*., 1998). Between *C. albicans* and other yeasts, a number of stress regulatory modules like the AP1-like transcription factors in *Saccharomyces cerevisiae Yap1* (Kuge *et al.*, 1997), S. *pombe Pap1* (Toone *et al.*, 1998), *Candida glabrata CgYap1* (Roetzer *et al.*, 2011) and *C. albicans Cap1* (Zhang *et al.*, 2000) have been conserved. They play similar roles in the activation of transcriptional responses to oxidative stress. *Saccharomyces cerevisiae* Hog1 is mainly involved in responses to osmotic stress, while *Candida albicans* Hog1 and *Saccharomyces pombe* Sty1 are involved in the diverse range of stress responses (Brewster *et al.*, 1993). Additional mitogen activated protein kinase (MAPK) cascades like the cell wall integrity Mpk1/Slt2 pathway (Navarro-Garcia, 1995) and the cell wall morphogenesis and pheromone signalling pathways involving the Cek1 and Cek2 MAPKs have been conserved in *S. cerevisiae* and *Candida albicans* (Chen *et al.*, 2002).

5) Thermotolerance by protoplast fusion

To increase thermotolerance and ethanol tolerance in *Saccharomyces cerevisiae* strain YZ1, the strategies of high-energy pulse electron beam (HEPE) and three rounds of protoplast fusion were explored. The resultant strain

Table 4: Activities of antioxidases in *Debaryomyces hansenii* and *Pichia membranaefaciens*. (Bang *et al*., 2012).

Yeast	*D. hansenii*			*P. membranaefaciens*		
CaCl2 (mM)	CAT (U mg-1 protein)	SOD (U mg-1 protein)	POD (U mg-1 protein)	CAT (U mg-1 protein)	SOD (U mg-1 protein)	POD (U mg-1 protein)
0	626.27 ± 45.63a	78.26 ± 2.45a	49.10 ± 10.01a	134.28 ± 32.05a	7.78 ± 1.77a	11.64 ± 3.42a
1	688.96 ± 2.70a	95.43 ± 4.79a	72.03 ± 2.53b	125.64 ± 34.30a	8.27 ± 2.40a	10.98 ± 4.30a
5	683.07 ± 44.71a	124.54 ± 24.33b	97.78 ± 24.35c	120.48 ± 9.84a	8.79 ± 1.49a	12.97 ± 4.06a

Antioxidase activities were determined in cells cultured in SD medium containing 0, 1, or 5 mM CaCl2. Values are the mean ± standard deviations from three independent experiments. Values followed by different letters are significantly different according to Duncan's multiple range test (P\0.05)

YF31 had the characteristics of resistance to high-temperature, high-ethanol concentration, exhibit rapid growth and high yield. The YF31 could grow on plate cultures up to 47 °C, containing 237.5 g L^{-1} of ethanol. In particular, the mutant strain YF31 generated 94.2 ± 4.8 g L-1 ethanol from 200 g/L glucose L-1 at 42 °C, which was 2.48 times higher than the production by the wild strain YZ1. These results demonstrated that the variant phenotypes from the strains screened by HEPE irradiation could be used as a parent stock for yeast regeneration and the protoplast fusion for combining suitable characteristics in a single strain for ethanol fermentation. Zhang *et al.* (2012) performed the mutation studies on cells by HEPE irradiation and found that, *S. cerevisiae* cells showed dose-dependent decreased cell viability ($P< 0.05$) after HEPE treatment. The dose response curve fits the quadratic model, which indicated that HEPE induces minor physiological damage to yeast cells. The absorbed dose-rate of HEPE on biomaterials is 1010 Gys^{-1}, which is much higher than that of c-rays (usually under 60 Gy s^{-1}) and the other radiation methods. With such high dose rate of HEPE, high-density radicals were generated in seconds, leading to DNA double-strand breaks in the samples (Zhu *et al.*, 2008). The mutants thus become variable due to the genetic background of the parent samples diverged by HEPE irradiation. The HEPE irradiation may achieve more useful mutations and enlarge initial population diversity, which could exhibit better performance on protoplast fusion. The single HEPE mutant strains could not be tolerant to all stresses. The protoplast fusion approach was thus used to combine multiple stress tolerance.

b) Applications of thermotolerant yeasts

1) Bioethanol production

Bioethanol, a renewable eco-friendly fuel, is now considered to be an alternative to conventional gasoline. *S. cerevisiae* is a facultative anaerobe and under anaerobic conditions can ferment glucose to ethanol. Improvements in ethanol production by using genetically engineered yeast cells during the fermentation process may lead to a boost in the bioethanol production industry (Hal *et al.*, 2006). Among the desirable traits of strains required for efficient bioethanol production, tolerance to high temperature, sugar, acidity and ethanol is crucial for reducing cooling and ethanol recovery costs and for minimizing the risk of contamination. Although considerable progress has been made with respect to engineering yeast strains for stress tolerance, a full understanding of the molecular mechanisms that confer tolerance to stress is lacking (Jeffries and Jin, 2004). Thermotolerant yeast strains offer the advantage of conducting the bioethanol production process at elevated temperatures. Further more, isolation and selection of strains from nature is a promising way to obtain superior strains exhibiting desirable phenotypes such as ability to grow at high temperatures (Paivi *et al.*, 2011). The optimum temperature for growth of *S. cerevisiae* ranges from 25 °C to 30 °C, and *S. cerevisiae* does not normally grow at temperatures higher than 40 °C. Recently, a high-temperature growth phenotype (Htg), which enables cells to grow at 41 °C, has been categorized as a quantitative trait that is controlled by multiple genes in *S. cerevisiae* (Nevoigt, 2008). Htg+, Hep+ and Acd+ phenotypes are desirable for yeast bioethanol producers because strains showing these multiple-tolerance traits can minimize the costs of maintaining an optimum temperature, ethanol recovery and risk of contamination (Suthee *et al.*, 2012).

2) Probiotics

Eukaryotic microorganisms such as yeasts exhibit characteristics like morphological diversity (budding, pseudo mycelia), nutritional flexibility (ability to utilize a broad range of nitrogen, carbon, and phosphorous sources), stress tolerance (to low pH/oxygen/water activity, high osmotic pressure), enzyme secreting potential (secrete a broad range of enzymes such as lipase, peptidase, amylase, invertase, phytase, etc.), anti-oxidative/antitumor/antimicrobial activity (effective against a wide range of pathogens) which contribute to their success as an ideal probiotic (Fredlund *et al.*, 2002). Strains of *S. cerevisiae* have been widely tested for probiotic properties such as the protection of bacterial translocation and preservation of gut barrier integrity (Generoso *et al.*, 2010). Additionally, probiotic yeasts may have inhibitory activity against pathogenic bacteria (Tiago *et al.*, 2009). *S. cerevisiae* var. *boulardii* is the only yeast with proven clinical effects and the only yeast with proven probiotic efficiency in double blind studies (Sazawal *et al.*, 2006). It is used for prevention and treatment of many different types of human gastrointestinal diseases (Zanello *et al.*, 2011).

CONCLUSIONS

Yeasts have been widely used by human beings for fermentation and baking purposes, but very little is known about their diversity. Knowing the yeast ecology, diversity and mechanism of adaptation will greatly help to understand the power of yeast genetics, ecological and evolutionary aspects. Following aspects have to be considered for utilization of their potential applications in the industry and human welfare:

- Combination of culture-dependent and independent molecular methods should be used to evaluate the influence of different chemical treatments on the abundance and diversity of yeast communities.
- Research need to be focused on glacier habitats, which are the largest unexplored and extreme biospheres for the prospect of biotechnology.
- Role of enzymes and protein secretions which are important for adaptation strategies in microbes
- Evaluation of sexual selection processes that help in shaping macro-evolutionary patterns and biodiversity.

- Exploitation of molecular methods such as RFLP of genomic DNA or mitochondrial DNA, microsatellite analysis, delta sequences or karyotyping and also establishing variation in the D1/D2 region of the large subunit (25S) rDNA which help in detecting the diversity of yeast.

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