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# Screening of natural yeast isolates under the effects of stresses associated with second-generation biofuel production

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**Robust microorganisms are required for sustainable second-generation biofuel production. We evaluated the growth and fermentation performance of six natural isolates that were derived from grape wine and medicinal herbs using a wide range of carbon sources, rice and wheat straw hydrolysates as well as stress conditions associated with second-generation ethanol production. Sequence analysis of the 5.8S internal transcribed spacer (ITS) and species-specific PCR amplification of the *HO* gene region assigned the natural isolates to *Saccharomyces cerevisiae*. Restriction fragment length polymorphism (RFLP) analysis of the mitochondrial DNA revealed that natural yeast isolates are genetically closer to the laboratory strain BY4741 than to the CEN.PK strains. Dextrose fermentation by a natural isolate, MTCC4780, under semi-anaerobic conditions produced maximum ethanol yields of 0.44 g/g and 0.39 g/g, respectively, with and without the stresses encountered during lignocellulosic ethanol fermentation. However, MTCC4780 produced ethanol yields of 0.48 g/g, 0.42 g/g and 0.45 g/g, respectively, with glucose, rice and wheat straw enzymatic hydrolysate fermentation in a bioreactor. The isolates MTCC4781 and MTCC4796 showed higher growth and fermentation performance than did MTCC4780 in the presence of elevated temperature and pre-treatment inhibitors. Taken together, the MTCC4780, MTCC4781 and MTCC4796 strains have the potential to serve as a platform for lignocellulosic ethanol production under stresses associated with second-generation biofuel production.**



[**Key words:** Lignocellulosic ethanol; Robust yeast; Inhibitor tolerance; Rice and wheat straw; Combined stress]

The production of second-generation biofuels from lignocellulose-based raw materials that are derived from agricultural, forest and municipal solid wastes can provide strategic, economic and environmental benefits (1). The increasing demand for food, feed, and energy has raised several concerns about the potential use of food-based biofuels and their future sustainability. Moreover, global warming and energy security concerns have intensified the search for safe and effective methods to commercially produce bioethanol from cellulose-based biomass. Bioethanol is completely renewable in nature with zero net carbon emissions (2). Microbial fermentation offers a promising alternative for the production of sustainable biofuels. Recently, *Saccharomyces cerevisiae* has received much attention as a vehicle for the production of ethanol and higher alcohols (3).

The production of cellulosic ethanol involves three major sequential steps: (i) the chemical and physicochemical pre-treatment of the lignocellulosic biomass, (ii) the hydrolysis of cellulose and hemicelluloses to fermentable sugars by cellulolytic enzymes, and (iii) microbial fermentation for the production of ethanol. Regardless of the process used for pre-treatment, various toxic compounds (weak acids, furan derivatives, and phenolics) are produced during pre-treatment that inhibit microbial growth, metabolism and ethanol yield (4,5). In addition, high temperature and concentrated ethanol are potent inhibitors of industrial lignocellulosic hydrolysate fermentation, which results in reduced growth and ethanol yield (6,7).

Several approaches have been applied to make cellulosic ethanol production technically and commercially feasible. The evolutionary adaptation of genetically engineered yeast strains to fermentation-related stresses is a powerful strategy, but it often results in the loss of other desirable traits (8). Genetic engineering is another powerful tool for developing robust microbes (9). Interestingly, genetic engineering approaches are mostly applied to laboratory strains, and these laboratory strains may be difficult to use in industrial processes because of their low industrial fitness and fermentation performance (10,11). An alternative approach is to select the yeast strains from industrial and natural resources because these yeast strains may show native resistance to inhibitors and improved fermentation performance. These selected strains could be further engineered to utilize pentose, which otherwise remains un-metabolized by native *S. cerevisiae*, as a carbon source for ethanol production. Pentose constitutes a major fraction of the lignocellulosic biomass, and bioengineering is required to incorporate the pentose metabolic pathway into native *S. cerevisiae* to allow for its conversion to ethanol (12,13).

A number of reports have been published on the pre-treatment and enzymatic hydrolysis of the lignocellulosic biomass (14,15). The previous approaches were mainly focused on selecting *S. cerevisiae* isolates that are tolerant to a single stress at a time (16,17). However, recently, a few reports have considered natural isolates for

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industrial adaptation based on their innate resistance and fermentation performance in the presence of two or more simultaneous stresses (18–21). Because the combined effect of inhibitors, high temperature and concentrated ethanol poses a major challenge for the successful production of bioethanol, selecting natural *S. cerevisiae* isolates with inherent resistance to these stress factors could be a more realistic strategy for second-generation biofuel production (22).

To select promising *S. cerevisiae* strains to serve as a platform for second-generation biofuel production, natural yeast isolates that were derived from grape wine and medicinal herbs from different regions of India were screened. The growth and fermentation performance of these isolates was evaluated in the presence of various stresses that are encountered during lignocellulosic hydrolysate fermentation. In addition, the fermentation performance of the selected isolates for ethanol production, using wheat and rice straw cellulosic hydrolysates, was evaluated.

## MATERIALS AND METHODS

**Strains and media** The *S. cerevisiae* laboratory strains and yeast natural isolates used in this study are listed in Table 1 (23). All of the strains were grown and maintained in YEPD broth (1% yeast extract, 2% peptone and 2% dextrose) and on YEPD plates (YEPD medium supplemented with 20 g/l agar), except where otherwise stated. Synthetic media (2 g/l yeast extract, 0.4 g/l $MgSO_4$, 2 g/l $(NH_4)_2SO_4$, 5 g/l $KH_2PO_4$) and glucose synthetic media (synthetic media with 100 g/l glucose) were used to test the fermentation ability of the yeast strains.

**DNA extraction** Total yeast DNA was extracted as described by Guillamon et al. (24). Briefly, the cells were grown overnight in YEPD broth and harvested by centrifugation at 4000 r.p.m. for 5 min. The cells were resuspended in 500 µl of SOE buffer (1 M sorbitol, 0.1 M EDTA-pH 7.5), transferred to 1.5 ml microfuge tubes and incubated for 2 h at 37°C with 100 T Zymolyase. The lysate was centrifuged for 1 min, the pellet was resuspended in 0.5 ml of TE buffer [50 mM Tris-HCL (pH7.4), 20 mM EDTA] and 50 µl of 20% SDS was added; the mixture was then incubated for 30 min at 65°C. After the addition of 0.2 ml potassium acetate, the mixture was placed on ice for 1 h and centrifuged for 5 min. The supernatant was transferred to a fresh tube, mixed with an equal volume of 2-propanol, and incubated for 5 min at room temperature. The pellet was air-dried and dissolved in sterile distilled water.

**Restriction fragment length polymorphism of mitochondrial DNA (mtDNA-RFLP)** The *S. cerevisiae* strains described in Table 1 were subjected to mtDNA restriction analysis (24). First, 20 µg of the total DNA was digested overnight at 37°C with 40 units of *HinfI* restriction endonuclease (Thermo Scientific, USA) in a final volume of 50 µl. Digested DNA fragments were separated on 1.2% agarose gel in 1X TBE buffer (Tris, Borate and EDTA), stained with ethidium bromide and visualized under a UV light. The correlation of similarities among banding profiles was analyzed using Unweighted Paired Group Average (UPGMA) cluster analysis based on the Dice coefficient, using PyElph software version 1.4.

**Species-specific PCR amplification** Species-specific PCR amplifications were performed as described by Pereira et al. (25). Briefly, two different sets of PCR primers were used to amplify the *HO* region from the genomic DNA samples of the laboratory strains and natural isolates listed in Table 1. One set of PCR primers, (ScHO-F and ScHO-R) generated a single amplicon of 400 bp when *S. cerevisiae* genomic DNA was used as template. The same primer set generated a 300 bp PCR amplicon with *Saccharomyces pastorianus* genomic DNA. However, a second set of PCR primers (LgHO-F/LgHO-R) generated a 700 bp PCR amplicon with *Saccharomyces bayanus* genomic DNA. These primers did not generate any PCR amplicons with non-*Saccharomyces* yeast genomic DNA.

**PCR amplification and sequence analysis of the internally transcribed spacer region** The 5.8S-internally transcribed spacer (ITS) rDNA regions (ITS1 and ITS2) of laboratory strains and natural isolates (Table 1) were PCR amplified from the genomic DNA using the ITS5 (5′-GGAGAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. PCR amplifications were performed in a 50 µl reaction volume supplemented with 50 ng of genomic DNA, 10 pmol of each primer, 10 µM of each dNTP and 1 unit of Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA). All the amplifications were programmed in a T100 thermo cycler (BIO RAD, India) as follows: 98°C for 3 min, followed by 35 cycles of 98°C for 30 s, 50°C for 30 s and 72°C for 1 min, with a final extension step at 72°C for 10 min. The amplified PCR products were separated on a 0.8% (w/v) agarose gel in 1X TAE (40 mM Tris-Acetate, 1 mM EDTA pH 8.0) buffer and detected by staining with ethidium bromide.

The amplified PCR products of the 5.8S-ITS rDNA regions were purified using the QIAquick_PCR Purification Kit (Qiagen, Germany) and sequenced using the ITS5 and ITS4 primers. A BLAST search of the sequences was performed using the Gene Bank data library, National Centre for Biotechnology Information (NCBI). Cluster analysis was conducted using a neighbor-joining method from the software package MEGA (Molecular Evolutionary Genetics Analysis version 5).

**Semi-anaerobic and anaerobic fermentation** Batch fermentation experiments under semi-anaerobic conditions were performed in air tight 50 ml sealed glass bottles containing 100 g/l dextrose or other carbon sources (listed in Table 2) in 10 ml synthetic media; the initial pH and shaking conditions were set to 5.0 and 150 rpm, respectively. Experiments under complete anaerobic conditions were performed in a benchtop fermenter (Biostat Q Plus, Sartorius, India) with a working volume of 250 ml. Anaerobic conditions were established by flushing with argon gas at a flow rate of 0.02 l/min throughout the fermentation experiment. The pH and stirring were maintained at 5 and 300 rpm, respectively, throughout the fermentation experiments. The temperature and inoculum size were kept at 30°C and 10% (v/v), respectively, for both the anaerobic and semi-anaerobic studies. The yeast strains were grown overnight in YEPD at 30°C before using 10% (v/v) as an inoculum for fermentation. Ethanol and sugar were estimated with a high performance liquid chromatography (HPLC) system (Agilent 1260 Series, USA) using an Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA, USA). The column temperature was maintained at 40°C, and the flow rate of the mobile phase (4 mM $H_2SO_4$) was 0.3 ml/min. All of the fermentation experiments were conducted in three replicates, and their standard deviation values are presented in the tables and represented as error bars in the figures.

**Wheat and rice straw fermentation** The wheat and rice straw biomass were washed, dried in the shade for 48 h and pulverized in a laboratory grinder (Bajaj Electronics, India) to a size range of 0.75–1 mm. For the acid pre-treatment of the biomass, wheat and rice straw with 10% w/v solid content were autoclaved with 2% sulfuric acid for 1 h at 120°C (103.4 KpaG). The pre-treated biomass was filtered with a double-layered muslin cloth to separate the acid hydrolysates from the solid biomass residue. The enzymatic hydrolysis of pretreated solid biomass was performed as described by Munjal et al. (26). Briefly, the biomass was hydrolyzed using an enzyme cocktail (100 FPU/g dry weight, Advanced Enzyme Technology Ltd.) in an Erlenmeyer flask (500 ml) with 5% (w/v) solid content in 50 mM citrate buffer (pH 4.8) at 50°C with continuous stirring in a rotary shaker at 150 rpm for 8 h. The pH of the enzymatic hydrolysate was adjusted to 6.3 using NaOH. Then, 250 ml of filtered sterilized enzymatic hydrolysates was supplemented with 2 g/l of yeast extract, 2 g/l $KH_2PO_4$, 0.5 g/l $NH_4Cl$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.25 g/l $(NH_4)_2HPO_4$, 0.1 g/l $FeCl_3{\cdot}2H_2O$ and 0.1 g/l $CaCl_2{\cdot}2H_2O$. The fermentation experiment was conducted in a 350-ml laboratory bench-top fermenter under completely anaerobic conditions for 24 h and was supplemented with a 10% inoculum of *S. cerevisiae* laboratory strains and natural isolates. The fermentation broth was centrifuged, and the supernatant was used for the quantification of ethanol and residual sugar. The total sugar in the enzymatic hydrolysates, as well as the residual sugar and ethanol contents in the fermentation broth, was estimated using HPLC.

**Growth and fermentation under multiple stresses** Fermentation under various stress conditions was studied in semi-anaerobic fermentation conditions. Thermotolerance was assayed by growing strains under varying temperature (38°C–42°C), whereas the tolerance of the strains to ethanol and toxic inhibitors was studied by adding a known amount of ethanol (6–10% v/v) and inhibitors

**TABLE 1.** List of strains used in this study.

| Strain | Type | Source | Isolated from | Reference |
|---|---|---|---|---|
| CEN.PK113-7D | Haploid laboratory strain | Peter Kötter (J.W. Goethe Universitat Frankfurt, Frankfurt, Germany) | – | 21 |
| CEN.PK112 | Diploid laboratory strain | Peter Kötter (J.W. Goethe Universitat Frankfurt, Frankfurt, Germany) | – | 21 |
| BY4741 | Haploid laboratory strain | ATCC | – | 21 |
| MTCC4780 | Natural isolate | Grape wine | Wantgu, Himachal Pradesh, India | This study |
| MTCC4781 | Natural isolate | Grape wine | Rekongpeo, India | This study |
| MTCC4787 | Natural isolate | Grape wine | Kardang, Himachal Pradesh, India | This study |
| MTCC4793 | Natural isolate | Grape wine | Kinnaur, Himachal pradesh, India | This study |
| MTCC4796 | Natural isolate | Grape wine | Khawangi, Himachal Pradesh, India | This study |
| MTCC6008 | Natural isolate | Medicinal herb | Kerala, India | This study |