Genome Shuffling for Improved Thermotolerance, Ethanol Tolerance and Ethanol Production of Saccharomyces cerevisiae 2013

Fredmoore L. Orosco\textsuperscript{1,2*}, Sean M. Estrada\textsuperscript{3}, Jessica F. Simbahan\textsuperscript{1,3}, Virgie A. Alcantara\textsuperscript{3} and Irene G. Pajares\textsuperscript{3}

\textsuperscript{1}Institute of Biology, University of the Philippines, Diliman, Quezon City 1101, Philippines
\textsuperscript{2}Institute of Biological Sciences, University of the Philippines Los Baños, Laguna 4031, Philippines
\textsuperscript{3}National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, Laguna 4031, Philippines

Bioethanol production has been the center of interest of scientists to generate a more sustainable, low cost and environment-friendly fuel compared to petroleum fuel products. However, a more robust and high performing ethanologen still needs to be developed to effectively convert sugars to ethanol while withstanding chemical and thermal stress. In this study, genome shuffling through ethyl methanesulfonate (EMS) mutagenesis and protoplast fusion was performed to develop superior strains of Saccharomyces cerevisiae 2013 with improved ethanol tolerance, thermotolerance and ethanol production. Results of the experiment obtained eight promising strains. These isolates exhibited higher ethanol yield compared to the parental strain at 30 °C and 42 °C. Furthermore, these isolates remained stable and viable up to 42 °C in 15 % (w v\textsuperscript{-1}) to 18 % (w v\textsuperscript{-1}) ethanol. Data on presence of trehalose content further supported these observations. Improved strain, F1D, was found to be the most promising isolate being able to grow at 42 °C and 18 % ethanol and yielding 10.83 % higher ethanol than parental strain 2013 and 15.16 % over the industrial strain \textit{S. cerevisiae} HBY3. Thus, genome shuffling is a powerful tool in developing \textit{S. cerevisiae} 2013 strains with improved fermentation qualities.

KEYWORDS
Ethanol production, ethanol tolerance, ethyl methanesulfonate, genome shuffling, \textit{Saccharomyces cerevisiae} 2013, thermotolerance

INTRODUCTION
Ethanol produced from renewable resources can provide environment-friendly transportation fuel, less dependence on foreign oil and improved rural employment (Chen et al. 2010). The Philippines is currently implementing the Biofuels Act of 2006, also known as Republic Act no. 9367, which requires that gasoline must be replaced with five percent locally-produced ethanol by 2008 and 10 % ethanol by 2010. Since local ethanol production cannot meet the demands for fuel ethanol substitution, research on the improvement of fuel production and utilization of alternative sources are very relevant. Nazari et al. (2005) noted that bacteria and fungi have been recognized for their capacity to convert sugars to ethanol. Although many microorganisms have been exploited for ethanol production, they still lack the necessary characteristics for on-farm use, such as tolerance to high and low temperatures, to optimize fuel production.
production, *Saccharomyces cerevisiae* is still the most effective organism in fermenting sugars to ethanol due to its capacity to ferment under aerobic and anaerobic conditions, metabolic versatility, relatively high ethanol tolerance (Ward and Singh 2002) and temperature tolerance. Experiments that will lead to improvement of these characteristics will potentially result to lower cost of production, higher fermentation rate and enhanced ethanol production (Panchal and Tavares 1990).

Fermentation of yeasts requires temperature to be maintained between 30–35 °C to maximize ethanol production (D’Amore et al. 1989). Since maintaining the optimum fermentation temperature requires expensive cooling systems, local distilleries usually dispense with these and conduct fermentations under ambient conditions where batch fermentation can reach temperatures of 40 °C or higher. Thus, it would be economically and technically advantageous to use a strain of yeast that can yield maximum ethanol levels at higher temperatures.

Ethanol is a chemical that degrades cell structure and affects physiology of all microorganisms (Mobini-Dekhordi et al. 2007). It destroys rigidity of cytoplasmic membranes, inhibits endocytosis, blocks proton motive forces of inner mitochondrion membranes, inactivates several essential enzymes such as protein kinase A and alcohol dehydrogenase, and prevents element translocation across membranes in cells (Mobini-Dekhordi et al. 2008). Thus, it is important to obtain yeast isolates that can tolerate high ethanol concentration while producing it.

Researchers have used the method of genome shuffling to obtain desirable characteristics (Chen et al. 2010; Gong et al. 2009; Hou 2009; Shi et al. 2009). Genome shuffling allows many parental strains with certain phenotypic improvements obtained from mutagenesis to be recombined through recursive protoplast fusion. A library of shuffled yeast strains with exchanged genetic characteristics is obtained by the repetition of this process (Shi et al. 2009). The method is efficient and reliable because it overcomes the laborious and time consuming process of regeneration as well as loss of mutations during selection of the initial mutant library (Hou 2009; Steensels et al. 2014).

This research aimed to produce *S. cerevisiae* strains with improved thermotolerance, ethanol tolerance and ethanol yield compared with the parental strain *S. cerevisiae* 2013 and industrial strain *S. cerevisiae* HBY3 through the use of genome shuffling.

**MATERIALS AND METHODS**

**Strain**

*Saccharomyces cerevisiae* 2013 was obtained from the culture collection of the Philippine National Culture Collection of Microorganisms (PNCM) at the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños, Laguna originally deposited by Ms. Elvira Guevarra. It was grown on agar slants of Yeast Extract Peptone Dextrose (YPED, 1.0 % yeast extract, 2.0 % peptone, 2.0 % glucose and 1.8 % agar) at 30 °C for 48 h. *S. cerevisiae* 2013 is a wild type strain, high ethanol yielding from molasses (Unpublished Data), isolated from sugarcane plant from Canlubang, Laguna.

**Protoplast Isolation and Regeneration**

Protoplasts of *S. cerevisiae* 2013 were isolated using a modified procedure by Taya et al. (1984), Magbanua (1992) and Bermejo (2006). A loopful of the parental strain was inoculated in 10 mL YEPD broth in a 50-mL Erlenmeyer flask. The flask was incubated for 20 h with shaking at room temperature (28–30 °C). One mL aliquots were transferred to fresh 1.5-mL microfuge tubes. The cells were centrifuged using Eppendorf Centrifuge 5415C at 12000 rpm (9000 X G) for 2 min, washed with 1 mL 0.8 M Tris-Sorbitol (TS) buffer thrice. The cells were then resuspended in 0.2 % 2-mercaptoethanol in 50 mM ethylenediaminetetraacetic acid (EDTA) and incubated for 30 min at room temperature. The cells were centrifuged at 5000 rpm (1600 X G) for 2 min and washed with 0.8 M TS buffer twice. After washing, the cells were resuspended in 0.10 mg mL⁻¹ Amsbio Zymolyase ®100T, Cat No.120493 (Abingdon, UK) solution and the tubes were incubated for 120 min at 37 °C with constant rotary shaking at 200 rpm.

Protoplasts were regenerated using Medium A (MA) composed of 1.0 % yeast extract, 2.0 % peptone, 2.0 % glucose and 2.0 % agar dissolved in 80 % D-sorbitol and 20 % distilled water (Magbanua 1992). The protoplasts were inoculated by spot plating at different dilutions (10⁻¹ – 10⁻⁴) to plates containing MA media with or without an osmotic stabilizer. The plates were then incubated at room temperature (28-30 °C) for 3-5 days, then protoplasting efficiency was computed using the equations:

\[
\% \text{Protoplasting Efficiency} = \frac{\text{count in media with stabilizer} - \text{count in media without stabilizer}}{\text{count in media with stabilizer}} \times 100\%
\]

**Genome Shuffling**

To construct the initial mutant library for genome shuffling, protoplast mutagenesis using ethylmethanesulfonate (EMS) was performed using a method from Mobini-Dekhordi et al. (2008). The protoplasts were then regenerated and screened for ethanol production, ethanol tolerance and thermostolerance. The superior strains after mutagenesis (M) and screening were selected to be the parental strains for protoplast fusion (F). Protoplasts of superior M strains were isolated and randomly fused based on the procedure by Spencer and Spencer (1988). The resulting fusants were again regenerated and screened for ethanol production, ethanol tolerance and thermostolerance. The overview of the whole protocol is summarized in Figure 1.

Figure 1. Overview of methodology and major results of improvement of *Saccharomyces cerevisiae* 2013 through genome shuffling.

**Screening of Putative Mutants and Fusants**

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Thermotolerance Test
Growth of the mutants and fusants was tested at 37 °C, 40 °C, 42 °C and 45 °C. Using a microtitration plate, a loopful was inoculated in each well containing 100 µL YEPD broth. On a fresh YEPD plate, 2 µL of suspension from each well was inoculated and incubated at the different temperatures mentioned above. Growth of different colonies was scored after 48 h of incubation.

Ethanol Tolerance Test
Growth of parental, mutant and fusants were tested in YEPD agar containing 10 %, 12 %, 15 %, 18 % and 20 % absolute ethanol. Using a microtitration plate, a loopful was inoculated in each well containing 100 µL YEPD broth. In a fresh YEPD plate containing the above concentrations of ethanol, 2 µL of suspension was inoculated and incubated at room temperature (28-30°C). Growth of each colony was scored after 48 h of incubation.

Testing for Phenotypic Stability
To check for stability, colonies from mutagenesis and protoplast fusion were picked and grown alternately on media under optimal conditions then on media under limiting conditions. For example, mutants and fusants were initially streaked on YEPD master plates and incubated at 30 °C. After 24 h the colonies were streaked on another YEPD plate containing ethanol and incubated at higher temperature. The isolates were then transferred back to YEPD plate and incubated at 30 °C then back to more stringent limiting conditions until most of the unstable and intolerant isolates were eliminated after five weeks (Panchal and Tavares, 1990).

Molasses-Based Flask Fermentation
A loopful of a pure culture of each isolate was streaked into YEPD agar slants and incubated at room temperature (28°C -30 °C) for 24 h. Five (5) mL aliquot from 30-mL Build-up Medium, BM (5 % equivalent total sugars, 0.14 % KH2PO4, 0.025 % MgSO4.7H2O and 0.1 % (NH4)2SO4, with pH adjusted to 4.5 with phosphoric acid) was dispensed into the test tube with 24-h old yeast culture. The growth on agar surface were scraped off using a sterile wireloop, shaken thrice and aseptically emptied back into the 125-mL flask containing the remaining BM. The flask was placed in a rotary shaker and incubated at room temperature (28 °C -30 °C) for 24 h. The resulting culture was used as yeast inoculum for fermentation.

Each yeast inoculum was transferred to separate microfuge tubes and centrifuged at 5000 rpm (1600 X G) for 5 min. The pellet was washed twice with sterile distilled water, resuspended in 1 mL sterile distilled water and diluted to x 40. The optical density of 2 mL sample was measured at 660 nm and used to determine the volume of the cell suspension needed to obtain an absorbance of 1.0. The corresponding volume of cells were inoculated to Fermentation Medium containing 20 % equivalent total sugars, 0.14 % KH2PO4, 0.025 % MgSO4.7H2O and 0.1 % (NH4)2SO4 (pH adjusted to 4.5 using phosphoric acid). After 48 h of incubation at 30 °C and 42 °C, the total ethanol produced was measured in duplicates using a gas chromatograph.

Determination of Ethanol Production by Gas Chromatography
Ethanol concentration was measured by injecting 1µL sample into a Shimadzu GC-8A gas chromatograph with the following settings: nitrogen gas as carrier at 60 mL min⁻¹; air pressure at 0.5 kg cm⁻²; H2 pressure at 0.7 kg cm⁻²; column temperature set at 180 °C; detector temperature set at 200 °C and Porapac Q as column material.

Determination of Trehalose Content
Trehalose content of the superior isolates from mutagenesis and genome shuffling and parental strain was detected using a modified method by Sharma (1997) and Trevelyan and Harrison (1956). The cells were grown in 50-mL Erlenmeyer flask containing 10 mL YEPD broth for 48-72 h in a rotary shaker at room temperature (28-30 °C). A 5 mL aliquot was obtained and washed twice with cold sterile distilled water. To each test tube, 4 mL 0.5 M cold tri-chloro acetic acid (TCA) were added and the contents were mixed at 10 min intervals for up to 30 min. Centrifugation at 3000 rpm (600 X G) was performed and supernatant was collected in a clean 50 mL-centrifuge tube. After adding TCA solution, centrifugation and supernatant collection were repeated. One mL of each sample was transferred to a clean test tube containing 5 mL of anthrone reagent solution (0.8g anthrone in 100 mL concentrated sulfuric acid). All tubes were placed in a boiling water bath for 10 min. Optical density at 620 nm of each solution was determined in duplicates using Shimadzu UV-PharmaSpec 1700. The trehalose content of each sample was interpolated from the standard curve of trehalose.

Statistical Analysis
Data were analyzed using Factorial ANOVA (P<0.05). Mean differences were established by Duncan’s Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

EMS Mutagenesis
Ethyl methylsulfonate mutagenized protoplasts of Saccharomyces cerevisiae 2013 regenerated in MA media with 93.02 % protoplasting efficiency. Of the 428 mutants picked, ten were selected based on the growth at media with 15 % ethanol and at 42 °C. Although yeast produces ethanol, it is still a chemical component that affects cell structure and physiology (Mobini-Dehkordi et al. 2007). This is the main reason why ethanol tolerance has a significant role in the fermentation of ethanol.

Only 14 colonies (3.27 %) remained viable at 42 °C. The parental strain, 2013, whose optimum temperature was at 35 °C, was non-viable at this temperature. A representative image showing viable and non-viable mutants is shown in Figure 2.
Four mutants, namely MA, MB, MC and MD, were found to produce the highest ethanol and remained viable in medium containing 15% ethanol at 42 °C. These strains were used as the parental strains for further genome shuffling by protoplast fusion.

**Genome Shuffling**

Indirect selection method by Spencer et al. (1980), Pina et al. (1986), and Amano (2007) allowed the selection of 141 colonies (20.38%) that grew on media containing 18% ethanol and 106 colonies (15.32%) that grew on YEPD agar incubated at 42 °C. The other 386 colonies were not able to grow at 42 °C. The resulting strains were 80% more ethanol tolerant than *S. cerevisiae* 2013 and 20% more ethanol tolerant than the F1 parental strains.

The ten F1 fusants that possessed highest growth at 18% ethanol and 42 °C were picked and evaluated for ethanol production in molasses-based flask fermentation at room temperature (Figure 4). Four mutants, namely F1A, F1B, F1C and F1D, produced ethanol at higher rate than HBY3. Among the four mutants, only F1D had statistically higher ethanol production than the parental strain 2013. Thus, thermotolerance, ethanol tolerance and ethanol production were improved by the genome shuffling technique compared to the parental strain 2013. The results of the present study also suggest that genome shuffling can rapidly improve the complex phenotypes of microorganisms while still maintaining their robust growth. Similar findings were also obtained by Shi et al. (2009) by obtaining isolates that can resist up to 55 °C, 25% (w v⁻¹) ethanol and produce 9.95% (w v⁻¹) ethanol.

**Ethanol Production at Elevated Temperature**

It was observed that F1D can produce ethanol up to 42 °C more efficiently than *S. cerevisiae* 2013 by 10.83%. Factorial ANOVA with 5% DMRT revealed a significant difference in ethanol yield of F1D among all other mutants, fusants and parental strain (Figure 5). Furthermore, F1D had significantly higher ethanol yield than the industrial strain *S. cerevisiae* HBY3 by 15.16%. Thus, F1D strain has the potential to replace parental strain by 10.83%. Factorial ANOVA with 5% DMRT revealed a significant difference in ethanol yield of F1D among all other mutants, fusants and parental strain (Figure 5). Furthermore, F1D had significantly higher ethanol yield than the industrial strain *S. cerevisiae* HBY3 by 15.16%. Thus, F1D strain has the potential to replace parental strain, 2013, had the lowest trehalose content (0.025 mg mL⁻¹), (Figure 6). Among the mutants and fusants, strain MA had the lowest trehalose content (0.0399 mg mL⁻¹). On the other hand, F1D had the highest trehalose content (0.0794 mg mL⁻¹) significantly higher than the parental strain by 217.6%.

It was also observed that as the ethanol tolerance of each improved strain increased from each generation, the trehalose content also increased. This observation conformed to the experiments performed by Sharma (1997) and Mobini-Dehkordi et al. (2008) that trehalose had a role in ethanol tolerance. Trehalose is a dimer of D-glucose with α-α bond, which affects tolerance of yeast to other stresses such as high osmotic, thermal and ethanol stress. It preserves the integrity of the biological membranes, stabilizes proteins in their native state and inhibits accumulation of denatured proteins during stress (Attfield 1987; Zhao and Bai 2009, Lucero et al. 2000).

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CONCLUSION

A method for genome shuffling which involved mutation and recursive protoplast fusion was used to create variants of Saccharomyces cerevisiae 2013 with improved thermostolerance, ethanol tolerance and ethanol production. With the use of increasing ethanol concentration as the selection pressure for each round of genome shuffling, the generated strains were phenotypically stable and amenable to further improvement. From the variants produced, F1D proved to be the most promising. It can grow at 42 °C in 18 % ethanol concentration and produce ethanol 10.83 % higher than the parental strain S. cerevisiae 2013. This strain must be further evaluated in large-scale conditions to assess the suitability for use in large ethanol distilleries in the Philippines. Further genetic characterization for these isolates using highly polymorphic molecular markers must be performed to prove genetic variation after genome shuffling.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Figure 4. Ethanol production of F1 shuffled strains, Saccharomyces cerevisiae 2013 and HBY3 in duplicates after 48 hours of incubation at room temperature (28-30°C) in 20% total sugar molasses medium [bars with same letters are not significantly different (P≤0.05) using Factorial ANOVA with 5% DMRT].

Figure 5. Ethanol production of Saccharomyces cerevisiae 2013 and its mutants and fusants in duplicates at 30°C and 42°C after 48 hours in 20% total sugar molasses medium [bars with the same letters are not significantly different (P≤0.05) using Factorial ANOVA with 5% DMRT].
CONTRIBUTIONS OF INDIVIDUAL AUTHORS

FLO conducted experiments and prepared the manuscript. SME conducted experiments as well. JFS conceptualized the study, did the experimental design, and edited the manuscript. VAA edited the manuscript. IGP conceptualized the study, did the experimental design, and prepared and edited the manuscript.

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Figure 6. Trehalose content of Saccharomyces cerevisiae 2013 and its mutants and fusants during stationary phase in duplicates [bars with same letters are not significantly different (P≤0.05) using Factorial ANOVA with 5% DMRT; labels above solid lines are concentration of ethanol that can be tolerated by these strains].

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Trehalose content (mg mL⁻¹)


