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A Thermostable *Candida molischiana* Mutant Capable of Ethanol Production at Elevated Temperatures

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The isolation of a mutant strain of *Candida molischiana* ATCC 2516 with increased thermotolerance compared to its parent strain was accomplished using nitrous acid mutagenesis and resistance to the glucose analogue 2-deoxy-D-glucose. Mutagenized ATCC 2516 cells were spread on a minimal medium containing raffinose and 2-deoxy-D-glucose with the resistant colonies being subsequently screened for thermotolerance at 45°C. A thermotolerant mutant strain was identified and its ability to utilize glucose at selected temperatures was compared to its parent strain. The mutant strain was capable of increased ethanol production using 20 g l^{-1} glucose as a carbon source at 35°C compared to the wild strain. The mutant strain also produced ethanol from glucose at 45°C while the parent strain did not. The isolation procedure involving 2-deoxy-D-glucose described in this study could provide a rapid method for the isolation of thermotolerant mutants from other yeast species with an increased ability to produce ethanol at higher temperatures.

Key words: Thermostable, Mutant, Glucose, Utilization, Ethanol fermentation, *Candida*.

If grain-based ethanol production is to be replaced by lignocellulosic ethanol production, strain improvement of ethanol-producing yeast species will be necessary. In particular, the use of ethanol-producing yeast species that are more nutritionally-versatile than *Saccharomyces cerevisiae* in utilizing cellulose degradation products for ethanol production is becoming a research focus. Further, it is also of interest to isolate mutants of such yeast species that exhibit an increase in thermotolerance since the processing of the lignocellulosic biomass during saccharification occurs at temperatures (45°C-50°C) much higher than most yeast species can tolerate. A yeast species that has engendered much interest is *Candida molischiana*. This yeast species has been shown in prior studies to utilize cyclodextrins to produce alcohol[1,2]. In addition, *C. molischiana* is one of the few yeast species capable of degrading cellobiose to glucose[2]. It has been determined that this yeast produces a beta-glucosidase that degrades cellobiose to glucose[3-5]. The ability of this yeast species to utilize cellulose degradation products for growth represents an advantage for lignocellulosic ethanol production since both glucose and cellobiose can be used as substrates for ethanol production by *C. molischiana*.

The objective of this work was to isolate a mutant strain of *C. molischiana* ATCC 2516 that exhibited greater thermotolerance than its parent strain. A protocol was devised to isolate such a mutant strain using chemical mutagenesis and resistance to a glucose analogue. The glucose analogue 2-deoxy-D-glucose was used because previous work has shown it affects the cell membrane of yeast[6-9]. Following the isolation of a mutant strain with increased thermotolerance, the

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strain was subsequently characterized for its ability to utilize glucose as well as its ability to produce ethanol from glucose. The mutant strain was found to have increased thermotolerance compared to its parent strain when grown on glucose. In addition, the mutant strain was capable of producing ethanol at 45°C unlike its parent strain.

MATERIALS AND METHODS

The yeast *Candida molischiana* ATCC 2516 was used during this investigation. YEPD medium contained 1% yeast extract, 2% peptone, and 2% glucose. Batch cultures (100 ml) in sterile 250 ml Erlenmeyer flasks were grown in a temperature controlled shaker (200 rpm) during this study. Glucose was added to the medium after sterilization. For solid medium, agar (2%) was added. To select the thermotolerant yeast mutants, a medium containing 0.17% yeast nitrogen base medium (with no amino acids or ammonium sulfate added), 0.5% ammonium sulfate, 2% raffinose and 0.1% 2-deoxy-D-glucose was used[10]. Raffinose was added following sterilization of the medium.

Using an overnight culture of *C. molischiana* ATCC 2516 grown in YEPD broth at 30°C, the cells were centrifuged at 10,005 x g at 25°C for 15 min. After washing the cells with sterile 0.85% NaCl and again being centrifuged at 10,005 x g at 25°C for 15 min, the cells were suspended in sterile 0.85% NaCl. An aliquot of cells (10^8 cells) was added to 0.1 M sodium acetate pH 4.5. After mixing, 3 mM sodium nitrite was added to the cell suspension[11]. The mutagenesis was performed for 240 min at 30°C. The mutagenized cells were collected, washed with 0.85% NaCl and resuspended in YEPD broth for 24 h at 30°C. Following outgrowth, an aliquot of cells was washed and suspended into sterile 0.85% NaCl. An aliquot of the suspension (about 10^7 cells) was spread onto solid medium plates. The solid medium contained 0.17% yeast nitrogen base (without amino acids or ammonium sulfate added), 0.5% ammonium sulfate, 2% raffinose, 0.1% 2-deoxyglucose and 2% agar. The plates were grown at 45°C for 24-48 hours. All colonies that grew at 45°C were selected and further analyzed. After screening potential mutants for growth in YEPD liquid cultures at 45°C, the most thermostable mutant was identified as strain MGW-13.

Both ATCC 2516 and MGW-13 were grown in cultures of YEPD medium (100 ml) in 250 ml Erlenmeyer flasks that were equipped with a rubber stopper and a needle with a filter on the top to allow for a microaerobic environment. To inoculate each culture, an inoculum (1%) of each strain grown for 24 h in YEPD was used. The cultures of each strain were shaken (200 rpm) at 30°C, 35°C, 40°C and 45°C. A sample (1 ml) was removed from each culture at selected time intervals to determine viable cell concentration using methylene blue staining and subsequent cell counting on a hemocytometer. The growth of ATCC 2516 and MGW-13 in YEPD liquid cultures at 45°C was also followed spectrophotometrically at 600 nm.

Samples (3 ml) were removed from each YEPD culture at 0, 48 and 72 h. Each sample was filtered using a 0.2 µm filter. The filtrate of each culture sample was placed in a vial for subsequent high performance liquid chromatographic (HPLC) analysis and stored in a freezer (-20°C) until all samples were collected. Once all the filtrates from the parent and mutant cultures had been sampled, the samples were analyzed for concentrations of glucose and ethanol using Waters HPLC unit equipped with a Waters 717 Plus autosampler and a Waters 2410 refractive index detector. After each sample (10 µl) was injected onto a heated 300 mm Aminex HPX-87H column (65°C), it was eluted from the column using a mobile phase of 5 mM sulfuric acid (flow rate of 0.6 ml min^{-1})[12]. All values represent the mean of three independent determinations involving three separate cultures. The Student's *t*-test was used during statistical analysis. All determinations were performed in triplicate.

RESULTS

Once a thermotolerant *C. molischiana* ATCC 2516 mutant was isolated, a comparison of cell growth of the parent strain and mutant strain in YEPD medium containing 2% glucose at 30°C, 35°C, 40°C and 45°C was undertaken. The generation times for ATCC 2516 at 30°C, 35°C and 40°C were 210 min, 225 min and 300 min, respectively. The generation times for the mutant strain on 2% glucose were the same at 30°C and 35°C (generation time 240 min) with both generation times being slower than ATCC 2516 at the same

temperatures. The generation time for the mutant strain (120 min) was more rapid than the generation time for ATCC 2516 on 2% glucose-containing YEPD medium at 40°C. At 45°C using glucose as a carbon source, the mutant strain grew rapidly (generation time 45 min) while its parent strain was able to grow for approximately a generation prior to cessation of cell growth (Fig. 1). The findings clearly indicated that mutant MGW-13 was more thermotolerant than its parent strain at 45°C.

Glucose utilization by the parent strain ATCC 2516 and the mutant strain were compared at 30°C, 35°C, 40°C and 45°C after 48 and 72 h. ATCC 2516 utilized glucose rapidly and was totally consumed from the YEPD medium at 30°C or 35°C after 48 h of growth (Fig. 2). Glucose utilization by ATCC 2516 was not complete at 40°C after 48 h of growth (Fig. 2) or 72 h of growth (Fig. 3). Very little glucose utilization by ATCC 2516 was observed after 48 h (Fig. 2) or 72 h (Fig. 3) at 45°C. The mutant strain exhibited a slightly different pattern of glucose utilization from the YEPD medium relative to its parent strain. Glucose consumption by the mutant strain was complete after 48 h at 35°C or 40°C (Fig. 2) and after 72 h (Fig. 3) at 30°C. Glucose utilization by the mutant strain compared to ATCC 2516 strain was significantly higher ($P<0.01$) at 45°C after 48 or 72 h of growth. Although glucose was

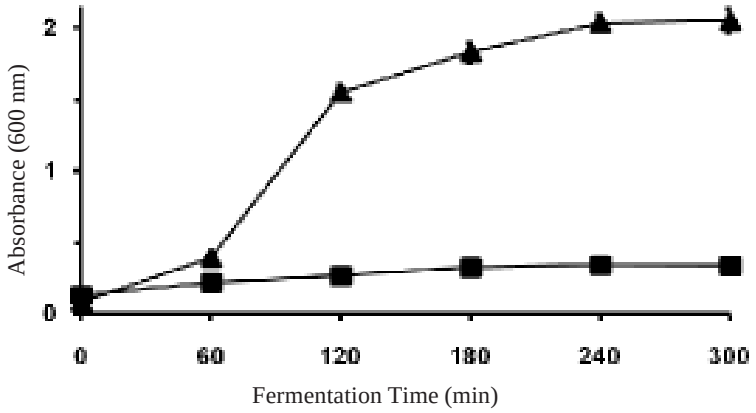

Fig. 1. Comparison of growth by *Candida molischiana* ATCC 2516 (■) and mutant strain MGW-13 (▲) on 2% glucose as a carbon source at 45°C by following the change in absorbance at 600 nm of the medium over a period of 300 min. The data represent the mean of three separate trials ± standard deviation (error bars)

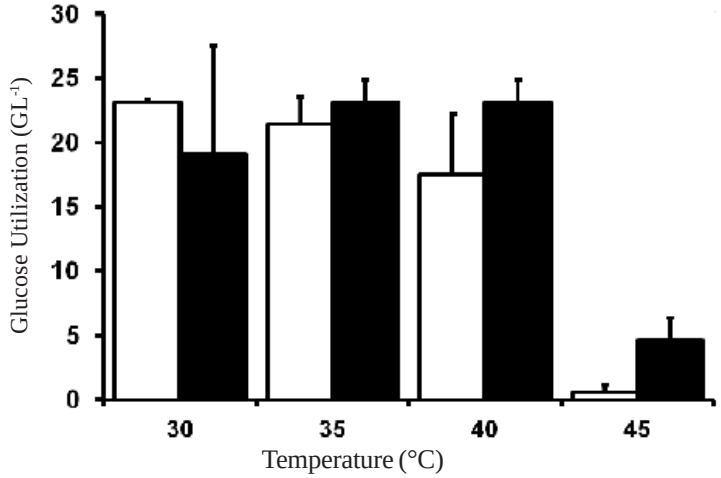

Fig. 2. Glucose utilization (g l^{-1}) by *Candida molischiana* ATCC 2516 (□) and mutant strain MGW-13 (■) using 2% glucose as a carbon source at 30°C, 35°C, 40°C and 45°C after 48 h of growth. The data represent the mean of three separate trials ± standard deviation (error bars)

utilized by the mutant strain at 45°C, approximately 83% of the 2% glucose in the medium remained after 72 h.

Ethanol production from the 2% glucose in the YEPD medium by ATCC 2516 and the mutant strain were next compared at 30°C, 35°C, 40°C and 45°C after 48 h of growth (Fig. 4) or 72 h of growth (Fig. 5). It was found that the mutant strain produced a 1.4-fold higher ethanol concentration than its parent strain after 72 h of growth (Fig. 5) on the glucose-containing medium at 30°C. At 35°C, the mutant strain produced a 1.6-fold or 1.4-fold higher ethanol concentration, respectively, than the parent strain did after growth on YEPD medium for 48 (Fig. 4) or 72 h (Fig. 5). The difference in ethanol production by the strains at 30°C or 35°C was statistically significant after 48 h ($P<0.01$) or 72 h ($P<0.05$). The mutant strain produced a 1.2-fold higher ethanol level than did ATCC 2516 after 48 h at 40°C (Fig. 4). The increased thermotolerance of the mutant strain improved ethanol production from glucose compared to ethanol production by the parent strain at 30°C, 35°C and 40°C. At 45°C, only the mutant strain produced low levels of ethanol from glucose. It is not clear why the thermotolerance of the mutant strain only improved ethanol production from glucose at the lower incubation temperatures tested relative to its parent

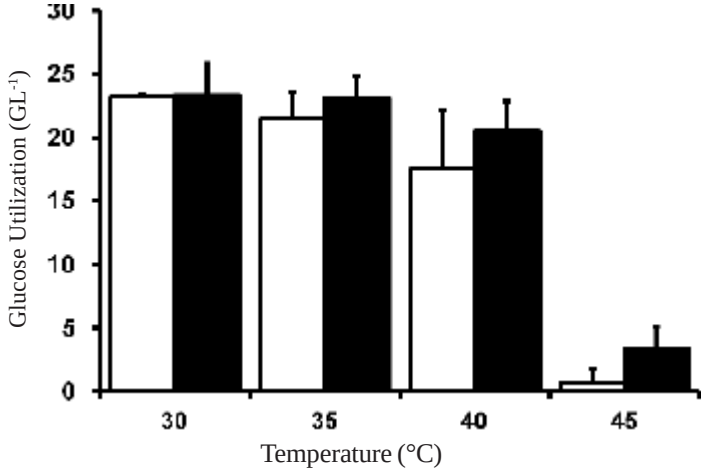

Fig. 3. Glucose utilization (g l^{-1}) by *Candida molischiana* ATCC 2516 (□) and mutant strain MGW-13 (■) using 2% glucose as a carbon source at 30°C, 35°C, 40°C and 45°C after 72 h of growth. The data represent the mean of three separate trials ± standard deviation (error bars)

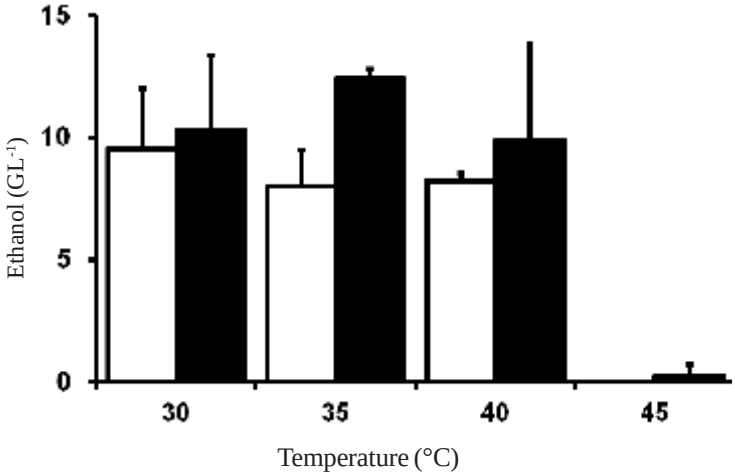

Fig. 4. Ethanol production (g l^{-1}) by *Candida molischiana* ATCC 2516 (□) and mutant strain MGW-13 (■) using 2% glucose as a carbon source at 30°C, 35°C, 40°C and 45°C after 48 h of growth. The data represent the mean of three separate trials ± standard deviation (error bars)

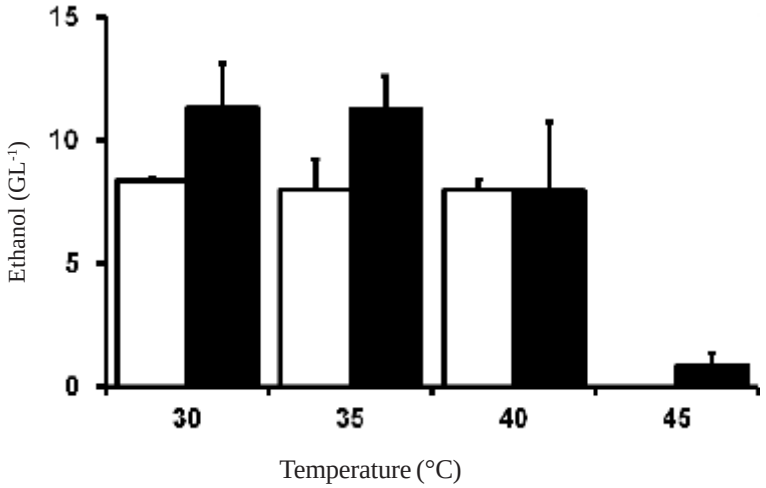

Fig. 5. Ethanol production (g l^{-1}) by *Candida molischiana* ATCC 2516 (□) and mutant strain MGW-13 (■) using 2% glucose as a carbon source at 30°C, 35°C, 40°C and 45°C after 72 h of growth. The data represent the mean of three separate trials ± standard deviation (error bars)

strain. The ethanol yield (g ethanol g^{-1} glucose) was determined to be 0.38, 0.37 and 0.40 for the parent strain at 30°C, 35°C and 40°C, respectively. The ethanol yield (g ethanol g^{-1} glucose) for the mutant strain was 0.49, 0.52, 0.47 and 0.03 at 30°C, 35°C, 40°C and 45°C, respectively. Relative to ethanol yield from glucose, the mutant strain was consistently higher than ATCC 2516.

DISCUSSION

It is known that at high temperatures changes in the fluidity of the *S. cerevisiae* cell membrane results in diminished fermentation ability[13]. The glucose analogue 2-deoxy-D-glucose was used in this work to isolate a thermotolerant mutant of *C. molischiana* because prior studies have shown that resistance to the analogue can result in the isolation of yeast and fungal mutants that have altered cell membrane properties[6-9,14,15]. It has been reported that a 2-deoxy-D-glucose resistant mutant of the yeast *Kluyveromyces marxianus* was capable of converting sucrose into ethanol[9]. Similarly, it has been previously reported that *S. cerevisiae* mutant strains resistant to 2-deoxy-D-glucose exhibited an increased fermentation ability to produce ethanol[10]. The increase in ethanol production by the strains in *S. cerevisiae* was attributed to a lack of catabolite repression by glucose[7]. It was also determined that mutants exhibited an increased uptake for the sugars glucose, maltose, sucrose and galactose[7]. By using 2-deoxy-D-glucose to select the *C. molischiana* mutants, it was thought the mutants isolated could have altered thermotolerance while maintaining fermentation efficiency.

The isolation of thermotolerant yeast strains have been reported previously. A mutant was isolated following ultraviolet mutagenesis of *S. cerevisiae* ATCC 26602 cells and subsequent extensive screening which produced a higher ethanol yield than its parent strain at 40°C when grown on a molasses-containing medium[16]. Progressive or thermal shock acclimatization has been used to isolate thermotolerant *S. cerevisiae* mutants[17]. The isolated mutants could grow and ferment glucose at 40°C but not at 45°C. At 40°C, the mutants produced ethanol for 24 h on lignocellulosic substrates at which time ethanol production ceased[17]. Another study reported the isolation of a thermotolerant mutant of *S. cerevisiae* using ultraviolet light mutagenesis[18]. Its growth rate and fermentation ability at 40°C was higher compared to its parent strain[18]. The advantage of the isolation procedure used in this study to isolate *C. molischiana* mutants is that mutants can be isolated directly from selection on solid medium rather than having to use successive cycles of progressive or thermal shock acclimatization for mutant selection or simply screening mutagenized colonies for thermotolerant mutants. The procedure was also advantageous

in that it allowed the rapid selection of a mutant strain that can utilize glucose to produce ethanol at 45°C.

Overall, a thermotolerant mutant strain of *C. molischiana* ATCC 2516 was isolated by chemical mutagenesis and by its resistance to the glucose analogue 2-deoxy-D-glucose. The mutant strain grew more rapidly on glucose at higher temperatures than did its parent strain. Ethanol production by the mutant strain was improved at lower temperatures compared to its parent strain.

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