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NOTE

Strategy for simultaneous saccharification and fermentation using a respiratory-deficient mutant of *Candida glabrata* for bioethanol production

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High temperature and agitation are advantageous for ethanol production from insoluble feedstock when using the simultaneous saccharification and fermentation (SSF) technique. To construct an effective SSF system, a respiratory-deficient mutant was isolated from the thermotolerant yeast *Candida glabrata*. Our results suggest that this respiratory-deficient mutant has higher ethanol production abilities in SSF.

[**Key words:** *Candida glabrata*; Bioethanol production; Thermotolerance; Respiratory-deficient mutant; SSF]

It has been shown that the simultaneous saccharification and fermentation (SSF) technique reduces the processing time, which in turn leads to increases in the production of ethanol (1). During the SSF process, in which liberated glucose is quickly converted to ethanol by fermenting microorganisms, the inhibition of cellulase by the reaction end-products is reduced and this single fermenter process eliminates a portion of the investment cost by reducing the number of fermenters required for start-up (2). In addition, the SSF process reduces the contamination risk due to the presence of ethanol in the medium (3). However, the performance of the SSF process is limited by the different optimum temperatures for enzymatic saccharification and microbial fermentation. The optimum temperature for cellulases is usually in the range of 40 to 50°C, while the optimum temperature of ethanol production for the most common ethanologenic yeast, *Saccharomyces cerevisiae*, is between 30 and 37°C. To overcome this notable discrepancy, thermotolerant yeast strains have been used in high temperature SSF processes (4, 5). In such cases, the performance of SSF with a thermotolerant yeast at temperatures closer to the optimum temperatures for cellulase activity could enhance saccharification, reduce the operation time and reduce cellulase dosage.

Agitation is considered an important factor in the SSF process due to the heterogeneous nature of the reaction mixture. Although excessive agitation leads to the inactivation of the cellulase, saccharification is enhanced by a relatively high agitation speed that results in efficient mass transport and adsorption of cellulase onto insoluble substrates (6). This agitation process also provides the yeast cells better access to sugar and nutrients. Therefore, agitation is an essential component to the SSF of insoluble cellulose materials. However, if excess aeration is applied to the medium by vigorous agitation under glucose-limited conditions, ethanol production may be suppressed by the increased aeration in a process known as the Pasteur effect (7). This drawback can be eliminated by using a respiratory-deficient mutant strain (petite mutant). Ethanol production using respiratory-deficient *S. cerevisiae* mutants has already been studied in detail (8, 9).

It is known that several yeast strains, such as *S. cerevisiae* and *Candida glabrata*, can produce ethanol even under aerobic conditions if there is a high concentration of glucose in the medium (Crabtree effect) (10, 11). In contrast, *Kluyveromyces marxianus*, a yeast known for its ability to produce ethanol at temperatures up to 50 °C, produces only a small amount of ethanol under aerobic conditions because it is a strictly Crabtree-negative yeast (11). In addition, the production of the petite mutant *K. marxianus* is difficult because the strain is a petite-negative yeast (12). In order to solve these problems, we propose the use of a respiratory-deficient mutant of the thermotolerant yeast *C. glabrata*, which is a petite-positive yeast (10). The use of this organism should improve ethanol production and allow for the use of any condition with respect to the temperature and the dissolved oxygen concentration in the fermentation media. We previously proposed that *C. glabrata* is suitable for bioethanol fermentation (13, 14). Because *C. glabrata* has higher stress tolerance to both acid and high temperatures in addition to possessing effective ethanol conversion capabilities, we believe this organism would be useful in the development of an effective fermentation process.

In the present report, we describe the possibility of designing an effective fuel ethanol production system by SSF using a respiratory-deficient mutant isolated from the thermotolerant yeast *C. glabrata* that is robust under high temperatures and aeration that is accompanied by agitation.

A strain of *C. glabrata* NFRI3164 (wild-type strain) and a derivative mutant isolated from *C. glabrata* NFRI3164 were used in

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this study while *S. cerevisiae* NBRC0224 was used as a control strain. The *C. glabrata* and *S. cerevisiae* strains were maintained on YPD agar plates (10 g/l yeast extract [Difco Laboratory, Detroit, MI, USA], 20 g/l peptone [Difco], 20 g/l glucose and 20 g/l agar). The *C. glabrata* respiratory-deficient mutants were obtained by exposure to ethidium bromide according to the following protocol. *C. glabrata* NFRI3164 was incubated on YPD agar plates containing ethidium bromide (5 μg/ml). After 3 days of incubation at 30°C, the respiratory-deficient mutants, which grew as small colonies, were selected. The ethanol production ability of the isolated respiratory-deficient mutant strains was determined and, based on these results, a respiratory-deficient mutant strain with a high ethanol production ability, designated as Cgrd1, was used for further analyses in the present study.

The respiratory deficiency was determined by growth on a solid synthetic media containing glycerol as the sole carbon source (1.7 g/l yeast nitrogen base lacking amino acids [Difco] and ammonium sulfate, 5.0 g/l $(NH_4)_2SO_4$ and 20 g/l glycerol) and by polymerase chain reaction (PCR) assays. The ethidium bromide-induced cytoplasmic respiratory-deficient mutants are defective in mitochondrial function and lack the ability to grow on non-fermentable sugar (15). The wild-type strain and Cgrd1 were examined by growth on glucose and glycerol agar. Cgrd1 showed a complete lack of growth on the glycerol-containing agar, while the wild-type strain was able to grow on both glucose and glycerol (Fig. 1A). These results suggested that Cgrd1 is a respiratory-deficient strain. To determine the status of the mitochondrial DNA in Cgrd1, a PCR assay was performed. The total DNA was extracted using a commercial kit (GenTLE-kun; TaKaRa Bio, Inc., Shiga, Japan) following the manufacturer's instructions. PCR was carried out using gene-specific primers and Taq DNA Polymerase (EX Taq; TaKaRa). The PCR primers for the mitochondrial DNA were designed for the large subunit of the ribosomal region in mtDNA (MtLrRNA) (16) and included ML7 (forward) 5′-GACCCTATG-CAGCTTCTACTG-3′ and ML8 (reverse) 5′-TTATCCCTAGCGTAACTTT-TATC-3′. As a control, amplification of the *C. glabrata ACT1* gene (*CgACT1*) encoded in the chromosome was performed using the primers A1Cg1 (forward) 5′-TGACATGGAAAAGATCTGGC-3′ and A1Cg2 (reverse) 5′-CCATCTGGCAATTCGTAGGA-3′. The *CgACT1* PCR primers were used in the amplification of the total DNA from both the wild-type strain and the Cgrd1 strain (lanes 1, 2) while the MtLrRNA PCR primers were used to amplify only from the wild-type template (lanes 3, 4) (Fig. 1B). No PCR products were amplified using the MtLrRNA primers when the total DNA isolated from Cgrd1 was used as a template. These results suggest that Cgrd1 partially or completely lacks mitochondrial DNA. Therefore, we conclude from these results that Cgrd1 is a respiratory deficient strain due mutation of its mtDNA.

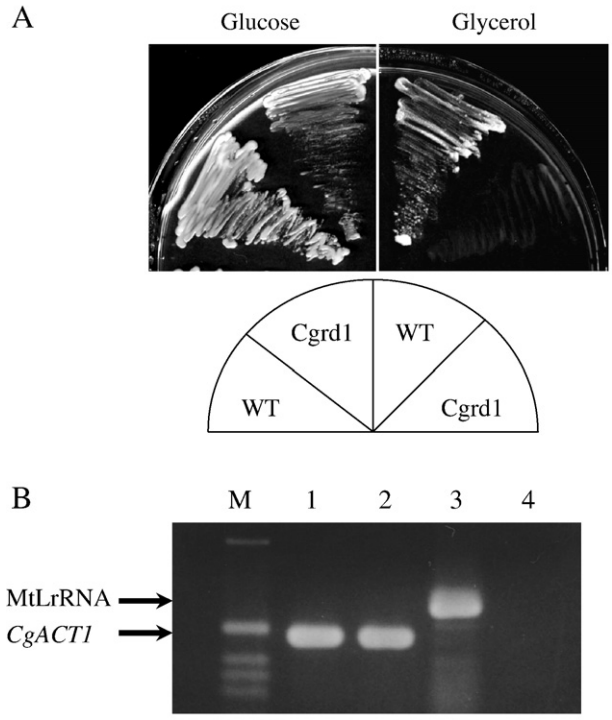

FIG. 1. (A) The growth phenotypes of the wild-type strain of *C. glabrata* NFRI3164 and the respiratory-deficient mutant Cgrd1 grown on glucose or glycerol at 30 °C (2 days). (B) The banding pattern of PCR products amplified from the total DNA of the wild-type strain and Cgrd1 using 2% agarose gel electrophoresis. Lanes 1 and 2, *CgACT1*; lanes 3 and 4, MtLrRNA.

To assess the influence of the mutation on the growth and ethanol production efficiency of Cgrd1, we measured time-dependent changes in the optical density (OD) at 600 nm (OD_{600}) in addition to the ethanol concentration in the YPD medium at 30°C and 42°C under aerobic conditions upon agitation at 150 rpm. Twenty-five milliliters of YPD liquid medium in a 100-ml cotton-plugged Erlenmeyer flask was inoculated using pre-culture grown in YPD medium at an initial OD_{600} of 0.1. Aliquots of the culture medium were subsequently removed at the indicated time points and the OD_{600} values were recorded. A second aliquot was analyzed for the ethanol and glucose concentrations using a high performance liquid chromatography (HPLC) system containing a refractive index detector (Prominence Series; Shimadzu, Kyoto, Japan) equipped with a fermentation monitoring column (Bio-Rad Laboratories, Hercules, CA, USA). The samples were eluted at a flow rate of 0.6 ml/min using 5 mM sulfuric acid as the mobile phase. The ethanol yield (g/g) was calculated as grams of ethanol produced per grams of total glucose consumed. In addition, the ethanol production abilities of the wild-type strain (*C. glabrata* NFRI3164) and of *S. cerevisiae* were examined. The results of these analyses are shown in Fig. 2A through D. After 12 h of incubation, the glucose was almost completely consumed in all cases with the exception of the *S. cerevisiae* experiment performed at 42 °C (data not shown). Cgrd1 had a slower growth rate than the wild-type strain, and the maximum cell density of Cgrd1 was approximately one-third of that measured for the wild-type strain (Fig. 2A and B). Although the slow growth rate is unfavorable for preparing seed cultures for ethanol fermentation, the growth rate and maximum cell density of Cgrd1 were higher than that of *S. cerevisiae* for the cultures incubated at 30°C during the first 12 h (Fig. 2A). The wild-type strain, Cgrd1 and *S. cerevisiae* strains produced 7.7 g/l of ethanol with a 0.39 ethanol yield (g-ethanol produced/g-glucose consumed), 8.6 g/l of ethanol with a 0.43 ethanol yield and 8.2 g/l of ethanol with a 0.41 ethanol yield, respectively, within 12 h at 30°C (Fig. 2C). At 42 °C, the wild-type strain and Cgrd1 produced 7.7 g/l of ethanol with a 0.45 ethanol yield and 8.6 g/l of ethanol with a 0.48 of ethanol yield, respectively, while within 12 h, *S. cerevisiae* produced only a small amount of ethanol (0.7 g/l) at 42 °C (Fig. 2D). These results suggest that Cgrd1 has almost the same fermentation ability as the wild-type strain at high temperatures. The maximum ethanol concentration in the Cgrd1 culture at both 30°C and 42°C was higher than that of its wild-type strain and remained relatively high until the end of the cultivation period (Fig. 2C and D). These observations suggest that the wild-type strain and the *S. cerevisiae* strain reassimilate the accumulated ethanol and continue to grow after glucose has been completely consumed. In contrast, Cgrd1 was unable to utilize ethanol due to its lack of mitochondrial function and consequent impairment of ethanol oxidation.

To assess the effects of Cgrd1 on ethanol production, SSF experiments were carried out in 100-ml cotton-plugged Erlenmeyer

flasks using 50 ml of medium consisting of 50 g/l Avicel PH101 microcrystalline cellulose (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 5 g/l yeast extract, 5 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4 and 0.5 g/l $MgSO_4 \cdot 7H_2O$ in a 50 ml citrate buffer (pH 5.0, 50 mM). The flasks were incubated at 30°C and 42 °C in the absence of shaking or on the rotary shaker at 150 rpm for 120 h. For the saccharification process, the cellulase enzyme complex from *Trichoderma reesei* (Accellerase 1500; Genencor Kyowa, Tokyo Japan) was added at 0.2 ml/g-Avicel. This enzyme has an activity, according to the manufacturer of the enzyme, of 440 to 560 CMC U/g-Avicel and 105 to 155 pNPG U/g-Avicel, respectively. For the fermentation process, inoculums were prepared from a YPD overnight culture wherein the cells were grown at 30°C in a rotary shaker (150 rpm) and were harvested by centrifugation prior to being washed twice with 0.9% NaCl. The washed cells were suspended at a cell concentration of approximately 0.5 g dry cell weight per liter. The ethanol content was determined by HPLC as described above, and the glucose concentration was measured with an enzyme-based biochemistry analyzer (YSI 2700 Select; YSI Japan, Ltd, Tokyo, Japan). The actual yield of ethanol was calculated based on the theoretical yield as described elsewhere (17), and the ethanol production rate (g/l h) was calculated as grams of ethanol produced per liter of medium per fermentation hour. The highest ethanol concentration was obtained when the SSF experiments were performed with Cgrd1 at 42 °C with shaking at 150 rpm (Fig. 3D). Under these conditions, Cgrd1 produced 17.0 g/l of ethanol within 48 h at 66.6% of its theoretical yield and with 0.35 g/l h productivity. The conditions involving agitation at higher temperatures increased the ethanol concentration for SSF with Cgrd1. This may be because 42°C is closer to the optimum temperature for the activity of both the cellulase enzyme and the thermotolerant characteristics of *C. glabrata*. Additionally, Cgrd1 was not susceptible to the Pasteur effect and the reassimilation of ethanol. The concentrations of glucose in the medium were low for all SSF experiments performed with Cgrd1 and its wild-type strain. However, during the last stage of the high temperature SSF experiment with Cgrd1, the glucose concentrations in the medium increased modestly.

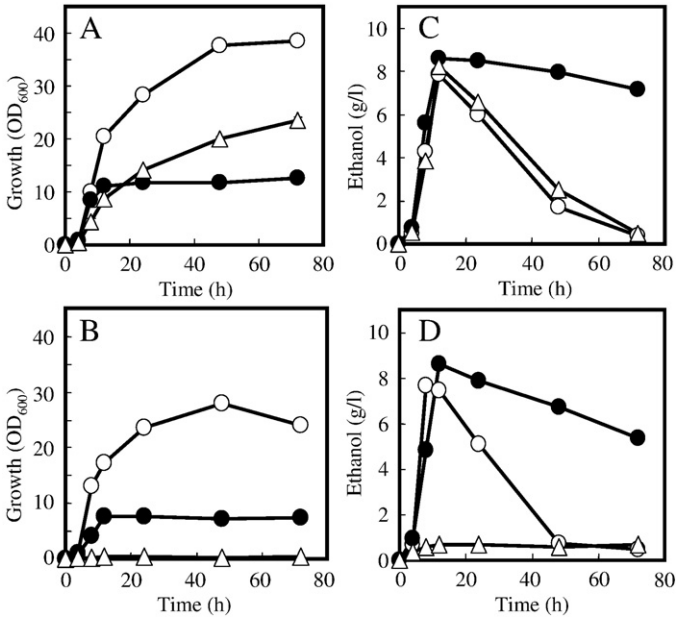

FIG. 2. The growth and ethanol production of the wild-type strain of *C. glabrata* NFRI3164 (open circles), the respiratory-deficient mutant Cgrd1 (closed circles) and *S. cerevisiae* (open triangles) in YPD medium. The time courses of cell growth at (A) 30 °C and (B) 42 °C and the ethanol production at (C) 30 °C and (D) 42 °C with an agitation rate of 150 rpm.

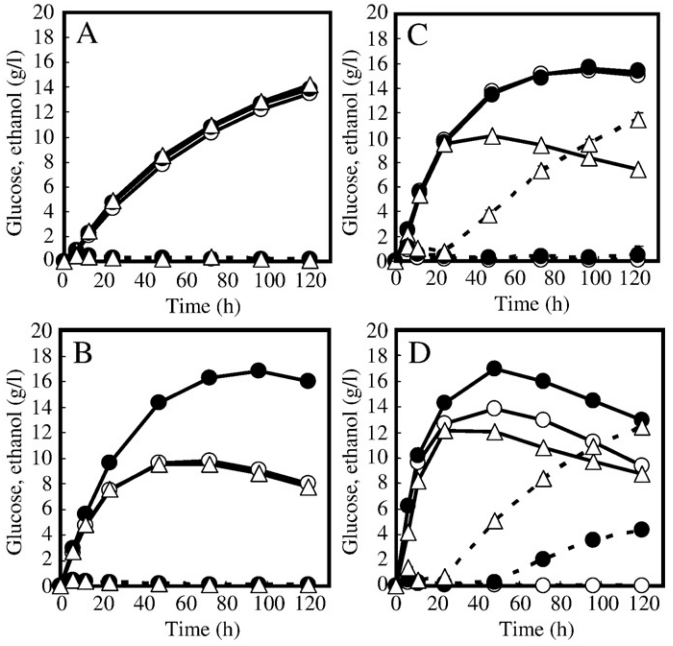

FIG. 3. Time course of ethanol (continuous line) and glucose (dotted line) production during the simultaneous saccharification and fermentation (SSF) experiments using the wild-type strain of *C. glabrata* NFRI3164 (open circles), the respiratory-deficient mutant Cgrd1 (closed circles) and *S. cerevisiae* (open triangles). SSF experiments were performed with 5% Avicel at 30 °C with agitation at (A) 0 rpm (static) or (B) 150 rpm and at 42 °C with agitation at (C) 0 rpm (static) or (D) 150 rpm. The wild-type strain of *C. glabrata* NFRI3164, the respiratory-deficient mutant Cgrd1 and *S. cerevisiae* NBRC0224 were cultivated in media for 120 h and the ethanol and glucose concentrations during SSF were monitored.

Gause and Kusovkova (18) reported that the respiratory-deficient mutant strains of *S. cerevisiae* were more thermosensitive under aerobic conditions than their parent strain. Likewise, it may be possible that Cgrd1 is more sensitive to high temperature than its wild-type strain. The maximum concentration of ethanol produced by *S. cerevisiae* was low and the glucose concentration increased throughout the experiments at 42°C (Fig. 3C and D) possibly due to its lower tolerance at the aforementioned temperature. In the case where SSF was performed at 30°C with an agitation rate of 150 rpm (Fig. 3B), the maximum yield of ethanol was reduced with the exception of Cgrd1. This decrease in the ethanol concentration is partially ascribed to the synergistic effect of the lower activity of the cellulase enzyme at 30°C and the aerobic respiration of the respiratory-competent yeast cells.

In conclusion, Cgrd1, a respiratory-deficient mutant of the thermotolerant yeast *C. glabrata*, was subjected to ethanol production by high-temperature SSF under aerobic conditions. The SSF of Cgrd1 at 42°C with an agitation speed of 150 rpm resulted in the highest ethanol yield observed from the experiments performed herein. Ethanol production by Cgrd1 increased as the temperature and the agitation rates were increased. Cgrd1 has some advantages over both its wild-type parental strain and *S. cerevisiae*. The Cgrd1 mutant, unlike *S. cerevisiae*, is not susceptible to the Pasteur effect at high temperatures; this mutant continues to produce ethanol without reassimilation due to its lack of respiration ability. Cgrd1 is able to produce ethanol effectively even if SSF is conducted at high temperatures under aerobic conditions.

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