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Isolation and Characterization of Ethanol-Producing *Schizosaccharomyces pombe* CHFY0201

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An ethanol-producing yeast strain, CHFY0201, was isolated from soil in South Korea using an enrichment technique in a yeast peptone dextrose medium supplemented with 5% (w/v) ethanol at 30°C. The phenotypic and physiological characteristics, as well as molecular phylogenetic analysis based on the D1/D2 domains of the large subunit (26S) rDNA gene and the internally transcribed spacer (ITS) 1+2 regions, suggested that the CHFY0201 was a novel strain of *Schizosaccharomyces pombe*. During shaking flask cultivation, the highest ethanol productivity and theoretical yield of *S. pombe* CHFY0201 in YPD media containing 9.5% total sugars were 0.59±0.01 g/l/h and 88.4±0.91%, respectively. Simultaneous saccharification and fermentation for ethanol production was carried out using liquefied cassava (*Manihot esculenta*) powder in a 5-l lab-scale jar fermenter at 32°C for 66 h with an agitation speed of 120 rpm. Under these conditions, *S. pombe* CHFY0201 yielded a final ethanol concentration of 72.1±0.27 g/l and a theoretical yield of 82.7±1.52% at a maximum ethanol productivity of 1.16± 0.07 g/l/h. These results suggest that *S. pombe* CHFY0201 is a potential producer for industrial bioethanol production.

Keywords: Ethanol fermentation, *Schizosaccharomyces pombe*, cassava (*Manihot esculenta*) powder, simultaneous saccharification and fermentation

With the wild fluctuation in the world market of conventional fossil fuels by the rapid growth of industries of many developing countries, there is a need for environment sustainable energy sources [2, 24]. Bioethanol is an attractive energy source to substitute for fossil fuel, and the increasing interest in fuel ethanol has resulted in strong demand for high yielding processes and easily accessible technology for the production of ethanol at reduced cost [19, 26]. Recently, the production of ethanol by microbial fermentation has received special attention because the world energy crisis has enhanced the interest in sustainable energy sources [30]. The economics of ethanol production by fermentation are significantly influenced by the cost of the raw materials, which accounts for more than half of the production cost [24]. For this reason, bioethanol production from renewable agricultural resources by several microorganisms (*e.g.*, *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Schizosaccharomyces pombe*, *Kluyveromyces marxianus*, and *Zymomonas mobilis*) has attracted considerable attention in recent years [8, 10, 15, 17, 21, 23]. More recently, there is growing interest in finding alternate bioresources for commercial ethanol production apart from sugar cane/beet molasses and starchy crops such as sweet sorghum, cassava (*Manihot esculenta*), and sweet potato [20, 27]. In particular, cassava powder is a cheap substrate that is easily available in tropical countries such as Southeast Asia and Africa [4, 5, 20]. There have been many reports of the potential applications of cassava powder and its hydrolysate as an alternative bioresource in simultaneous saccharification and fermentation [1, 22, 29]. On the other hand, the most important aspect of bioethanol fermentation is the ethanol yield, or more specifically the industrial yield [7]. The yield is dependent on many factors, such as the fermentative capacity of the cell population, the microbial activity, and the resistance of those industrial cells to stress conditions, particularly those of yeasts [7, 11, 26]. For several decades, *Schizosaccharomyces pombe* has proved an invaluable model organism to study biological ethanol fermentation

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processes [6, 10, 12]. Although many studies are needed to improve the strain and fermentation process to produce more ethanol, *S. pombe*, an efficient ethanol producer, has many advantages such as a well-studied genetic and physiological background, faster fermentation rates with the ability to grow under both aerobic and anaerobic conditions, flocculation ability, a high tolerance to ethanol and osmotic tolerance, ease of manipulation and safety for foods [10–13]. Hence, *S. pombe* has significant potential in environmentally friendly ethanol fermentation processes. Therefore, this novel ethanol-fermenting yeast requires isolation and study.

In the present study, we report on the screening and isolation for ethanol-resistant yeast cultures, which resulted in the isolation of a novel yeast strain, CHFY0201, capable of ethanol fermentation. The isolate was identified by its morphological and physiological characteristics, as well as by phylogenetic analysis of the internal transcribed spacer (ITS1+2) regions and the D1/D2 domains of the large-subunit (26S) rDNA coding gene. The analyses showed that both newly isolated yeasts are novel strains of *Schizosaccharomyces pombe*. The suitability of the cassava root for ethanol production, through enzymatic liquefaction and simultaneous saccharification and fermentation, was also investigated. The results show that *S. pombe* CHFY0201 is capable of ethanol production from cassava powder hydrolysate through batch fermentation. Therefore, this novel strain has potential industrial applications for bioethanol production.

Materials and Methods

Isolation and Screening of Yeast Strains for Ethanol Production

The yeasts were isolated from soil samples collected from Changhae ethanol factories and the Changhae Institute of Cassava and Ethanol Research in Jeon-ju (latitude 35°85'N, longitude 127°08'E), South Korea. Five g of a soil sample was added to 25 ml of deionized water, stirred intermittently for 1 h, and then allowed to stand for 0.5 h. Each sample was spread on a yeast peptone dextrose (YPD) (1% yeast extract, 2% peptone, 2% glucose, 2% agar) medium containing 50 mg of chloramphenicol to inhibit bacterial growth and incubated for 48 h at 30°C. The number of colony forming units (CFU) was recorded. A single colony of each isolate was grown on YPD agar plates and stored at 4°C. The ethanol tolerance of the yeast strains was selected based on their growth performance at 30°C in a YPD broth containing 5% ethanol for 24 h with constant shaking at 200 rpm. The successful cultures were collected and further screened for their ethanol production efficiency by flask cultivation.

The screening for high ethanol production was carried out at 30°C in 1,000-ml Erlenmeyer flasks containing 250 ml of a YPD (1% yeast extract, 2% peptone, and 9.5% glucose) medium with the pH adjusted to 4.5 with 10 N H_2SO_4. The inocula were prepared by transferring one loopful of a 24-h culture grown on a slant of YPD agar to an Erlenmeyer flask containing 50 ml of a YPD medium. After incubation on a rotary shaker at 30°C for 24 h, the inocula were transferred to the screening medium at a rate of 5%, followed by incubation for 68 h at 30°C in a rotary shaking incubator (Dasol DS310C2; Seoul, Korea).

Morphological and Physiological Characterizations of Selected Yeast Strains

The morphological and physiological characteristics were examined according to the methodology reported by Yarrow [32]. The cell morphology was examined by bright field microscopy (Nikon Eclipse 80i; Tokyo, Japan). The maximum growth temperature was determined in a YPD broth using metal block baths (ISOCAL-6; Isotech, Southport, England). The utilization of various carbon sources and other physiological characteristics were determined using a YT microplate (Biolog, Hayward, CA, U.S.A.) according to the manufacturer's instructions.

rDNA Sequencing and Molecular Phylogenetic Analysis

The DNA was extracted and purified using a Wizard Genomic DNA purification kit (Promega, Madison, WI, U.S.A.) using the standard protocol. The D1/D2 domains of the 26S rDNA, and the internal transcribed spacer regions 1 and 2 of the rDNA gene were sequenced directly from the PCR products generated using the primer pairs ITS5w (5'-GGA ASTA AAA GTC GTA ACA AT-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') [31]. The LSU D1/D2 gene was sequenced using the primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') [31]. The ITS1–5.8S rDNA gene–ITS2 region was sequenced using the primers ITS5w (5'-GGA ASTA AAA GTC GTA ACA AT-3') and ITS4p (5'-TCC TCC GCT TAT TGA TAT GC-3') [31]. The purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, U.S.A.) and an Applied Biosystems 310 automatic sequencer (Applied Biosystems). The 26S rDNA D1/D2 sequences were compiled using SeqMan software (DNASTAR, Madison, WI, U.S.A.). The D1/D2 and ITS sequences of the related taxa were retrieved from GenBank. The phylogenetic analyses were based on an analysis of 588 base pairs of D1/D2 and 462 base pairs of ITS sequences. Multiple alignments were performed using the program CLUSTAL_X [28]. The evolutionary distances were calculated using the Kimura's two-parameter model [14]. Phylogenetic analysis was performed using the neighbor-joining method [25] with the program MEGA3 [16] and bootstrap analysis based on 1,000 replicates [9].

Ethanol Fermentation in a Jar Fermenter

As a carbon source for ethanol production in a batch bioreactor, cassava powder hydrolysate medium was prepared by two-step enzymatic hydrolysis. For liquefaction, α-amylase (120 kU/g; Novozyme, Bagsvaerd, Denmark) from *Bacillus licheniformis* was added to 0.1% (w/w) of cassava powder, and the cassava mixtures were hydrolyzed at 95°C for 3 h with mild agitation (100 rpm). After, liquefaction, the pH was adjusted to 4.5. Glucoamylase (21 kU/g; Novozyme) was then added to the 0.18% (w/w) cassava mash, and the simultaneous saccharification and batch fermentation was conducted at 32°C for 66 h, with mild agitation (120 rpm). The batch fermentations were carried out in a 5-l jar fermenter (BIOFLO IIC, New Brunswick, NJ, USA) containing 3 l of cassava powder hydrolysate (*i.e.*, the total sugar concentration was ≈19.5%) medium. A seed culture was grown at 30°C for 24 h in a 500-ml flask

containing 150 ml of a YPD medium and then inoculated into a 5-l jar fermenter. During batch fermentation, the temperature was maintained at 32°C, and the agitation speed was 100 rpm. Samples were taken during the course of 66-h fermentation to monitor the ethanol and residual sugar concentrations, as well as the dry cell weight.

Assay Methods

The cell mass concentrations were determined from duplicate 10-ml samples, which were centrifuged for 10 min at 3,000 rpm (Hanil SUPRA 22 K; Seoul, Korea). The pellets were washed with deionized water, recentrifuged, dried at 75°C for 36 h, and weighted. The total sugar concentration in the cassava powder hydrolysate was diluted and analyzed by HPLC (Waters, Milford, NJ, U.S.A.). The column was a Rspack KC-811 (8×300 mm) column (Showa Denko, Tokyo, Japan) and the mobile phase was 0.25% (v/v) sulfuric acid and 99.75% DI water. The temperature was kept at 60°C with a flow rate of 1.0 ml/min, and the sample volume was 20 μl. The effluent from the column was monitored using a RI detector (Waters 2414; Millipore, Milford, NJ, U.S.A.). The ethanol concentration was analyzed by gas chromatography (Agilent 4890; Palo Alto, U.S.A. Column: Supelco 6.6% CARBOWAX 20M; detector: flame ionization detector; carrier gas: nitrogen; oven temperature: 100°C; injection temperature: 200°C, flame ionization detector temperature: 250°C; Agilent Chemstation Data Analysis System, Palo Alto, CA, U.S.A.), and isopropanol was used as the internal standard. The ethanol productivity, which is defined as grams of ethanol per liter per hour, was calculated from the final ethanol concentration. The ethanol conversion yield was calculated based on the initial sugar concentration and is reported as a percentage of the theoretical yield.

RESULTS AND DISCUSSION

Isolation of Yeast Strains and Ethanol Fermentation in Erlenmeyer Flask

From the soil samples, approximately 77 yeast strains were isolated from the previous selection scheme. In order to achieve efficient ethanol fermentation, it is necessary to use efficient yeast strains that can tolerate high ethanol concentrations. Therefore, 5% (w/v) ethanol was added to the YPD medium to obtain yeast strains that can tolerate high ethanol concentrations. Among these, 16 strains were screened for their ability to grow in a YPD broth containing 5% ethanol, and were then tested for their fermentation ability in an ethanol production medium containing 9.5% glucose. Although there was considerable ethanol production

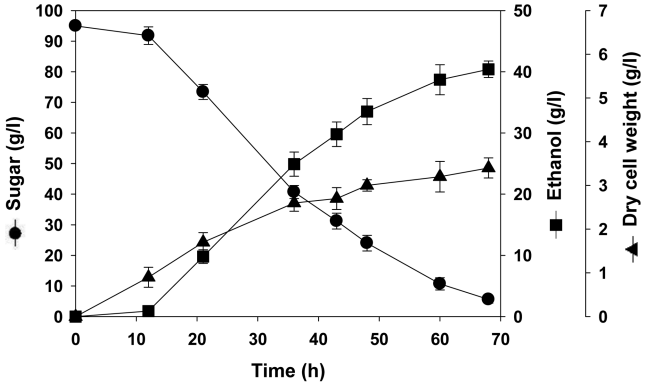

Fig. 1. Time-course profiles of ethanol production (square), sugar concentration (circle), and dry cell weight (triangle) during flask cultivation of CHFY0201.

observed for all the strains selected, the results of shaking flask cultivation were that one strain, namely CHFY0201, produced high ethanol concentrations at 30°C after 68 h fermentation, as shown in Fig. 1. At 30°C, strain CHFY0201 produced 4.04±0.14% (w/v) ethanol. As shown in Table 1 and Fig. 1, the highest productivity and the dry cell weight of CHFY0201 were 0.59±0.01 g/l/h and 3.40±0.23 g/l, respectively, in a medium containing 9.5% glucose. In addition, the percentage of the theoretical yield [*i.e.*, conversion rate (%) of sugar into ethanol] of CHF0201 was 88.4±0.91%. The remaining 15 strains produced ethanol at relatively lower concentrations ranging from 0.05–1.07% (w/v), at 30°C (data not shown). Therefore, the CHFY0201 strain was selected for further studies.

Morphological and Physiological Characteristics

On YPD agar media, the streak culture of the colonies of the isolate, CHFY0201, had a cream to tan and butyrous surface after 24 h at 30°C. Microscopically, the CHFY0201 strain formed yeast-like colonies that reproduced by splitting, and was 3.0–4.4 μm×6.4–13.4 μm in size. The colonies were observed as singles, pairs, or in groups (Fig. 2). Physiological characteristics of the CHFY0201 and comparisons with the other *S. pombe* strains are presented in Table 2. The CHFY0201 strain could ferment glucose. The maximum growth temperature of the CHFY0201 strain was 35°C, and the diazonium blue B reaction was negative. Interestingly, the CHFY0201 strain could be

Table 1. Ethanol production from CHFY0201 in a yeast peptone dextrose (YPD) medium by flask cultivation for 68 h at 30°C.

Yeast	Initial sugar concentration (g/l)	Final ethanol [% (w/v)]	Ethanol yield[a] (g/g)	Volumetric product productivity[b] (g/l/h)	Theoretical yield (%)
CHFY0201	~95	4.04±0.14	0.43±0.01	0.59±0.01	88.4±0.91

[a]Mass of ethanol formed per mass of total sugar consumed.
[b]Ethanol formed per liter of YPD medium per hour.

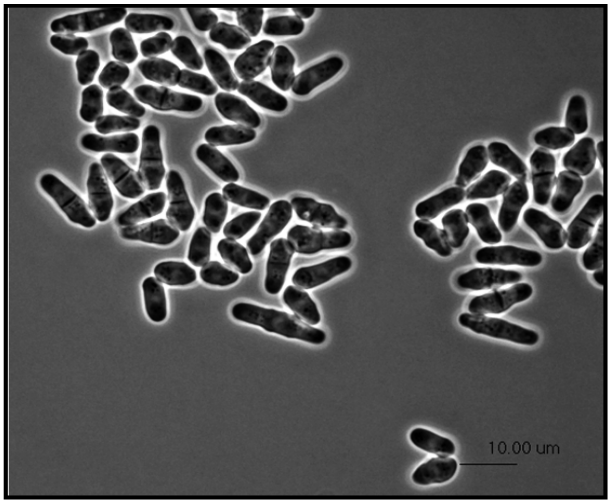

Fig. 2. Phase contrast light microscopy image showing the cell morphology of CHFY0201 when grown in a YPD broth for 1 day at 30°C. Bar, 10 μm.

differentiated from the other *S. pombe* strains because it was able to assimilate D-ribose. Since the CHFY0201 could not be identified as *S. pombe* based on its carbon compound assimilation, molecular taxonomic analysis was also investigated using the results of sequencing analysis of the D1/D2 domains of the large subunit (26S) rDNA and the ITS1+2 regions in the CHFY0201 strain.

rDNA Gene Sequence Analysis

The sequence of D1/D2 26S rDNA of the CHFY0201 strain was amplified by PCR using the genomic DNA of this strain as a template. The phylogenetic relationships obtained by comparing the 26S rDNA D1/D2 sequences of the CHFY0201 strain with the 26S rDNA D1/D2 sequences of the 12 members of Ascomycota. Some species of the Saccharomycetaceae were used as an outgroup (Fig. 3A). As a result, the nucleotide sequences of the DNA fragment of the 26S rDNA D1/D2 gene clearly demonstrate that strain CHFY0201 belongs to the genus *Schizosaccharomyces*, with the highest identity of 99.8% and 99.6% to the *S. pombe* strain 972 and *S. pombe* strain Y-12796, respectively. On the other hand, the ITS1+2 regions were characterized by a lesser degree of evolutionary conservation than the 26S rDNA gene, and they showed far greater interspecific differences. The phylogenetic relationships were obtained by comparing the ITS1+2 regions sequences of the CHFY0201 strain with the ITS1+2 regions sequences of 7 members of *Schizosaccharomyces* of the Schizosaccharomycetaceae. Some species of *Saccharomyces* of the Saccharomycetaceae were used as an outgroup. From the multiple sequence alignments, the sequence of the ITS1+2 regions of CHFY0201 was identified as *S. pombe*, and was phylogenetically closely related to several species of *S. pombe* on the ITS1+2 sequence tree, as

Table 2. Comparison of physiological characteristics of *Schizosaccharomyces pombe* CHFY0201 and reference strains of *Schizosaccharomyces pombe*.

	S. pombe CHFY0201	*S. pombe* strains[a]
Assimilation of carbon compounds		
α-D-Glucose	+	+
Sucrose	+	+
Maltose	+	+, –
Acetic acid	–	+, –
Propionic acid	–	–
D-Gluconic acid	+	+, –
Cellobiose	–	–
D-Melibiose	–	+, –
D-Raffinose	+	+
Xylitol	–	–
Glycerol	–	–
Succinate	–	–
2-Keto-D-gluconic acid	–	+, –
Inulin	–	+, –
D-Melezitose	–	–
D-Trehalose	–	–
N-Acetyl-D-glucosamine	–	–
D-Galactose	–	D, –
L-Rhamnose	–	–
L-Sorbose	–	–
α-Methyl D-glucoside	V	+, –
Arbutin	–	–
Salicin	–	–
D-Mannitol	–	–
D-Arabitol	–	–
i-Erythritol	–	–
L-Arabinose	–	–
D-Arabinose	–	–
D-Ribose	V	–
D-Xylose	–	–
Quinic acid	–	–
D-Glucuronic acid	–	–
α-D-Lactose	–	–
m-Inositol	–	–
Propane-1,2-diol	–	–
Growth at		
25°C	+	+
30°C	+	+
35°C	+	+
40°C	–	+, –
45°C	–	–

+, Positive; –, negative; V, variable; D, delayed positive.
[a][32].

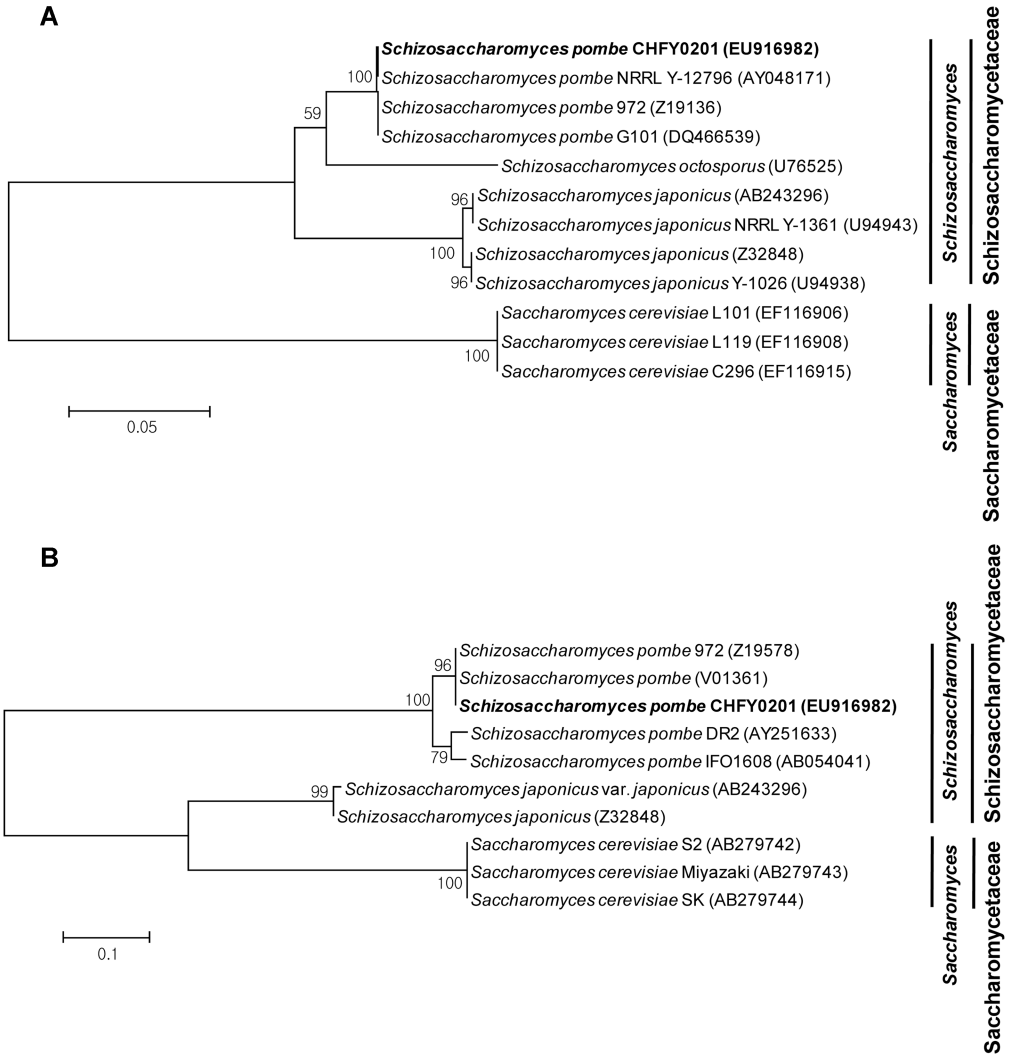

Fig. 3. Phylogenetic relationships of *Schizosaccharomyces* species and related taxa based on D1/D2 domains of the large subunit (26S) rDNA sequence (**A**) and the ITS1+2 regions (**B**).
The branching pattern was generated by the neighbor-joining method using Kimura's two-parameter model with the software package MEGA (Molecular Evolutionary Genetics Analysis) version 3.0 [16]. Percentage bootstrap values of 1,000 replicates are given; values greater than 40% are shown. The topology was rooted with some species of *Saccharomyces* of the Saccharomycetaceae. Bar, sequence divergence with Kimura two-parameter correction [25]. Sequences (partial 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and partial 26S rRNA gene) of *Schizosaccharomyces pombe* CHFY0201 were submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/) on July 22, 2008.

shown in Fig. 3B. Strain CHFY0201 was closely related to the *S. pombe* strain 972 (Fig. 3B) but differed from the latter species by 1 substitution in the ITS1+2 regions with 99.7% sequence homology. The overall ITS sequence similarity between *S. pombe* strains was more than 85%. Therefore, CHFY0201 was found to be a novel strain of *S.*

Table 3. Ethanol production by *Schizosaccharomyces pombe* CHFY0201 in a cassava powder hydrolysate medium by 5-l jar fermentation for 66 h at 32°C.

Yeast	Initial sugar concentration (g/l)	Final ethanol [% (w/v)]	Ethanol yield[a] (g/g)	Volumetric product productivity[b] (g/l/h)	Theoretical yield (%)
CHFY0201	~195	7.21±0.27	0.37±0.02	1.09±0.03	82.7±1.52

[a]Mass of ethanol formed per mass of total sugar consumed.
[b]Ethanol formed per liter of cassava powder hydrolysate medium per hour.

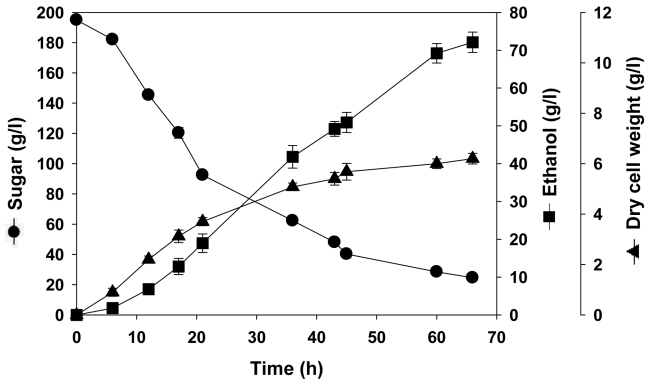

Fig. 4. Time-course profiles of ethanol production (square), sugar concentration (circle), and dry cell weight (triangle) during 5-l jar fermentation by CHFY0201.

pombe based on the morphological, physiological, and molecular data.

Ethanol Production by Batch Fermentation in Cassava Powder Hydrolysate Medium in a Jar Fermenter

An alternative renewable carbon source in industrial fermentation is a key requirement for effective ethanol production, because the utilization of a cheaper substrate such as cassava can make bioethanol more competitive with fossil fuel [10, 17, 18]. In this study, α-amylase and glucoamylase were used to pre-treat the cassava powder before fermentation, as described in Materials and Methods. After liquefaction, the cassava hydrolysate was used for simultaneous saccharification and fermentation using *Schizosaccharomyces pombe* CHFY0201. A 5% yeast inoculum was added and cultivated at 32°C for 66 h. As shown in Fig. 4 and Table 3, the *Schizosaccharomyces pombe* CHFY0201 produced 72.1±0.27 g/l ethanol at 66 h. From 3 l of cassava powder hydrolysate containing 585±3.7 g of sugars, 511±2.8 g of sugars was used by the *S. pombe* CHFY0201 to produce 216±2.1 g of ethanol, and the remaining 24.6±1.65 g of sugars was unutilized (Fig. 4 and Table 3). This gave an ethanol yield (*i.e.*, g ethanol/g sugar) of 0.37±0.02 g/g, a theoretical yield of 82.7±1.52%, and a productivity of 1.09±0.03 g/l/h. On the other hand, the dry cell weight and maximum ethanol productivity of CHFY0201 were 6.20±0.21 g/l and 1.16±0.07 g/l/h, respectively.

The results of this study demonstrated that the newly isolated *Schizosaccharomyces pombe* CHFY0201 could be efficiently employed for bioethanol production when cassava powder hydrolysate was used as a raw material. Further detailed studies for increasing the ethanol yield of the fed-batch and continuous fermentations from *Schizosaccharomyces pombe* CHFY0201 are currently being undertaken, and the results of these will be important for the development of new simultaneous saccharification and fermentation processes for efficient bioethanol production from cassava powder hydrolysate.

Refferences

1. Amutha, R. and P. Gunasekaran. 2001. Production of ethanol from liquefied cassava starch using co-immobilized cells of *Zymomonas mobilis* and *Saccharomyces diastaticus*. *J. Biosci. Bioeng.* **92:** 560–564.
2. Bai, F. W., W. A. Anderson, and Moo-Young M. 2008. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.* **26:** 89–105.
3. Barnett, J. A., R. W. Payne, and D. Yarrow. 2000. Yeast, pp. 678–679. *In* J. A. Barnett, R. W. Payne, and D. Yarrow (eds.). *Characteristics and Identification.* Cambridge University Press, U.K.
4. Brauman, A., S. Keleke, M. Malonga, E. Miambi, and F. Ampe. 1996. Microbiological and biochemical characterization of cassava retting, a traditional lactic acid fermentation for foo-foo (cassava flour) production. *Appl. Environ. Microbiol.* **62:** 2854–2858.
5. Cock, J. H. 1982. Cassava: A basic energy source in the tropics. *Science* **19:** 755–762.
6. Crichton, P. G., C. Affourtit, and A. L. Moore. 2007. Identification of a mitochondrial alcohol dehydrogenase in *Schizosaccharomyces pombe*: New insights into energy metabolism. *Biochem. J.* **401:** 459–464.
7. Da Silva Filho, E. A., H. F. de Melo, D. F. Antunes, S. K. dos Santos, R. A. do Monte, D. A. Simões, and M. A. de Morais Jr. 2005. Isolation by genetic and physiological characteristics of a fuel-ethanol fermentative *Saccharomyces cerevisiae* strain with potential for genetic manipulation. *J. Ind. Microbiol. Biotechnol.* **32:** 481–486.
8. Deshpande, V., H. Sivaraman, and M. Rao. 1983. Simultaneous saccharification and fermentation of cellulose to ethanol using *Penicillium funiculosum* cellulase and free or immobilized *Saccharomyces uvarum* cells. *Biotechnol. Bioeng.* **25:** 1679–1684.
9. Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.
10. Flores, C. L., C. Rodriguez, T. Petit, and C. Gancedo. 2000. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. *FEMS Microbiol. Rev.* **24:** 507–529.
11. Ge, X. M., X. Q. Zhao, and F. W. Bai. 2005. Online monitoring and characterization of flocculating yeast cell flocs during continuous ethanol fermentation. *Biotechnol. Bioeng.* **90:** 523–531.
12. Hu, C. K., F. W. Bai, and L. J. An. 2003. Enhancing ethanol tolerance of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* by Mg^{2+} *via* reduction in plasma membrane permeability. *Biotechnol. Lett.* **25:** 1191–1194.
13. Humberto de Queiroz, J., J. L. Uribelarrea, and A. Pareilleux. 1993. Estimation of the energetic biomass yield and efficiency of oxidative phosphorylation in cell-recycle cultures of *Schizosaccharomyces pombe*. *Appl. Microbiol. Biotechnol.* **39:** 609–614.
14. Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16:** 111–120.

15. Krishnan, M. S., F. Taylor, B. H. Davison, and N. P. Nghiem. 2000. Economic analysis of fuel ethanol production from corn starch using fluidized-bed bioreactors. *Bioresour. Technol.* **75:** 99–105.
16. Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief. Bioinform.* **5:** 150–163.
17. Limtong, S., C. Sringiewa, and W. Yongmanitchai. 2007. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour. Technol.* **98:** 3367–3374.
18. Lin, Y. and S. Tanaka. 2006. Ethanol fermentation from biomass resources: Current state and prospects. *Appl. Microbiol. Biotechnol.* **69:** 627–642.
19. Najafpour, G., H. Younesi, and Ku. I. K. Syahidah. 2004. Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. *Bioresour. Technol.* **92:** 251–260.
20. Nguyen, T. L., S. H. Gheewala, and S. Garivait. 2007. Full chain energy analysis of fuel ethanol from cassava in Thailand. *Environ. Sci. Technol.* **1:** 4135–4142.
21. Pan, Y. C. and W. C. Lee. 2005. Production of high-purity isomalto-oligosaccharides syrup by the enzymatic conversion of transglucosidase and fermentation of yeast cells. *Biotechnol. Bioeng.* **30:** 797–804.
22. Paolucci-Jeanjean, D., M. P. Belleville, N. Zakhia, and G. M. Rios. 2000. Kinetics of cassava starch hydrolysis with termamyl enzyme. *Biotechnol. Bioeng.* **68:** 71–77.
23. Rhee, S. K., G. M. Lee, C. H. Kim, Z. Abidin, and M. H. Han. 1986. Simultaneous sago starch hydrolysis and ethanol production by *Zymomonas mobilis* and glucoamylase. *Biotechnol. Bioeng. Symp.* **17:** 481–493.
24. Roble, N. D., J. C. Ogbonna, and H. Tanaka. 2003. A novel circulating loop bioreactor with cells immobilized in loofa (*Luffa cylindrica*) sponge for the bioconversion of raw cassava starch to ethanol. *Appl. Microbiol. Biotechnol.* **60:** 671–678.
25. Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
26. Sree, N. K., M. Sridhar, K. Suresh, I. M. Banat, and L. V. Rao. 2000. Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. *Bioresour. Technol.* **72:** 43–46.
27. Swain, M. R., S. Kar, A. K. Sahoo, and R. C. Ray. 2007. Ethanol fermentation of mahula (*Madhuca latifolia* L.) flowers using free and immobilized yeast *Saccharomyces cerevisiae*. *Microbiol. Res.* **162:** 93–98.
28. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25:** 4876–4882.
29. Ueda, S., C. T. Zenin, D. A. Monteiro, and Y. K. Park. 1981. Production of ethanol from raw cassava starch by a nonconventional fermentation method. *Biotechnol. Bioeng.* **23:** 291–299.
30. Ward, O. P. and A. Singh. 2002. Bioethanol technology: Developments and perspectives. *Adv. Appl. Microbiol.* **51:** 53–80.
31. White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.). *PCR Protocols: A Guide to Methods and Applications.* Academic Press, San Diego, U.S.A.
32. Yarrow, D. 1998. Methods for the isolation, maintenance and identification of yeasts, pp. 77–100. *In* C. P. Kurtzman, and J. W. Fell (eds.). *The Yeasts: A Taxonomic Study.* Elsevier Science BV, Amsterdam, The Netherlands.