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ORIGINAL RESEARCH PAPER

Direct utilization of purple sweet potato by sake yeasts to produce an anthocyanin-rich alcoholic beverage

Ja-Yeon Lee · Young-Kum Im · Hyun-Mi Ko · Jong-Eon Chin · Il-Chul Kim · Hwanghee Blaise Lee · Suk Bai

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Abstract
Objective To produce an alcoholic beverage containing anthocyanins that can act as antioxidants and have anticarcinogenic activities and antihypertensive effects.
Results High starch-assimilating sake yeast strain of *Saccharomyces cerevisiae* co-expressing the glucoamylase and α-amylase genes from *Debaryomyces occidentalis* using the double rDNA-integration system was developed. The new strain grew substantially using 5 % (w/v) purple sweet potato flour as the sole carbon source. Its cell yield reached 14.5 mg ml^{-1} after 3 days. This value was 2.4-fold higher than that of the parental wild-type strain. It produced 12 % (v/v) ethanol from 20 % (w/v) purple sweet potato flour and consumed 98 % of the starch content in purple sweet potato flour after 5 days of fermentation.
Conclusion We have produced a health-promoting alcoholic beverage abundant in anthocyanins from purple sweet potato.

Keywords Alcoholic beverage · Amylolytic sake yeasts · Anthocyanin · Purple sweet potato · *Saccharomyces cerevisiae* · Sake yeast · Starch

Introduction

Widely used raw materials for ethanol fermentation processes are mainly derived from various starch crops including rice, wheat, barley, potato, sweet potato, and cassava. Notably, purple sweet potato (*Ipomoea batatas* cultivar Ayamurasaki), a special type of sweet potato cultivar, not only contains starch and its partially hydrolyzed products, but also contains a high content of anthocyanin pigments in its roots (Ray et al. 2012). It is suitable for the production of a health-promoting alcoholic fermented beverage with good aroma and color (Saigusa et al. 2005). Anthocyanins possess biological functions such as antioxidant properties scavenging free radicals, antimutagenicity, and antihypertensive effects, and prevent carcinogenesis (Lila 2004). Furthermore, anthocyanins from purple sweet potatoes are more stable than those from other plants (Kano et al. 2005). *Saccharomyces cerevisiae,* including beer yeast, wine yeast and sake yeast, cannot metabolize starch naturally without

J.-Y. Lee · Y.-K. Im · I.-C. Kim · H. B. Lee · S. Bai (✉)
Department of Biological Sciences, College of Natural Sciences, Chonnam National University, Gwangju 500-757, South Korea
e-mail: sukbai@chonnam.ac.kr

H.-M. Ko
Department of Microbiology, College of Medicine, Seonam University, Namwon, Chunpook, South Korea

J.-E. Chin
Department of Cosmetology, Dongkang College University, Gwangju, South Korea

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liquefaction and saccharification processes by adding large amounts of exogenous commercial glucoamylase and α-amylase (Ray et al. 2012).

To alleviate these costly and complex processes, recombinant *S. cerevisiae* strains capable of expressing starch-hydrolyzing enzymes have been developed (Dohmen et al. 1990; Lin et al. 1998; Kim et al. 2010). However, the development of an efficient starch-fermenting *S. cerevisiae* strain that secretes amylolytic enzymes is still needed (Kim et al. 2011). Of the starch hydrolyzing enzymes tested to date, glucoamylase from the ascosporogenous yeast, *Debaryomyces occidentalis* (formerly *Schwanniomyces occidentalis*) is the only amyloglucosidase that exhibits both α-1,4-hydrolase activity and a significant α-1,6-debranching activity (Dohmen et al. 1990). A strain of *S. cerevisiae* that could produce both glucoamylase and α-amylase or a debranching enzyme would be more advantageous for the production of both fuel and potable ethanol (Eksteen et al. 2003).

In this study, we have developed high starch-assimilating sake yeasts of *S. cerevisiae* for the production of anthocyanin-rich alcoholic beverages using starch from purple sweet potato flour. The *D. occidentalis* glucoamylase gene (*GAM1*) was expressed in sake yeasts using the rDNA-integration system to obtain the starch assimilation ability. Subsequently, the α-amylase gene (*AMY*) from *D. occidentalis* (Kang et al. 2003) was co-expressed in the corresponding yeast expressing *GAM1*–encoded glucoamylase to increase the starch assimilation ability. We compared different sake yeast strains, expressing *GAM1* and/or *AMY,* for their amylolytic enzyme activities, starch assimilation, and improvement of ethanol production from purple sweet potato.

Materials and methods

Strains and plasmids

Escherichia coli DH5α was used for plasmid construction and transformation. The yeast strain used as a host for the yeast transformation experiment was a sake yeast strain, *Saccharomyces cerevisiae* KCTC 7904 from Korean Collection for Type Cultures (KCTC). YIpδAURSAδ plasmid (Kang et al. 2003) was used to clone the *AMY* gene. The YIpSGCU2rD plasmid (Park et al. 2014) served as the backbones of the double rDNA-integrative system.

Media and culture conditions

Saccharomyces cerevisiae was grown on YPD medium [1 % (w/v) yeast extract, 2 % (w/v) Bacto-peptone, and 2 % (w/v) glucose]. Yeast transformants were grown on YNBD plates [0.67 % yeast nitrogen base, 2 % (w/v) glucose, and 2 % (w/v) bacto-agar] containing 0.4 mM $CuSO_4$ (Park et al. 2014) and then transferred onto YPDS3 plates [YPD containing 3 % (w/v) soluble starch] and incubated for 4 days at 30 °C. Purple sweet potato was peeled and thinly cut followed by drying at 60 °C for 2 days. The dried chips were then ground to obtain the purple sweet potato flour. Yeast cells were grown in YPS medium [YP containing 2 % (w/v) soluble starch] or YPPS medium [YP to which purple sweet potato flour sterilized separately was added to final concentrations of 2–6 % (w/v)] at 30 °C for 5 days to assay the glucoamylase and α-amylase activities present in the culture supernatants. Residual starch was assayed via the starch-iodine reaction (Ghang et al. 2007). All experiments were conducted three to five times independently. The results are presented as the mean ± standard deviation of the mean (SD) of three different experiments.

DNA manipulation and yeast transformation

DNA manipulations and the *E. coli* transformation followed standard protocols. Integrative transformation of yeast was conducted by the method of Gietz et al. (1992).

Construction of double rDNA-integrative systems

To construct the double rDNA system containing the *AMY* gene, a 2.1 kb fragment harboring the *ADC1p* and *AMY* gene was excised from YIpδAURSAδ and inserted into the *Sph*I-*Mlu*I sites of YIpSGCU2rD lacking the *ADC1p* and *GAM1* gene, thereby generating the YIpSACU2rD (Fig. 1). On the other hand, to construct the double rDNA system containing the *GAM1* and *AMY* genes, the *ADC1p-AMY-CYC1*$_T$ cassette was amplified by polymerase chain reaction (PCR) using the oligonucleotides 5′-AAC*CTCGAG*G-CATGCAACTTCTTTTCTTTTTTT-3′ and 5′-AG

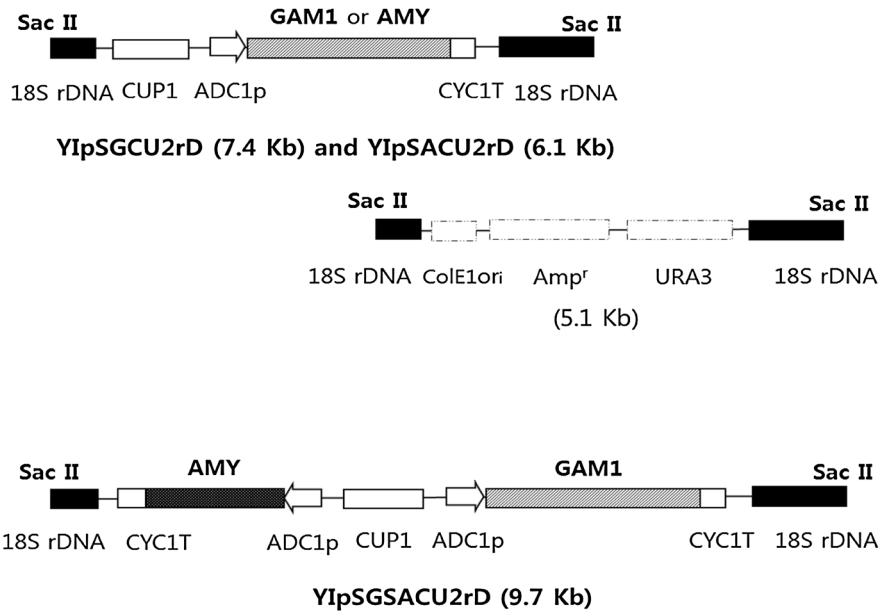

Fig. 1 Plasmid maps of YIpSGCU2rD, YIpSACU2rD, and YIpSGSACU2rD showing relative size, restriction sites, and location of the inserted DNA. Each rDNA-integration vector was linearized by digestion with *Sac*II. The bacterial vector DNA sequences, the ampicillin resistance gene and the ColE1 (pUC) origin, and the *URA3* gene (5.1 kb) were excised

G*TCGCGA*GGCCGCAAATTAAAGCC-3′. These primers were designed using the published nucleotide sequences of the *S. cerevisiae ADH1* gene (GenBank accession No. J01313) and pYES2 *CYC1 terminator* (Catalog No. V825-20, Invitrogen), respectively. To construct the double rDNA system containing the *GAM1* and *AMY* genes, a 2.3 kb amplified DNA fragment containing the entire *AMY* gene cassette of YIpδAURSAδ, which underwent digestion with *Xho*I and *Nru*I, was inserted using the same sites in YIpSGCU2rD to generate YIpSGSACU2rD (Fig. 1). rDNA-integration system, multi-copy integration method, is the most suitable method for overexpressing foreign genes in brewer's yeasts (Park et al. 2014).

Enzyme assays

Glucoamylase and α-amylase activities were quantified at pH 5.5 and 40 °C using the PGO/ODAD assay (Sigma) and dinitrosalicylic acid method, respectively (Ghang et al. 2007). All assays were repeated three times, and the means were calculated.

Ethanol fermentation and assay

The evaluation of ethanol production via fermentation was conducted using the method described by Ma et al. (2000). In brief, yeast cells were grown aerobically in 5 ml YPD or YPS medium at 30 °C with shaking at 200 rpm for 24 h. The culture was inoculated into 50 ml of 20 % (w/v) purple sweet potato flour in a 100 ml closed flask equipped with a bubbling CO_2 outlet, and was incubated at 30 °C for 7 days with agitation at 100 rpm. The supernatants were then assayed for ethanol content using the QuantiChrom ethanol assay kit (BioAssay Systems).

Results and discussion

Expression and secretion of glucoamylase and α-amylase in sake yeasts

YIpSGCU2rD and YIpSACU2rD were linearized by digesting the 18S rDNA sequences with *Sac*II (Fig. 1). The fragments (7.4 and 6.1 kb) harboring the *GAM1* gene cassette and the *AMY* gene cassette flanked by the 18S rDNA sequences were then integrated into the 18S rDNA sequences dispersed throughout the sake yeast genome to construct amylolytic sake yeasts (Lin et al. 1998; Nieto et al. 1999). The double rDNA system generates a smaller unneeded fragment (5.1 kb) that was eliminated before transformation (Fig. 1). The integrative cassette-harboring yeasts were designated KCTC 7904/YIpSGCU2rD (KCTC 7904SG) and KCTC 7904/YIpSACU2rD (KCTC 7904SA), respectively.

Table 1 Glucoamylase and α-amylase activities in cell-free supernatants from *S. cerevisiae* transformants

Yeast strains	Glucoamylase activity (U ml^{-1})[a]	α-Amylase activity (U ml^{-1})
S. cerevisiae KCTC 7904	ND	ND
KCTC 7904/YIpSGCU2rD (KCTC 7904SG)	0.35 ± 0.05[b]	ND
KCTC 7904/YIpSACU2rD (KCTC 7904SA)	ND	0.33 ± 0.1
KCTC 7904/YIpSGSACU2rD (KCTC 7904SGSA)	0.67 ± 0.06	7.45 ± 0.3

ND not detected

[a] Yeast cells were grown in the YPS medium at 30 °C for 3 days

[b] Values represent means of results from three independent experiments and were expressed in amylolytic activities present in the culture supernatants

As a dominant selection marker, the yeast *CUP1* gene was used in integrative systems for safe commercial beverage production (Park et al. 2014). In preliminary experiments (data not shown), sake yeast transformants expressing the *GAM1* or *AMY* gene (KCTC 7904SG or KCTC 7904SA) were grown in medium containing starch as the sole carbon source to test their capacities to hydrolyze and assimilate starch. KCTC 7904SG could utilize starch and its cell concentration reached a maximal value of 8.8 mg ml^{-1} after 3 days of growth in the medium containing 2 % (w/v) soluble starch, whereas KCTC 7904SA could not grow well.

In previous studies, most *S. cerevisiae* expressing only α-amylase gene could assimilate starch because they can assimilate maltose produced from starch digestion by α-amylase (Nieto et al. 1999; Marin et al. 2001; Kang et al. 2003). However, the ability of some sake yeast strains to assimilate and ferment maltose is known to be inferior to that of other *Saccharomyces* spp. (Baba et al. 1974). Accordingly, KCTC 7904SA displayed a relatively low growth rate (0.27 mg ml^{-1} day^{-1}) because the major starch hydrolysis products released by the *AMY*-encoded α-amylase were maltose and maltotriose (Ghang et al. 2007). In contrast, KCTC 7904SG efficiently degraded starch to glucose by using the *GAM1*-encoded glucoamylase (Dohmen et al. 1990).

To promote the starch assimilation ability of KCTC 7904SG, linearized YIpSGSACU2rD (9.7 kb) was integrated into the sake yeast genome, generating KCTC 7904/YIpSGSACU2rD expressing both *GAM1* and *AMY* genes (KCTC 7904SGSA). Kim et al. (2010) reported that *S. cerevisiae* expressing both *GAM1* and *AMY* genes for the production of bioethanol from starch was constructed by repeated transformations (δ-integration of *AMY* gene and rDNA-integration of *GAM1* gene) using antibiotic-resistance genes such as the G418 resistance gene as the dominant selection markers. In contrast, KCTC 7904SGSA for the production of safe alcoholic beverage from starch was constructed by one-step transformation (rDNA-integration of both *GAM1* and *AMY* genes) using the yeast-derived *CUP1* gene as the selection marker. Glucoamylase and α-amylase activities were examined in the culture supernatants from transformants grown in YPS medium. The clones with the highest level of activity were selected from the transformants and used for subsequent analyses.

As shown in Table 1, the glucoamylase activity exhibited by KCTC 7904SGSA was 1.9-fold higher than that of KCTC 7904SG. Moreover, KCTC 7904SGSA showed approximately 23-fold higher α-amylase activity than KCTC 7904SA. The improved enzyme activities in KCTC 7904SGSA are positively correlated with its high starch assimilation and growth rate, due to the efficient degradation of starch to glucose by the cooperative interaction between glucoamylase and α-amylase. In contrast, KCTC 7904SA secreting only α-amylase showed a low level of starch assimilation and growth, which lead to a very low enzyme activity (Kang et al. 2003). Time course analyses of the enzyme activities, cell growth, and starch hydrolysis for KCTC 7904SGSA over 5 days were compared with those of KCTC 7904SG and KCTC 7904SA to examine the improvement of the starch assimilation capability (Fig. 2). KCTC 7904SGSA utilized 100 % of the starch in the culture medium containing 2 % (w/v) starch during 2 days of growth, whereas KCTC 7904SG utilized 85 % of the starch and KCTC 7904SA did not hydrolyze the starch well in the corresponding culture medium, indicating that glucoamylase and α-amylase acted synergistically (Kim et al. 2010).

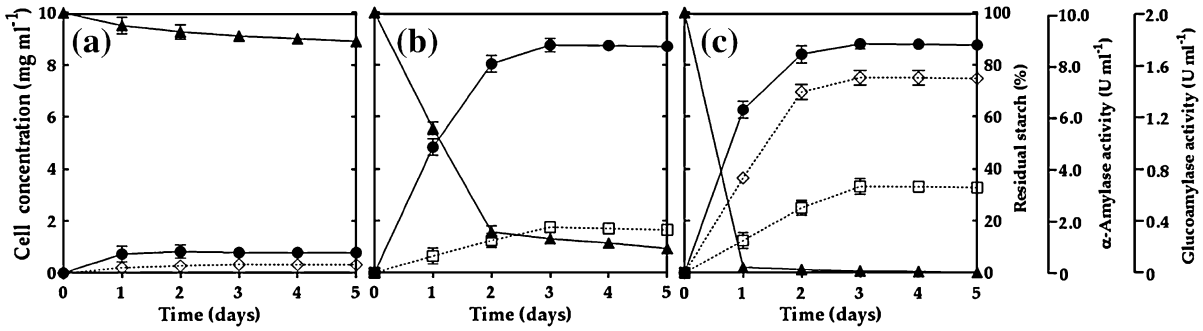

Fig. 2 Time courses of the growth, starch degradation, and extracellular glucoamylase and α-amylase activities produced by KCTC 7904SA (**a**), KCTC 7904SG (**b**), and KCTC 7904SGSA (**c**) at 30 °C in YPS medium. Growth was measured on different days based on cell dry weight. Glucoamylase and α-amylase activities were measured in culture supernatants. The remaining starch results are presented as percentages, considering the soluble starch in the uninoculated medium as 100 %; mg ml^{-1} cell concentration (*circles*); U ml^{-1} glucoamylase activities (*squares*); U ml^{-1} α-amylase activities (*diamonds*); % residual starch (*triangles*). Data (mean ± SD) are from three independent experiments performed in triplicate

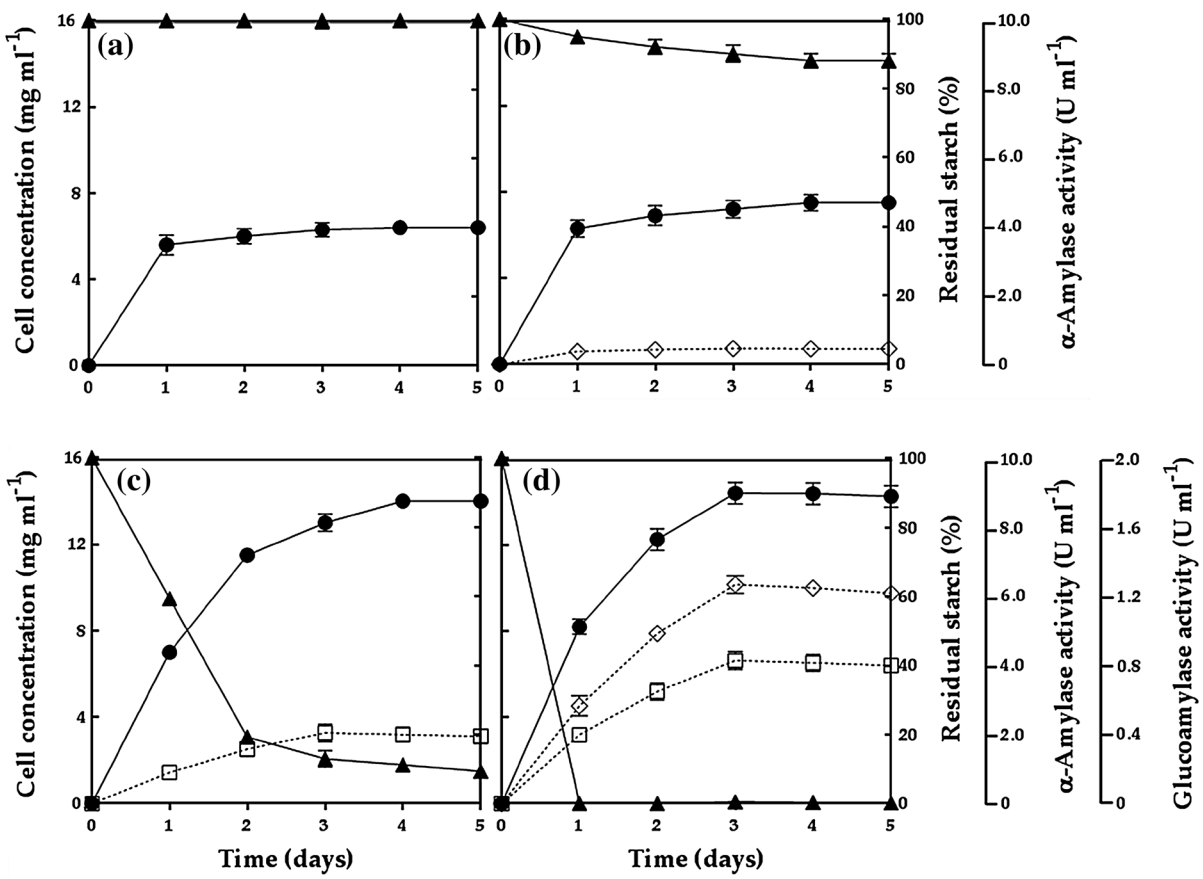

Fig. 3 Growth curves, time courses of starch hydrolysis, and extracellular glucoamylase and α-amylase activities produced by *S. cerevisiae* KCTC 7904 (**a**), KCTC 7904SA (**b**), KCTC 7904SG (**c**), and KCTC 7904SGSA (**d**) at 30 °C in YPPS medium containing 5 % (w/v) purple sweet potato flour. Growth was measured on different days based on cell dry weight. Enzyme activities were measured in culture supernatants. The remaining starch results were presented as percentages considering the starch in the uninoculated medium as 100 %; mg dry cell wt ml^{-1} (*circles*); glucoamylase activities, U ml^{-1} (*squares*); α-amylase activities U ml^{-1} (*diamonds*); % residual starch (*triangles*). Data (mean ± SD) are from three independent experiments performed in triplicate

Assimilation and fermentation of purple sweet potato flour by amylolytic sake yeasts

To determine which concentration was the most appropriate for a high level of expression and secretion of glucoamylase and α-amylase and cell growth, various concentrations of purple sweet potato flour were tested. More than 4 % (w/v) purple sweet potato flour gave the most promising results and 5 % (w/v) concentration was chosen for further investigations (data not shown). As shown in Fig. 3, sake yeast transformants expressing *GAM1* and/or *AMY* genes were grown in medium containing 5 % (w/v) purple sweet potato flour as the sole carbon source. The parental wild-type strain (*S. cerevisiae* KCTC 7904) and KCTC 7904SA displayed moderate growth because they could only utilize glucose and limited maltose in purple sweet potato flour. In contrast, KCTC 7904SG and KCTC 7904SGSA exhibited more extensive growth, and their cell concentrations after 3 days of growth were 13.2 and 14.5 mg ml^{-1}, respectively.

This observation indicated that they consumed the available starch in purple sweet potato flour besides utilizing glucose and maltose. KCTC 7904SG, KCTC 7904SA, and KCTC 7904SGSA glucoamylase and α-amylase activities as well as starch hydrolysis from purple sweet potato flour over 5 days were compared. KCTC 7904SGSA displayed a more significant hydrolysis of the starch and a twofold higher glucoamylase activity than that of KCTC 7904SG. This phenomenon may be due to the co-expression of *GAM1* and *AMY* genes in KCTC 7904SGSA (Eksteen et al. 2003; Kim et al. 2010).

KCTC 7904SGSA and the parental wild-type strain were used for direct ethanol fermentation from 20 % (w/v) purple sweet potato flour under anaerobic conditions (Fig. 4). As expected, the wild-type strain generated 6.3 % (v/v) ethanol after 2 days of fermentation, reaching a maximal value of 6.8 % (v/v) after 3 days of fermentation with no decrease in starch content in purple sweet potato flour. KCTC 7904SGSA directly fermented starch to ethanol. After 2 days of fermentation, 8.2 % (v/v) ethanol was produced and the maximal concentration of 12 % (v/v) ethanol was attained after 5 days. Up to 98 % of the starch content in purple sweet potato flour was consumed during the same period, which led to an increased ethanol productivity. Previously, alcoholic fermented beverages produced from purple sweet potato that contained large amount of anthocyanins and had a deep red color were developed by pretreating purple sweet potato with commercial glucoamylase from *Aspergillus niger* or both glucoamylase from *A. niger* and α-amylase from *Bacillus licheniformis* for saccharification and liquefaction processes (Saigusa et al. 2005; Ray et al. 2012).

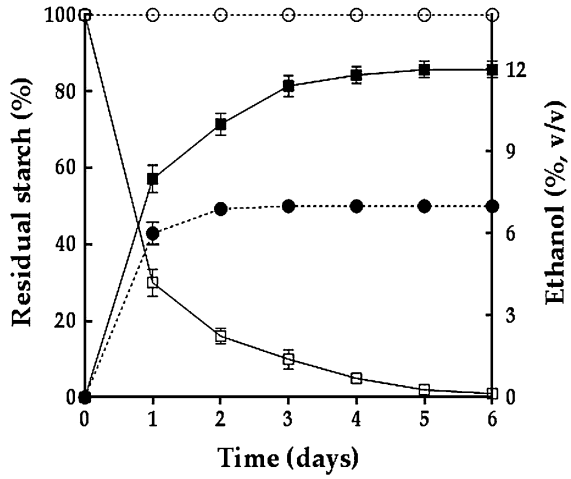

Fig. 4 Time courses of direct ethanol production via fermentation of 20 % (w/v) purple sweet potato flour by *S. cerevisiae* KCTC 7904 (*circles*) and KCTC 7904SGSA (*squares*). *Open* and *filled symbols* indicate relative residual starch content and ethanol concentration, respectively. Data (mean ± SD) are from three independent experiments

In the present study, a starch-fermenting sake yeast strain (KCTC 7904SGSA) was constructed to achieve direct liquefaction, saccharification, and fermentation for a one-step conversion of starch from purple sweet potatoes, which can provide a cheaper alternative to large quantities of commercial enzymes and simplify starch fermentation processes (Kim et al. 2011). Further studies are in progress to construct integrants displaying increased amylase activities for efficient fermentation. This study can lead to the development of various amylolytic sake yeasts, wine yeasts, and beer yeasts using the constructed integrative systems.

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