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Direct and efficient ethanol production from high-yielding rice using a *Saccharomyces cerevisiae* strain that express amylases

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ABSTRACT

Efficient ethanol producing yeast *Saccharomyces cerevisiae* cannot produce ethanol from raw starch directly. Thus the conventional ethanol production required expensive and complex process. In this study, we developed a direct and efficient ethanol production process from high-yielding rice harvested in Japan by using amylase expressing yeast without any pretreatment or addition of enzymes or nutrients. Ethanol productivity from high-yielding brown rice (1.1 g/L/h) was about 5-fold higher than that obtained from purified raw corn starch (0.2 g/L/h) when nutrients were added. Using an inoculum volume equivalent to 10% of the fermentation volume without any nutrient supplementation resulted in ethanol productivity and yield reaching 1.2 g/L/h and 101%, respectively, in a 24-h period. High-yielding rice was demonstrated to be a suitable feedstock for bioethanol production. In addition, our polyploid amylase-expressing yeast was sufficiently robust to produce ethanol efficiently from real biomass. This is first report of direct ethanol production on real biomass using an amylase-expressing yeast strain without any pretreatment or commercial enzyme addition.

1. Introduction

The finite nature of fossil fuels combined with the environmental problems associated with their extraction and combustion, such as global warming and acid rain, has promoted an interest in biofuel production. Ethanol can be produced from sucrose and from starchy or lignocellulosic biomass [1], and numerous studies on ethanol production from lignocellulosic biomass have recently been conducted. The attraction of these materials for fuel production is that they are abundant and cheaper than sucrose or starchy biomass [1–3]. However, there are numerous limitations associated with using lignocellulosic biomass to produce ethanol, such as the slow rate of enzymatic degradation and high cost of enzymes [1]. As a consequence, starch biomass is still the most commonly used feedstock for ethanol production.

Practical ethanol production from starchy biomass such as cassava, rice, sweet sorghum, and sweet potato have been reported [4–7]; of these, corn is the most commonly used starchy feedstock for bioethanol production [1]. In Japan, agricultural policies adopted by the government have resulted in the existence of extensive areas of unutilized rice fields. This has resulted in numerous initiatives being launched to produce ethanol using high-yielding rice that has been cultivated in these underutilized paddies [8]. Thus, in addition to developing ethanol production processes using commonly used starchy feedstocks such as corn, it is also important to develop cost effective and efficient processes using alternative starchy feedstocks like high-yielding rice which are common in the region.

Despite its efficiency as an ethanol producer, *Saccharomyces cerevisiae* cannot produce ethanol from raw starch directly because it lacks the ability to degrade raw starch into glucose. This is because conventional ethanol production from raw starch requires the following three steps: liquefaction of starch by heating and addition of α-amylase, enzymatic saccharification of the low-molecular liquefaction products to glucose, and fermentation of glucose to ethanol. The liquefaction process, which accounts for 30–40% of the total energy used for ethanol production, combined with the large quantities of enzymes that are required to convert the raw starch into glucose [5,9], both contribute to making conventional ethanol production an expensive and complex process. However, co-utilization of commercial enzymes and/or microorganisms and use of yeast capable of degrading raw starch can be used to reduce the costs of ethanol production from raw starch [4–7,10]. We previously constructed a high-performance, starch-degrading yeast capable of direct ethanol production from purified raw corn starch by com-

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bining δ-integration and polyploidization with high ethanol yield [11]. The polyploid characteristics of this yeast strain meant that it is an efficient ethanol producer as well as being robust in culture. In this study, we attempted to produce ethanol from high-yielding rice harvested in Japan. To our knowledge, this would be first report describing direct ethanol production from real biomass using an amylase-expressing yeast. Importantly, the low-cost and efficient ethanol production process described here was performed without supplementing the growth media with nutrients such as yeast extract or peptone.

2. Materials and methods

2.1. Yeast strains and media

The α-amylase and glucoamylase expressing tetraploid strain of *S. cerevisiae*, MNIV/δGS, was used for ethanol production [11]. The yeast was aerobically cultured in YPS medium (10 g/L of yeast extract (Nacalai Tesque, Kyoto, Japan), 20 g/L of Bacto-peptone (Difco Laboratories, Detroit, MI, USA), 50 g/L of soluble starch (Nacalai Tesque, Kyoto, Japan)). The control yeast strain, MT8-1, which is unable to synthesize amylase, has also been used to produce ethanol [12]. The control yeast strain was also aerobically cultured in YPD medium (10 g/L of yeast extract, 20 g/L of Bacto-peptone, 20 g/L of glucose (Nacalai Tesque)). Ethanol fermentation was performed using two kinds of medium, one containing a carbon source that was supplemented with nutrients (100 g/L of carbon source, such as purified raw corn starch and high-yielding rice, 10 g/L of yeast extract, 20 g/L of Bacto-peptone), and the other medium containing only a carbon source without any nutrients (200 g/L of high-yielding brown rice).

2.2. High-yielding rice

High-yielding rice was harvested in Hyogo prefecture, Japan. The rice was either left in its harvested state (brown rice) or polished (white rice) before being ground to produce flour with a particle size of approximately 0.5 mm using a laboratory disintegrator (Sansho Industry Co., Ltd., Osaka, Japan) for use as carbon sources for ethanol fermentation. According to the analysis of the Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries (Hyogo, Japan), the high-yielding brown rice was composed of 12.8% water, 75.3% carbohydrate, 8.3% protein, 2.3% lipid, and 1.3% ash.

2.3. Ethanol fermentation from purified raw corn starch or high-yielding rice

Yeast cells were cultured aerobically in YPS medium at 30 °C for 72 h. Yeast cells were harvested by centrifugation at 3000 × *g* for 5 min, washed twice with water, and re-suspended in 50 mL of medium containing the carbon source supplemented with nutrients. Raw corn starch or high-yielding rice was used as a carbon source. The initial cell concentration was adjusted to 50 g of wet cells/L, and wet cell weight was determined by weighing cells that had been pelleted by centrifuging at 3000 × *g* for 5 min. The estimated dry cell weight was approximately 0.15-fold the wet cell weight (data not shown).

For the low-cost fermentation, yeast cells (initial O.D.600 = 0.05) were also cultured aerobically in YPS medium at 30 °C for 72 h. The culture broth was inoculated into the fermentation medium containing only a carbon source. The inoculated volume ranged between 5, 10, 20% of the volume of the fermentation medium. The total volume of the fermentation medium was adjusted to same volume in each inoculum size.

Ethanol fermentation proceeded in a working volume of 50 mL incubated at 30 °C with mild agitation in 100 mL bottles equipped with a bubbling CO_2 outlet, or in a working volume of 1 L agitated at 500 rpm in 2 L jar fermenters.

2.4. Analytical methods

Ethanol concentration was determined by high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a Shim-pack SPR-Pb column (Shimadzu, Kyoto, Japan). The operating conditions were 80 °C with a water mobile phase and flow rate of 0.6 mL/min. The ethanol concentration was determined using a refractive index detector (Shimadzu RID-10A). Culture supernatant was separated from the fermentation broth by centrifugation at 14,000 × *g* for 10 min and then analyzed by HPLC. The activities of the glucoamylase and α-amylase in the fermentation broth were assayed as described previously using kits (Kikkoman Corp., Chiba, Japan), respectively [13].

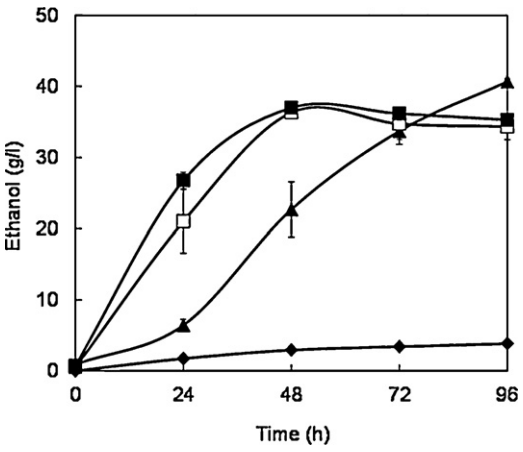

Fig. 1. Ethanol production from fermentation of various carbon sources supplemented with nutrients. Raw corn starch (closed triangles), polished white rice (open squires), brown rice (closed squares), and brown rice fermented with the yeast strain that did not produce amylase (closed diamonds). Data are averages of three independent experiments.

3. Results and discussion

3.1. Fermentation of raw corn starch or high-yielding rice carbon sources supplemented with nutrients

Using an amylase-expressing tetraploid yeast strain, we performed direct ethanol fermentation tests with high-yielding rice as a carbon source.

Fig. 1 shows the fluctuation in the ethanol concentration during the fermentation of raw, high-yielding rice and raw, purified corn starch. The ethanol productivity, ethanol yield, and amylase activities are summarized in Table 1. The ethanol productivity obtained over a 24 h period on brown rice (1.1 g/L/h) was about 5-fold higher than that obtained on purified raw corn starch (0.2 g/L/h). The ethanol yield obtained using brown rice (96%) was higher than the yields obtained on polished white rice or raw corn starch. In the yeast strain that did not produce amylase, the ethanol productivity and yield were 0.1 g/L/h and 10%, respectively, indicating that the expressed amylases facilitated ethanol fermentation on both corn starch and high-yielding rice.

Compared with the other carbon sources, the activities of glucoamylase and α-amylase were highest on brown rice (16.3 U/mL and 22.6 U/mL). Rice is known to contain relatively high concentrations of metal ions [14], and it is likely that ions stabilized α-amylase in the presence of the high ethanol concentrations [15–17]. Such stabilization would ensure the continued functioning of the α-amylase and may account for the relatively higher ethanol yield obtained using brown rice.

Even in the case using control yeast strain, only glucoamylase activity (2.4 U/mL) was detected after fermentation on brown rice; nonetheless, this activity was 1.6-fold higher than that obtained on raw corn starch. Taken together, these findings imply that high-yielding rice contains relatively large amounts of glucoamylase, the activity of which is likely to increase as the rice is degraded by α-amylase, resulting in high ethanol yields after fermentation.

3.2. Direct and cost-effective fermentation of brown rice not supplemented with nutrients

Direct inoculation of pre-cultured medium into the fermentation medium without nutrients is a simple way to achieve cost-effective ethanol fermentation. To evaluate the effect of initial inoculum size on ethanol productivity, ethanol fermentation on brown rice lacking any nutrient supplements was performed

Table 1
Fermentation of various carbon sources supplemented with nutrients.

Condition		Ethanol productivity in 24 h (g/L/h)	Ethanol yield to the theoretical yield (%)	Maximum glucoamylase activity (U/mL)	Maximum α-amylase activity (U/mL)
Biomass	Yeast				
Brown rice	MNIV/δGS	1.1	96[a]	16.3	22.6
Polished white rice	MNIV/δGS	0.9	95[a]	12.8	19.2
Raw corn starch	MNIV/δGS	0.2	91[b]	1.5	1.2
Brown rice	MT8-1	0.1	10[a]	2.4	0

[a]Carbohydrate-based theoretical yield
[b]Weight-based theoretical yield

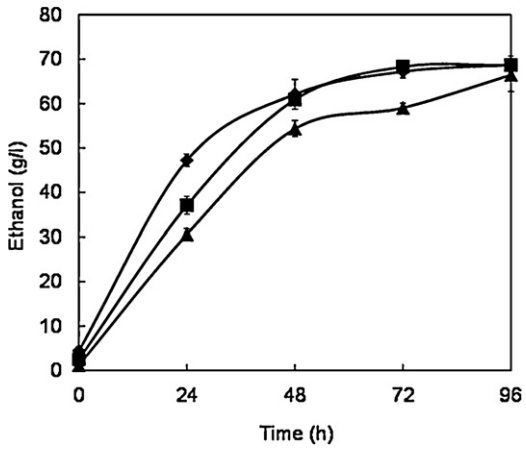

Fig. 2. Ethanol production from fermentation of brown rice as the only carbon source without any nutrients. Size of inoculum relative to volume of fermentation medium: 5% (closed triangles), 10% (closed squires), and 20% (closed diamonds). Data are averages of three independent experiments.

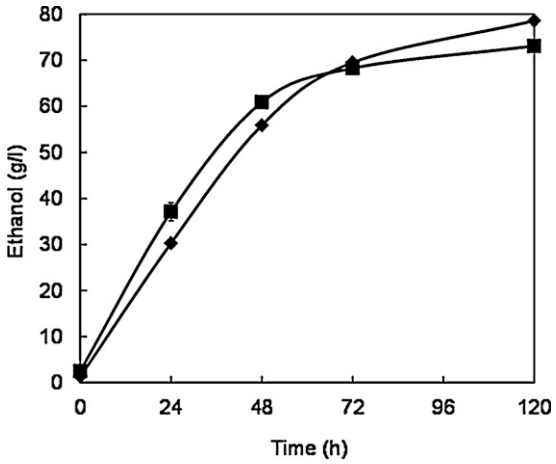

Fig. 3. Ethanol production from fermentation of carbon source without any nutrients in different fermenter volumes. 100 mL glass bottle (closed squires) and 2 L jar fermenter (closed diamonds). Data are averages of three independent experiments.

by varying the inoculum size of the pre-culture medium. Fig. 2 shows the change in the ethanol concentration during the fermentation of brown rice obtained using a variety inoculum sizes. Although ethanol productivity over a 24 h period was higher for the 20% inoculum (1.8 g/L/h) compared with the 10% inoculum, the ethanol yield obtained using the 10% inoculum (86%) was higher. These results clearly show that direct and cost-effective ethanol fermentation can be achieved using high-yielding rice without any nutrients.

Kadam and Newma reported that, to achieve low cost ethanol fermentation, at least 0.3% of corn steep liquor and 2.5 mM of $MgSO_4 \cdot 7H_2O$ are required to maintain yeast fermentation ability [18]. According to the Standard Tables of Food Composition in Japan [14], approximately 10 g/L of protein and 10 mM of magnesium derived from high-yielding rice were included in the medium. In addition to facilitating low-cost fermentation, the constituents of high-yielding rice are well suited for use as feedstock for low cost bioethanol production.

The ethanol productivity was improved by increasing the volume of the pre-culture inoculum (Table 2). In the initial stage of ethanol fermentation from starch, endo-acting α-amylase plays an important role in producing the reducing ends of starch chains which are then subsequently attacked by glucoamylase [19]. It therefore appears that ethanol productivity could be improved by increasing inoculum size.

As shown in Fig. 3, although ethanol productivity in a jar fermenter (1.2 g/L/h) over a 24 h period was slightly lower than that in a bottle (1.4 g/L/h), the ethanol yield (101%) obtained using the ethanol fermenter was significantly higher than that obtained in the bottle (86%). These findings suggest the ethanol production process established in this study can be scaled up relatively easily and does not require pretreatment with enzymes or nutrients. Furthermore, compared with previous reports [4–7], the high ethanol productivity (1.2 g/L/h) and yields (101%) obtained in this study indicate that the process described here is more efficient for producing ethanol. In addition, since our amylase-expressing yeast could be used for more than 10 repeated batch fermentations [15], further reductions in the cost of ethanol production could be affected by recycling yeast cells in a repeated batch fermentation.

In conclusion, we established a direct and efficient ethanol production process using high-yielding rice without requiring any pretreatment, addition of enzymes or nutrient supplements. The omission of any pretreatment is considered beneficial as compounds derived from such processes often inhibit fermentation [1].

Table 2
Fermentation of brown rice as a carbon source without any nutrients.

Condition		Ethanol productivity in 24 h (g/L/h)	Ethanol yield to the theoretical yield (%)	Maximum glucoamylase activity (U/mL)	Maximum α-amylase activity (U/mL)
Inoculum size (%)	Fermenter size				
5	100 mL	1.2	84	4.7	1.8
10	100 mL	1.4	86	5.3	2.1
20	100 mL	1.8	85	4.8	2.4
10	2 L	1.2	101	3.3	1.2

In addition, the high-yielding rice used in this study would be well suited for use as a feedstock of bioethanol production, and our polyploid, amylase-expressing yeast strain was sufficiently robust and capable of producing ethanol efficiently from real biomass. To our knowledge, this is first report of direct ethanol production from real biomass using amylase-expressing yeast without any pretreatment or addition of commercial enzymes.

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