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Production of Ethanol Directly from Potato Starch by Mixed Culture of *Saccharomyces cerevisiae* and *Aspergillus niger* Using Electrochemical Bioreactor

Jeon, Bo Young, Dae Hee Kim[1], Byung Kwan Na, Dae Hee Ahn[1], and Doo Hyun Park*

Department of Biological Engineering, Seokyeong University, Seoul 136-704, Korea
[1]Department of Environmental Engineering and Biotechnology, Myongji University, Kyonggi-do 449-728, Korea

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When cultivated aerobically, *Aspergillus niger* hyphae produced extracellular glucoamylase, which catalyzes the saccharification of unliquified potato starch into glucose, but not when grown under anaerobic conditions. The K_m and V_{max} of the extracellular glucoamylase were 652.3 mg/l of starch and 253.3 mg/l/min of glucose, respectively. In mixed culture of *A. niger* and *Saccharomyces cerevisiae*, oxygen had a negative influence on the alcohol fermentation of yeast, but activated fungal growth. Therefore, oxygen is a critical factor for ethanol production in the mixed culture, and its generation through electrolysis of water in an electrochemical bioreactor needs to be optimized for ethanol production from starch by coculture of fungal hyphae and yeast cells. By applying pulsed electric fields (PEF) into the electrochemical bioreactor, ethanol production from starch improved significantly: Ethanol produced from 50 g/l potato starch by a mixed culture of *A. niger* and *S. cerevisiae* was about 5 g/l in a conventional bioreactor, but was 9 g/l in 5 volts of PEF and about 19 g/l in 4 volts of PEF for 5 days.

Keywords: Electrochemical bioreactor, mixed culture, *Aspergillus niger*, *Saccharomyces cerevisiae*, ethanol fermentation, pulsed electric field (PEF)

For biological production of ethanol from starch, a three-step process must be employed: liquefaction of starch with α-amylase, enzymatic or biological saccharification of the liquefied starch, and fermentation of the sugars [1, 19]. The liquefaction and saccharification of starch using commercial amylases (frequently those produced by *Aspergillus* species) may significantly increase costs because additional bioreactors and processes are required [23]. As a substitute for the conventional multistage process, amylolytic yeasts that are capable of fermenting starch directly into ethanol were developed by genetic recombination techniques [19, 22, 27]. However, the recombinant yeast species with amylolytic function were generally not suitable for industrial ethanol production because they have low tolerance for ethanol and exhibit slow fermentation rates [4, 11].

Recently, genetic engineering techniques have been applied to improve the ethanol tolerance and fermentation rates of the recombinant yeasts [3, 5, 11, 16, 20]. The development of a respiration-deficient recombinant, a *Saccharomyces cerevisiae* strain that excretes a bifunctional fusion protein of *Bacillus subtilis* amylase and *Aspergillus awamori* glucoamylase, is a new approach for direct ethanol fermentation from starch [24]. Kim *et al.* [15] transformed *Saccharomyces* sp. into a strain capable of producing extracellular glucoamylase and α-amylase, which may be valuable in the bioconversion of starch to ethanol in coculture with *S. cerevisiae* [27]. The coculture of two *Saccharomyces* strains is more reasonable than coculture of *Saccharomyces* and *Aspergillus*, because oxygen is absolutely required for *Aspergillus* but not for ethanol fermentation by *Saccharomyces* [2]. This is applicable to ethanol production from starch in industrial systems by simultaneous saccharification and fermentation [25]; however, the additional cost for soluble starch must be considered, because the soluble starch is produced by catalysis of α-amylase. In the coculture of *Saccharomyces* and *Aspergillus*, oxygen concentration is a critical factor for optimal growth of two organisms coupled to ethanol fermentation. Abouzied and Reddy [1] reported that *A. niger* and *S. cerevisiae* were aerobically cocultivated for 24 h and then anaerobically cocultivated for 6 days under nitrogen atmospheric conditions. This may not be considered a true coculture from the viewpoint of bioconversion of starch to ethanol because *A. niger* was cultivated for different times and under different conditions than *S. cerevisiae*, albeit in the same reactor.

*Corresponding author
Phone: 82-2-940-7190; Fax: 82-2-919-0345;
E-mail: baakdoo@skuniv.ac.kr

In this study, we established bioconversion of potato starch to ethanol by mixed culture of *A. niger* and *S. cerevisiae* using an electrochemical bioreactor without enzymatic liquefaction and saccharification. *A. niger* and *S. cerevisiae* were cultivated in a pulsed electric field (PEF), and the conditions for ethanol production by the mixed culture of *A. niger* and *S. cerevisiae* were electrochemically optimized. The PEF functions as an activator for ethanol fermentation by *S. cerevisiae* [21], and oxygen generated by electrolysis of water in the PEF may be required for respiration of *A. niger* hyphae.

Materials and Methods

Organisms

Aspergillus niger KACC41858 was kindly provided by the Korean Agricultural Culture Collection, and *S. cerevisiae* ATTCC26603 was purchased from the America Type Culture Collection. To prepare inoculum, *A. niger* hyphae were aerobically cultivated in broth medium composed of 20 g/l potato starch, 3 g/l $NaNO_3$, and 2.72 g/l KH_2PO_4 at 30°C for 3 days. *S. cerevisiae* was anaerobically cultivated in medium composed of 20 g/l glucose and 5 g/l yeast extract at 30°C for 2 days.

Bioreactors

As shown in Fig. 1, the electrochemical bioreactor was designed to control electrolysis of water without a membrane to separate the anode and cathode. The total and working volumes of the bioreactor were adjusted to 2.0 l and 1.5 l, respectively. Three to 6 volts of DC electricity were charged to the electrodes, and the electric poles were periodically exchanged at 30-s intervals to prevent polarization of the anode and cathode, by which PEF was induced. The medium for mixed culture of *A. niger* and *S. cerevisiae* was composed of 50 g/l potato starch, 3 g/l $NaNO_3$, 2.72 g/l KH_2PO_4, and 5 g/l yeast extract. Before inoculation, DC electricity was charged to generate oxygen and hydrogen, by which the medium can be changed to oxygenic conditions because the solubility of oxygen (0.046 g/l at 20°C in Merck Index) is higher than that of hydrogen (0.0018 g/l at 0°C in Merck Index) in water. The oxidation-reduction potential of the medium was +250 ~ +460 mV (*vs.* Ag/AgCl) when 3–6 volts of DC electricity were charged to the electrode before inoculation. The optimal voltage for maximal ethanol production in the electrochemical bioreactor was determined by correlating the voltage and ethanol production. Five percent (v/v) of precultivated *A. niger* hyphae and *S. cerevisiae* were inoculated into the medium for mixed culture, and ethanol production was analyzed at 1-day intervals. The same bioreactor as the electrochemical bioreactor was used for a control test; no electricity was charged in the control, but the culture was agitated at 120 rpm. This bioreactor was named as the conventional bioreactor. The agitation speed was determined on the basis of glucose consumption by growing fungal hyphae for 72 h. In the conventional bioreactor, residual glucose (9.8 g/l) was about half of the starch (20 g/l) consumed during 72-h of incubation.

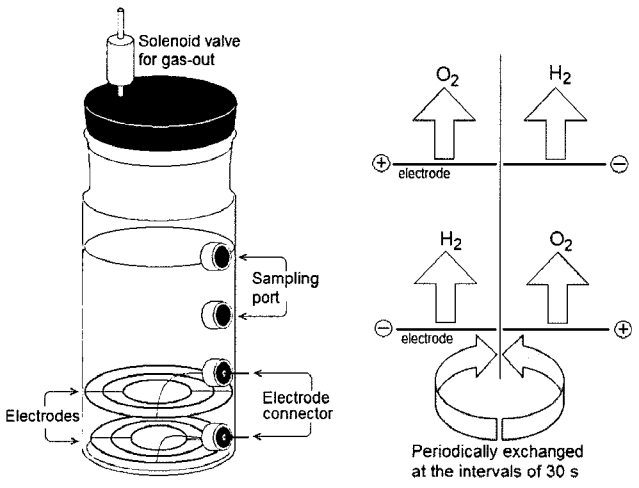

Fig. 1. Schematic structure of the electrochemical bioreactor used to produce ethanol in a mixed culture of *Aspergillus niger* and *Saccharomyces cerevisiae* (left) and mechanism for protection against polarization (right).
The electrode was made of platinum wire (0.5 mm diameter×314 mm) and the distance between the two electrodes was 30 mm. The lower electrode was located 30 mm from the bottom. DC electricity was charged to the electrodes, and the electric poles were periodically exchanged at 30-s intervals to protect against polarization of the anode (-) and cathode (+), by which the pulsed electric field between the two electrodes was induced.

Extracellular Enzyme of *A. niger*

A culture of *A. niger* hyphae that had been incubated for 3 days was 50-times concentrated with an ultrafiltration system equipped with a membrane filter (molecular cutoff size: 10,000) after removal of hyphae by centrifugation at 5,000 ×*g* and 4°C for 30 min. Fifty-times concentrated *A. niger* culture (crude enzyme solution) was used as a sample for saccharification activity assays, SDS-PAGE, and activity staining. Michaelis constant (K_m) and maximal reaction velocity (V_{max}) of the crude enzyme preparation were determined by Lineweaver-Burk plot, and the specific activity was expressed based on glucose produced from starch by enzymatic reaction for 10–60 min.

Analytical Methods

Glucose and ethanol were analyzed by HPLC with an HPX-87H column (BioRad, U.S.A.) and a refractive index detector. Samples were prepared by filtration with membrane filters (0.22 μM pore) before injection into HPLC, by which glucose and ethanol were simultaneously analyzed. HPLC also has a low probability of injection error because the injection volume is precisely controlled by the sampling loop. When HPLC was calibrated with standard ethanol from 10 to 100 mM, the linearity was more than 99%, which was more accurate than the 96% obtained when GC was calibrated. The protein concentration was determined with Bradford reagent as a coloring agent and bovine serum albumin as a protein standard [6]. The residual starch concentration in a 100-μl sample of undiluted culture broth or of culture broth appropriately diluted with water was determined as glucose by the phenol-sulfuric acid method [10]. Gases in the head space of the bioreactor were analyzed by gas chromatography with a Carboxen 1000 column (Supelco, U.S.A.). Argon (99.999%) was used as the mobile phase, and the flow rate was adjusted to 30 ml/min. The temperature of the column, injector, and detector was adjusted to 50°C, and the oven temperature was gradually increased to 220°C at a rate of 30°C min^{-1}

to clean the column. A gas sample isolated from the head space of the electrochemical reactor was directly injected into the injector with a gas-tight syringe.

Electrophoresis

SDS-PAGE was carried out according to Laemmli [18]. Native gel electrophoresis (SDS-free PAGE) was used for activity staining of extracellular enzyme produced by *A. niger* [28]. After electrophoresis, the gel was soaked in 0.5% soluble-starch solution for 1 h at 30°C. An iodine solution was then spread on the gel to induce starch-iodine reaction.

RESULTS

Extracellular Enzyme Secreted by *A. niger* hyphae

The extracellular enzyme produced by *A. niger* hyphae was characterized by electrophoresis. The protein pattern of crude enzyme solution was analyzed by SDS-PAGE and activity-staining technique. As shown in Fig. 2, two protein bands were observed in SDS-PAGE, but a single activity band was observed in native gel electrophoresis. The bright spot on the native gel showed that starch was hydrolyzed to sugar by an extracellular enzyme. The dark red background was developed by the reaction of starch with iodine, and the zone without starch was relatively brighter than the background. Based on this qualitative analysis, the enzymatic activity of crude enzyme solution was determined as shown in Fig. 3. The specific activity, V_{max}, and K_m of crude enzyme solution for saccharification of potato starch into glucose were 200.2 mg/l/min (glucose) per mg protein, 253.3 mg/l/min (glucose), and 652.4 mg/l (starch), respectively. Based on the V_{max}, the theoretical maximal amount of glucose produced from potato starch was found to be 7.3 g/l [0.253×60 (min)×24 (h)÷50 (times)].

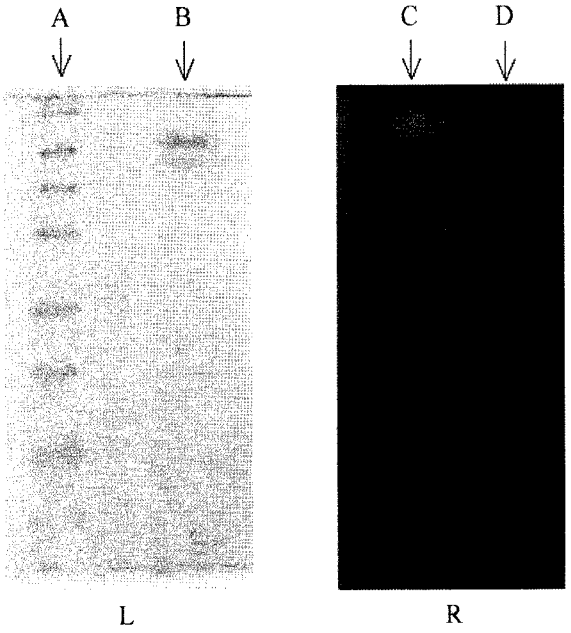

Fig. 2. SDS-PAGE pattern (L) and activity-staining (R) of concentrated *A. niger* culture. The molecular weight of the extracellular proteins (B) determined with the molecular weight marker (A) was about 96,000 and 76,000.

Activity staining was performed by native gel electrophoresis without SDS. Samples were prepared from concentrated culture of *A. niger* (AACC 41858) hyphae (C), and *S. cerevisiae* (D), which was the control experiment. After electrophoresis, the gel was soaked and incubated in 0.5% soluble-starch solution for 1 h at 30°C, and iodine solution was spread on the gel to induce starch-iodine reaction. The dark red background was developed by reaction of starch with iodine, and the zone without starch was relatively brighter than the background. MW of protein markers from top to bottom were 175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5 kDa.

Correlation Between Biomass and Glucose Production

Fungal hyphae growing in aerobic condition with plentiful oxygen may actively saccharify starch and consume glucose in coupling with increase of biomass. The residual glucose in fungal hyphae culture after 5 days of incubation may reflect the correlation between biomass and glucose production. Theoretically, the growth condition that is capable of inducing minimal fungal growth but producing maximal glucose would be the most ideal for ethanol production by mixed culture of fungal hyphae and yeast cells. As shown in Table 1, the residual glucose concentration (3.9 g/l) was greatly lower than the biomass concentration (7.1 g/l) in the conventional bioreactor under aerobic conditions. A relatively small amount of glucose (0.3 g/l) was produced by the extracellular glucoamylase contained in the inoculum under anaerobic conditions. The residual glucose concentrations showed the maximum at 4 volts of

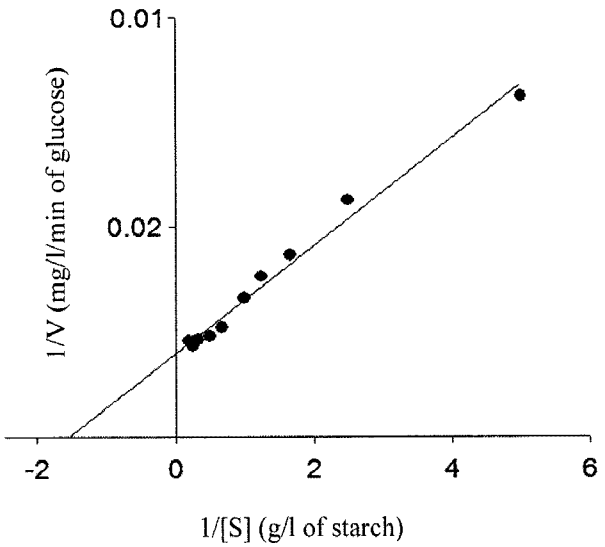

Fig. 3. Determination of K_m and V_{max} for extracellular glucoamylase excreted from *Aspergillus niger* KACC41858.

K_m and V_{max} were 652.3 mg/l (starch) and 253.3 mg/l/min (glucose), respectively, and the specific activity was 200.2 mg/l/min (glucose) per mg protein.

Table 1. Optimization for glucose production and biomass increase of *A. niger* (KACC 41858) hyphae.

Voltage of pulsed electric field	Growth condition	Dry mass of fungal hyphae (g/l)	Residual glucose in culture (g/l)
0	Agitated	7.1	3.9
0	Not agitated	0.2	0.3
3	Not agitated	1.2	7.1
4	Not agitated	2.6	16.4
5	Not agitated	3.4	12.8
6	Not agitated	4.2	9.4

Starch was adjusted to 20 g/l and the fungal culture was incubated for 5 days in a conventional bioreactor under general condition without agitation, or aerobic condition maintained by agitation at 120 rpm. Three, 4, 5, and 6 volts of pulsed electricity were charged to the electrochemical bioreactor.

Table 2. Optimization for ethanol production in a mixed culture of *Saccharomyces cerevisiae* (ATCC26603) and *Aspergillus niger* (KACC41858).

Voltage of pulsed electric field	Current (mA) between anode and cathode	Theoretical oxygen production (ml) for 1 h	Ethanol production (mM) (g/l)
0	0	0	0.0 (0.0)
3	35	7.3*	61 (2.8)
4	95	19.9	191 (8.8)
5	150	31.3	134 (6.2)
6	222	46.4	93 (4.3)

PEF was charged to electrodes equipped in the bioreactor. Starch was adjusted to 20 g l^{-1} and the mixed culture was incubated for 5 days without agitation.

*Example: [(0.035 A×3,600 s)÷96,487 coulomb]×0.25 [(one oxygen molecule/(2 valence electrons×2 oxygen atoms)]=7.31 ml

PEF. The amount of glucose (16.4 g/l) produced from starch under the condition was significantly higher than in the conventional bioreactor under aerobic conditions (3.9 g/l).

Optimal Voltage for Ethanol Production from Starch

Theoretically, ethanol production by *S. cerevisiae* is proportional to glucose concentration under anaerobic condition, but this may not be the case in the mixed culture of *A. niger* and *S. cerevisiae,* because fungal hyphae grow aerobically, but ethanol fermentation of yeast may be inhibited in aerobic conditions. Accordingly, experimental data have to be obtained on the basis of ethanol production, instead of glucose production. Optimal conditions were determined by correlating cultivation conditions and ethanol production. As shown in Table 2, when a mixed culture of *A. niger* and *S. cerevisiae* was incubated in the conventional and the electrochemical bioreactors for 5 days, ethanol production was the highest with 4 volts of PEF, corresponding with the residual glucose in Table 1. Electric voltages lower than 3 volts were not tested because the electric currents generated with 1 volt and 2 volts of PEF were 2.2 mA and 6.3 mA, respectively, which are too low to induce electrolysis of water. The oxygen generated by electrolysis might not be enough for fungal growth, but was completely consumed by *A. niger* hyphae growing around the electrodes, suggesting that ethanol fermentation was not inhibited.

Ethanol Production from Starch

Glucose (7.3 g/l/day) produced by the reaction of starch with crude enzyme solution was higher than that (3.3 g/l/day) produced by growing *A. niger* hyphae with 4 volts of PEF (Table 1). This indicates that the part of the glucose produced by starch hydrolysis was consumed by the growing hyphae, which might have increased in proportion to the oxygen in the culture broth. As shown in Fig. 4, ethanol production directly from potato starch was

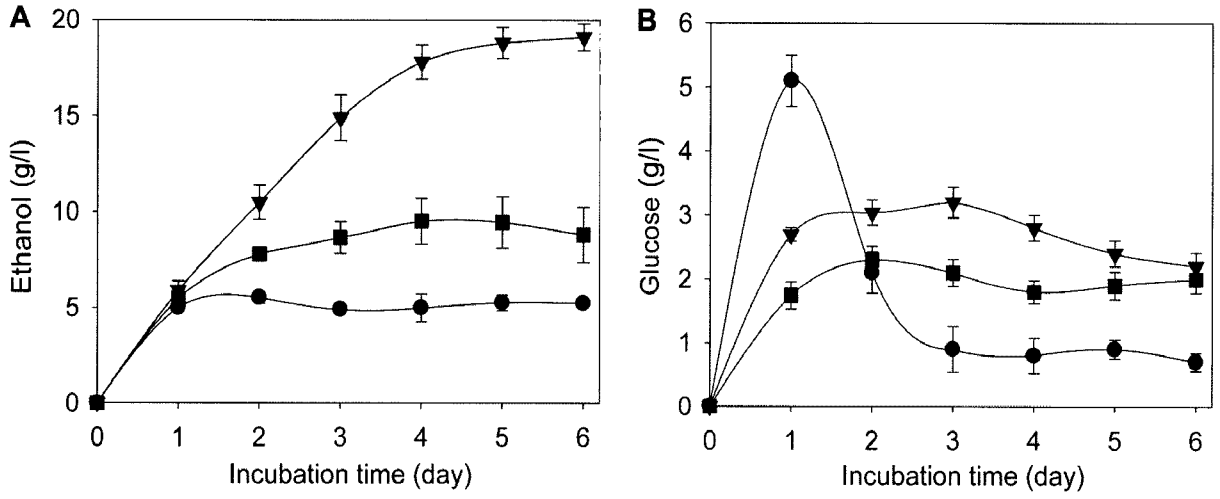

Fig. 4. Ethanol (**A**) and glucose (**B**) produced in a mixed culture of *Saccharomyces cerevisiae* and *Aspergillus niger.*
DC 4 volts (▼) and 5 volts (■) of electricity were charged to electrodes equipped in the bioreactor. For control experiments, electricity was not charged to the bioreactor (●); instead, the culture was agitated at 120 rpm. Initial starch was precisely adjusted to 5% (w/v).

the highest in the electrochemical bioreactor with 4 volts of PEF, but was the lowest in the conventional bioreactor shaken at 120 rpm (Fig. 4A). The residual glucose was relatively lower in the conventional bioreactor (Fig. 4B) than in the electrochemical bioreactor, and the residual glucose in 4 volts of PEF was higher than in 5 volts of PEF.

DISCUSSION

Extracellular enzymes secreted by *A. niger* hyphae consisted of two protein bands on SDS-PAGE and one band in native gel electrophoresis for activity staining, indicating that *A. niger* hyphae secrete mainly a specific enzyme for saccharification of potato starch [13]. Broth medium containing 20 g/l of potato starch was changed from opaque to completely transparent in 2 days, and that containing 50 g/l of potato starch was charged in 4 days. On the basis of two protein bands on SDS-PAGE and direct glucose production from unliquified potato starch, the extracellular proteins are possibly glucoamylase and α-amylase. Theoretically, residual starch in *A. niger* hyphae culture may be less than 1.0 g/l, based on the K_m value, which is a useful indicator for efficiency of direct ethanol fermentation from starch by mixed culture. When *A. niger* hyphae were aerobically cultivated in medium containing 20 g/l starch for 5 days, residual starch in the medium was lower than 2 g/l (data not shown). *A. niger* is a typical aerobe, but the ethanol fermentation by *S. cerevisiae* is typically activated in anaerobic conditions [8, 12, 17]. In the mixed culture of two microorganisms that grow in different conditions, optimization for maximal growth of both organisms is difficult [7]. However, optimization of maximal ethanol production from starch is possible by electrochemical control of the oxygen generation without agitation or shaking. In the mixed culture of *A. niger* and *S. cerevisiae*, the most important and difficult factor to control is oxygen, because it is an essential factor for *A. niger* growth as well as a limiting factor for ethanol fermentation of *S. cerevisiae*. In single culture of *A. niger* hyphae, glucose produced by *A. niger* hyphae can be accumulated in growth-limiting conditions, whereas the glucose accumulated was mostly consumed in oxygen-abundant condition (Table 1). Growing *A. niger* hyphae produce extracellular enzymes for saccharification of starch and take up glucose in coupling with respiratory metabolism. Accordingly, oxygen control is critical for the minimal uptake but maximal production of glucose by fungal hyphae, and also for maximal production of ethanol from starch in the mixed culture of fungal hyphae and yeast. Abouzied and Reddy [1] aerated the coculture of *A. niger* and *S. cerevisiae* for 24 h to activate fungal growth and then stopped aeration to induce ethanol fermentation of yeast for 6 days. This shows how difficult it is to optimize minimal glucose consumption by *A. niger*, but maximal ethanol production by *S. cerevisiae*, in a mixed culture. In this study, we observed more residual glucose accumulation in the single culture of *A. niger* hyphae and more ethanol production in the mixed culture of *A. niger* and *S. cerevisiae* in the electrochemical bioreactor than in a conventional bioreactor agitated at 120 rpm. Dissolved oxygen in the culture can be controlled by agitation speed and an oxygen sensor in the conventional bioreactor; however, the up-down circulation or fluctuation of the culture cannot be controlled. The microsized hydrogen and oxygen bubbles generated from the electrode cannot perturb the culture, and the oxygen produced in limited amount may be consumed by fungal hyphae growing around the electrode before diffusing into the medium. In a conventional bioreactor, both fungal hyphae and yeast cells are up-down circulated and frequently contact with oxygen, and both organisms may competitively consume glucose and oxygen coupled to the respiratory metabolism, thereby possibly limiting saccharification and ethanol fermentation. In the electrochemical bioreactor, however, only yeast cells growing at the bottom of the reactor have difficulty to contact with oxygen generated from the electrodes that are located 30 mm from the bottom, and fungal hyphae floating around the electrodes may consume most of the oxygen generated from the electrodes. Therefore, fungal hyphae can grow in proportion to oxygen, and alcohol fermentation of the yeast may be not inhibited. Oxygen produced on the electrode can precisely be controlled because the oxygen/hydrogen production by electrolysis is completely proportional to current consumption [7]. The limited oxygen produced on the electrode may be consumed by hyphae growing around the electrode before diffusing into the medium. In these conditions, it may be difficult for yeast cells to come into contact with the oxygen. The higher ethanol production in 4 *vs.* 5 volts of PEF is thought to be caused by a difference in oxygen generation: 19.9 ml/h and 31.3 ml/h of oxygen were generated in 4 and 5 volts of PEF, respectively. The difference in oxygen generation with 4 *vs.* 5 volts of PEF, 11.4 ml/h, may activate growth of fungal hyphae, which then consume more glucose. The lower residual glucose in 5 volts than in 4 volts of PEF suggests that *A. niger* hyphae may consume more glucose, therefore inhibiting ethanol fermentation by *S. cerevisiae*. When gases in the head space of the electrochemical bioreactor were analyzed with GC, more than 99.9% carbon dioxide and less than 0.1% hydrogen were detected; however, oxygen was not detected, and the oxidation-reduction potential of mixed culture in the electrochemical bioreactor was −407±15 mV (*vs.* Ag/AgCl). This is much lower than the oxidation-reduction potential, +200 ~ +360 mV (*vs.* Ag/AgCl), of the medium before inoculation. This result shows that the oxygen generated from the electrode was completely taken up by the fungal

hyphae, whereas hydrogen influenced the oxidation-reduction potential of the culture [14]. In this environment, fungal hyphae grew in proportion to the oxygen generated by electrolysis of water, and glucose was produced in proportion to glucoamylase produced by the fungal hyphae. Therefore, the ethanol fermentation of yeast cells might be continuously maintained by glucose produced by the fungal hyphae. The redox potential of the mixed culture in a conventional bioreactor agitated at 120 rpm was 120±20 mV (*vs.* Ag/AgCl), which could inhibit alcohol fermentation.

Consequently, the biomass of fungal hyphae and yeast cells cannot be measured separately, but ethanol production per starch consumption can be measured in the mixed culture. Accordingly, maximal ethanol production per starch consumption is indicative for optimization of the mixed culture of fungal hyphae and yeast cells. The electrochemically generated PEF is a useful device to control electrolysis of water through adjustment of electric voltage charged to the electrode or electrode surface area, and has been proven to activate yeast fermentation by *S. cerevisiae* in a previous study [9]. We do not know the mechanism of how the PEF activates ethanol fermentation by *S. cerevisiae*; nevertheless, we know that the PEF is more compatible to the bioreactors than agitation, aeration, or a shaking apparatus to control the mixed culture of fungal hyphae and yeast. Urszula and Jamroz [26] reported that 500 volts of PEF charged to resting cells of *S. cerevisiae* activated selenium accumulation in the cytoplasm, which can be an example to show the effect of PEF on yeast physiology, but it cannot explain how PEF activated ethanol fermentation.

In the present research, about 20 g/l of ethanol was produced while 50 g/l of starch was consumed, which is much higher than previously reported results and nearly 80% of the maximal yield. Theoretically, about 180 g/l of glucose could be produced by saccharification of 180 g/l of starch, and a maximal 92 g/l of ethanol could be produced from 180 g/l of glucose. Accordingly, the theoretical ethanol yield is maximally 51%. The ethanol yield in this research was about 40%, which corresponds to about 80% of the theoretical yield. To the best of our knowledge, this is the first research ever performed using an electrochemical bioreactor for direct ethanol production from potato starch in the mixed culture of *A. niger* (which is capable of producing extracellular enzyme for direct saccharification of unliquified starch) and *S. cerevisiae,* without separation of the liquefaction and saccharification processes.

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