Comparison of Bioethanol Production by *Candida molischiana* and *Saccharomyces cerevisiae* from Glucose, Cellobiose, and Cellulose

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Bioethanol has attracted much attention in recent decades as a sustainable and environmentally friendly alternative energy source. In this study, we compared the production of bioethanol by *Candida molischiana* and *Saccharomyces cerevisiae* at different initial concentrations of cellobiose and glucose. The results showed that *C. molischiana* can utilize both glucose and cellobiose, whereas *S. cerevisiae* can only utilize glucose. The ethanol yields were 43–51% from different initial concentrations of carbon source. In addition, different concentrations of microcrystalline cellulose (Avicel) were directly converted to ethanol by a combination of *Trichoderma reesei* and two yeasts. Cellulose was first hydrolyzed by a fully enzymatic saccharification process using *T. reesei* cellulases, and the reducing sugars and glucose produced during the process were further used as carbon source for bioethanol production by *C. molischiana* or *S. cerevisiae*. Sequential culture of *T. reesei* and two yeasts revealed that *C. molischiana* was more efficient for bioconversion of sugars to ethanol than *S. cerevisiae*. When 20 g/l Avicel was used as a carbon source, the maximum reducing sugar, glucose, and ethanol yields were 42%, 26%, and 20%, respectively. The maximum concentrations of reducing sugar, glucose, and ethanol were 10.9, 8.57, and 5.95 g/l, respectively, at 120 h by the combination of *T. reesei* and *C. molischiana* from 50 g/l Avicel.

Keywords: Bioethanol, *Candida molischiana*, *Saccharomyces cerevisiae*, *Trichoderma reesei*, Avicel

Introduction

As industry development accelerates and fossil fuels decline rapidly, energy demand continues to rise and extensive research has been done on alternative, sustainable and environmentally friendly energy sources. Among them, crop-based bioethanol is the most widely used biofuel with production of 106 billion liters in 2017 [1]. Abuse of arable land for bioethanol production has been a concern for many years. Therefore, the scientific community is working extensively to produce bioethanol using microorganisms from inexpensive wastes such as lignocellulose [2, 3]. Their use can reduce greenhouse gas emissions by 30–85% compared to gasoline [4]. The agricultural wastes generated during or after the processing of crops are generally rich in lignocellulosic biomass. Lignocellulose consists of cellulose and hemicellulose firmly bonded to lignin [5]. Second-generation bioethanol

is produced by saccharification of lignocellulosic biomass, followed by microbial fermentation and product recovery [6].

Saccharomyces cerevisiae strains are commonly used in the food industry and in the laboratory because they are easy to grow and genetically tractable. In addition, they are used as model organisms for studying eukaryotic cellular processes. They are comparatively superior to other fungi and bacterial strains for bioethanol production at an industrial level. They can withstand a wide pH range and optimal acidic pH is more effective in suppressing contamination-related problems [7, 8]. In addition, they are GRAS (generally regarded as safe) for human consumption [9]. *Candida molischiana* is one of the few yeast species capable of degrading cellobiose into glucose [10]. Geiger *et al.* [11] isolated a mutant strain with increased thermotolerance (up to 45°C) compared to parent strain. Because *C. molischiana* has strong resistance to ethanol and heat, this yeast may be

more effective when introduced into industrial cellulose ethanol plants [11].

Recently, high-efficiency biodegradation of cellulosic biomass through microbial co-culture or complex communities has been proposed [12]. *S. cerevisiae* was co-cultured with other microorganisms for optimal bioethanol production in a shorter period of time [13]. In our previous study, *Trichoderma reesei* and *C. molischiana* were sequentially cultured to produce bioethanol directly from α -cellulose [14]. In this study, microcrystalline cellulose (Avicel) was used as a carbon source and three fungal species, *T. reesei*, *C. molischiana*, and *S. cerevisiae*, were used for bioethanol production from cellulose. First, growth of *C. molischiana* and *S. cerevisiae* and their bioethanol production were compared at different initial concentrations of glucose and cellobiose as carbon sources. Direct production of bioethanol from Avicel was then carried out by a combination of *T. reesei* and *C. molischiana* or *S. cerevisiae*. Cellulose was hydrolyzed by a fully enzymatic saccharification process using *T. reesei* cellulases and the produced reducing sugars and glucose were further utilized for ethanol production by *C. molischiana* or *S. cerevisiae*. The yields and concentrations of the produced reducing sugar, glucose, and ethanol by different microbial combinations were compared from various initial concentrations of Avicel.

Materials and Methods

Microorganisms

Three fungal strains were used in this study. *Trichoderma reesei* RUT-C30 (KCTC 6968) and *Saccharomyces cerevisiae* (KCTC 7928) were procured from Korean Collection for Type Cultures (Daejeon, Korea). *Candida molischiana* (ATCC 2516) was procured from American Type Culture Collection (USA).

Media and Cultivation

The *T. reesei* stock culture was aseptically grown on potato dextrose agar (PDA) medium consisting of 24 g/l potato dextrose broth and 20 g/l agar and incubated at 30°C for 7 days until sporulation was sufficient and then stored at 4°C. *T. reesei* pre-culture medium consisted of (per L): Avicel PH101 (Sigma-Aldrich) 10 g, $(\text{NH}_4)_2\text{SO}_4$ 1.4 g, KH_2PO_4 2 g, CaCl_2 0.3 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5×10^{-2} g, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ 1.56×10^{-2} g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.4×10^{-2} g, and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 2×10^{-2} g. The pre-culture was grown in a shaking incubator at 30°C, 200 rpm for 48 h and inoculated into (10% v/v) 250 ml flasks containing 100 mL medium of different initial concentrations of Avicel (10, 20, 30, 40, and 50 g/l). The initial pH was adjusted to 7 using 3 N NaOH.

C. molischiana and *S. cerevisiae* stock cultures were aseptically cultivated in YM agar petri plates consisting of (per L): 3 g yeast

extract, 3 g malt extract, 5 g peptone, 10 g glucose, and 20 g agar incubated at 30°C for 2 days and then stored at 4°C. *C. molischiana* and *S. cerevisiae* pre-cultures were grown in YM broth in a shaking incubator at 30°C and 200 rpm for 24 h and inoculated into (5% v/v) 250 ml flasks containing 100 ml medium of different initial concentrations of glucose or cellobiose (20, 50, and 100 g/l). The initial pH was adjusted to 6 using 3 N NaOH. For sequential culture, *T. reesei* was first grown in 250 ml flasks containing 100 ml medium of the pre-culture medium with different initial concentrations of Avicel (10–50 g/l) in a shaking incubator at 30°C, pH 7, and 200 rpm. Then, 5 ml of *C. molischiana* or *S. cerevisiae* culture was added to 100 ml of *T. reesei* cultured medium after 84 h for initial Avicel concentrations of 10, 20, and 30 g/l and after 120 h for initial Avicel concentrations of 40 and 50 g/l, respectively.

Analytical Methods

Growth (OD at 600 nm) was measured using a UV-visible spectrophotometer (UV mini-1240, Shimadzu, Japan). Reducing sugar was measured by dinitrosalicylic acid (DNS) method [15]. Glucose and ethanol concentrations were measured by HPLC system (YL 9100, Young-Lin, Inc., Korea). Samples used for HPLC analysis were centrifuged and filtered through 0.2 μm filters. Each sample was injected into a Biorad Aminex hpx-87h column (USA) at 55°C with a refractive index detector and was eluted from the column using a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. One unit (U) of cellulase was defined as the amount of enzyme that produced 1 μmol of reducing sugar (glucose equivalent) per min at 30°C and pH 7 [16].

Results and Discussion

Bioethanol is one of the most suitable renewable, alternative energy sources to replace fossil fuels. In order to solve the energy crisis and promote its use, it is essential to reduce the production cost by using cheap substrate along with increasing production efficiency using suitable microorganisms. In this study, we investigated the growth of *C. molischiana* and *S. cerevisiae* at different initial concentrations of glucose and cellobiose (20, 50, and 100 g/l) as carbon sources (Fig. 1). Growth of the yeast was quantified by optical density measurement (OD at 600 nm). In the case of *C. molischiana*, the growth rate in glucose medium was slightly higher than in cellobiose medium, but the final OD was similar for both carbon sources (Fig. 1A). The maximum OD values for both carbon sources were 11–13, indicating that *C. molischiana* can grow well at various concentrations of glucose and cellobiose. In the case of *S. cerevisiae*, growth in medium containing 20 and 50 g/l initial glucose concentration was similar to that of *C. molischiana* in glucose medium at the same concentration

Fig. 1. Growth of (A) *C. molischiana* and (B) *S. cerevisiae* using different initial concentrations of glucose and cellobiose.

(Fig. 1B). The final OD of *S. cerevisiae* culture at 100 g/l initial glucose concentration was higher than that of *C. molischiana* culture and reached 16 after 36 h. However, the growth of *S. cerevisiae* in cellobiose medium was remarkably slow and the final OD was much lower than that in glucose medium. This indicates that *S. cerevisiae* did not utilize cellobiose as carbon source and light growth occurred by consuming nutrients contained in YM media (yeast extract, malt extract, and peptone).

Figs. 2 to 4 show the sugar consumption profiles during the fermentation period of two yeast strains. Ethanol production by *C. molischiana* and *S. cerevisiae* was studied

Fig. 2. Ethanol production by *C. molischiana* and *S. cerevisiae* using 20 g/l of glucose and cellobiose. (A) Glucose, (B) cellobiose.

using 20 g/l of glucose or cellobiose as a carbon source (Fig. 2). Two yeast strains converted most of the glucose to ethanol (Fig. 2A). Glucose was completely consumed to produce ethanol within 12 h by *C. molischiana* and *S. cerevisiae*, with a yield of 46% and 50%, respectively. Unlike *C. molischiana* which completed cellobiose consumption within 28 h with a yield of 43%, *S. cerevisiae* was unable to metabolize cellobiose to ethanol (Fig. 2B). *C. molischiana* fermented glucose faster than cellobiose. Similar results were obtained when the substrate concentration was increased to 50 g/l and 100 g/l (Figs. 3 and 4). Fermentation time increased with increasing carbon source concentration. Table 1 summarizes bioethanol production yield from different initial concentrations of cellobiose and glucose. The ethanol yields were 43–51% from different initial

Fig. 3. Ethanol production by *C. molischiana* and *S. cerevisiae* using 50 g/l of glucose and cellobiose. (A) Glucose, (B) cellobiose.

concentrations of carbon source. High ethanol concentrations of 51 g/l and 49 g/l were obtained by *C. molischiana* from 100 g/l glucose and cellobiose, respectively. *S. cerevisiae* produced 50 g/l ethanol from 100 g/l glucose, whereas

Table 1. Summary of ethanol production by *C. molischiana* and *S. cerevisiae* using different initial concentrations of glucose and cellobiose.

Yeast	Substrate concentration (g/l)	Ethanol yield from glucose (g/g)	Ethanol yield from cellobiose (g/g)
<i>Candida molischiana</i>	20	0.46	0.43
	50	0.51	0.51
	100	0.51	0.49
<i>Saccharomyces cerevisiae</i>	20	0.50	0
	50	0.48	0
	100	0.50	0

Fig. 4. Ethanol production by *C. molischiana* and *S. cerevisiae* using 100 g/l of glucose and cellobiose. (A) Glucose, (B) cellobiose.

ethanol was not detected from cellobiose.

Based on the basic characteristics of *C. molischiana* and *S. cerevisiae*, sequential cultivations of *T. reesei* and two yeasts were performed to produce ethanol from cellulose. *T. reesei* is known to be an overproducer of cellulolytic enzymes such as exoglucanases (cellobiohydrolases), endoglucanases, and β -glucosidases [17, 18]. Direct production of ethanol from α -cellulose by a microbial combination of *T. reesei* and *C. molischiana* has therefore been proposed [14]. Avicel was used as a source for cellulose in this study. Different initial concentrations of Avicel were used in the range of 10–50 g/l to compare the ethanol yield from cellulose and determine the optimal initial substrate concentration (Figs. 5 to 9). Bioconversion of the substrate to ethanol by *T. reesei* and two yeasts was investigated for 132 h (Figs. 5 to 7) and 156 h (Figs. 8 and 9), respectively. The results showed that as the initial Avicel concentration

Fig. 5. Reducing sugar and glucose production by *T. reesei* and ethanol production by *C. molischiana* and *S. cerevisiae* using 10 g/l Avicel.

Arrow indicates the inoculation time of yeast strains in the *T. reesei* cultured medium.

Fig. 7. Reducing sugar and glucose production by *T. reesei* and ethanol production by *C. molischiana* and *S. cerevisiae* using 30 g/l Avicel.

Arrow indicates the inoculation time of yeast strains in the *T. reesei* cultured medium.

increased, more time was required to hydrolyze the cellulose. Table 2 summarizes the maximum concentrations and yields of reducing sugar, glucose, and ethanol. The ethanol yield by the combination of *T. reesei* and *C. molischiana* was relatively higher than that by the combination of *T. reesei* and *S. cerevisiae*. Therefore, it can be concluded that

C. molischiana is more efficient for the production of bioethanol from cellulose than *S. cerevisiae*.

On the other hand, maximum reducing sugar and glucose concentrations were obtained from fermentation of 50 g/l Avicel and the maximum ethanol concentration was similar at 30, 40, and 50 g/l Avicel (Figs. 7 to 9 and Table 2).

Fig. 6. Reducing sugar and glucose production by *T. reesei* and ethanol production by *C. molischiana* and *S. cerevisiae* using 20 g/l Avicel.

Arrow indicates the inoculation time of yeast strains in the *T. reesei* cultured medium.

Fig. 8. Reducing sugar and glucose production by *T. reesei* and ethanol production by *C. molischiana* and *S. cerevisiae* using 40 g/l Avicel.

Arrow indicates the inoculation time of yeast strains in the *T. reesei* cultured medium.

Fig. 9. Reducing sugar and glucose production by *T. reesei* and ethanol production by *C. molischiana* and *S. cerevisiae* using 50 g/l Avicel. Arrow indicates the inoculation time of yeast strains in the *T. reesei* cultured medium.

This means that high concentration of Avicel was not efficiently hydrolyzed by *T. reesei* used in this study. As the initial Avicel concentration increased beyond 20 g/l, the ethanol yield gradually decreased. Although the concentration of reducing sugar, glucose, and ethanol was not the highest at 20 g/L Avicel, the final ethanol yield was highest at this Avicel concentration. Cellulase activities by *T. reesei* at different initial concentrations of Avicel were also calculated with time (Figs. 5 to 9). Maximum cellulase activities appeared 36–48 h after *T. reesei* inoculation. The highest cellulase activity was 180 U/100 ml at 36 h when the initial Avicel concentration was 20 g/l (Fig. 6). This indicates that the optimal initial Avicel concentration is 20 g/l in terms of cellulase activity and ethanol yield.

The highest ethanol yield from 20 g/l Avicel was 20% for

the combination of *T. reesei* and *C. molischiana* and 13% for the combination of *T. reesei* and *S. cerevisiae*. The ethanol yield obtained with the combination of *T. reesei* and *C. molischiana* is higher than the 15% previously obtained with the same combination from α -cellulose [14]. Higher ethanol yield in this study can be related to the type of cellulose used. The weight average degree of polymerization (DPw) of Avicel PH101 was reported to be 200–240 anhydroglucose units (AGU) compared to 2140–2420 AGU for α -cellulose [19]. α -Cellulose is a solid residue of lignocellulose after extraction with strong alkali. In contrast, Avicel PH101 is further processed from α -cellulose to change crystallinity, degree of polymerization, and porosity [19]. Similar results were obtained with Avicel as a substrate compared to other cellulosic substrates for the higher cellulase activity of *T. reesei* Rut C-30 [20], *Sporocytophaga rnyxococcoides* [21], and *Saccharophagus degradans* [22].

C. molischiana has been reported to ferment celloextrins with a degree of polymerization of 2 to 6 to ethanol [23]. The higher ethanol yield by the combination of *T. reesei* and *C. molischiana* than by the combination of *T. reesei* and *S. cerevisiae* can be due to the additional consumption by *C. molischiana* of reducing sugars in addition to glucose produced by *T. reesei*, while *S. cerevisiae* can utilize only glucose. Although a high ethanol yield of 41% was reported using metabolically engineered strains of *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* [24], the ethanol yield (20%) obtained in this study is similar to or higher than previously reported for wild-type strains [25–28]. It is noteworthy that *C. molischiana* and *S. cerevisiae* can perform ethanol fermentation in the presence of *T. reesei*. It suggests that there are not enough detrimental enzymes such as chitinase, which can have harmful effects on yeast to prevent alcohol accumulation [14]. By isolating the hyper cellulose producer and developing a highly productive bioreactor operation, direct ethanol production from cellulose may be possible by a microbial combination.

Table 2. Summary of reducing sugar, glucose, and ethanol production using different initial concentrations of Avicel.

Avicel (g/l)	Reducing sugar		Glucose		Ethanol (<i>T. reesei</i> + <i>C. molischiana</i>)		Ethanol (<i>T. reesei</i> + <i>S. cerevisiae</i>)	
	Maximum conc. (g/l)	Yield (g/g)	Maximum conc. (g/l)	Yield (g/g)	Maximum conc. (g/l)	Yield (g/g)	Maximum conc. (g/l)	Yield (g/g)
10	2.68	0.27	2.13	0.21	1.04	0.10	0.88	0.09
20	8.34	0.42	5.20	0.26	4.08	0.20	2.55	0.13
30	8.93	0.30	7.52	0.25	5.58	0.19	3.69	0.12
40	9.70	0.24	7.55	0.19	5.63	0.14	3.54	0.09
50	10.9	0.22	8.57	0.17	5.95	0.12	3.86	0.08

In conclusion, this study demonstrated that the use of *C. molischiana* was superior to *S. cerevisiae* for bioethanol production from cellobiose or Avicel as a carbon source. Ethanol yield from glucose and cellobiose was 43–51% in aerobic environment. The optimal initial Avicel concentration for cellulase activity and ethanol production was 20 g/l. Maximum yields of reducing sugar, glucose, and ethanol were 42%, 26%, and 20%, respectively, using the combination of *T. reesei* and *C. molischiana* under this condition.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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