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Direct ethanol production from cellulose by consortium of *Trichoderma reesei* and *Candida molischiana*

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Abstract: Industrial cellulosic ethanol production is a challenge due to the high cost of cellulases for hydrolysis when lignocellulosic materials are used as feedstock. In this study, direct ethanol production from cellulose was performed by consortium of *Trichoderma reesei* and *Candida molischiana*. Cellulose was hydrolyzed by a fully enzymatic saccharification process using *Trichoderma reesei* cellulases. The produced reducing sugar was further utilized by *Candida molischiana* for ethanol production. Because the optimal temperature for the cellulase system is approximately 50°C, the effect of temperature rise from 30°C to 50°C on cellulose hydrolysis was investigated. The results showed that the temperature rise from 30°C to 50°C after 36 h of cultivation was the best for reducing sugar and glucose production. Under these conditions, the maximum concentrations of reducing sugar and glucose produced by *T. reesei* were 8.0 g/L and 4.6 g/L at 60 h, respectively. The maximum production of ethanol by *C. molischiana* was 3.0 g/L after 120 h.

Keywords: bioethanol; *Candida molischiana*; cellulose hydrolysis; *Trichoderma reesei*

1 Introduction

The ongoing global dependence on fossil fuels has produced serious energy crises and environmental problems. Renewable energy sources such as organic waste are attractive alternatives to fossil fuels [1]. New technologies to convert plant biomass into alternative biofuels, such as bioethanol, are being developed [2]. Fermentation-derived ethanol can be produced from sugar, starch, or lignocellulosic biomass. Sugar and starch-based feedstocks are currently predominant at the industrial level and are economically advantageous [3]. Lignocellulosic materials are important for the production of bioethanol due to their abundance [4]. Industrial cellulosic ethanol production is still a challenge because of the high processing cost, for example, the high cost of cellulase for hydrolysis after using lignocellulosic materials as feedstock [5-7]. During enzymatic hydrolysis, cellulase converts cellulose into soluble sugars.

Cellulose is a linear polymer of glucose units, which are hydrolyzed by endoglucanase, cellobiohydrolase, and β-glucosidases [8,9]. Some fungal strains produce large amounts of cellulase. For more than five decades, different *Trichoderma reesei* strains have been screened for their potential production of cellulases [9]. *T. reesei* cellulases are widely used to hydrolyze lignocellulosic biomass to fermentable sugars. These enzymes synergistically convert cellulose because the action of one enzyme is utilized as a substrate by another enzyme, which leads to the production of glucose [10].

The existing ethanol production process includes a pretreatment step in which the sugar in the raw material is separated and fed to the fermenter as a substrate. *Candida molischiana* is one of the few yeast species capable of degrading cellobiose into glucose [11]. Because *C. molischiana* is highly resistant to ethanol and heat, this yeast is likely to be more effective when introduced in industrial cellulose ethanol plants [12]. One mutant strain of *C. molischiana* could tolerate up to 4% ethanol in the medium when grown at 45°C for 48 h [12]. Recently, the highly efficient biodegradation of cellulosic biomass through microbial co-cultures or complex communities has been proposed [4]. In this study, *T. reesei* and *C. molischiana* were sequentially cultured to produce ethanol from cellulose without acidic, ionic, or chemical pretreatments. First, the effect of temperature changes on

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cellulosic hydrolysis by *T. reesei* from 30°C to 50°C were investigated. The reducing sugars and glucose obtained were further used for ethanol production by *C. molischiana* to develop a fully enzymatic hydrolysis process from cellulose.

2 Materials and methods

2.1 Microorganisms

The fungus *T. reesei* RUT-C30 (KCTC 6968) and yeast *C. molischiana* (ATCC 2516) were purchased from Korean Collection for Type Cultures (Daejeon, Korea) and American Type Culture Collection (Manassas, VA, USA), respectively.

2.2 Media and cultivation

The *T. reesei* stock culture was aseptically grown on potato dextrose agar 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. *T. reesei* was cultivated in chemically defined medium consisting of (per L): glucose 10 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2 g, $CaCl_2$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 5×10^{-2} g, $MnSO_4 \cdot 5H_2O$ 1.56×10^{-2} g, $ZnSO_4 \cdot 7H_2O$ 1.4×10^{-2} g, and $CoCl_2 \cdot 6H_2O$ 2×10^{-2} g. The pre-culture was incubated at 30°C and 175 rpm for 48 h. Next, 10 mL of the pre-culture was inoculated into 100 mL of the same chemically defined medium containing 10 to 30 g/L of α-cellulose (Sigma) instead of 10 g/L glucose in a 250 mL flask.

C. molischiana stock culture was cultivated in YM agar consisting of (per L): 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, and 20 g agar. It was incubated at 30°C for 2 days. *C. molischiana* was then inoculated into YM broth and grown in a shaking incubator at 30°C and 175 rpm for 48 h. For ethanol production, 10 mL of *C. molischiana* culture was added to 100 mL of *T. reesei* cultured medium in a 250 mL flask at 30°C after 60 h.

2.3 Effect of temperature rise time on cellulose hydrolysis

The cellulose hydrolysis process was optimized for temperature rise times (24 to 60 h). The temperature rise time refers to the period of cultivation at 30°C prior to increasing to 50°C for reducing sugar and glucose production. The initial pH was adjusted to 7 using 3 N NaOH. *T. reesei* was grown in a 250 mL flask with 100 mL of chemically defined medium containing cellulose.

2.4 Analytical methods

Reducing sugar was measured by the dinitrosalicylic acid (DNS) method as described previously [13]. Glucose concentration was measured using an Asan set glucose kit (Asan Pharm, Co. Ltd., Seoul, Korea). Ethanol concentration was measured by HPLC system (YL 9100, Young-Lin, Inc., Anyang, Korea) using a Biorad Aminex hpx-87h column (Hercules, CA, USA) with a refractive index detector.

3 Results and discussion

Cellulases from *T. reesei* fungi have been used in the biofuels industry to degrade vegetable feedstocks into substrates that can be used for other processes. Another promising microorganism for bioethanol production is *C. molischiana*, which produces ethanol from substrates containing glucose, fructose, and sucrose [11]. Glucose production using *T. reesei* RUT-C30 fermentation is a complex system [14]. Many factors influence glucose productivity, including pre-incubation time, initial pH, and initial cellulosic concentration. In a preliminary study, fermentation was performed up to 5 days, and the production of reducing sugar and glucose was monitored at 24-h intervals. The optimal conditions for pre-culture incubation time, initial pH, and initial cellulosic concentration were 2 days, pH 7 and 20 g/L, respectively, in terms of reducing sugar and glucose production from cellulose. Under these conditions, the maximum reducing sugar and glucose production reached 4.1 and 2.6 g/L, respectively, after 2 days of incubation.

T. reesei RUT-C30 is an overproducer of cellulolytic enzymes, including cellulases and xylanases [14]. Complete cellulose hydrolysis to glucose requires exoglucanases (also cellobiohydrolases), endoglucanases, and β-glucosidases. The extracellular cellulolytic system of *T. reesei* is composed of 60 to 80% cellobiohydrolases or exoglucanases, 20 to 36% endoglucanases, and 1% β-glucosidases, which act synergistically to convert cellulose to glucose [15]. The optimal temperature for this cellulase system is approximately 50°C [16]. Therefore, the effect of increasing the temperature from 30°C to 50°C

on cellulose hydrolysis to produce reducing sugar and glucose was investigated at 24, 36, 48, and 60 h (Figure 1).

Figures 1A and 1B show changes in the production of reducing sugar and glucose over time when the temperature was increased to 50°C. At constant temperature at 30°C (control), the maximum reducing sugar and glucose production reached 4.1 and 2.7 g/L, respectively. Sugar production gradually increased at a temperature rise time of 24 to 36 h, while sugar production decreased at a temperature rise time of 48 to 60 h. When the temperature was increased at 36 h, the maximum reducing sugar and glucose production reached 8.0 and 4.6 g/L, respectively, which were 95% and 70% higher, respectively, than control. Thus, the incubation time of 36 h was used for further ethanol production experiments. Because 20 g/L of initial cellulose was used, the total yields of reducing sugar and glucose from cellulose were 40% and 23%, respectively. This is higher than a previous report using carboxymethyl cellulose (CMC) as the carbon source and performed in *T. reesei* with a total yield of 25.7% reduction sugar [17].

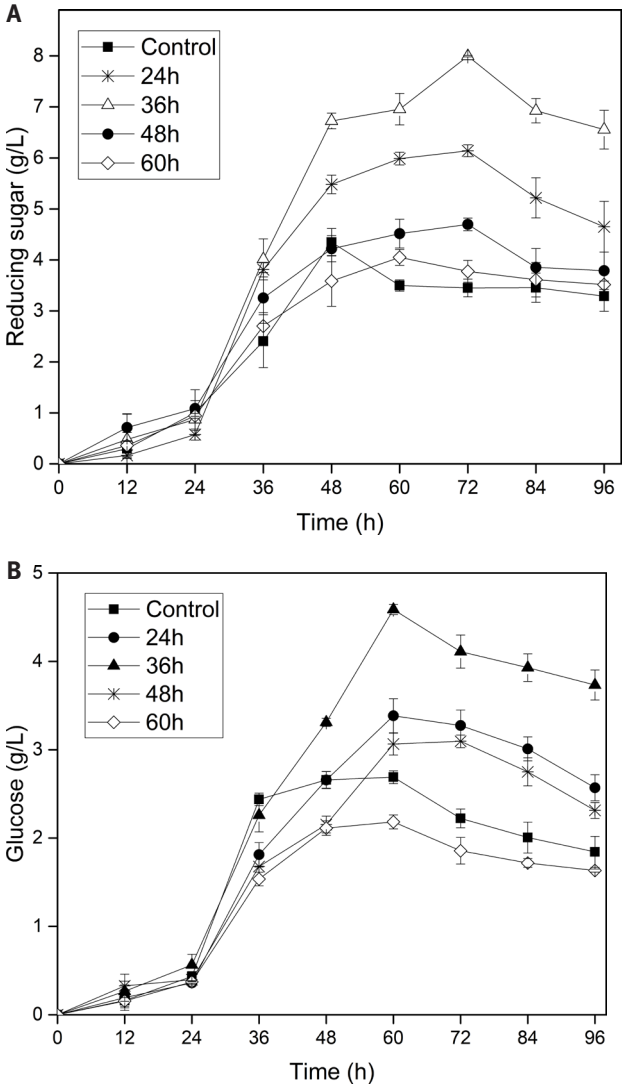

Figure 1: Time courses of cellulose hydrolysis by *T. reesei* to (a) reducing sugar production and (b) glucose production at different temperature rise times (24 to 60 h). The temperature rise time refers to the period of cultivation at 30°C prior to increasing to 50°C. For example, the 24 h temperature rise time means that *T. reesei* was first grown at 30°C and the temperature was risen to 50°C after 24 h. Control means constant temperature operation at 30°C (no temperature change).

The following optimized conditions were used for the conversion of cellulose into reducing sugar and glucose: pre-culture of *T. reesei* for 2 days, 10% (v/v) inoculum in cultivation medium, initial pH of 7, initial cellulose concentration of 20 g/L, and temperature rise from 30°C to 50°C at 36 h (Figure 2). After 72 h of *T. reesei* cultivation, the temperature was again lowered to 30°C, and *C. molischiana* was inoculated. The maximum ethanol concentration was 3.0 g/L at 120 h, resulting in a yield of 0.15 g of ethanol per g of cellulose. Considering that the maximum glucose production was 4.6 g/L at 60 h, the ethanol yield based on glucose (0.66 g of ethanol per g of glucose) was remarkably higher than the typical yield of 0.48 g of ethanol per g of glucose by *Saccharomyces cerevisiae*. This result reflects that *C. molischiana* utilizes cellodextrins as well as glucose. This yeast produces β-glucosidase that degrades cellobiose to glucose [11]. *C. molischiana* ferments cellodextrins with degree of polymerization 2 to 6 to ethanol [18]. The ability of this yeast to utilize cellulose degradation products for growth is advantageous for the production of lignocellulosic

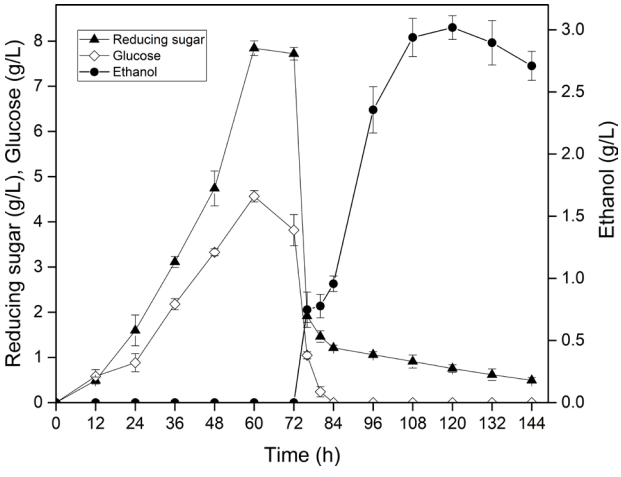

Figure 2: Ethanol production from cellulose by consortium of *T. reesei* and *C. molischiana*. *T. reesei* was initially grown at 30°C and the temperature was risen to 50°C after 36 h. After 72h, the temperature was again lowered to 30°C, and *C. molischiana* was inoculated.

Table 1: Summary of ethanol production from cellulosic substrates by consortium of microorganisms.

Consortium	Substrate	Reducing Sugar Yield (%)[a]	Glucose Yield (%)[b]	Ethanol Production Time (h)	Ethanol Yield (%)[c]	References
Acremonium cellulolyticus and *Saccharomyces cerevisiae*	Solka–Floc 50–300 g/L	–	–	72	12–19	[22]
Clostridium phytofermentans and *Saccharomyces cerevisiae* cdt–1 with added endoglucanase	α–Cellulose 100 g/L	–	–	400	22	[20]
Clostridium sp. and *Thermoanaerobacter* sp.	Avicel 11.1 g/L	–	–	–	17	[23]
Metabolically engineered *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum*	Avicel 92 g/L	–	–	146	41	[19]
Fusarium oxysporum and recombinant *Saccharomyces cerevisiae*	Pretreated wheat straw 110 g/L	–	–	48	4.5	[21]
Trichoderma reesei, *Aspergillus niger* and *Zymmomonas mobilis*	CMC 10 g/L	25.7	–	24	5.6	[17]
Trichoderma reesei and *Candida molischiana*	α–Cellulose 20 g/L	40	23	120	15	This study

[a] (g reducing sugar per g substrate supplied) × 100 (%)
[b] (g glucose per g substrate supplied) × 100 (%)
[c] (g ethanol per g substrate supplied) × 100 (%)

ethanol because not only glucose but also cellobiose and cellodextrins can be used as substrates for ethanol production. It is noteworthy that *C. molischiana* can perform the fermentation in the presence of the *T. reesei*, suggesting that there are not sufficient detrimental enzymes such as chitinases that would contribute to detrimental effects on the yeast to prevent the alcohol accumulation. This sequential approach does not require that *T. reesei* have alcohol tolerance as would be required for a simultaneous co-culture.

Table 1 compares ethanol production from cellulosic substrates by consortium of microorganisms. High ethanol yields were obtained using metabolically engineered strains of *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* [19] or by adding endoglucanase [20]. The combination of *Fusarium oxysporum* and recombinant *S. cerevisiae* produced 4.5% ethanol from 110 g/L pretreated wheat straw [21]. For wild-type strains, the ethanol yield (15%) obtained in this study is similar to or higher than previously reported. The co-culture of a hyper cellulase producer, *Acremonium cellulolyticus* C-1, and *S. cerevisiae* yielded 12 to 19% ethanol [22]. A thermophilic anaerobic *Clostridium* sp. isolated from a Himalayan hot spring yielded 17% ethanol by co-culture with *Thermoanaerobacter* sp. from Avicel [23]. The lower ethanol yield of 5.6% obtained by co-culture of *T. reesei* with *Aspergillus niger* and *Zymmomonas mobilis* from CMC [17] suggests that *C. molischiana* may have superior performance to *Z. mobilis* for ethanol production using some cellulose hydrolysates as carbon sources.

In summary, by increasing the temperature from 30°C to 50°C at 36 h for cellulose hydrolysis by *T. reesei*, reducing sugar and glucose production were improved by 95 and 70%, respectively, compared with no temperature rise. When the cellodextrin-utilizing yeast *C. molischiana* was inoculated at 60 h, the ethanol yield increased to 15% in 120 h. This study shows that direct ethanol production from cellulose may be possible by consortium of microorganisms when the hyper cellulose producer is available in a highly productive bioreactor mode of operation.

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