Consolidated Bioprocessing Using *Clostridium thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* Co-culture for Enhancing Ethanol Production from Corn Straw

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A co-culture consisting of *Clostridium thermocellum* ATCC 27405 and *Thermoanaerobacterium thermosaccharolyticum* DSM 571 was employed to improve the ethanol yield from microcrystalline cellulose (MCC) and corn straw substrates. An ethanol concentration of 1.29 g/L (26.1% ethanol yield) was obtained with 98.6% cellulose degradation when MCC was used as substrate in fermenter tanks. The ethanol yield obtained in fermenter tanks was 13.9% higher than that obtained *via* an anaerobic process performed in bottles. An ethanol concentration of 0.45 g/L, corresponding to 55.6% cellulose degradation and 11.2% ethanol yield, was achieved with corn straw as substrate in fermenter tanks. This ethanol yield was 28.2% higher than that formed in anaerobic bottles. Surprisingly, a 40.7% hemicellulose degradation was achieved *via* fermentation tanks, which was 127% higher than that obtained from anaerobic bottles.

Keywords: Co-culture; Consolidated bioprocessing; Corn straw; Ethanol; Clostridium thermocellum ATCC 27405; Thermoanaerobacterium thermosaccharolyticum DSM 571

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INTRODUCTION

China is one of the largest producers of agricultural-based products (Jiang *et al.* 2012). According to some statistics, about 800 million tons of agricultural crop residues are generated per year in China. Most of these residues are burned or returned to the field directly, which leads to environmental pollution and the waste of natural resources (Wang *et al.* 2010). Corn straw, an agricultural byproduct from corn crop, has attracted increased attention in the field of biofuel production in recent years (Li *et al.* 2016). Microbial fermentation of crop residues has some advantages such as low cost, high availability, and its environmentally friendly properties (Liu *et al.* 2016). Currently, various technologies, such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP), *etc.*, are employed for the concentration processes of cellulosic ethanol (Hahn-Hägerdal *et al.* 2006). A schematic diagram is shown in Fig. 1. Consolidated bioprocessing converts lignocellulose

to biofuels in a single step and with no additional enzyme consumption in the hydrolysis step (Lynd *et al.* 2005). The process is known as direct microbial conversion. It means that a single microbial community integrates all the processes of enzymes production, enzymatic saccharification, and fermentation. The CBP method represents a potential technological advance that could lead to lower costs and higher hydrolysis efficiency (Horisawa *et al.* 2015). However, the ethanol yield obtained is lower because of both the inability of the microbe to solubilize lignocellulose and produce the desired biofuels efficiently. Hence, effective cellulolytic microbes with the above properties are essential for the CBP method. *Clostridium thermocellum* is a thermophilic anaerobe that efficiently degrades lignocellulose for producing biofuels (Yang *et al.* 2009). This microbe was used as a typical strain for CBP technology. However, only a low bio-ethanol yield was obtained with real lignocellulose substrate because it could not efficiently catabolize pentose derived from hemicellulose. Engineering the organism may be an effective method to overcome this obstacle. Furthermore, the addition of a partner-microbe, which can utilize pentose and produce ethanol at high concentrations, is also a good method. *Thermoanaerobacterium thermosaccharolyticum* is a good candidate for a partner-microbe and when used with *C. thermocellum* can successfully grow on hexose and pentose sugars. Moreover, it is consistent with *C. thermocellum* growth conditions such as temperature, pH value, and oxygen demand (Chimtong *et al.* 2011).

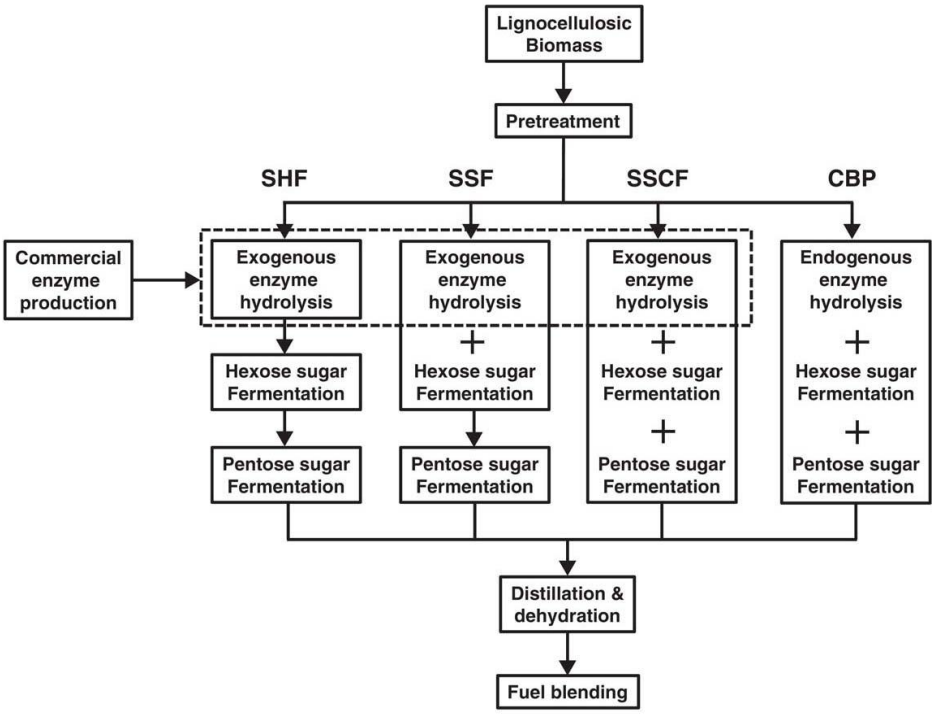

Fig. 1. Schematic diagram of SHF, SSF, SSCF, and CBP technology (Den *et al.* 2015)

A co-culture system has been used to improve hydrogen, ethanol, and butanol concentrations, as shown in previous reports (Chou *et al.* 2011; Cheng and Zhu 2013; Li *et al.* 2013). A co-culture of cellulolytic *C. thermocellum* NBRC 103400 and *C. saccharoperbutylacetonicum* strain N1-4 was used to produce butanol (5.5 g/L) with alkali-pretreated rice straw (40 g/L) as the carbon source, while the exoglucanase activity

was enhanced in the co-culture system (Kiyoshi *et al.* 2015). A co-culture system was established to increase the cellulose degradation ratio of *C. thermocellum via* the combination of bacteria W2-10. The results showed that the cellulose degradation ratio increased 37.8% with alkaline-treated wheat straw and 72.4% with filter paper as the carbon source. Additionally, the endoglucanase activity and biomass of *C. thermocell*um was significantly increased (Lu *et al.* 2013). A co-culture of *C. cellulolyticum* and hydrogen-producing bacteria with steam-exploded corn stover yielded a higher hydrogen concentration (51.9 L H_2 kg^{-1} total solid) than that from a monoculture (Zhang *et al.* 2016). Valdez-Vazquez *et al.* (2015) co-cultured *Enterococcus* and *Clostridium* to produce acetone, butanol, ethanol, and hydrogen through a consolidated two-stage bioprocessing system. First, *Enterococcus* was used to produce hydrogen with wheat straw as the carbon source. Secondly, 5.4 g/L of acetone, 14.2 g/L of butanol, and 3.7 g/L of ethanol were produced with a co-culture of *C. beijerinckii* 10132 and *C. cellulovorans* 35296 from treated wheat straw, which indicated the synergic effect between these species (Valdez-Vazquez *et al.* 2015). Li *et al.* (2012) operated a co-culture system of *C. thermocellum* and *C. thermosaccharolyticum* to enhance the hydrogen concentration with cornstalk as the carbon source. The co-culture yielded approximately 68.2 mL of hydrogen per gram of corn stalk, which was 94.1% higher than that of a monoculture. Furthermore, 74.9 mL of hydrogen per gram of cornstalk was obtained when fermentation was performed in an 8-L continuously stirred tank reactor, which was 28.24% higher than that obtained from anaerobic bottles (Li and Liu 2012).

In this work the authors examined the fermentation performance of microcrystalline cellulose (MCC) and corn straw when treated with *C. thermocellum* in the presence of *T. thermosaccharolyticum*. The objective was to study the co-culture system for increased ethanol yield and efficient lignocellulose degradation ratio compared to the monoculture when grown on real substrate, untreated corn straw.

EXPERIMENTAL

Materials

Corn straw was collected from the suburbs of Hohhot City (China). The corn straw was crushed, and the fraction passing through a 40-mesh screen was used in the subsequent tests. The microcrystalline cellulose (MCC) was (Avicel® PH-101, catalogue number: 11363). It was purchased from Sigma-Aldrich Chemicals Company (St. Louis, MO, USA).

Microorganisms and media

The microorganism strains were provided by Lee Lynd's lab at Dartmouth College, (Hanover, NH, USA). The strain of *C. thermocellum* was cultured with 5 g/L cellobiose as substrate in a modified medium for thermophilic clostridia (MTC). For the *T. thermosaccharolyticum*, 2.5 g/L of xylose and 2.5 g/L of cellobiose were added to the modified MTC medium as the carbon source (Shao *et al.* 2009). The MOPS sodium salt (100 g/L) was used to adjust the initial pH value. Stock solutions containing A, B, C, D, E, and F were deoxygenated with nitrogen gas and then sterilized following the reported procedure (Shao *et al.* 2011). All of the chemicals used were reagent-grade and were obtained from Sigma-Aldrich Chemicals Company (St. Louis, MO, USA), unless indicated otherwise.

Methods

Co-culture experiments in anaerobic bottles

All experiments were performed in 100-mL anaerobic bottles with 30 mL modified MTC medium, at 55 ºC, and at 150 rpm. Each bottle was sealed with a butyl rubber stopper and a screw cap, purged with 100% nitrogen gas, and degassed before autoclaving at 120 ºC for 21 min.

The sample strains *C. thermocellum* and *T. thermosaccharolyticum* were separately cultured in modified MTC medium and its initial pH value was adjusted to 7.5. The cell concentrations of *C. thermocellum* and *T. thermosaccharolyticum* were determined *via* spectrophotometry in an inoculation step. The culture conditions, *C. thermocellum* fermentation time, *C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio, and *T. thermosaccharolyticum* inoculation time for co-cultures were optimized with MCC and corn straw as carbon sources, in that order.

Fermentations with MCC and corn straw as substrates

To compare and confirm the performance of the co-culture fermentation system completely, MCC was used as a pure carbon source, and corn straw was used as a real lignocellulose feedstock. Approximately 5 g/L of MCC and 15 g/L of untreated corn straw were added to 100-mL serum bottles in a working volume of 30 mL. When the carbon source used was MCC, the test samples were taken in 12 h, 24 h, 36 h, 48 h, and 60 h time periods by withdrawing the fermentation liquid with a syringe from the serum bottle while shaking. For *T. thermosaccharolyticum* inoculation time, *T. thermosaccharolyticum* was inoculated into the liquid culture medium at 0 h, 4 h, 8 h, or 12 h after *C. thermocellum* inoculation.

When corn straw was used as the carbon source, the serum bottles were sampled at 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h, respectively. For *T. thermosaccharolyticum* inoculation time, *T. thermosaccharolyticum* was inoculated into the liquid culture medium at -48 h, -24 h, 0 h, 24 h, and 48 h of *C. thermocellum* inoculation. The *T. thermosaccharolyticum* cultures grown at the end of the logarithmic growth period were used along with *C. thermocellum* to inoculate the liquid medium at *T. thermosaccharolyticum* inoculation ratios of 1:1, 5:1, 10:1, 1:5, and 1:10. The inoculum volume of *C. thermocellum* in both mono- and co-cultures was 5% (MCC as substrate) and 15% (corn straw as substrate) of the liquid culture medium.

Scaling up of co-culture experiments

The INFORS HT Multifors 2 cell bioreactor (Swiss INFORS Biological Technology Co., Ltd., Beijing, China) with a working volume of 1 L was used to scale up the co-culture system. The reaction mixture was continuously stirred *via* magnetic stirring during fermentation. The sterilized fermenter tanks were flushed with nitrogen gas for 2 h. Then, the modified MTC medium containing 5 g/L of MCC and 15 g/L of corn straw was added to each fermenter tank in turn and autoclaved at 115 ºC for 30 min. Approximately 1 mL of corn oil was added as an antifoaming agent. Finally, *C. thermocellum* and *T. thermosaccharolyticum* were inoculated during the exponential-growth phase in the fermenter tanks under the optimum cultural condition. To keep the pH value of the fermentation liquid at 7.0, 2 M NaOH was added and the other fermentation was performed without any pH value regulation.

Methods

Analysis

Fermentation broths were centrifuged at 3500 × g for 10 min. Then the solid phase was washed 3 times with distilled water. The remaining sugar and ethanol contents were determined using a Waters high performance liquid chromatograph (HPLC) system (model: #2695, Milford, MA, USA), using a differential refractometer (model: e2414, Waters, Milford, MA, USA) and a Bio-Rad HPX-87H column (Hercules, CA, USA), which was operated with 0.01% (v/v) H_2SO_4 as the mobile phase at 40 ºC. The degradation products of cellulose and hemicellulose were measured using quantitative saccharification, as described by Shao *et al.* (2009). The cellulose degradation ratio and hemicellulose degradation ratio were calculated according to our previously reported protocol (Pang *et al.* 2017). Conversion ratios were calculated as a percentage of the original contents of solubilized glucan and xylan based on an analysis of residual solids. As previously reported, 36.3% of glucan and 14.9% of xylan in corn straw were obtained (Pang *et al.* 2017). The ethanol yields from MCC and corn straw were calculated based on the ethanol concentration in combination with the cellulose and hemicellulose degradation ratio values according to Eq. 1,

$$\text{Ethanol yield from MCC/ corn straw} = \frac{M_{\text{ethanol}}}{M_{\text{consumed}}} \times 100\% \quad (1)$$

where M_{ethanol} is the amount of ethanol present in the supernatant (g) and M_{consumed} is the amount of MCC/ corn straw consumed in the fermentation broth (g).

$$\text{Ethanol yield from MCC} = \frac{C_{\text{ethanol}} \times V_2}{C_i \times V_1 \times a \times c} \times 100\% \quad (2)$$

$$\text{Ethanol yield from corn straw} = \frac{C_{\text{ethanol}} \times V_2}{C_i \times V_1 \times a \times c + C_i \times V_1 \times b \times d} \times 100\% \quad (3)$$

where a is the percentage of glucan in MCC or corn straw (36.3%), b is the percentage of xylan in corn straw (14.9%), c is the glucan solubilization, d is the xylan solubilization, C_i is the initial concentration of MCC (5 g/L) or corn straw (15 g/L), C_{ethanol} is the ethanol concentration (g/L), V_1 is the broth volume after inoculation (L), and V_2 is the initial culture medium (L).

Statistical analysis

All of the experiments were conducted in triplicate, and the data are presented as mean values ± standard deviation. An analysis of variance (ANOVA) of the obtained results was performed using SAS 9.0 software (SAS Institute Inc., Cary, NC USA).

RESULTS AND DISCUSSION

Effect of *C. thermocellum* Culture Time on Ethanol Production

Microbes usually have different growth rates and product formation rates with various substrates. To confirm the optimal culture time of *C. thermocellum* with MCC and corn straw substrates, the ethanol concentration, cellulose/hemicellulose degradation ratio, and ethanol yield with time were monitored. The results are shown in Fig. 1. From Fig. 2(a), when MCC was used as the carbon source, the difference between 48 h and 60

h periods was not noticeable. The maximum cellulose degradation ratio, ethanol concentration, and ethanol yield obtained were 98.4%, 0.70 g/L, and 14.1%, respectively, at 48 h. From Fig. 2(b), when corn straw was used as the carbon source, the maximum ethanol concentration and yield were 0.20 g/L and 5.7%, respectively, with 56.9% cellulose degradation ratio and 16.6% hemicellulose degradation ratio when the culture time was 120 h.

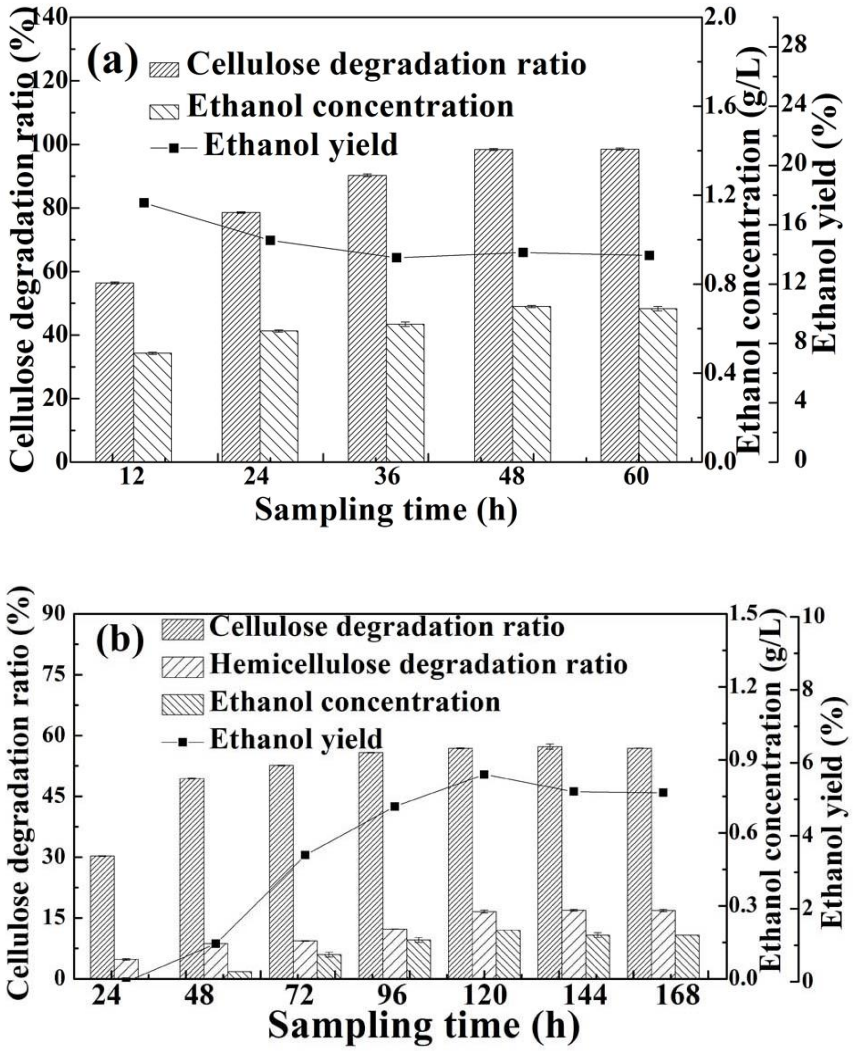

Fig. 2. Effects of culture time of *C. thermocellum* for fermentation performance with MCC (a) and corn straw (b) as substrates

Effect of Inoculation Time of *T. thermosaccharolyticum* on Ethanol Production

In order to achieve optimal ethanol production, the inoculation time of *T. thermosaccharolyticum* was explored in the co-culture system. As shown in Fig. 3(a), the highest ethanol concentration and yield were obtained when *T. thermosaccharolyticum* and *C. thermocellum* were inoculated at the same time in MCC. In this case, the maximum ethanol concentration and yield obtained were 0.83 g/L and 16.7%, respectively, with a 98.7% cellulose degradation ratio. As shown in Fig. 3(b), the optimum ethanol concentration and yield attained were 0.24 g/L and 7.08%, respectively,

with a cellulose degradation ratio of 56.4%, hemicellulose degradation ratio of 14.9% with corn straw as the carbon source when *C. thermocellum* and *T. thermosaccharolyticum* were inoculated simultaneously.

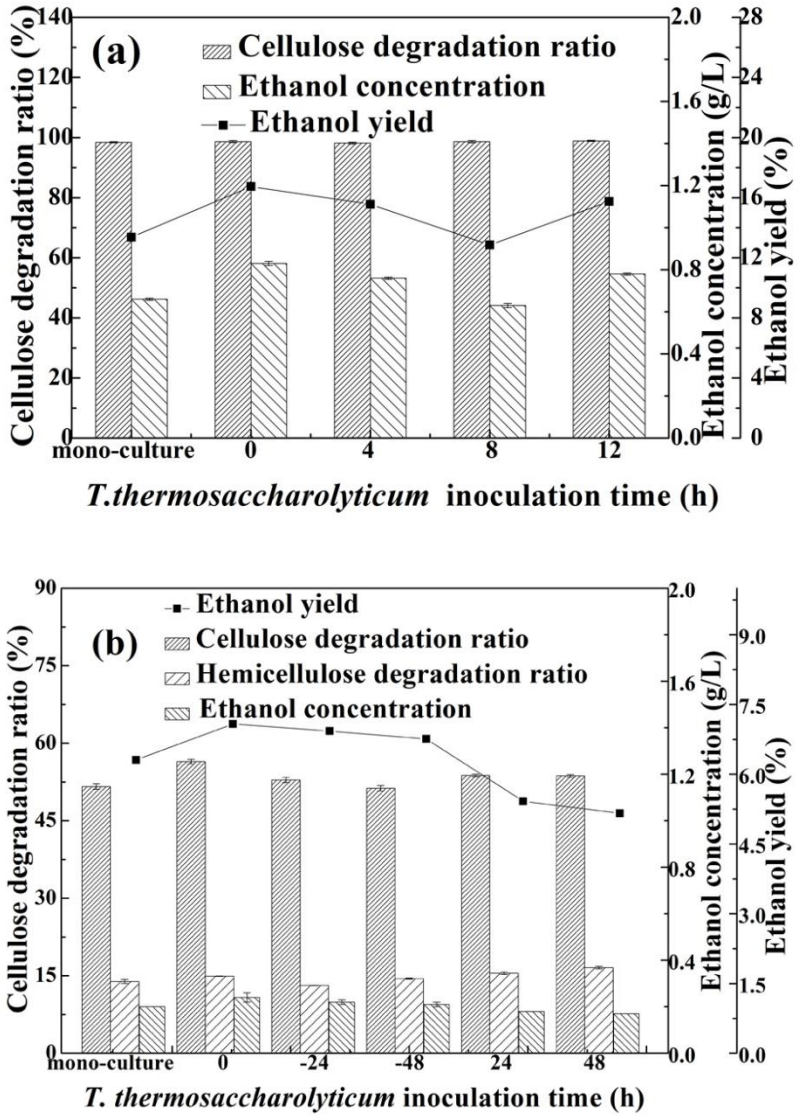

Fig. 3. Effects of inoculation time of *T. thermosaccharolyticum* for fermentation performance with MCC (a) and corn straw (b) as substrates

Effect of the Inoculation Ratio of *C. thermocellum* to *T. thermosaccharolyticum* on Ethanol Production

Various inoculation ratios of *C. thermocellum* to *T. thermosaccharolyticum* were attempted for maximum ethanol concentration. As shown in Fig. 4(a), the optimum ethanol concentration and yield found were 0.89 g/L and 18.10% with the cellulose degradation ratio of 98.7% with MCC as the carbon source when the inoculation ratio of *C. thermocellum* to *T. thermosaccharolyticum* was at 1:5. As shown in Fig. 4(b), the maximum ethanol concentration and yield were 0.29 g/L and 8.51%, respectively, with a cellulose degradation ratio of 54.7%, and a hemicellulose degradation ratio of 17.6% with

corn straw as the carbon source when the inoculation ratio of *C. thermocellum* to *T. thermosaccharolyticum* was at 1:5. The above results indicated that the ethanol concentration and yield were improved significantly ($P < 0.01$) in the co-culture compared to the monoculture. The ethanol yield was 37.6%, which was 49.8% higher than that from the monoculture of *C. thermocellum* for the same substrates. In general, the ethanol concentration from corn straw was lower than that from MCC due to its physical barriers and the recalcitrant nature of lignin present (Li and Liu 2012).

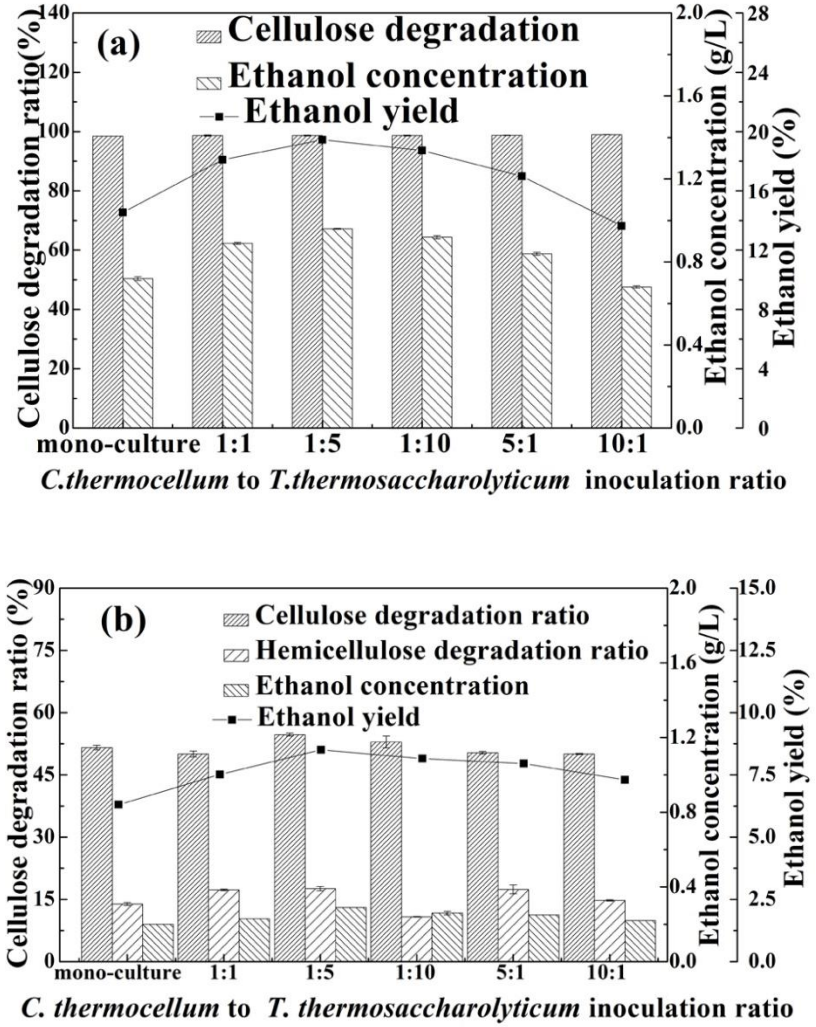

Fig. 4. Effects of inoculation ratios of *C. thermocellum* to *T. thermosaccharolyticum* for fermentation performance with MCC (a) and corn straw (b) as substrates

Scaling up of Co-culture Experiments

Finally, the co-culture system was scaled-up from the 100-mL anaerobic bottles to a fermenter tank with a working volume of 1 L using the same co-culture strategy founded in the serum bottle. A higher ethanol yield was obtained in the fermenter tanks when compared to that in anaerobic bottles due to more efficient mixing. As shown in Fig. 5(a), with MCC as the substrate, 1.29 g/L of ethanol and 26.1% yield was obtained. Approximately 98.6% cellulose was consumed when the pH value in the tanks was kept at 7.0 during fermentation. The ethanol yield in the fermentation tanks was 13.9% higher

than that in the anaerobic bottles, which was a noteworthy improvement compared to the unadjusted pH value conditions during fermentation. As shown in Fig. 5(b), with corn straw as the substrate, 0.45 g/L ethanol and 11.2% ethanol yield were obtained in fermentation tanks when the pH value of fermentation broth was adjusted and stabilized at 7.0. The ethanol yield was 28.2% higher than that in anaerobic bottles. The cellulose degradation ratio was 55.6%, which was not improved significantly.

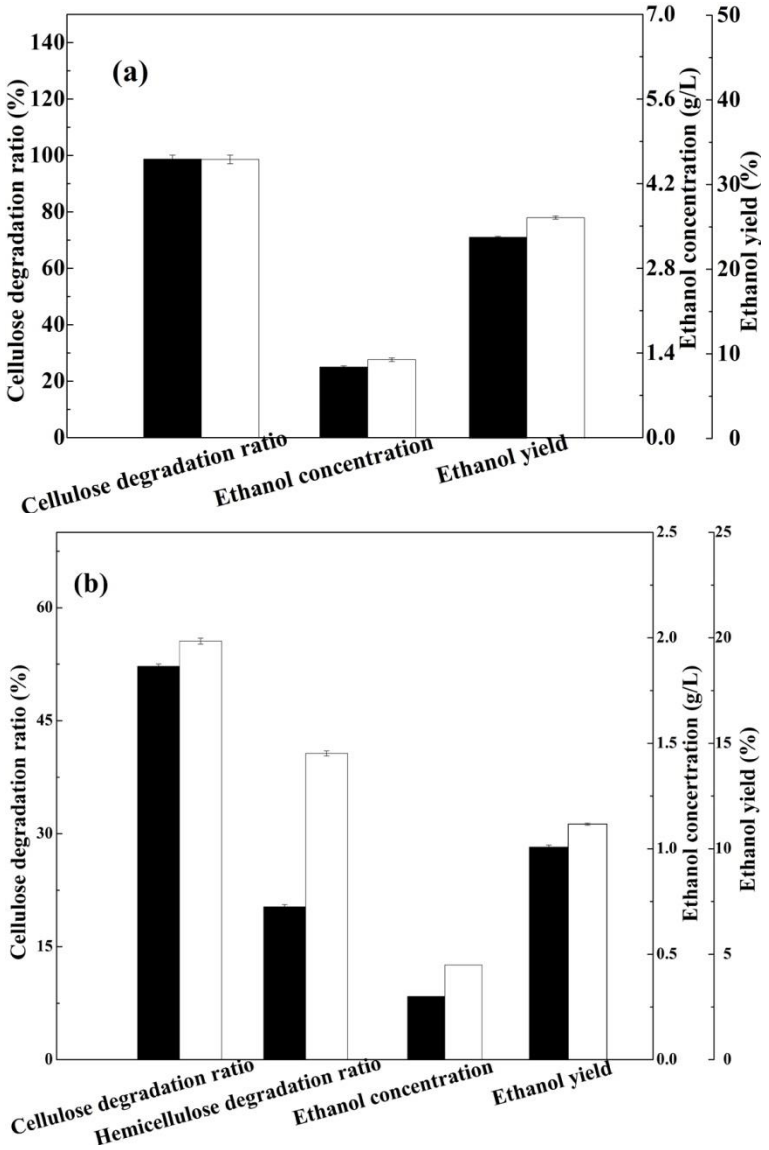

Fig. 5. Co-culture of *C. thermocellum* and *T. thermosaccharolyticum* with MCC (a), and corn straw (b) as substrates in fermentation tanks (black bar chart represents no pH adjustment; white bar chart represents pH adjusted medium)

Amazingly, the hemicellulose degradation ratio was 40.7%, which was approximately 127% higher than that from anaerobic bottles. Maybe the hemicellulase gene content increased its expression when the pH value was stable at 7.0 during fermentation. Alternatively, the cell growth was inhibited, and thus the production of hemicellulase decreased with the decline in pH value when the pH value was not

adjusted. The relevant mechanism about the improvement of hemicellulose degradation ratio will be researched in a future study. During fermentation, some kinds of acids (mainly acetic acid) are produced when *C. thermocellum* utilizes fermentable sugars. The pH value of the fermentation broth had a great influence on the ethanol concentration of *T. thermosaccharolyticum* (Li and Liu 2012). Therefore, adjusting the pH value of the fermentation broth during fermentation was essential. This was due to the cell growth and fermentation efficiency being affected when the pH value of the medium was changed. Furthermore, the pH value of the fermentation broth showed a decreasing trend that may restrain ethanol production (Zhu and Yang 2004). Additionally, the accumulation of the acetate and butyrate may affect the enzyme activities. All the above factors changed the ethanol yield and concentration. Sydney *et al.* (2014) optimized the pH value of the medium during the fermentation and obtained a higher H_2 concentration. Furthermore, 3.59 g/L of butanol was obtained from palm kernel cake by *C. saccharoperbutylacetonicum* N1-4 at optimum conditions when the pH value, temperature, and inoculum size were all varied (Shukor *et al.* 2014).

The ethanol concentration and yield was considerably enhanced when the co-culture system was scaled up from the anaerobic bottles to fermenter tanks. The adhesion between the cell and the substrate was increased with efficient stirring in the fermenter tank, and thus the degradation ratio of the lignocellulose and ethanol concentration increased. Li and Liu (2012) drew a similar conclusion and the hydrogen yield significantly increased when the co-culture system scaled-up from an anaerobic bottle to a bioreactor. *Enterobacter aerogenes* produced higher ethanol and hydrogen concentrations in the tank reactors than the small bottles from biodiesel-based glycerol (Jitrwung and Yargeau 2015). In previous research, Karagöz and Özkan (2014) co-cultured *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* to produce ethanol from wheat straw in batch and continuous systems. The ethanol concentration in the co-culture system was better than that in the monoculture *S. cerevisiae* (Karagöz and Özkan 2014). A co-culture system of *Fibrobacter succinogenes* A3c and *Prevotella ruminicola* H2b was established to degrade mature orchard grass. The cellulose degradation ratio was not changed, but the hemicellulose degradation ratio was significantly improved, which is consistent with the results from this study (Fondevila and Dehority 1996). The butanol concentration, yield and volumetric productivity significantly increased when *C. beijerinckii* and *C. tyrobutyricum* were co-cultured in free-cell, immobilized-cell fermentation, and continuous immobilized-cell mode (Gupta and Verma 2015).

CONCLUSIONS

1. A co-culture system consisting of *C. thermocellum* and *T. thermosaccharolyticum* was established to produce ethanol from microcrystalline cellulose (MCC) and corn straw *via* CBP.
2. The optimal co-culture conditions were obtained in anaerobic bottles and then scaled-up in fermenter tanks for higher ethanol concentration. Ethanol concentration was improved with co-culture comparing with mono-culture.
3. The hemicellulose degradation ratio was significantly improved with corn straw as substrate on optimal co-culture conditions in fermenter tanks.

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