Enhancing the Ethanol Yield from *Salix* Using a *Clostridium thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* Co-Culture System

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A strategic method that utilizes the co-culture of *Clostridium thermocellum* ATCC 27405 and *Thermoanaerobacterium thermosaccharolyticum* DSM 571 was developed to improve the ethanol yield from the thermophilic fermentation of *Salix*. The co-culture conditions of the two strains were optimized using single factor and response surface experiments to enhance the ethanol yield. An even higher ethanol yield was obtained under the optimum co-culture conditions in fermenter tanks than what was observed in pre-experiments in serum bottles. The maximal ethanol concentration and yield were 0.2 g/L and 11.1%, respectively, and with a 26.4% cellulose degradation ratio and 13.8% hemicellulose degradation ratio when the pH was kept stable at 7.0 in fermenter tanks.

Keywords: Clostridium thermocellum ATCC 27405; Thermoanaerobacterium thermosaccharolyticum DSM 571; Co-culture; Salix; Fermenter tank; Ethanol

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INTRODUCTION

Utilization of biomass as feedstock for production of sustainable transportation fuels is of interest (Yamada *et al.* 2013), with lignocellulosic biomass being of particular interest in this context (Gupta and Verma 2015; Li *et al.* 2016). The primary obstacle impeding the widespread production of energy from lignocellulosic biomass is the absence of a low-cost technology for overcoming the recalcitrance of these materials (Dias *et al.* 2011).

Consolidated bioprocessing (CBP), in which the production of cellulolytic enzymes, biomass hydrolysis, and fermentation of resulting sugars to desired products occur in a single process step, represents a potential technological advance that could lead to lower costs and higher efficiency of cellulosic bio-ethanol production (Olson *et al.* 2012; Schuster and Chinn 2012; Yamada *et al.* 2013; Horisawa *et al.* 2015). CBP-enabling microorganism(s) must be able to effectively solubilize lignocellulosic biomass substrates and produce the desired products at high yields and titers under industrial conditions. *C. thermocellum* can rapidly solubilize cellulose, and it has often been considered for use in a CBP process configuration. However, *C. thermocellum* does not utilize the pentoses derived from hemicellulose as carbon source, which means that it cannot utilize 30% to

40% of the total carbohydrates in plant biomass. As a result, improving the utilization of pentoses is a key factor for making *C. thermocellum* CBP commercially viable.

Co-culture of different types of microorganisms is one strategy to overcome the limitations of particular microbes, and there are some reports about the co-culture of different kinds of microorganisms as ways to improve hydrogen, ethanol, and butanol production (Chou *et al.* 2011; Cheng and Zhu 2013; Li *et al.* 2013). However, the results of these studies varied greatly with different microorganisms in co-culture systems. Cheng and Zhu (2013) established a co-culture of *C. thermocellum* and *T. aotearoense* for bio-hydrogen production, using sugarcane bagasse (SCB) that was pretreated under mild alkali conditions, demonstrating the synergy and the economic advantages of the co-culture over monocultures of either *C. thermocellum* or *T. aotearoense*. In a progress report from Daniel I. C. Wang, Charles L. Cooney and their colleagues, *C. thermocellum* and *C. thermosaccharolyticum* were co-cultured to produce ethanol with real biomass, corn stover as substrate. The ethanol productivity was improved when the corn stover was treated with 1% alkali; however the dry weight of the substrate was lost (Wang *et al*. 1980). Co-cultures of *Candida shehatae* and *Saccharomyces cerevisiae* showed ethanol yields (YP/S) of 0.42 and 0.51 in synthetic medium and in rice hull hydrolysate (RHH), respectively, while pure cultures of *C. shehatae* produced slightly lower ethanol yields of 0.40 (Hickert *et al.* 2013). *C. beijerinckii* and *C. tyrobutyricum* were co-cultured in free-cell, immobilized-cell fermentation, and continuous immobilized-cell mode, which significantly enhanced butanol production, yield, and volumetric productivity (Gupta and Verma 2015). In nature, the rumen microorganism is a typical example of co-culture. Perhaps unsurprisingly, when cellulolytic bacteria and non-cellulolytic rumen bacteria were co-cultured to observe the changes of cellulose degradation, the results differed greatly for different combinations of microorganisms (Fondevila and Dehority 1996; Shi *et al.* 1997; Chen and Weimer 2001). *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, and *Ruminococcus albu*s 7 were co-cultured in the presence or absence of the non-cellulolytic ruminal bacteria *Selenomonas ruminantium* or *Streptococcus bovis*, and the relative abundance of the three strains as well as the relative yields of fermentation products were studied. *S. ruminantium* altered the relative proportions of the cellulolytic species. *R. albus* and *R. flavefaciens* were found to produce inhibitors that suppressed growth of *R. flavefaciens* and *F. succinogenes*, respectively (Chen and Weimer 2001). The interactions between *Fibrobacter succinogenes*, *Prevotella ruminicola*, and *Ruminococcus flavefaciens* in the digestion of cellulose from Forages were also studied. When the non-cellulolytic *P. ruminicola* was co-cultured with either of the two cellulolytic species (*F. succinogenes* or *R. flavefaciens*), forage cellulose digestion numerically increased over that of the cellulolytic species alone. However, when *F. succinogenes* and *R. flavefaciens* were co-cultured, cellulose digestion was reduced compared to *F. succinogenes* alone (Fondevila and Dehority 1996). Complementary action between *Butyrivibrio fibrisolvens* D1 and either of the two *F. succinogenes* strains (*Fibrobacter succinogenes* S85 and BL2) was identified with respect to co-culture growth and carbohydrate utilization. With the addition of *B. fibrisolvens*, the solubilization of cell walls of both untreated wheat straw and sulfur-dioxide-treated wheat straw did not change, whereas the concentration of cells increased when *Fibrobacter succinogenes* BL2 was used in the co-cultures (Shi *et al.* 1997). Past research has also reported the co-culture of *C. thermocellum* and C5 sugar fermenting thermophilic ethanologenic bacteria of the genera *Thermoanaero bacterium* and *Thermoanaerobacter*

(Argyros *et al*. 2011). Co-cultures of cellulolytic *C. thermocellum* with non-cellulolytic *Thermoanaerobacterium* strains (X514 and 39E) significantly improved ethanol yield by a striking 194% to 440%. Strain X514 enhanced ethanolic fermentation much more effectively than strain 39E in co-cultivation, with ethanol production in X514 co-cultures being at least 62% higher than in 39E co-cultures (He *et al.* 2011). In a similar study, a co-culture of *C. thermocellum* and *C. thermohydrosulfuricum* actively fermented MN300 cellulose, microcrystalline cellulose, Solka floc, SO_2-treated wood, and steam-exploded wood, with a threefold increase in the ethanol production rate compared to a monoculture of *C. thermocellum* (Ng *et al.* 1981).

Salix as a type of biomass resource plays an important role in breaking wind and fixing sands, purifying air, and conserving water and resources in Inner Mongolia. However, large numbers of deadwood are produced after the process of stumping rejuvenation ever year, which causes environmental pollution and waste of resources. Therefore, new ideas are proposed to reuse *Salix* based on the regional features. In this study, a co-culture system of *C. thermocellum* and *T. thermosaccharolyticum* was established to increase ethanol yield from abundant *Salix* in Inner Mongolia. The strains' co-culture conditions were optimized to enhance the ethanol yield using single-factor and response surface experiments. The results were confirmed by scaling up from 100 mL anaerobic bottles to 1 L fermenter tanks.

EXPERIMENTAL

Materials

Untreated *Salix psammophila* was collected in autumn from the suburbs of Hohhot City, P.R. China, and ground to pass through a 0.425 mm screen. The cellulose and hemicellulose contents of the resulting biomass were 38.4% and 12.2%, respectively.

Microorganisms and media

C. thermocellum and *T. thermosaccharolyticum* were kindly provided by Lee Lynd, Dartmouth College, USA. Seed cultures of *C. thermocellum* were grown for 24 h at 55 ºC under constant orbital shaking at 180 rpm. *T. thermosaccharolyticum* was cultured under the same conditions for 30 h. To ensure the consistency of mono- and co-cultures, both *C. thermocellum* and *T. thermosaccharolyticum* were grown in 150 mL serum bottles in modified MTC medium prepared as described by Zhang and Lynd (2003), with the exception of the addition of MOPS. Stock solutions comprising 100 g/L MOPS sodium salt was adjusted to different pH values using 72% H_2SO_4. Solution A contained the respective carbon sources supplemented with an appropriate amount of distilled water. For seed cultures of *C. thermocellum*, 5 g/L Avicel PH105 was added to the modified MTC medium as the carbon source. For seed cultures of *C. thermocellum*, 5 g/L cellobiose (*T. thermosaccharolyticum*, 2.5 g/L xylose and 2.5 g/L cellobiose) were used as carbon source (Shao *et al.* 2009). The initial concentration of Avicel PH105 was 5 g/L, and untreated *Salix* was used at a concentration of 15 g/L. Solutions A, B, C, D, E, and F were mixed, purged with nitrogen gas, and sterilized, according to a published protocol (Shao *et al.* 2011).

Methods

Growth curve of both bacteria in monocultures

Growth curves of *C. thermocellum and T. thermosaccharolyticum* were inoculated into seed cultures and the cell concentration were determined based on the absorbing value of 600 nm every 2 h.

Co-culture of C. thermocellum and T. thermosaccharolyticum in serum bottles

The co-culture experiments were executed in 100 mL serum bottles (Cang Zhou Ming Jie Leechdom Co., Ltd., China) with an active volume of 30 mL, and N_2-gassed glass containers with screw-top sealable metal lids before being autoclaved at 120 ºC for 21 min. To explore the optimal *C. thermocellum* fermentation time, *T. thermosaccharolyticum* inoculation time, and *C. thermocellum* to *T. thermosaccharolyticum inoculation ratio* for ethanol yield and the experiments using *Salix* as carbon source, *C. thermocellum* and *T. thermosaccharolyticum* were separately cultured in modified MTC medium without resazurin.

The concentration of *Salix* was 15 g/L, and a 10% (v/v) inoculum of *C. thermocellum* was inoculated in the liquid culture medium in both mono- and co-cultures. To optimize the *C. thermocellum* fermentation time, samples were taken at 96, 120, 144, and 168 h, by withdrawing fermentation liquid from the serum bottle using a syringe while shaking. To optimize the inoculation time of *T. thermosaccharolyticum*, the bacteria were inoculated at -48, -24, 0, 24, or 48 h of *C. thermocellum* inoculation. *T. thermosaccharolyticum* cultures grown for 30 h 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 optimization criterion was maximum ethanol yield.

Response surface analysis

Culture conditions were optimized *via* response surface methodology (RSM) based on Box-Behnken designs (Box and Behnken 1960). The variables containing X_1 (*T. thermosaccharolyticum* inoculation time, h), X_2 (*C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio) and X_3 (*C. thermocellum* sampling time, h) were carried out to grope the optimum condition *via* single-factor test. Subsequently, a three-factor two-level analysis of the response surface experiments was designed to study the optimal values of the three factors and the interactions between them according to the Box-Behnken central composite principle. The response surface experimental design of variables is shown in Table 1. In this study, the experimental design contained fifteen trials.

Table 1. The Response Surface Experimental Design of Variables

Factor	Level		
	-1	0	1
X_1	-12	0	12
X_2	1:1	1:5	1:10
X_3	96	120	144

Note: X_1, *T. thermosaccharolyticum* inoculation time (h); X_2, *C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio; X_3, *C. thermocellum* sampling time (h)

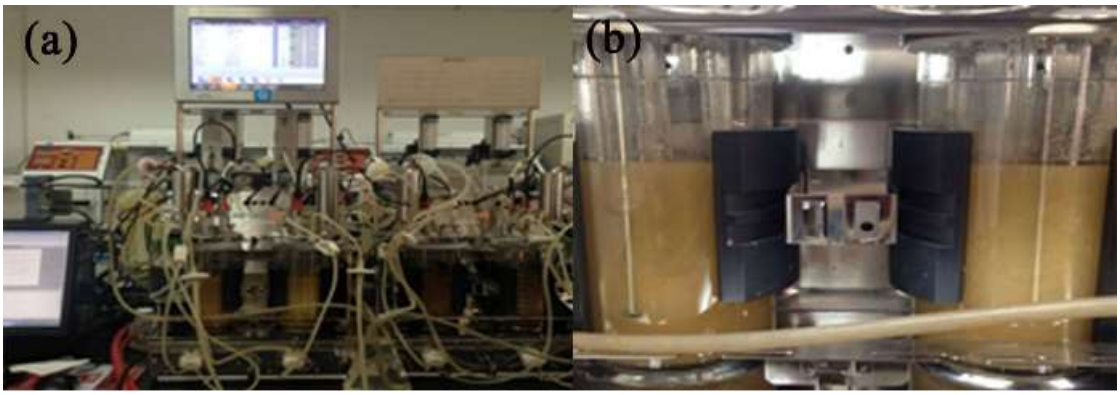

Fig. 1. The fermenter tanks were used in this study. a: overall configuration; b: detail of the stirred tanks

Co-culture of C. thermocellum and T. thermosaccharolyticum in fermenter tanks

The INFORS HT Multifors 2 Cell bioreactor (Swiss infors (INFORS) biological technology (Beijing Co., Ltd, China) with an active volume of 1 L was used to extend the co-culture system (Fig. 1). A magnetic stirrer was used continuously to ensure sufficient mixing. An initial load comprising 0.56 L of distilled water with 15 g/L of *Salix* was added to each fermenter tank and autoclaved at 115 ºC for 30 min. The sterilized fermenter tanks were flushed with nitrogen gas for 2 h prior to adding 72 mL of 100 g/L MOPS buffer pH 7.5, 72 mL yeast extract, 32 mL of solution B, and 16 mL of each of the solutions D, E, and F to each one. Finally, 1 mL of corn oil was added as an antifoaming agent. All chemicals used were molecular biology or analytical grade, unless indicated otherwise. Exponential-phase cultures of *C. thermocellum* and *T. thermosaccharolyticum* were used to inoculate the fermenter tanks according to the inoculation times, fermentation time and ratios shown in section 2.4. One fermentation experiment was performed with automated addition of 2M NaOH, in order to keep the pH at 7, and another was performed without active pH regulation.

Equations

The solid and liquid phases were separated by centrifugation at 3500 × g for 10 min, after which the solid phase was washed three times with distilled water. The remaining sugar titer and the concentrations of the main fermentation products in the supernatant were determined using a Waters HPLC system (#2695, Milford, MA, USA), equipped with a differential refractometer (e2414, Waters USA) and a Bio-Rad HPX-87H column (Hercules, CA, USA), which was operated with 0.01% (v/v) H_2SO_4 as mobile phase, and kept at 40 ºC. The degradation of cellulose and hemicellulose was measured using quantitative saccharification, as described by Shao *et al.* (2009, 2011). Briefly, 1 mL of 72% (w/v) H_2SO_4 was added to a 28 mL aliquot of the supernatant and autoclaved at 121 ºC for 60 min. After filtering through a centrifugal filter cartridge (0.22 um, Shan Yu Technologies Co. Ltd., China), the product concentrations were determined using the Waters HPLC system. Conversions were calculated as percentages of the originally present solubilized glucan and xylan, based on analysis of residual solids. Glucan and xylan solubilization ratios were calculated according to Eqs. 1 and 2. The degradable components of *Salix* comprise 38.4% glucan and 12.2% xylan. *Salix* degradation was calculated *via*

glucan solubilization and xylan solubilization using the method of Jian Pang (Pang *et al.* 2017). The ethanol yield from *Salix* was calculated based on the ethanol concentration in combination with the cellulose and hemicellulose solubilization values,

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

where M_{ethanol} is the amount of ethanol in the fermentation supernatant (g), and $M_{\text{cellulose consumed}}$ is the amounts of *Salix* consumed in the fermentation broth (g).

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

In Eq. 2, a is the percentage of glucan in *Salix* (38.42%), b is the percentage of xylan in *Salix* (14.86%), c is glucan solubilization, d is xylan solubilization, C_i is the initial concentration of *Salix* (15 g/ L), V_1 is the broth volume after inoculation (L), and V_2 is the initial volume of culture medium (L).

Statistical Analysis

All 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 conducted with SAS 9.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Co-Culture Experiments in Serum Bottles

Previous studies showed that co-fermentation of ethanol-producing bacteria with cellulolytic bacteria can significantly improve ethanol production (Da Cunha-Pereira *et al.* 2011). In this study, cheap and abundant *Salix* waste was used to produce ethanol by co-culture of *C. thermocellum* and *T. thermosaccharolyticum*. The growth curves of *C. thermocellum* and *T. thermosaccharolyticum* in monocultures were shown in Fig. 2 by measuring optical density at 600 nm. From the Fig. 2, the exponential phase of *C. thermocellum* and *T. thermosaccharolyticum* was made and microorganisms' biomass was quantified by determining the OD value at 600 nm.

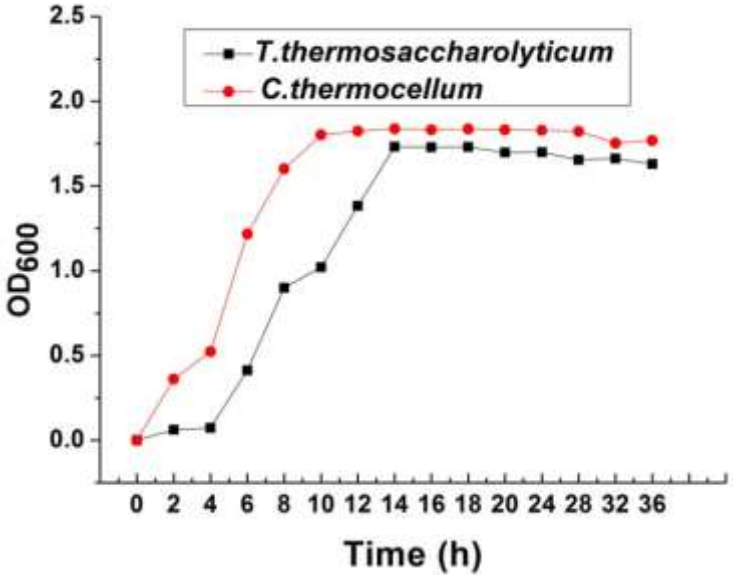

Fig. 2. The growth curves of *C. thermocellum* and *T. thermosaccharolyticum* in monocultures

Effects of C. thermocellum fermentation time, T. thermosaccharolyticum inoculation time, and C. thermocellum to *T. thermosaccharolyticum inoculation ratio on ethanol yield*

As shown in Fig. 3a, after fermentation for 120 h with *Salix* as carbon source, the maximum cellulose and hemicellulose degradation ratios, ethanol concentration, and ethanol yield were 27.2%, 14.4%, 0.13 g/L, and 7.3%, respectively. It led to low ethanol yield that substrates were not adequately utilized. The highest ethanol yield was measured at the beginning of the culturing process because the degradation ratios of cellulose and hemicellulose were very low at the start. The reasons why substrates were not adequately utilized were supposed as the following. Firstly, substrate, *Salix*, is composed of cellulose, hemicellulose, and lignin. The cellulose and hemicellulose are covered by lignin. Lignin is very complex heterogeneous mixture of phenolic compounds. Hence the natural barrier, lignin, hindered the contact between cellulase and cellulose or hemicellulose. As a result, the substrate without pretreatment would not be adequately degraded and utilized. Secondly, with the increase in concentration of some end metabolites, for example acetate and formate, pH value decreased. As a result, cell growth declined, ethanol concentration decreased, and a low ethanol yield was achieved. Lastly, the feedback inhibition caused by ethanol also led to inadequate substrate utilization.

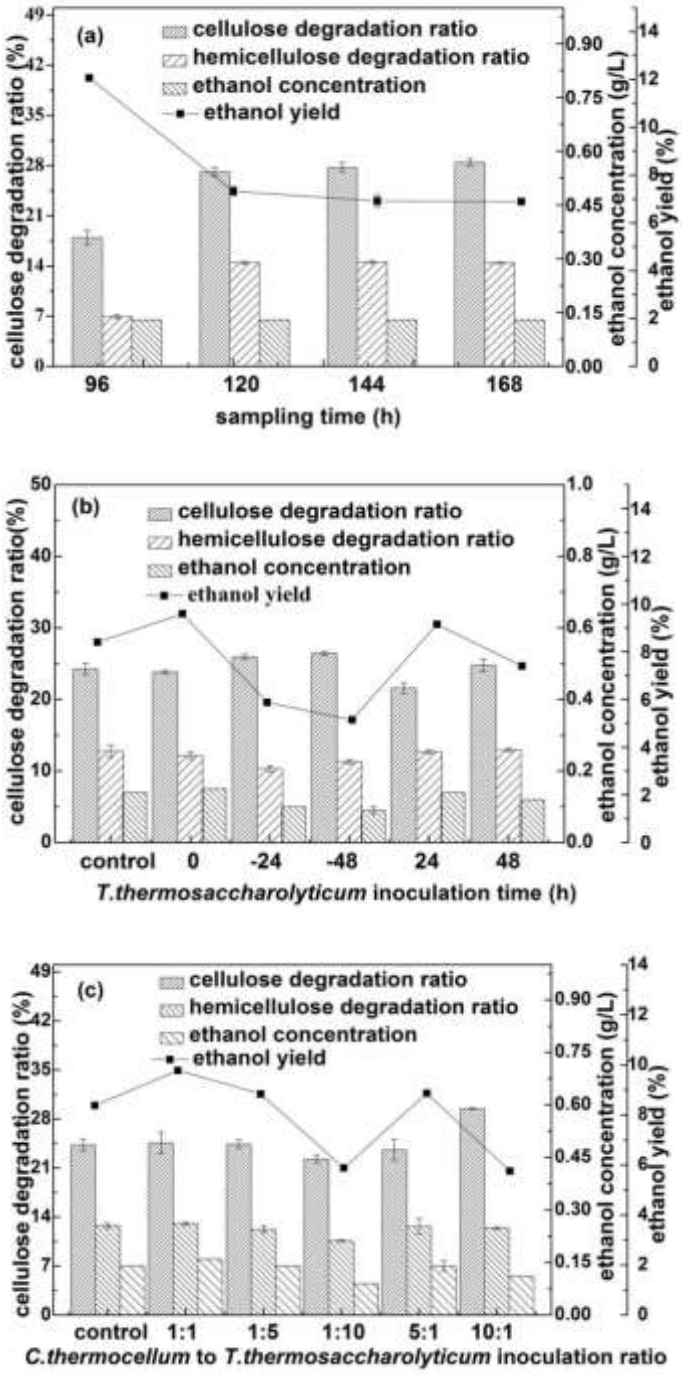

Fig. 3. Co-culture performance of *C. thermocellum and T. thermosaccharolyticum* in anaerobic bottles: (a) *C. thermocellum* sampling time; (b) inoculation time of *T. thermosaccharolyticum*; and (c) *C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio

The timing of *T. thermosaccharolyticum* inoculation in anaerobic bottles was studied due to the rationale that the rapid growth of *C. thermocellum* could synthesize cellulosome to hydrolyze *Salix*. As shown in Fig. 3b, ethanol production and yield were limited when inoculation of *T. thermosaccharolyticum* was followed by *C. thermocellum*.

In this case, the maximum ethanol concentration and yield were 0.15 g/L and 9.6%, respectively, with 23.9% of cellulose and 12.2% of hemicellulose consumed.

As shown in Fig. 3c, ethanol yield and production were increased in the co-culture process relative to a monoculture of *C. thermocellum*. Among the combination ratios of *C. thermocellum* to *T. thermosaccharolyticum* tested, the maximal ethanol concentration and yield (0.16 g/L and 9.8%, respectively) were obtained at the ratio of 1:1 (v/v), with 24.6% cellulose and 13.1% hemicellulose degradation ratios. The ethanol yield was improved significantly ($P < 0.01$) in the co-culture compared to the mono-culture, with a 33.4% higher ethanol yield.

In general, the biodegradation of lignocellulosic *Salix* is difficult due to physical barriers and the recalcitrant crystalline structure of lignin. In this study, the cellulose and hemicellulose degradation ratios from *Salix* showed little change because of the distinctive structure and complexity of this recalcitrant carbon source, which may explain why ethanol production from this complex substrate was lower than from microcrystalline cellulose (Li and Liu 2012). There are two ways to increase degradation ratios for recalcitrant crystalline substrates. Usually a pretreatment method has been used to increase degradation ratios for recalcitrant crystalline substrates (Rouches *et al*. 2016). The cost will be raised and pollution will result, although high degradation ratios can be achieved. In the present study, a second way was applied. Degradation ratio was increased by reducing the hydrolysate concentration of substrate. *C .thermocellum* cannot utilize pentose, the hydrolysate of hemicellulose. As a result, the high concentration pentose decreased degradation ratios by feedback inhibition. *T. thermosaccharolyticum* was used as a partner to relieve the feedback inhibition because it can ferment pentose to ethanol and other products.

Response Surface Analysis to Determine the Optimal Culture Conditions

Predicted and analysis of response surface model.

Response surface methodology was used to explore the interactions of the variables containing *T. thermosaccharolyticum* inoculation time (X_1), *C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio (X_2), and *C. thermocellum* sampling time (X_3). The results of the Box-Behnken design experiment with microcrystalline cellulose as substrate are shown in Table 2. The response surface model of response value (Y) was expressed *via* multiple regression analysis according to Eq. 3, based on the experimental data,

$$Y = 0.164333 + 0.00875\,X_1 - 0.00825\,X_2 + 0.0135\,X_3 - 0.024167\,X_1X_1 - 0.0015\,X_1X_2 + 0.005\,X_1X_3 - 0.020667\,X_2X_2 - 0.0045\,X_2X_3 - 0.030167\,X_3X_3 \quad (3)$$

where Y is the ethanol concentration, whereas X_1, X_2, and X_3 are the coded variables for *T. thermosaccharolyticum* inoculation time, *C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio, and *C. thermocellum* sampling time, respectively.

Table 2. Box–Behnken Experimental Design with Three Independent Variables

RUN	Coded and Noncoded Variable Levels			Y_1/Ethanol concentration (g/L)
	X_1/ *T. thermosaccharolyticum* inoculation time (h)	X_2/ *C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio	X_3/ *C. thermocellum* sampling time (h)	
1	-1 (-12)	-1 (1:5)	0 (120)	0.11
2	-1 (-12)	1 (5:1)	0 (120)	0.10
3	1 (12)	-1 (1:5)	0 (120)	0.13
4	1 (12)	1 (5:1)	0 (120)	0.11
5	0 (0)	-1 (1:5)	-1 (96)	0.10
6	0 (0)	-1 (1:5)	1 (96)	0.13
7	0 (0)	1 (5:1)	-1 (96)	0.09
8	0 (0)	1 (5:1)	1 (144)	0.10
9	-1 (-12)	0 (1:1)	-1 (96)	0.08
10	1 (12)	0 (1:1)	-1 (96)	0.09
11	-1 (-12)	0 (1:1)	1 (144)	0.10
12	1 (12)	0 (1:1)	1 (144)	0.13
13	0 (0)	0 (1:1)	0 (120)	0.16
14	0 (0)	0 (1:1)	0 (120)	0.17
15	0 (0)	0 (1:1)	0 (120)	0.17

Note: The center point was replicated three times and the others were replicated twice

Table 3. ANOVA for Ethanol Production

Source	Statistic				
	(Degrees of freedom) DF	(Sum of squares) SS	(Mean square) MS	(F-value) F	Prob>F
Model	9	0.01	0.00	104.78	0.00
X_1	1	0.00	0.00	64.25	0.00
X_2	1	0.00	0.00	57.12	0.00
X_3	1	0.00	0.00	152.94	0.00
X_1X_1	1	0.00	0.00	226.20	0.00
X_1X_2	1	0.00	0.00	0.94	0.38
X_1X_3	1	0.00	0.00	10.49	0.02
X_2X_2	1	0.00	0.00	165.42	0.00
X_2X_3	1	0.00	0.0	8. 50	0.03
X_3X_3	1	0.00	0.00	352.46	0.00
Error	5	0.00	0.00		
Total	14	0.01			

Note: R^2=0.9947; C.V. (coefficient of variation) = 2.70; F = 104.77; Values of "Prob>F" lower than 0.05 were significant

The adequacy and significance of response surface model was tested by ANOVA, and results of corresponding quadratic model fitting for ethanol production are shown in Table 3. The independent variables and quadratic terms significantly affected the ethanol concentration. The results also showed that the interaction between X_1, X_2, and X_3 was significant.

Analysis of the response surface and identification of optimal co-culture conditions for maximal ethanol production

As shown in Fig. 4, the response surface model with two-dimensional contours and three-dimensional representation shows a connection between independent and dependent variables. The variables had different interactions when the contours displayed different shapes. The interactions between variables were significant when the elliptical contour plot appeared.

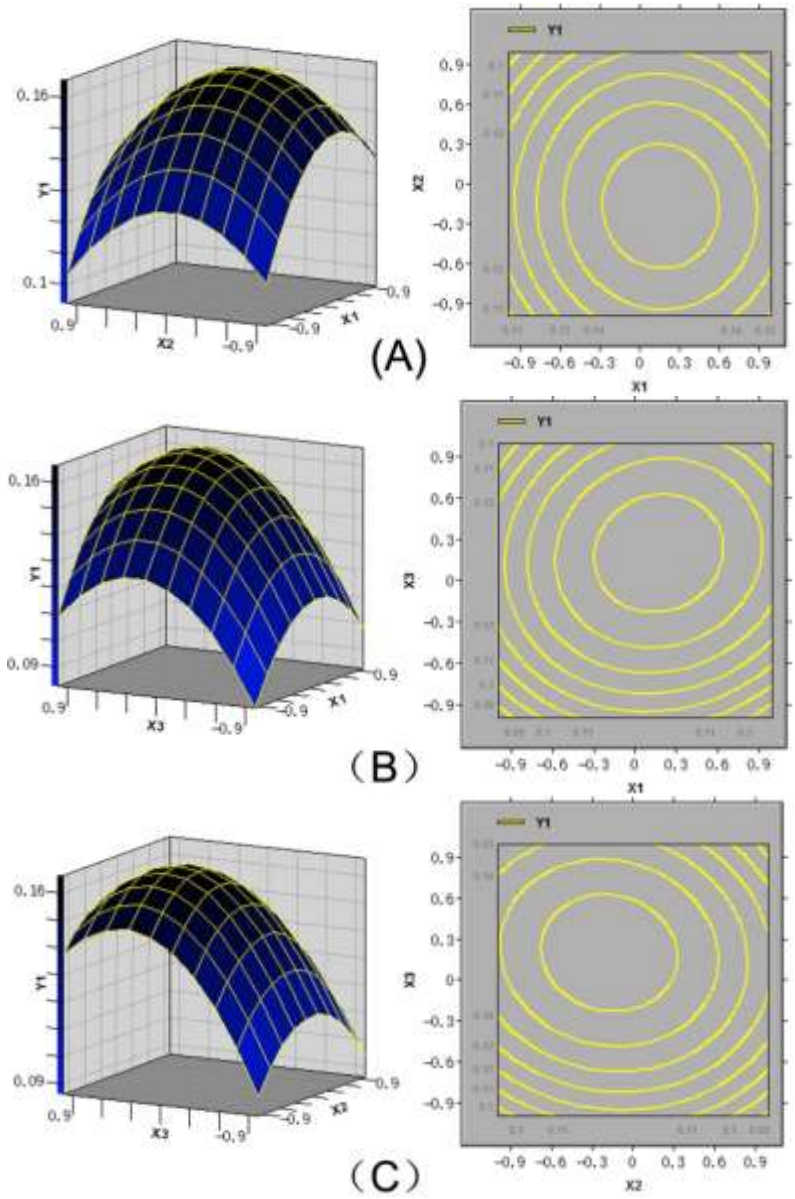

Fig. 4. Two- and three-dimensional contour plots indicate maximum ethanol concentration. The Response surface methodology plots were formed based on the data shown in Table 2. The 15 experimental fermentative runs executed according to the conditions of Box-Behnken design. (A) Ethanol concentration (g/L) as an index of *T. thermosaccharolyticum* inoculation time and *C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio. (B) Ethanol concentration (g/L) as an index of *T. thermosaccharolyticum* inoculation time and *C. thermocellum* sampling time. (C) Ethanol concentration (g/L) as an index of *T. thermosaccharolyticum* to *C. thermocellum* inoculation ratio and *C. thermocellum* sampling time.

As shown in the figure, the two- and three-dimensional contour plots indicating ethanol concentration versus X_1 (*T. thermosaccharolyticum* inoculation time) and X_2 (*C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio), X_1 (*T. thermosaccharolyticum* inoculation time) and X_3 (*C. thermocellum* sampling time), as well as X_2 (*C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio) and X_3 (*C. thermocellum* sampling time) were all elliptical with elongated diagonals. This indicates that the interactions between the independent variables and ethanol concentration (Y) were significant. Therefore, optimum values of the independent variables were obtained by calculating the maximum value of the regression equation (Y), as shown in Table 4.

Table 4. Predicted Ethanol concentration under Optimum Conditions

Substrate	*T. thermosaccharolyticum* inoculation time (h)	*C. thermocellum* to *T. thermosaccharolyticum* inoculation ratio	*C. thermocellum* sampling time (h)	*Y*/ Predicted ethanol concentration (g/L)
Salix	At the same time	1:0.815	117.26	0.16

As shown in Fig. 5, the ethanol concentration under these conditions was 0.17 g/L and the ethanol yield was 10.2% ($P < 0.01$), with a 24.6% cellulose degradation ratio and a 13.1% hemicellulose degradation ratio. The ethanol yield was thus 39% higher than without optimization.

It is essential to find optimal co-culture conditions *via* response surface methodology to increase ethanol yield. In previous studies, *Enterobacter aerogenes* KKU-S1 was able to produce ethanol from waste glycerol; when several effect factors were optimized through central composite design of response surface methodology, ethanol production was improved (Reungsang *et al.* 2013). To increase hydrogen production from *Rhodobacter capsulatus* JP91fermentation glucose, the independent variables were optimized by response surface methodology (Ghosh *et al.* 2012). Additionally, Chen *et al.* (2012) optimized an extraction technology of soluble polysaccharides from *Boletus edulis* mycelia *via* response surface methodology, yielding results that were well matched with the predicted yield. These examples illustrate that response surface methodology is a reliable tool that can be used to eliminate the interactions of independent variables.

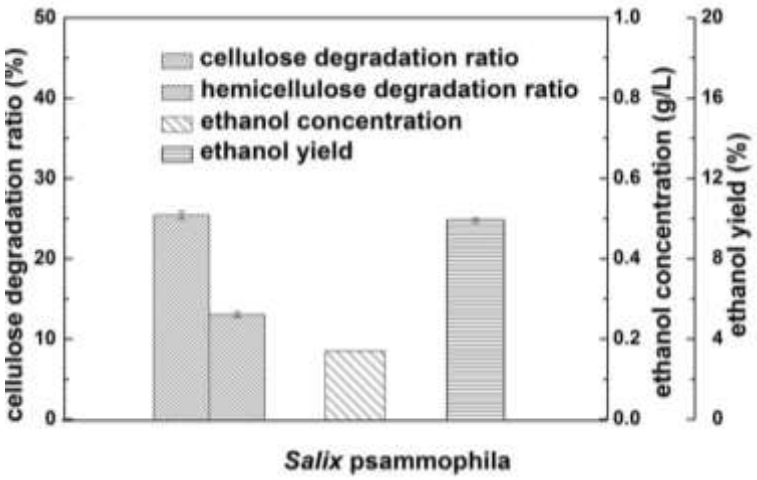

Fig. 5. Co-culture performance of *C. thermocellum* and *T. thermosaccharolyticum* at optimum conditions in serum bottles

Co-Culture Experiments in Fermenter Tanks

Higher ethanol concentration and yield was obtained in the fermenter tanks compared to the anaerobic bottles, which may be at least partly due to more efficient mixing. As shown in Fig. 6, the maximal ethanol concentration and yield was 0.2 g/L and 11.1%, with 26.4% cellulose degradation ratio and 13.8% hemicellulose degradation ratio, when the pH was kept at 7 during fermentation. The ethanol yield in the stirred-tank bioreactor was 11.9% better than in the serum bottles, and control of pH led to a further significant increase.

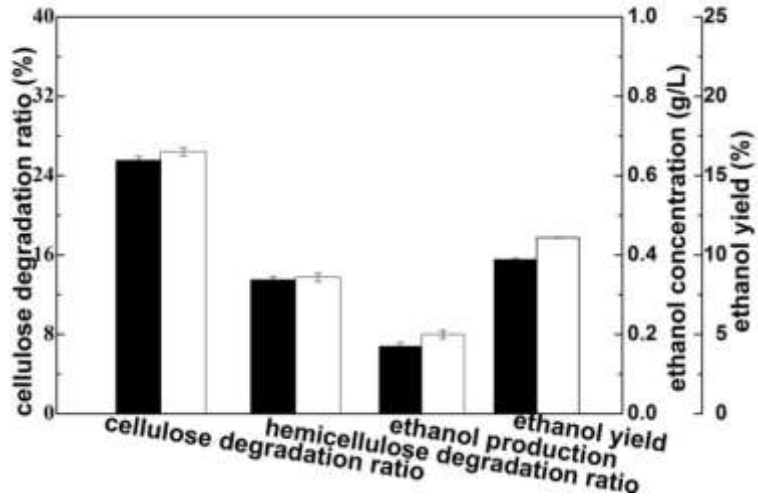

Fig. 6. Co-culture processes of *C. thermocellum* and *T. thermosaccharolyticum* in fermenter tanks. Black represent non-regulated pH; White represent actively regulated pH.

During fermentation, significant amounts of acetate and butyrate accumulated during the fermentation period, which inhibited ethanol yield from *Salix*. Furthermore, keeping the pH of fermentation stable using 2 M NaOH was beneficial for the synthesis of ethanol. When the cellulolytic bacterium *Fibrobacter succinogenes* A3c and the non-cellulolytic bacterium *Prevotella ruminicola* H2b were co-cultured on mature orchard

grass (*Dactylis glomerata*), immature orchard grass, mature alfalfa, and immature alfalfa, cellulose digestion was not improved, but hemicellulose digestion was improved significantly on immature orchard grass and mature alfalfa (Fondevila and Dehority 1996). Since the ethanol yield was improved markedly when the pH was adjusted during fermentation in a bioreactor, it was speculated that the physiological activity of the strains was at its best during active growth, or when the negative effects of the *C. thermocellum* acid metabolism pathways were repressed because the *C. thermosaccharolyticum* cultures utilized the corresponding carbon sources to metabolize organic acids, hydrogen, and others (Li and Liu 2012). Therefore, the pH of the fermentation broth greatly affected the ethanol yield capacity of ethanol-producing bacteria. Additionally, fermentation in continuous stirred-tank reactors is also a major strategy for improving the yield of the target product. For instance, the use of a co-culture of *C. thermocellum* and *C. thermosaccharolyticum* to improve hydrogen production was similar to our studies, and the hydrogen yield in the bioreactor was 9.8% higher than that in serum bottles (Li and Liu 2012). Fermentation of cornstalks using *C. thermocellum* 7072 to produce hydrogen was researched, and the yield was again higher in the bioreactor system than in anaerobic bottles (Cheng and Liu 2011). A co-culture system of *C. beijerinckii* and *C. cellulovorans* was established to produce ethanol (0.87 g/L) from alkali-corn cob using consolidated bioprocessing (Wen *et al.* 2014).

CONCLUSIONS

1. Co-culture of *C. thermocellum* and *T. thermosaccharolyticum* showed its advantages over the monoculture of *C. thermocellum* in ethanol yield, using cheap and abundant untreated *Salix* as the substrate.
2. A higher ethanol yield was obtained under the optimized conditions in fermenter tanks than in anaerobic bottles. However, the cellulose and hemicellulose degradation ratios were not improved significantly. The mechanisms responsible for the improvement of ethanol yield thus merit further study.
3. *T. thermosaccharolyticum* is a good partner microorganism for *C. thermocellum* for cellulose degradation and fuel ethanol yield.

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