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Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of *Clostridium beijerinckii* and *Clostridium cellulovorans*

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Abstract

Background: Butanol is an industrial commodity and also considered to be a more promising gasoline substitute compared to ethanol. Renewed attention has been paid to solvents (acetone, butanol and ethanol) production from the renewable and inexpensive substrates, for example, lignocellulose, on account of the depletion of oil resources, increasing gasoline prices and deteriorating environment. Limited to current tools for genetic manipulation, it is difficult to develop a genetically engineered microorganism with combined ability of lignocellulose utilization and solvents production. Mixed culture of cellulolytic microorganisms and solventogenic bacteria provides a more convenient and feasible approach for ABE fermentation due to the potential for synergistic utilization of the metabolic pathways of two organisms. But few bacteria pairs succeeded in producing biobutanol of high titer or high productivity without adding butyrate. The aim of this work was to use *Clostridium cellulovorans* 743B to saccharify lignocellulose and produce butyric acid, instead of adding cellulase and butyric acid to the medium, so that the soluble sugars and butyric acid generated can be subsequently utilized by *Clostridium beijerinckii* NCIMB 8052 to produce butanol in one pot reaction.

Results: A stable artificial symbiotic system was constructed by co-culturing a cellulolytic, anaerobic, butyrate-producing mesophile (*C. cellulovorans* 743B) and a non-cellulolytic, solventogenic bacterium (*C. beijerinckii* NCIMB 8052) to produce solvents by consolidated bioprocessing (CBP) with alkali extracted deshelled corn cobs (AECC), a low-cost renewable feedstock, as the sole carbon source. Under optimized conditions, the co-culture degraded 68.6 g/L AECC and produced 11.8 g/L solvents (2.64 g/L acetone, 8.30 g/L butanol and 0.87 g/L ethanol) in less than 80 h. Besides, a real-time PCR assay based on the 16S rRNA gene sequence was performed to study the dynamics of the abundance of each strain during the co-culturing process, which figured out the roles of each strain at different periods in the symbiosis.

Conclusion: Our work illustrated the great potential of artificial symbiosis in biofuel production from lignocellulosic biomass by CBP. The dynamics of the abundance of *C. beijerinckii* and *C. cellulovorans* revealed mechanisms of cooperation and competition between the two strains during the co-culture process.

Keywords: ABE fermentation, Consolidated bioprocessing, Co-culture, Dynamics, Lignocellulose, Optimization

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Background

As the main product of acetone-butanol-ethanol (ABE) fermentation, butanol is considered to be a promising gasoline substitute on account of its superior characteristics over ethanol; for example, butanol is less hygroscopic, less volatile, and has an energy density closer to that of gasoline [1]. Renewed attention has been paid to butanol production on account of the depletion of oil resources and increasing gasoline prices.

Traditional ABE fermentation methods employ corn, cassava or molasses as the substrate. However, with the rising price of these substrate materials, the feedstock cost has become a major factor determining the total economics of the ABE fermentation industry [2]. Therefore, alternative low-price renewable feedstock, including energy crops, agricultural residues, forestry and food processing wastes have been assessed for the production of ABE. Recently, various biomass hydrolysates have been used as substrates in batch ABE fermentations by numerous *Clostridium* strains [3-8]. Unfortunately, the cost of cellulase used to hydrolyze the cellulose fraction of biomass has made these attempts economically uncompetitive, in respect that solventogenic clostridia are not able to utilize lignocellulose as a raw material directly [9].

Consolidated bioprocessing (CBP) has been suggested as an efficient and economical method for biofuels production from low-price renewable feedstock, which combines cellulase production, cellulose hydrolysis and fermentation in one reactor and offers the potential for lower costs and higher efficiency than processes featuring dedicated cellulase production [10]. To realize the potential, a single microorganism or microbial system must be developed to utilize lignocellulose at a high rate of conversion and produce solvents at high yields and titers.

Cellulase (or cellulosome) genes have been cloned into *C. beijerinckii* and *C. acetobutylicum* to produce butanol from cellulose in one pot reaction, but the level of heterologous cellulase (or cellulosome) expression was rather low [11-14]. Efforts to implant the (iso)butanol biosynthetic pathway in cellulolytic clostridia did not come up to desired objective either, and only small quantities of (iso)butanol was produced [15,16]. It was very difficult to produce butanol efficiently from lignocellulose directly by pure culture.

Mixed culture of cellulolytic microorganisms and solventogenic bacteria is a more convenient and feasible approach to ABE fermentation by CBP. In the community, microorganisms may develop the potential for synergistic utilization of the metabolic pathways from interspecies. Attempts at artificial symbiosis have been carried out by co-culturing solventogenic *C. acetobutylicum* and cellulolytic *Clostridium cellulolyticum* or *Clostridium thermocellum* [17,18]. Nevertheless, the results were far from satisfactory, requiring the addition of butyric

acid to induce the solventogenic phase of *C. acetobutylicum*. In view of the phenomenon, to avoid the addition of butyric acid, *C. saccharoperbutylacetonicum* strain N1-4, whose induction mechanism of butanol production somewhat differs from that of other butanol-producing clostridia, was chosen as the partner for *C. thermocellum* [19]. This bacteria pair succeeded in manufacturing 7.9 g/L of butanol from 40 g/L of avicel cellulose in 11 days without adding butyrate. However, since the culture temperature of *C. thermocellum* (60°C) doesn't match that of *C. saccharoperbutylacetonicum* (37°C), the CBP has to be carried out in two stages, that is, thermophilic and subsequent mesophilic stage, exhibiting a low ABE productivity of 0.0375 g/L/h. Further more, the crude lignocellulosic biomass (e.g. AECC) usually composed of cellulose, hemicellulose and lignin, which made CBP based on lignocellulose more complicated than that using pure cellulose. It was of high necessity to develop a novel CBP for real lignocellulosic biomass utilization.

The aim of this work was to construct a stable artificial symbiosis for efficient biobutanol production by CBP using AECC, an abundant agricultural residue after a simple pretreatment, as a low-cost feedstock. In the symbiosis, *Clostridium cellulovorans* 743B, an anaerobic, cellulolytic and butyrate-producing mesophile [20], was selected to saccharify lignocellulose and produce butyric acid, instead of adding cellulase and butyric acid to the medium, so that the soluble sugars and butyric acid generated can be utilized by solventogenic bacteria to produce butanol. Meanwhile, *C. beijerinckii* NCIMB8052, one of the most conventional solventogenic clostridia, was introduced as the production strain for ABE fermentation. As *C. beijerinckii* can co-ferment hexose and pentose sugars [21], studies on lignocellulosic biomass-based ABE production by *C. beijerinckii* have intensified in the last few years [4-7,22], but no study on ABE production with AECC involving the co-culture of two strains above has been reported. The present work involved the construction of symbiotic system for ABE fermentation using AECC as the raw material by co-culture of *C. beijerinckii* and *C. cellulovorans*, and the dynamics of the abundance of each strain during the co-culture process.

Results and discussion

Construction of symbiosis by co-culture of *C. beijerinckii* and *C. cellulovorans* with AECC as the sole carbon source

The single culture of *C. beijerinckii* displayed very poor growth on AECC. At an initial concentration of 70 g/L, less than 10% of the substrate was utilized even after 7 days of incubation, and the ABE output was rather low (0.34 g/L of ethanol, butanol and acetone not detected). These agreed with the observations of López-Contreras et al. [11] and indicated that *C. beijerinckii* is unable to produce ABE from AECC directly.

As a cellulolytic microorganism, *C. cellulovorans* grows well on lignocelluloses as it can produce cellulosome, a cellulose-degrading multi-enzyme complex, which can decompose cellulose more efficiently than free cellulase by adhering to the substrate and providing multi-cellulase synergy [23]. In the single culture of *C. cellulovorans*, 67.2 g/L AECC was added as the sole carbon source and the pH was kept at 7.0 throughout. *C. cellulovorans* produced 6.72 g/L of butyric acid, 3.17 g/L of acetic acid and 0.29 g/L of ethanol and degraded 59.7% of the substrate in 100 h (Figure 1A and B), about 75% faster than AECC degraded by *C. thermocellum* strains SS21 and SS22 [24]. It was worth noting that the total sugars accumulated from the beginning of fermentation and finally achieved 11.7 g/L, including glucose, cellobiose, xylose, xylobiose, arabinose and some other kinds of fermentable sugars (Figure 1C). The accumulation of fermentable sugars showed that the rate of AECC saccharification exceeded that of glycolysis. In fact, cellulolytic clostridia such as *C. cellulovorans*, *C. cellulolyticum* and *C. thermocellum* can only use small quantities of carbohydrates due to early inhibition of metabolism and growth [25]. Moreover, some papers have reported that butyric acid and acetic acid accumulated in the broth are very useful in ABE fermentation, and can be transformed into solvents during solventogenesis of *C. beijerinckii* [26,27]. Efficient degradation of AECC as well as sufficient accumulation of fermentable sugars

and organic acids made *C. cellulovorans* a superior candidate for co-culture with *C. beijerinckii*.

Taking the results above into account, we established a co-culture method to produce ABE from AECC directly. *C. beijerinckii* and *C. cellulovorans* were inoculated simultaneously with an inoculum of 0.5% (v/v) and 10% (v/v), respectively, by volume from an exponentially growing culture. During the first 36 h, the pH was kept at 7.0, the optimal pH for growth of *C. cellulovorans*. Figure 2 describes the time course of co-culture. The co-culture exhibited typical two-phase fermentation with acidogenesis followed by solventogenesis. Acetic and butyric acid accumulated during the initial 48 h, and then butanol began to be produced. During solventogenesis, the pH slowly rebounded from 5.83 to 6.54 with the re-assimilation of butyrate and acetate, which were speculated to be used as co-substrates with fermentable sugars to produce solvents [27].

In 100 h, 5.68 g/L of ABE (acetone 1.11, butanol 4.11 and ethanol 0.46 g/L), about 10 times higher than from the single culture with *C. beijerinckii*, was produced from 46.8 g/L of AECC (Figure 2A and B) without adding butyrate. Compared with the single culture of *C. cellulovorans*, the accumulation of total sugars decreased by 51.9%, but AECC degradation increased by 16.7%, which implied that the fermentable sugars were utilized by *C. beijerinckii* and the degradation of the substrate by *C. cellulovorans* was promoted

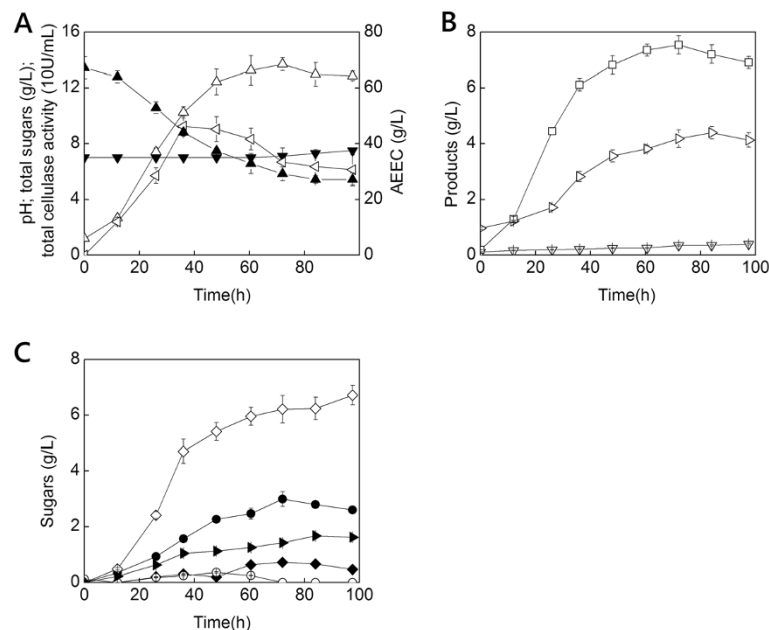


Figure 1 Time courses of single culture of *C. cellulovorans* with AECC as sole carbon source. AECC was decomposed and converted to soluble sugars and organic acids, which could be used to produced solvents in ABE fermentation. **(A)** Time courses of pH, total cellulase activity, total sugars accumulation and AECC degradation, **(B)** Time courses of organic acids and solvents production, **(C)** Time courses of monosaccharides and disaccharides accumulation. ▲ AECC, △ total sugars, ▼ pH, ★ ABE, ◁ total cellulase activity, ☆ acetone, ■ butanol, ▽ ethanol, ▷ acetic acid, □ butyric acid, ● cellobiose, ○ glucose, ◆ xylobiose, ◇ xylose, ► arabinose.

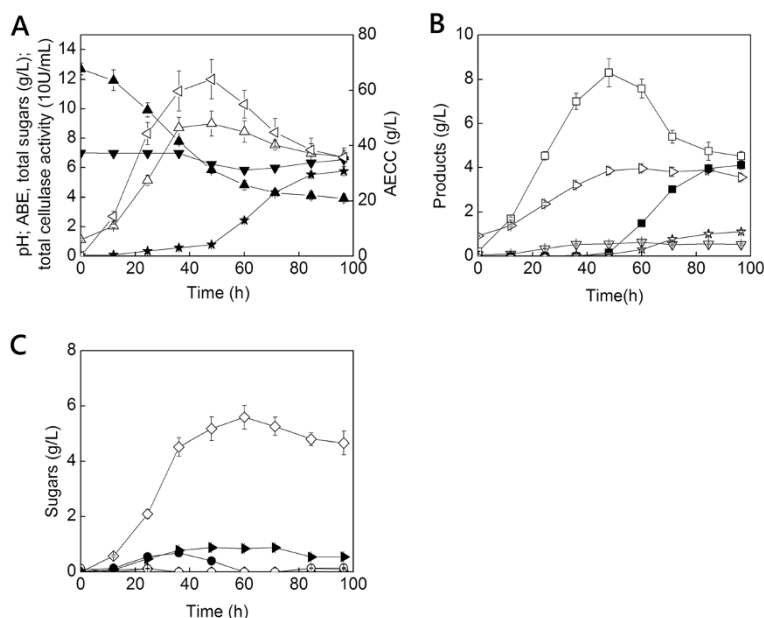


Figure 2 Time courses of co-culture of *C. cellulovorans* and *C. beijerinckii* with AECC as sole carbon source. The artificial symbiosis succeeded in ABE production from AECC by CBP. **(A)** Time courses of pH, total cellulase activity, total sugars accumulation and AECC degradation, **(B)** Time organic acids and solvents production, **(C)** Time courses of monosaccharides and disaccharides accumulation. ▲ AECC, △ total sugars, ▼ pH, ★ ABE, < total cellulase activity, ☆ acetone, ■ butanol, ▽ ethanol, ▷ acetic acid, □ butyric acid, ● cellobiose, ○ glucose, ◆ xylobiose, ◇ xylose, ► arabinose.

with the removal of total sugars. That was confirmed by the changes of mixed sugars components in Figures 1C and 2C. In Figure 2C, the preferred carbon sources like glucose and cellobiose almost depleted, whereas xylose and arabinose accounted for most of the residual sugars, which could be attributed to the carbon catabolite repression (CCR) mechanism [28] and inefficient pentose utilization of *C. beijerinckii* [29]. Besides, enhancement in the total cellulase activity also contributed to the improved AECC decomposition (Figures 1A and 2A).

In the symbiotic system, *C. cellulovorans* provided fermentable sugars and organic acids for *C. beijerinckii* to produce solvents. At the same time, the consumption of various sugars by *C. beijerinckii* alleviated the feedback inhibition of cellulase and improved the degradation of AECC [30]. The cooperation between the two strains was enhanced by the two-stage pH control strategy and butyrate accumulation in the broth. The neutral pH during the first 36 h guaranteed vigorous growth of *C. cellulovorans* to degrade AECC efficiently and produced sufficient organic acids, especially butyrate. With a pH decline from 7 to 5.83 since 36 h, the butyrate “feeding” to *C. beijerinckii* induced solventogenesis, which thereby prevented acidogenic fermentation caused by neutral pH performing throughout [31].

Although ABE fermentation from AECC without addition of butyric acid was accomplished by co-culture of *C. beijerinckii* and *C. cellulovorans*, only very small quantities of solvents were produced. It was subsequently

improved by optimizing some important parameters during co-culture.

Optimization of the co-culture conditions

As described above, in the symbiotic system, *C. beijerinckii* and *C. cellulovorans* collaborated in AECC decomposition and ABE production. However, they also competed for carbon sources and other nutrients to grow and metabolize. Accordingly, it was critical to ease competition and enhance cooperation by regulating some important culture conditions during the fermentation process.

Many important parameters such as inoculation timing, inoculation ratio, and the initial concentration of yeast extract and the substrate have been discussed for co-cultures [19,32,33]. However, few studies have focused on the effects of pH control in co-cultures. In the following studies, the effects of inoculation timing, inoculation ratio and the duration of pH control on co-cultures were investigated to improve ABE production and AECC degradation.

Effects of *C. beijerinckii* inoculation timing on co-culture

Because *C. beijerinckii* was unable to efficiently produce solvents from AECC directly, it was necessary to hydrolyze AECC by a single culture of *C. cellulovorans* before inoculation of *C. beijerinckii* into the co-culture. In this study, *C. beijerinckii* was added to the medium after inoculation and cultivated with *C. cellulovorans* for 0, 12, 24, 36 and 48 h (pH controlled at 7.0 during the first 36 h, inoculation ratio of 0.5:10 (v/v) between *C. beijerinckii*

and *C. cellulovorans*), after which butyric acid accumulation, butanol production and AECC degradation were measured at 100 h.

Figure 3A show that delayed inoculation of *C. beijerinckii* from 0 to 48 h decreased butanol production from 4.07 to 1.10 g/L, and AECC decomposition from 39.7 to 32.8 g/L, respectively. In other words, the simultaneous addition of *C. cellulovorans* and *C. beijerinckii* offered the best results, which was distinguished from co-cultures of *C. thermocellum* and *C. acetobutylicum* [18] or *C. saccharoperbutylacetonicum* strain N1-4 [19]. Since the optimal incubation temperature of *C. cellulovorans* matched that of solventogenic bacterium, cellulase production by *C. cellulovorans* and ABE fermentation by *C. beijerinckii* could be carried out simultaneously. Moreover, the inoculation of two strains in series may make *C. beijerinckii* disadvantaged in competition with *C. cellulovorans*, which negatively affected the utilization of fermentable sugars and the transformation of organic acids into solvents (Figure 3A).

Effects of the inoculation ratio between *C. beijerinckii* and *C. cellulovorans* on co-culture

AECC decomposition and ABE production depended mainly on the population and relative abundance of two strains. It has been reported that the relative abundance of *C. thermocellum* and *C. thermopalmarium* monitored by real-time quantitative PCR varied at different periods of co-culture [32], but it was principally determined by the initial

inoculation ratios. Therefore, the effects of the inoculation ratio between *C. beijerinckii* and *C. cellulovorans* on ABE fermentation were assessed.

To ensure the repeatability and accuracy of experiments, cell density of *C. beijerinckii* (OD_{600} 1.2-1.5) was previously normalized to 1.0 of OD_{600} before inoculating with gradient volumes. For *C. cellulovorans* pre-cultured for 24 h in 200 ml serum bottle with AECC as sole carbon source, the cell protein content was measured and calculated as 20.7 mg/L on average. That is, cell density of *C. cellulovorans* used to inoculation was 36.3 mg/L. In order to facilitate understanding, inoculum ratio was expressed on a volumetric basis.

Butanol production, total sugars accumulation and AECC degradation were compared among co-cultures with *C. beijerinckii* /*C. cellulovorans* inoculation ratios of 0:10 (single culture of *C. cellulovorans*), 0.25:10, 0.5:10, 1:10 and 2:10 (v/v) for 100 h, at pH 7.0 during the first 36 h (Figure 3B). Butanol production and AECC degradation increased greatly from 0 to 5.08 g/L and 34.7 to 49.9 g/L, respectively, with an increased abundance of *C. beijerinckii* in the inoculums. Solvents production was not apparently promoted when the ratio exceeded 1:10, thus, an economical inoculation ratio of 1:10 was adopted in our subsequent studies.

Effects of pH control duration on co-culture

C. cellulovorans is very sensitive to pH, with an optimal pH for growth at about 7.0, and a range of 6.4 to 7.8 [20].

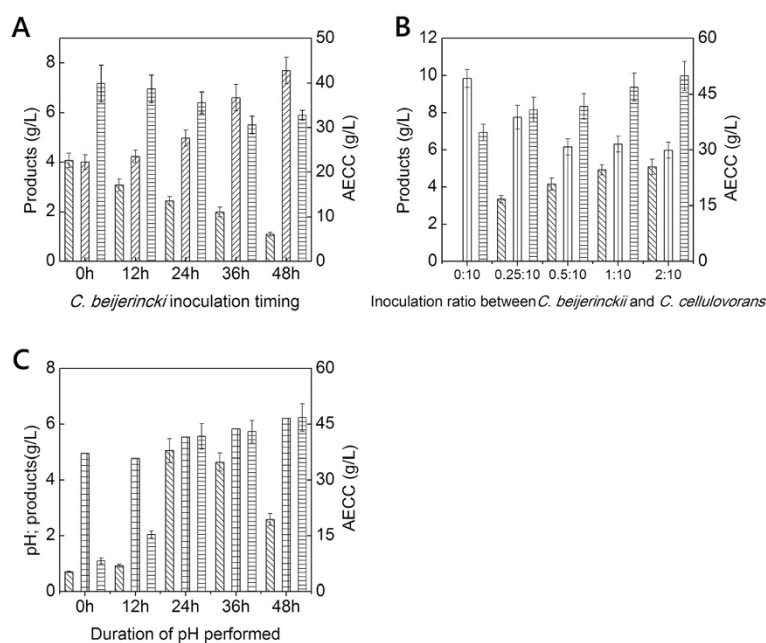


Figure 3 Effects of *C. beijerinckii* inoculation timing (A), inoculation ratio (B) and pH control strategy on co-culture (C). The co-culture conditions were investigated and optimized as follows: simultaneous inoculation, inoculation ratio of 1:10(v/v) between *C. beijerinckii* and *C. cellulovorans*, pH control at 7.0 during the first 24 hours. butanol, butyric acid, AECC degraded, total sugars, pH value.

However, low pH is a prerequisite for solvents production in ABE fermentation, because when ABE fermentation is performed at a pH close to neutrality throughout, acids are the predominant products [31]. Therefore, during co-culture, a two-stage pH control strategy was employed, in which the pH in the first stage was regulated at 7.0 to ensure vigorous growth of *C. cellulovorans*, so that cellulosomes could be produced sufficiently to hydrolyze AECC efficiently. In the second stage, the pH was not controlled. With the accumulation of undissociated acids, the pH declined to induce the solventogenesis and prevent acidogenic fermentation [31].

The duration of pH control was investigated by varying the runtime for 0, 12, 24, 36 and 48 h (Figure 3C). Uncontrolled pH in an earlier period, for example 12 h, was not favorable for AECC decomposition and butanol production, by reason that there were not sufficient cellulosomes synthesized and secreted by *C. cellulovorans* to degrade AECC, which was supported by the total cellulase activity during the first 48 h in Figures 1A and 2A. ABE fermentation was severely restricted by the limited supply of carbon sources. Although a prolonged pH control stage for 48 h contributed to a high amount of substrate consumed (46.8 g/L), organic acids were main products. There was only a minor decline in pH from 7.0 (only 0.79) attained, which caused rather low butanol production and solvents yield (2.58 g/L and 0.076 g/g, respectively) [31]. Although butanol production was not significantly different between cultures with the pH not controlled after 24 h and 36 h, the former showed a lower pH minimum (5.54) during fermentation, which was more conducive to the production of solvents. In the following studies, the pH was controlled at 7.0 during the first 24 h.

Few studies have paid attention to the effects of pH control on substrate utilization and butanol production during co-culture, and the pH is usually not regulated or optionally performed at compromise values for two strains, which always leads to slow lignocellulose decomposition or acidogenic fermentation [17,33]. The two-stage pH control strategy here provided a viable solution to promote substrate degradation and prevent acidogenic fermentation. Alternatively, the strategy could be carried out by adding cheap calcium carbonate or others with buffering capacity of the medium to reduce costs of pH control [34].

ABE fermentation from AECC under optimized co-culture conditions

The performance of fed-batch ABE fermentation from AECC under the optimized co-culture conditions are described in Figure 4. Compared with the time courses shown in Figure 2, a double inoculum, 1% (v/v) of *C. beijerinckii* had no obvious effects on AECC decomposition and total

sugars accumulation during the first 24 h; nevertheless, solventogenesis was triggered 12 h earlier, owing to a shortened duration of pH control, which together made for a shorter fermentation time of 80 h. Earlier recovery of *C. beijerinckii* due to large inocula as well as a shorter runtime of pH control slowed the accumulation of total sugars and thus promoted the utilization of AECC. The feeding of AECC provided more adhesion sites for *C. cellulovorans* to colonize and extra enzymatic domain for cellulosomes [35], which thus increased the total cellulase activity and the supply of carbon sources for *C. beijerinckii*. With the slowing down of AECC degradation after 60 h, the accumulated sugars began to be consumed. Although *C. beijerinckii* can utilize hexoses and pentoses simultaneously [21], the uptake of xylose and arabinose was rather poor and inefficient (Figure 4C) [29], which limited significantly the supply of ATP for organic acids transformation to solvents [27], hence there were some pentose, butyrate and acetate residual in final broth.

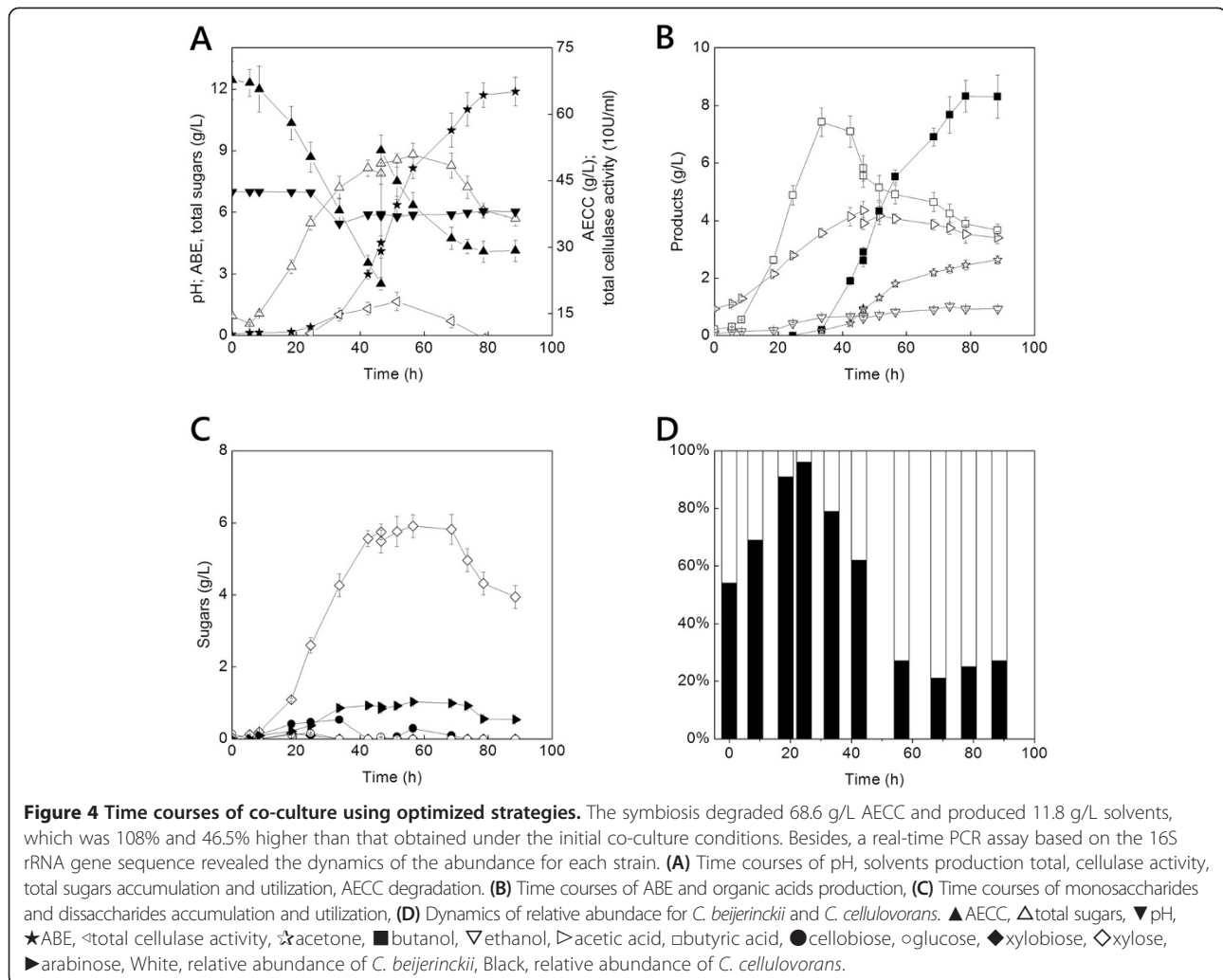
The co-culture produced 11.8 g/L of ABE (acetone 2.64, butanol 8.30 and ethanol 0.87 g/L) and degraded 68.6 g/L of AECC, which was 108% and 46.5% higher than that obtained under the initial unoptimized co-culture conditions. Comparing with attempts to engineer a native cellulolytic or solventogenic microbe, with the goal of developing the combined ability in an industrial microorganism to produce butanol (isobutanol) from lignocellulose in one pot reaction, the strategy of co-culture provided distinct advantages in terms of output and productivity (Table 1). Yet co-culture with different bacteria pairs and substrates also exhibited varied results. ABE production in the present work was 19.2% higher than that by a sequential co-culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* strain N1-4, and the solvents productivity increased by 3.0-fold [19].

However, compared with the established ABE fermentation process from starch or sugars with other clostridia, the co-culture here still has a long road ahead in the ABE output, productivity and yield [2], which may be improved by promotion for the symbiosis in AECC saccharification, pentose uptake and utilization, organic acids reassimilation.

Dynamics of the abundance of *C. beijerinckii* and *C. cellulovorans* during the co-culture process

In the symbiotic system, *C. beijerinckii* and *C. cellulovorans* developed mutualistic relationships overall, at the same time commensal or competitive relationships as well in partial stages. It was necessary to figure out the roles of each strain at different periods. The population and relative abundance dynamics of both strains were quantified by real-time PCR (Figure 4D).

During the first 24 h after simultaneous inoculation, *C. cellulovorans* became more dominant in the mixed



population. In other words, *C. beijerinckii* grew far slower than *C. cellulovorans*, which could be partly due to the lack of sufficient carbon sources (xylose and arabinose accumulated mainly) available. Moreover, interspecific competition from *C. cellulovorans* also limited the growth of *C. beijerinckii*, which explained why higher *C. beijerinckii*/*C. cellulovorans* inoculation ratios did not significantly affect ABE production (Figure 3B). *C. beijerinckii* need a period to adapt to the growth environment stresses, and simultaneous inoculation led to a better adaptation and growth. In the stage of pH control at 7.0, the environment benefited *C. cellulovorans* and there was more competition than cooperation.

From 24 to 56 h, with the reduction of pH value, the growth of *C. cellulovorans* was negatively affected and slowed down [20], while *C. beijerinckii* seized the opportunity to proliferate rapidly and even exceeded *C. cellulovorans*. The low pH limited the growth and metabolism of *C. cellulovorans* and thus eased the competition from *C. cellulovorans* [20], which dramatically facilitated

C. beijerinckii. Furthermore, AECC degradation was greatly promoted by higher cellulase (cellulosome) activity at a low pH and the mitigation of feedback inhibition owing to the consumption of soluble sugars (Figures 2A and 4A). At 36 h, the low pH induced a switch in the metabolism of *C. beijerinckii* from acidogenesis to solventogenesis [38], in which the organic acids produced by *C. cellulovorans* were re-assimilated and transformed into solvents. The two-stage pH control strategy and butyrate accumulation by *C. cellulovorans* recovered *C. beijerinckii* and succeeded in shifting from competition to cooperation.

In the late fermentation period, AECC decomposition almost ceased and both strains were forced to compete for finite carbon source and nutrition. *C. beijerinckii* maintained dominance over *C. cellulovorans*, but the proportion declined as a result of accumulating butanol and rising pH with the re-assimilation of organic acids.

Competition and cooperation between *C. cellulovorans* and *C. beijerinckii* was preliminarily revealed by the dynamics of the abundance of both strains during the co-culture

Table 1 Comparison of butanol (isobutanol or ABE) production with varied lignocellulose by CBP

Strain	Strategy	Substrate	Isobutanol (g/L)	Butanol (g/L)	ABE (g/L)	ABE productivity (g/L/h)	Reference
<i>C. cellulolyticum</i>	Expressing enzymes that direct the conversion of pyruvate to isobutanol	Crystalline cellulose	0.660		ND	ND	[16]
<i>Echerichia coli</i>	Expressing enzymes that involve biomass digestion capabilities and butanol synthesis pathways	Ionic liquid-treated switchgrass		0.028	ND	ND	[15]
<i>Trichoderma reesei</i> and <i>E. coli</i>	Co-culture of <i>T. reesei</i> and an <i>E. coli</i> strain metabolically engineered to produce isobutanol	Pretreated corn stover	1.88		ND	ND	[36]
<i>C. thermocellum</i> and <i>C. acetobutylicum</i>	Sequential co-culture and feeding butyrate	Cellulose solka floc		2.40	3.90	0.0232	[18]
<i>C. thermocellum</i> and <i>C. acetobutylicum</i>	Sequential co-culture	Crystalline cellulose		0.60	1.4	0.0053	[19]
<i>C. thermocellum</i> and <i>C. saccharoperbutylacetonicum</i> strain N1-4	Sequential co-culture	Crystalline cellulose		7.9	9.9	0.0375	[19]
<i>C. thermocellum</i> and <i>C. beijerinckii</i>	Sequential co-culture	Crystalline cellulose		2.1	3.0	0.0114	[19]
<i>C. cellulolyticum</i> and <i>C. acetobutylicum</i>	Co-culture	Cellulose solka floc		0.80	1.1	0.0051	[17]
<i>Bacillus cellulolyticus</i> and <i>C. acetobutylicum</i>	Co-culture	Pretreated palm pressed fiber		0.49		ND	[33]
<i>C. thermocellum</i> and <i>C. beijerinckii</i>	Sequential co-culture	AECC		8.75	16.0	0.0889	[37]
<i>C. cellulovorans</i> and <i>C. beijerinckii</i>	Co-culture	AECC		8.30	11.8	0.148	this work

ND not determined.

process. The abundance of each strain was mainly regulated by the culture conditions, for example the pH value and nutrient availability. However, the molecular mechanism of competition and cooperation in the symbiotic system remained unclear.

It is known that quorum sensing (QS) plays an important role in determining the local bacterial concentration and proportion [39,40], but the genes in both strains involved in QS have not been verified with certainty (Gene ID: 5295733 and 9610822 in Genbank). Further research for the production and recognition of signaling molecules within species and interspecies may contribute to understand how the symbiosis worked.

Conclusion

In this report, we developed a stable mutualistic system of *C. cellulovorans* and *C. beijerinckii* for ABE fermentation from AECC in one pot reaction. Optimization strategies were confirmed by investigating the effects of the co-culture conditions on butanol production and AECC degradation. Under the optimized conditions, the co-culture produced 11.8 g/L of ABE and degraded 68.6 g/L of AECC in 80 h, which were 108% and 46.5% higher than those obtained under the initial co-culture conditions. The dynamics of the abundance of *C. beijerinckii* and

C. cellulovorans revealed mechanisms of cooperation and competition between the two strains during the co-culture process.

These preliminary results illustrate the great potential of artificial symbiosis in biofuel production from lignocellulosic biomass by CBP. However, the mechanism of inter-specific competition and cooperation in the community based on molecular is still unclear. The dynamics during co-culture remains to be studied further.

Methods

Alkali extracted deshelled corn cobs (AECC)

Deshelled corn cobs were cut into pieces approximately 1 cm in length, and were then ground to pass through 30–40 mesh (450–600 μm). Alkali extracted fractions were prepared by autoclaving 6% (w/v) corn cobs at 121°C for 20 min with 1% (w/v) NaOH, followed by neutralization with 1% (w/v) H₂SO₄. These fractions were thoroughly washed with distilled water and dried at 80°C for 24 h [24].

The component of AECC was determined using a raw fiber extractor, FIWE 3 (Velp Scientifica/Goodwill (HK) Technology Ltd., Hong Kong, China) [41]. The cellulose, hemicellulose and lignin contents of AECC were 69.8, 27.4 and 1.47% (w/w), respectively, compared to 44.9, 33.2 and 14.5% before alkali extraction.

Strain and medium

Clostridium cellulovorans 743B was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The stock culture was maintained in 25% glycerol and frozen at -80°C .

Clostridium beijerinckii NCIMB 8052 was kindly provided by Professor Sheng Yang, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences (CAS). The stock culture was maintained in 25% glycerol and frozen at -80°C . *C. beijerinckii* was anaerobically pre-cultured in TGY medium (1 L TGY medium contained 5 g of tryptone, 3 g of yeast extract, 2 g of glucose, 0.25 g of L-cysteine HCl and 0.001 g of resazurin, pH 6.5). It was incubated under static conditions at 37°C for 12–16 h, at which point the log phase was reached.

C. cellulovorans were anaerobically pre-cultured in 200 mL of medium with AECC as the sole carbon source under static conditions at 37°C for 24 h before inoculation. The medium was slightly modified compared to that described before [20]. One liter of medium (pH 7.0) contained 70.0 g of AECC, 1.5 g of NH_4Cl , 1 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.5 g of KCl, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.65 g of CH_3COONa , 0.5 g of yeast extract, 0.5 g of tryptone, 0.5 g of L-cysteine HCl, 0.001 g of resazurin, 20 mL of a trace metal solution [42], 100 μg of *p*-aminobenzoic acid, 100 μg of thiamine and 1 μg of biotin. NH_4Cl , CH_3COONa , *p*-aminobenzoic acid, thiamine and biotin were filter-sterilized using a 0.22 μm pore size filter, and the other materials were autoclaved at 121°C for 20 min followed by cooling to room temperature under 100% N_2 . The medium was also used for single and co-culture of *C. beijerinckii* and *C. cellulovorans*.

ABE fermentation from AECC

Single and co-culture of *C. beijerinckii* and *C. cellulovorans* were carried out in a 5 L bioreactor (Biotech-5BGH, Baoxing Bio-engineering Equipment Co. Ltd., Shanghai, China) with 2 L working volume with pH control if necessary by the automatic addition of 5 N NaOH. Agitation was kept constant at 100 rpm and the temperature was maintained at 37°C . Samples were taken at regular intervals for the analysis of biomass, substrate and products concentration.

The optimization of co-culture conditions was carried out in 500 mL shaken flasks with a 400 mL working volume by a reequipped six-channel refrigerated pH control feed shaker (SHpH6 shaker incubator, Shanghai Guoqiang Bioengineering Equipment Co. Ltd., Shanghai, China). Samples were taken at the end of fermentation for analysis and comparison.

The inoculum size of *C. cellulovorans* in all co-cultures was 10% (v/v) if not otherwise indicated.

Real-time PCR

Total RNA was isolated from freshly collected culture samples using Trizol (Invitrogen, California, USA) following the

manufacturer's instructions for bacteria. cDNA was synthesized from isolated RNA separately from reverse transcription PCRs using a PrimeScript™ RT reagent Kit (for SYBR Green Assay, Takara, Shiga, Japan), following reaction conditions: 37°C for 15 min followed by 85°C for 5 s. The resultant RNA and cDNA were aliquot and stored at -80°C .

An Eppendorf Mastercycler Realplex 4S system (Eppendorf, Hamburg, Germany) was used to quantify the copy number of 16S rRNA genes from each strain. For the 16S rRNA of *C. cellulovorans* (16S *clocel*), amplification using the forward primer 5'- ACGGCTTTGAGGTGACAGGA -3' and reverse primer 5'- ACCGAACTAACATAAGGGTTGC-3' resulted in a 105 bp fragment. For the 16S rRNA of *C. beijerinckii* (16S *cbei*), amplification with the forward primer 5'- ACCCTTCGGGGCAGGAA-3' and reverse primer 5'- GCGAGTGCTCAACTAAATGGTAGC-3' gave a 125 bp fragment. A SYBR Premix Ex Taq kit (Takara, Shiga, Japan) was used under the following reaction conditions: 90°C for 10 s followed by 40 cycles of 90°C for 15 s and 60°C for 15 s. All assays were performed at least in triplicate. Those products with copy numbers between 10^4 and 10^{13} were used as gradient templates to generate standard curves [32].

Since 16S *clocel* (amplification fragment) and 16S *cbei* (amplification fragment) are expressed with 9 and 14 copies in the genomes of *C. cellulovorans* and *C. beijerinckii*, respectively, the abundance of each strain in the co-culture system was determined by Equations (1) and (2).

$$\begin{aligned} & \text{Abundance of } C. \textit{cellulovorans} \\ &= \frac{16\text{Sclocelcopynumber}/9}{16\text{Sclocelcopynumber}/9 + 16\text{Scbeicopynumber}/14} \end{aligned} \quad (1)$$

$$\begin{aligned} & \text{Abundance of } C. \textit{beijerinckii} \\ &= \frac{16\text{Scbeicopynumber}/14}{16\text{Sclocelcopynumber}/9 + 16\text{Scbeicopynumber}/14} \end{aligned} \quad (2)$$

Analysis

Cell growth on glucose or cellobiose was determined by the measurement of optical density at 600 nm (OD_{600}) by a spectrophotometer (UV mini-1240, Shimadzu Corporation, Tokyo, Japan). Cell density of *C. beijerinckii* ($\text{OD}_{600} 1.2-1.5$) was previously normalized to 1.0 of OD_{600} before inoculating with various volumes.

On particles of AECC, the cell mass was estimated based on bacterial cell protein measurement. The cell dry weight-protein correlation was established for bacteria grown on cellobiose, and this correlation was assumed to be the same for cells grown on AECC. That is: cell mass = pellet protein/ $f_{P/CDW}$, where $f_{P/CDW} = (\text{pellet protein})/(\text{cell dry$

weight). A value of 0.57 was calculated [43]. The pellet protein measurement was described previously [44]. The AECC concentration was calculated by subtracting the estimated cell mass from the pellet dry weight.

Total cellulase activity was measured as indicated before [44] with a minor modification. 10 ml sample was centrifuged (8,000 g for 15 min at 4°C) and washed twice with 0.9% (w/v). The pellet was resuspended in 10 ml 25 mM phosphate buffer (pH 7.0). The incubation was performed at 37°C for 30 min using AECC as the substrate. Liberation of reducing sugars was measured by dinitrosalicylic acid method with glucose as the standard. One unit of total cellulase activity was defined as the amount of enzyme which released 1 μmol of total sugars per min. [45] Total cellulase activity was represented by the pellet cellulase activity, because the supernatant cellulase activity was rather low (lower than 0.05U throughout in the work) compared with the pellet cellulase activity [44].

ABE and organic acids were measured by gas chromatography (GC; 6820; HP-INNOWAX (19091 N-113) capillary chromatographic column; temperature programming [46]; Agilent Technologies, California, USA).

The concentration of total sugars was estimated using the phenol-sulfuric acid method as previously described [47]. Glucose, cellobiose, xylose, xylobiose, arabinose and other sugars were measured using a Dionex Ultimate™ 3000 HPLC (Thermo fisher scientific, Massachusetts, USA) equipped with a corona-charged aerosol detector (CAD). The HPLC column (HPX-87C; Aminex Resin-based) was purchased from BioRad (California, USA). Column temperature was 76°C, and the mobile phase (pure water) flow rate was maintained at 0.4 mL/min.

Reactor productivity was estimated as the total ABE produced in g/L divided by the fermentation time and was expressed as g/L/h. ABE yield was calculated as g of ABE produced per g of AECC used.

The reported results are the average values of three experiments carried out on different occasions. The experimental variation between parallel samples from different reactors under the same conditions was less than 10%.

Abbreviations

ABE: Acetone-butanol-ethanol; AECC: Alkali extracted deshelled corn cobs; CBP: Consolidated bioprocessing.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZW, MW and JL carried out the studies and drafted the manuscript, and YL, LY, PC participated in the project design and manuscript preparation. All authors read and approved the final manuscript.

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