Interactions between *Bacillus cereus* CGMCC 1.895 and *Clostridium beijerinckii* NCIMB 8052 in co-culture for butanol production under non-anaerobic conditions

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Abstract

Low oxygen tolerance and substrate restriction continues to hamper the process of bio-butanol industrialization. In this work, butanol fermentation with co-cultures of *Bacillus cereus* CGMCC 1.895 and *Clostridium beijerinckii* NCIMB 8052 under non-anaerobic conditions was investigated, and the interactions between these two strains were examined. The addition of *B. cereus* CGMCC 1.895 resulted in higher oxygen tolerance and a wider range of substrate utilization, compared with the pure culture of *C. beijerinckii* NCIMB 8052. Butanol concentration reached 10.49 g/L with an

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/bab.1522.

optimized inoculation size of 90% under non-anaerobic conditions, and this concentration was close to that of pure *C. beijerinckii* NCIMB 8052 culture under anaerobic conditions. Dynamic relative abundance analysis demonstrated that the ratio of *C. beijerinckii* NCIMB 8052 accounted for nearly 99% of the co-cultured cells. Furthermore, the substrate utilization range was expanded, allowing the use of corn mash for butanol production. The final concentration of butanol and total solvents was 6.78 g/L and 10.52 g/L, respectively. Co-culture also was performed successfully in 5L fermenter and 8.75 g/L butanol was obtained. Dynamic dissolved oxygen analysis demonstrated that *B. cereus* consumed the dissolved oxygen in the broth, and resulted to the anaerobic condition for *C. beijerinckii*.

1 Introduction

Butanol is an important industrial chemical and a promising gasoline substitute. It is superior to ethanol due to its higher energy density, lower corrosivity, higher octane number and mutual solubility with gasoline [1]. Bio-butanol production by fermentation with Clostridia is also known as acetone–butanol–ethanol (ABE) fermentation, in which its main end-products consist of acetone, butanol and ethanol. Bio-butanol is considered as an ideal, renewable alternative biofuel to diminishing fossil fuel [2]. However, the industrialization of bio-butanol remains to be determined in the foreseeable future due to its high cost, low conversion rate, and other factors. According to molecular genetics, there are four types of butanol-producing strains. All these strains are obligate anaerobic Clostridium [3]. Butanol fermentation was performed under strict anaerobic conditions, which require special anaerobic equipment, a complicated operation and long operation time. This obligate anaerobic characteristic is one of the disadvantages impeding the bio-butanol process. Metabolic engineering has been adapted to improve oxygen tolerance of the butanol-producing strains, and the recombinants could not grow in ambient air conditions [4, 5]. Furthermore, the strains responsible for butanol production were not multifunctional, which can only utilize simple and digestible carbohydrates (glucose, xylose) as substrate, due to the shortage of some key enzymes [6]. Furthermore, amylase is also insufficient in some types of strains, such as *Clostridium beijerinckii* (*C. beijerinckii*) NCIMB 8052, which is therefore incapable of utilizing starch-based materials [7]. In

addition, the metabolism restriction of these strains also block the development of bio-butanol industrialization.

The cooperation between different microorganisms is thought to be an ideal solution to overcome these disadvantages [8]. Co-cultures had been successfully used to guarantee the aerobic fermentation of cellulose and bio-hydrogen, both of which are produced by obligate anaerobic Clostridium species [9, 10]. In 1988, Stevens *et al.* [11] reported that the co-culture of *C. beijerinckii* and *Bacillus cereus* could utilize cheese to produce higher concentrations of butanol than that of pure Clostridium. However, this work has been largely overlooked for the past 20 years. Recently, one native symbiotic system, TSH06, that consisted of two strains (obligate anaerobic *C. Acetobutylicum* TSH1 and facultative anaerobic *B. cereus* TSH2), was isolated in our lab [12]. Compared with traditional ABE fermentation, TSH06 can produce butanol under non-anaerobic conditions. On the other hand, an artificial co-culture was employed again for butanol production in recent years [13, 14]. These studies broke the restriction of the obligate anaerobic cultivation for Clostridium [13,14,15]. However, interactions and details such as the optimal strain ratio, strain relationship and function of the artificial co-culture have never been clearly explored [16, 17].

In this study, the co-culture of *B. cereus* CGMCC 1.895 with amylase activity and *C. beijerinckii* NCIMB 8052 with butanol-producing ability was investigated. In this co-culture, the strain relationship was assumed more complex and interesting than TSH06, the native symbiotic system obtained previously [12]. The optimal inoculation ratios, the dynamic relative abundance of these two strains, and the dynamic variation of dissolved oxygen in the broth were discussed. The possible interactions of these two strains in the co-culture were also explored. These results would be helpful in further understanding of the metabolism of these two strains in co-culture for butanol production. Furthermore, these would be useful for the further optimization of butanol production under non-anaerobic conditions on co-cultures.

2 Materials and Methods

2.1 Strains and medium

The *C. beijerinckii* NCIMB 8052 and *B. cereus* CGMCC 1.895 strains used in this study were purchased from the American Type Culture Collection (ATCC) and the China General Microbiological Culture Collection Center (CGMCC), respectively.

Reinforced *Clostridium* medium (RCM) containing 10.0 g/L of beef extract, 3.0 g/L of yeast extract, 10.0 g/L of tryptone, 5.0 g/L of glucose, 1.0 g/L of starch, 3.0 g/L of sodium acetate, 0.5 g/L of L(+)-cysteine and 5.0 g/L of NaCl was used for the *C. beijerinckii* NCIMB 8052 culture. Lysogeny broth (LB) liquid medium containing 5.0 g/L of yeast extract, 10.0 g/L of tryptone and 10.0 g/L of NaCl was used for the *B. cereus* CGMCC 1.895 culture.

The P2 medium and corn mash medium were used for the fermentation. The P2 medium contained 40-70 g/L of glucose, 1.0 g/L of yeast extract, 0.5 g/L of KH_2PO_4, 0.5 g/L of K_2HPO_4, 2.2 g/L of ammonium acetate, 0.2 g/L of $MgSO_4.7H_2O$, 0.01 g/L of $MnSO_4.H_2O$, 0.01 g/L of $FeSO_4.7H_2O$, 0.01 g/L of NaCl, 0.001 g/L of p-aminobenzoate, 0.001 g/L of thiamin, and 0.00001 g/L of biotin. The corn mash medium contained 6.5% corn flour (w/w), which was boiled for 50 minutes and sterilized at 121°C for 15 minutes.

All reagents and chemicals used in this study were purchased from Sigma-Aldrich, unless mentioned otherwise. The corn flour was purchased from Huyu supermarket (Changping District, Beijing, China).

2.2 Culture condition

The *C. beijerinckii* NCIMB 8052 seed culture was performed in a 100-mL shake flask (with rubber stopper) containing 60 mL of RCM, and was placed in an anaerobic and static condition in an anaerobic chamber (Plas Labs, USA) at 37°C for 24 hours. The *B. cereus* CGMCC 1.895 seed culture was performed in a 100-mL shake flask containing 20 mL of LB liquid medium in aerobic conditions, centrifuged at 200 rpm, and kept at 37°C for 12 hours.

Fermentation was performed in a 100-mL shake flask (with rubber stopper) containing 60 mL of P2 medium or corn mash medium at 37°C in static conditions. Inoculation ratio was 7%. Then, cells were collected at 0, 10, 24, 48, 72 and 96 hours.

The co-culture was performed when the two strains were separately cultured, and when OD_{600nm} reached 2. Then, cells were inoculated into P2 medium or corn mash medium for fermentation. Inoculation ratio was 7%. Cells were collected at 0, 10, 24, 48, 72 and 96 hours.

Bioreactor fermentation was performed in a 5L fermenter containing 3 L of P2 medium at 37°C in static conditions. Inoculation size was 7%. Before inoculation, the broth was saturated by air at 1.0 vvm and centrifuged at 200 rpm, and the initial dissolved oxygen was set as 100%. The fermentation process was performed statically without aeration and agitation.

All the fermentation of co-culture were performed under non-anaerobic conditions with the ordinary incubator or fermenter and no nitrogen or anaerobic device were needed.

2.3 Real-time PCR

Cells were harvested by centrifugation, washed and suspended in 100 mM of Tris-hydrochloride buffer (pH 7.6). The genome was extracted from fresh cells using a Genome Extraction Kit (TaKaRa, Shiga, Japan).

Real-time PCR of 16S rRNA was performed to quantify the relative number of these two strains in the co-culture. For *C. beijerinckii* NCIMB 8052, the following primers were used to amplify the 249-bp fragment: 5'-CGGGCTTAACCTGGGTGCTG-3' and 5'-GCGGCGGCACAGAGGTCAT-3'. For *B. cereus* CGMCC 1.895, the following primers were used to amplify the 158-bp fragment: 5'-TAGTTGAATAAGCTGGCACC-3' and 5'-CGTGGGCTTTCACATCAGA-3'. The reaction system was a 20-μL solution containing 10 μL of SYBR Premix Ex Taq (Takara, Shiga, Japan), 0.5 μL of each primer (5 μmol/L), and 1 μL of DNA template (20 μg/μL). The reaction was performed with a CFX connect PCR machine (Bio-Rad, USA) under the following reaction conditions: 95°C for three minutes (initial denaturation), followed by 40 cycles of 95°C for 10 seconds (denaturation), 55°C for

30 seconds (primer annealing), and primer extension. The dissolution curve increased at 0.5°C per second, from 65°C to 95°C. All samples were repeated three times.

2.4 Analytical methods

Solvents including ethanol, acetone and butanol were analyzed by gas chromatography (GC 2010, Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector (FID) and a glass column (KB-5MS,25 m×0.53 mm×1.00 μm, Kromat Corporation, USA). The temperature of the detector and injector were maintained at 260°C and 240°C, respectively.

The concentration of glucose and acids (acetate and butyrate) were assayed using high-performance liquid chromatography (HPLC) analysis (LC-20AT, Shimadzu Co, Kyoto, Japan). A HPX-87 H column (300×7.8 mm, Bio-Rad, USA) was used with a mobile phase of 5 mM of sulfuric acid and a flow rate of 0.80 mL/min at 65°C. The oxygen concentration in the medium was measured with an oxygen electrode (Hamilton OXYFERM VP 225, Bonaduz, Switzerland).

3 Results

3.1 Co-culture of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052

B. cereus CGMCC 1.895 is aerobic and does not have the butanol-producing ability, while *C. beijerinckii* NCIMB 8052 is anaerobic and has the butanol-producing ability. In order to investigate the effect of *B. cereus* CGMCC 1.895 in the butanol fermentation of *C. beijerinckii* NCIMB 8052, a co-culture was performed on these two strains with different inoculation ratios. The experimental design is shown in Table 1. The inoculation ratio of *B. cereus* CGMCC 1.895 was set from 0% to 100%. The pure *C. beijerinckii* NCIMB 8052 cultured under non-anaerobic conditions was assigned as group T1, the pure *C. beijerinckii* NCIMB 8052 cultured under anaerobic conditions was assigned as group T2, the co-culture of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 under non-anaerobic conditions was assigned as groups T3-T7, the pure *B. cereus* CGMCC 1.895 cultured under non-anaerobic conditions was assigned as group T8, and the pure *B. cereus* CGMCC 1.895 cultured under anaerobic conditions was assigned as group T9. Results are shown in Figure 1.

The culture of pure *C. beijerinckii* NCIMB 8052 could not grow aerobically (group T1) and produce butanol, because it was strictly anaerobic and sensitive to oxygen [18]. In addition, pure *B. cereus* CGMCC 1.895 cells could not ferment glucose to produce ABE in glucose-based P2 medium under any conditions (groups T8-T9). However, for co-cultures (groups T3-T7), all cells grew well and synthesized butanol under non-anaerobic conditions (Fig. 1). The fermentation process in groups T3-T7 was similar to that in the pure *C. beijerinckii* NCIMB 8052 under anaerobic culture (group T2). These results indicate that co-cultures had higher tolerance to oxygen than in the pure culture of *C. beijerinckii* NCIMB 8052 under non-anaerobic conditions.

From groups T3 to T7, with the increasing inoculation ratio of *B. cereus* CGMCC 1.895, butanol production by *C. beijerinckii* NCIMB 8052 was not affected under such condition. Hence, *B. cereus* CGMCC 1.895 strain in the co-culture protected the anaerobic characteristic of *C. beijerinckii* NCIMB 8052 from oxygen damage during the whole fermentation process. Pure *B. cereus* CGMCC 1.895 could not grow in P2 medium, especially in anaerobic conditions. However, it could keep it active in the co-culture, and plays an important role once the co-culture is exposed to air.

3.2 Dynamic relative abundance analysis of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 in co-culture

Since *B. cereus* CGMCC 1.895 was observed to play an important role in the co-culture, the dynamic relative abundance of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 during fermentation was further investigated, in order to determine the relationship between *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 in the co-culture. The 16S rRNA of the co-culture groups (groups T3-T7) was analyzed by Real-time PCR. The different initial cell ratios of *B. cereus* CGMCC 1.895 are presented in Table 2.

The dynamic relative abundance of the two strains in each group is shown as Figure 2**.** The ratio of *C. beijerinckii* NCIMB 8052 cells increased during fermentation, while the ratio of *B. cereus* CGMCC 1.895 cells decreased in the co-culture groups. At the end of the fermentation, the ratio of these two strains reached a stable level for all cases. The ratio of *C. beijerinckii* NCIMB 8052 reached nearly

99%, while the ratio of *B. cereus* CGMCC 1.895 was near to 1%. Furthermore, the higher the initial inoculation ratio of *B. cereus* CGMCC 1.895 was, the faster the cell growth of *C. beijerinckii* NCIMB 8052 became. These results indicate that the presence of *B. cereus* CGMCC 1.895 was helpful for *C. beijerinckii* NCIMB 8052 cell growth under non-anaerobic conditions, and was also beneficial for the synthesis of solvents. When the initial inoculation ratio of *B. cereus* CGMCC 1.895 was higher than 70%, cell growth and solvents synthesis of the co-culture under non-anaerobic conditions was similar with that under anaerobic conditions, which could explain why the fermentation results in group T7 (inoculation ratio of *B. cereus* CGMCC 1.895 was 90%) was close to that in group T2 (pure *C. beijerinckii* NCIMB 8052 cultured anaerobically).

3.3 Benefit of *C. beijerinckii* NCIMB 8052 from amylase activity of *B. cereus* CGMCC 1.895 in co-culture with starch-based medium

B. cereus CGMCC 1.895 has amylase, which helps degrade starch into glucose [19, 20]. However, pure *B. cereus* CGMCC 1.895 could not utilize glucose to produce ABE. *C. beijerinckii* NCIMB 8052 could not utilize starch due to amylase shortage [21]. Hence, pure *B. cereus* CGMCC 1.895 or pure *C. beijerinckii* NCIMB 8052 does not produce ABE with corn mash medium under any anaerobic or non-anaerobic conditions. The co-culture of *C. beijerinckii* NCIMB 8052 and *B. cereus* CGMCC 1.895 was performed with corn mash medium as the substrate. These results are illustrated in Figure 3.

As indicated in Figure 3, when pure *C. beijerinckii* NCIMB 8052 was cultured in corn mash medium, a small amount of glucose (0.09 g/L) and ABE (acetone 0.06 g/L, butanol 0.8 g/L and acetone 1.43 g/L) was detected. For the case of pure *B. cereus* CGMCC 1.895 culture, 3.71 g/L of glucose was obtained, but the final ABE concentration was very low (acetone 0.02 g/L, butanol 0.46 g/L and acetone 0.86 g/L), which were 8.75 g/L and 12.75 g/L, respectively.

Dynamically dissolved oxygen analysis (Fig. 5) suggests that dissolved oxygen was kept constant and did not change during the whole process for controls, because pure *C. beijerinckii* NCIMB 8052 cells could not grow under non-anaerobic conditions. However, for co-cultures, dissolved oxygen rapidly

decreased to 0% within 60 minutes, and was kept stable during the fermentation process. These results demonstrate that *B. cereus* CGMCC 1.895 consumed the dissolved oxygen and provided an anaerobic environment for *C. beijerinckii* NCIMB 8052 cell growth and solvents production. These results coincide with the conclusion of the dynamic relative abundance analysis.

4 Discussion

The co-culture of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 appeared to have obvious advantages compared with the pure culture of *B. cereus* CGMCC 1.895 or *C. beijerinckii* NCIMB 8052. The co-culture could produce butanol under non-anaerobic conditions, and also utilize starch as the substrate to produce butanol. *B. cereus* CGMCC 1.895 plays an important role in the co-culture. Under non-anaerobic conditions, with increasing *B. cereus* CGMCC 1.895 inoculation size, the final concentration of butanol and solvents also increased. The dynamic analysis of the relative abundance of *C. beijerinckii* NCIMB 8052 and *B. cereus* CGMCC 1.895 in the co-culture illustrates that the major strain in the broth was *C. beijerinckii* NCIMB 8052 at the end of the fermentation. The highest butanol concentration was obtained from the least proportion of *Clostridium*.

Aerobic co-culture was beneficial to the obligate anaerobic *Clostridium* [9, 10]. Due to its obligate anaerobic character, *C. beijerinckii* NCIMB 8052 cells could not grow under aerobic conditions. *B. cereus* CGMCC 1.895 consumed the oxygen in the co-culture and provided anaerobic conditions for *C. beijerinckii* NCIMB 8052. *C. beijerinckii* NCIMB 8052 cells grew faster and oxygen was exhausted faster with the increasing inoculation ratio of *B. cereus* CGMCC 1.895. On the other hand, P2 medium maybe not suitable for aerobic *B. cereus* CGMCC 1.895 cell growth. Hence, the ratio of *B. cereus* CGMCC 1.895 in the co-culture system quickly dropped after inoculation. However, it was the small part of *B. cereus* CGMCC 1.895 that played an important role during the whole fermentation. Actually, in this study, samples were taken and dealt with under aerobic conditions, which could be observed as an oxygen shock for *C. beijerinckii* NCIMB 8052. However, the small part of *B. cereus* CGMCC 1.895 in the co-culture protected *C. beijerinckii* NCIMB 8052 from oxygen damage.

Another important role of *B. cereus* CGMCC 1.895 in the co-culture was that *B. cereus* CGMCC 1.895 with amylase activity could provide glucose for *C. beijerinckii* NCIMB 8052 to produce butanol when using a starch-based substrate. Due to enzyme shortage, the substrate of *Clostridium* is restricted to simple or soluble carbohydrates. Introducing another strain can help it with unavailable substrates. The co-culture of *C. beijerinckii* and *Clostridium cellulovorans* (*C. cellulovorans*) can use alkali extracted from deshelled corn cobs to produce ABE, which is a step to consolidated bioprocessing (CBP) [22]. In our study, *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 revealed a cooperation in the co-culture. Corn starch could be degraded into glucose by pure *B. cereus* CGMCC 1.895, but this could not be further transferred to acids or solvents. With glucose accumulation, *B. cereus* CGMCC 1.895 was inhibited and the decomposition reaction stopped. In the co-culture, glucose was immediately transferred by *C. beijerinckii* NCIMB 8052. Hence, both starch degradation and butanol fermentation could be continued. These results demonstrate that ABE production by the co-culture of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 with a starch-based medium was technically feasible.

This study provides more detailed information on the strain relationship between *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 in co-culture under non-anaerobic conditions, which could be helpful for further optimizing butanol production with co-cultures. Since for the co-culture, all the strain activation, seed culture, fermentation were performed under normal culture condition. No anaerobic equipment and N_2 was needed during these process. The cost of anaerobic equipment was saved. Moreover, the operation of the whole process was simplified greatly.

5 Acknowledgements

This work was supported by National Natural Science Foundation of China (NO. 21176141), Tsinghua University Initiative Scientific Research Program (NO.2012THZ02289) and National Energy Administration (NO.20131448926).

6 References

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Figure legends

Fig.1 Time course of glucose consumption and solvents production by pure *C. beijerinckii* NCIMB 8052 and co-culture. (A) Butanol production; (B) Acetone production; (C) Ethanol production; (D) Total solvents production; (E) Glucose consumption. Symbols: (■) Group T2 (pure *C. beijerinckii* 8052 under anaerobic condition); (◇) Group T3 (10% *B. cereus* CGMCC 1.895); (▲) Group T4 (30% *B. cereus* CGMCC 1.895); (▽)Group T5 (50% *B. cereus* CGMCC 1.895); (★) Group T6 (70% *B. cereus* CGMCC 1.895); (□) Group T7 (90% *B. cereus* CGMCC 1.895).

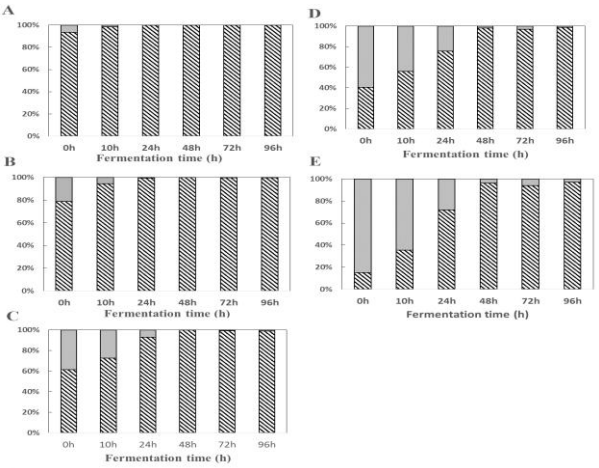

Fig. 2. Dynamic relative abundance of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052 in co-culture. (A) Group T3 (10% *B. cereus* CGMCC 1.895); (B) Group T4 (30% *B. cereus* CGMCC 1.895); (C) Group T5 (50% *B. cereus* CGMCC 1.895); (D) Group T6 (70% *B. cereus* CGMCC 1.895); (E) Group T7 (90% *B. cereus* CGMCC 1.895). Symbols: (▨) cell ratio of *B. cereus* CGMCC 1.895; (▩) cell ratio of *C. beijerinckii* NCIMB 8052.

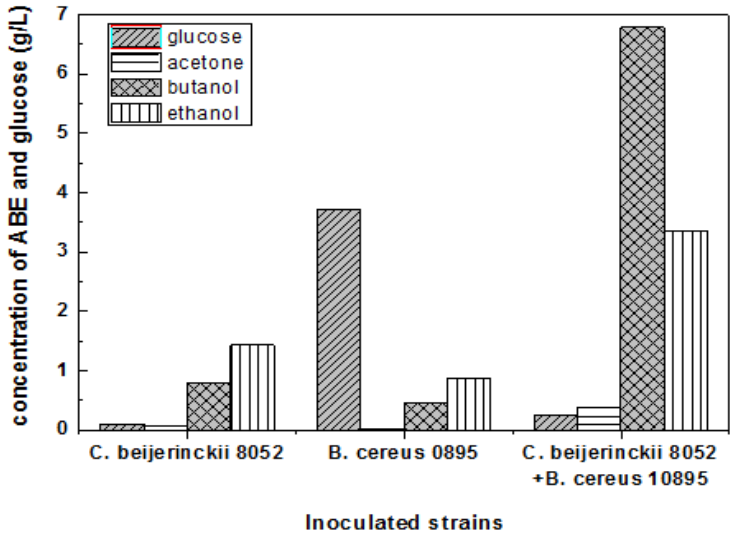

Fig. 3. Glucose consumption and solvents production with corn mash medium by co-culture of *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052. Symbols: (▨)glucose; (▤)acetone; (▩)butanol; (▥) ethanol.

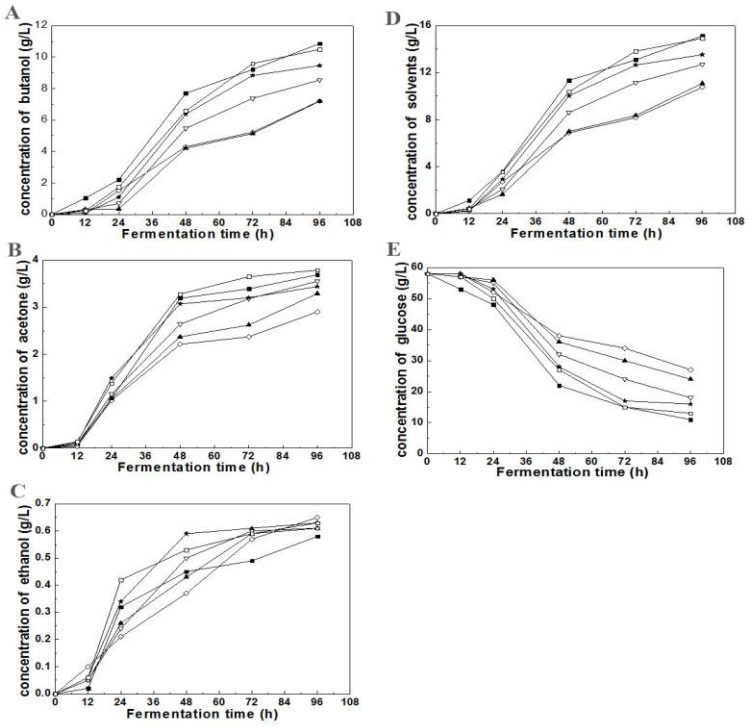

Fig. 4. Butanol fermentation of the co-culture in 5L fermenter. Symbols: (◇)OD_{600nm}; (▲) ethanol; (□) acetone; (◆) butanol; (■) solvents; (△) glucose.

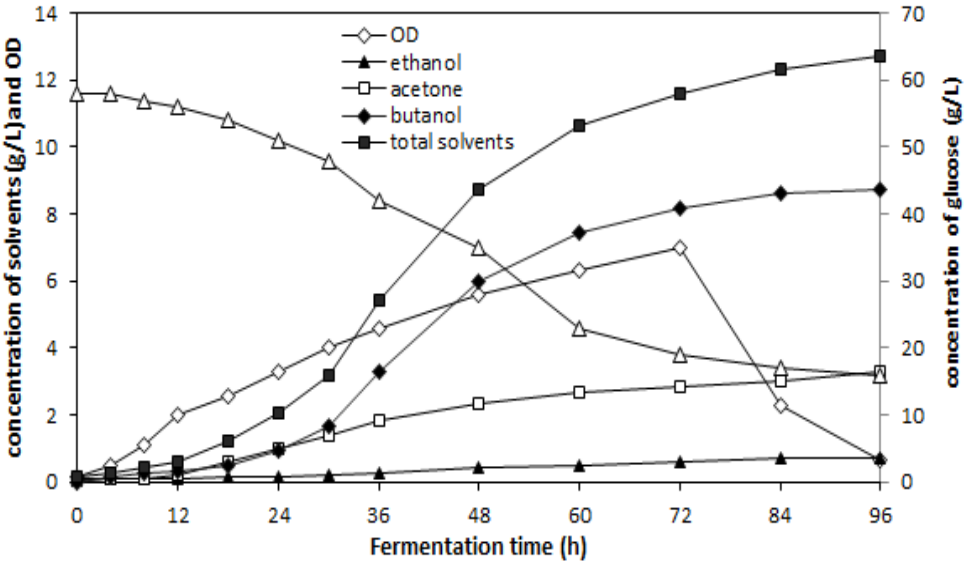

Fig. 5. Dynamic variation of dissolved oxygen (DO) of pure *Clostridium* (control) and co-culture in 5L fermenter. Symbols: (△) control; (◆) co-culture.

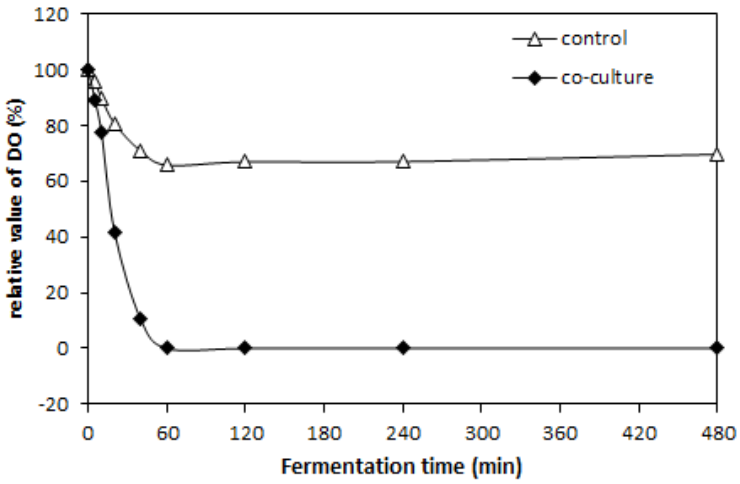

Table 1. Experimental design of different inoculation ratio of *B. cereus* CGMCC 1.895

Group number	Inoculation ratio of *B. cereus* 1.895 (%)	Anaerobic or non-anaerobic [a, b]	*B. cereus* 1.895 (mL)	*C. beijerinckii* 8052 (mL)	Total (mL)
T1	0	- -	0	4.20	4.20
T2	0	++	0	4.20	4.20
T3	10	- -	0.42	3.78	4.20
T4	30	- -	1.26	2.94	4.20
T5	50	- -	2.10	2.10	4.20
T6	70	- -	2.94	1.26	4.20
T7	90	- -	3.78	0.42	4.20
T8	100	- -	4.20	0	4.20
T9	100	++	4.20	0	4.20

[a)] "++" means the fermentation was under anaerobic condition.

[b)] "- -" means the fermentation was under non-anaerobic condition.

Table 2. Initial cell ratio of *B. cereus* CGMCC 1.895 in co-culture

Group number	Inoculation ratio of *B. cereus* 1.895 in volume (%)	Initial cell ratio of *B. cereus* 1.895 (%)
T3	10	6.53
T4	30	21.23
T5	50	38.61
T6	70	59.47
T7	90	84.99