Improvement of 1,3-Propanediol Production From Crude Glycerol by Co-cultivation of Anaerobic and Facultative Microbes Under Non-strictly Anaerobic Conditions

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Research

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Abstract

Background: Natural microbial consortia could efficiently produce 1,3-propanediol, the most promising bulk biochemical derived from glycerol that can be used as a monomer in the synthesis of polytrimethylene terephthalate (PTT). While natural microbial communities are made up of a diverse range of microbes with frequently unknown functions, the construction of synthetic microbial consortia allows for creating more defined systems with lower complexity.

Results: In this study, the synthetic microbial consortia were constructed by combing facultative microbes of *Klebsiella pneumoniae* DUT2 (KP) and/or *Escherichia coli* DUT3 (EC) cultures with the strict anaerobic microbe of *Clostridium butyricum* DUT1 (CB) cultures under micro-aerobic conditions. The function of EC and KP during the fermentation process was to deplete oxygen and provide an anaerobic environment for CB. Furthermore, KP competes with CB to consume crude glycerol and produce 1,3-PDO. The interaction of commensalism and competition resulted in synthetic microbial consortia that could efficiently convert crude glycerol to 1,3-PDO even under micro-aerobic conditions. In a batch fermentation, the synthetic CB:KP co-culture at an initial abundance ratio of 92.5:7.5 yielded a maximum 1,3-PDO concentration of 52.08 g/L, with a yield of 0.49 g/g and a productivity of 1.80 g/(L.h), which increased by 10%, 9%, and 12%, respectively, when compared to the CB mono-culture under strictly anaerobic conditions. Compared to the KP mono-culture, the final 1,3-PDO concentration, yield, and productivity by the synthetic CB:KP consortia increased by 16%, 19%, and 84%, respectively. The synthetic CB:KP:EC co-culture achieved the highest 1,3-PDO flux of 49.17% at an initial abundance ratio of 85:7.5:7.5, while 7.43%, 5.77%, 3.15% 4.24%, and 2.13% of flux was distributed to butyric acid, acetic acid, lactic acid, ethanol, and succinic acid pathways. In a fed-batch fermentation, synthetic CB:KP:EC co-culture demonstrated a maximum 1,3-PDO concentration of 77.68 g/L with a yield of 0.51 g/g which is 30% and 13% higher than the production by the CB mono-culture at 0.02 vvm N_2 supply. The initial abundance of CB guaranteed to be at least 85% facilitates 1,3-PDO production from crude glycerol efficiently by the development of synthetic microbial consortia.

Conclusion: Under micro-aerobic conditions, the synthetic microbial consortia demonstrated excellent performance on 1,3-propanediol production via the interaction of commensalism and competition. The experimental results demonstrated the potential benefit of using the synthetic microbial consortia to produce 1,3-propanediol from crude glycerol.

Background

Since thousands of years, microbial consortia have been widely used in traditional food and beverage fermentation due to their robustness against environmental fluctuations and excellent performances for complex tasks [1, 2]. The use of natural microbial communities in the bioconversion of raw materials to biochemicals such as 1,3-propanediol, lactic acid, ethanol, and propionate has recently received a lot of attention [3–6]. All of these microbial consortia demonstrate excellent utilization of complex carbon sources and high efficiency in producing target products.

In order to improve raw glycerol metabolism to 1,3-propanediol (1,3-PDO), the most promising bulk biochemical that can be used as a monomer for the synthesis of polytrimethylene terephthalate (PTT), several studies focusing on natural microbial consortium has been developed [4, 7–12]. In fed-batch cultivation, a mixed culture from a municipal wastewater treatment plant produced 70.0 g/L 1,3-PDO with a productivity of 2.6 g/(L.h) [8]. The microbial consortium DL38 with 95.57% abundance of *Klebsiella pneumoniae* achieved a relatively high 1,3-PDO titer of 81.4 g/L with a yield of 0.52 g/g [9]. The effect of initial pH on a batch mixed culture fermentation of glycerol was also investigated [10]. And the results indicated that the highest 1,3-PDO production yield of 0.64 mol/mol was obtained at pH 7 and 8 by the predominant bacteria from *Clostridiaceae*, *Enterococcaceae*, and *Enterobacteriaceae* families. Furthermore, an anaerobic microbial consortium C2-2M enriched from anaerobic activated sludge with 94.64% abundance of *Clostridium butyricum* produced 60.61 and 82.66 g/L 1,3-PDO in batch and fed-batch fermentation, with the productivity of 3.79 and 3.06 g/(L.h), respectively [7]. Performances of continuous fermentations were also investigated by consortium C2-2M and the highest 1,3-PDO production of 57.86 g/L was achieved with a productivity of 5.55 g/(L·h) at a dilution rate of 0.096 h^{-1} and an initial glycerol concentration of 130 g/L [11]. Furthermore, under micro-aerobic conditions, a novel consortium DUT08 primarily comprised of a strictly anaerobic microbe of *C. butyricum*, facultative microbes of *K. pneumoniae* and *E. coli* could efficiently convert crude glycerol to 1,3-PDO [4]. In a batch fermentation without nitrogen supply, 43.20 g/L 1,3-PDO with a yield of 0.39 g/g and a productivity of 0.98 g/(L.h) was obtained.

Despite the fact that these natural microbial consortia could efficiently convert crude glycerol to 1,3-PDO, there are still significant limitations in answering the fundamental ecological and evolutionary questions, as well as the interaction mechanism surrounding natural microbial consortia. Even the simplest natural microbial consortia characterized to date contain tens to thousands of species, making it difficult to experimentally verify which species in such characterizations are actively part of the community or perform key functions [13]. The creation of artificial microbial consortia that retain the key features of their natural counterparts is a promising way to overcome the difficulties associated with studying natural communities. The construction of synthetic microbial consortia enables generation of defined systems with reduced complexity.

In our previous work, 1,3-PDO producing microbes of anaerobic *C. butyricum* DUT1 and facultative *K. pneumoniae* DUT2 were isolated from natural microbial consortium DUT08 [4]. In this study, the synthetic microbial consortia was successfully constructed and developed for 1,3-PDO production from crude glycerol. Co-culture systems containing a strict anaerobic microbe of *C. butyricum* and facultative microbes of *K. pneumoniae* and/or *E. coli* were investigated in order to improve the high oxygen tolerance of the strict anaerobes under micro-aerobic conditions. Furthermore, metabolic flux distribution and abundance of synthetic microbial consortia were discussed.

Results And Discussion

Comparison of 1,3-PDO production between mono- and co-culture of C. butyricum **or/and** K. pneumoniae

C. butyricum (CB) and *K. pneumoniae* (KP), having a significant ability of 1,3-PDO production, attracted much attention. In this study, we chose a CB:KP co-culture to take advantage of these two 1,3-PDO producing microorganisms. *K. pneumoniae* is a facultative strain that can grow in aerobic, micro-aerobic, and anaerobic conditions on glycerol. As a result, we hope that the growth of *K. pneumoniae* will create an anaerobic environment that will benefit the growth of *C. butyricum*. The production of 1,3-PDO using crude glycerol under the mono-culture and the CB:KP co-culture at anaerobic serum vial level was tested, and the results are presented in Table 1. *C. butyricum* can grow well with 0.2 vvm nitrogen supply for 3 min, yielding 10.79 g/L 1,3-PDO with a yield to glycerol of 0.54 g/g. The mono-culture of *C. butyricum* produced no 1,3-PDO in the absence of N_2. At different inoculation ratios, The CB:KP co-culture produced nearly identical amounts of 1,3-PDO. After 24 h cultivation without N_2, 6.84 g/L 1,3-PDO was produced at the optimal ratio of 3:2. The level of 1,3-PDO production in the CB:KP co-culture was comparable to that in the KP mono-culture but lower than that in the CB mono-culture. Many byproducts were produced for the KP mono-culture and the CB:KP co-culture, including acetic acid, lactic acid, succinic acid, and ethanol. Butyric acid, on the other hand, was not detected in the CB:KP co-culture. It showed that dissolved oxygen in the medium inhibited the growth of *C. butyricum* in the CB:KP co-culture, even though *K. pneumoniae* was inoculated to create the anaerobic condition. *K. pneumoniae* was primarily responsible for the ability of the CB:KP co-culture to produce 1,3-PDO without N_2 supply. The performance of *K. pneumoniae* in producing 1,3-PDO under micro-aerobic conditions was inferior to that of *C. butyricum* under anaerobic conditions. As a result, the synthetic microbial consortium of *C. butyricum* and *K. pneumoniae* under micro-aerobic condition showed a weak 1,3-PDO production ability that was not comparable to the CB mono-culture under anaerobic condition.

Table 1
Comparison of 1,3-PDO production by the mono-culture and the CB:KP co-culture at different inoculation ratios under anaerobic serum vial level (20 g/L initial glycerol)

Microorganisms	RG (g/L)	Products (g/L)					
		1,3-PDO	Butyrate	Acetate	Lactate	Succinate	Ethanol
C. butyricum DUT1*	0.35	10.79	2.49	-	0.23	-	-
C. butyricum DUT1	19.88	-	-	-	-	-	-
K. pneumoniae DUT3	1.25	6.72	-	1.03	2.16	0.71	1.09
Syn-CB:KP (4:1)	0.78	6.41	-	0.56	1.72	0.32	0.89
Syn-CB:KP (3:2)	1.56	6.84	-	0.43	2.31	0.34	0.91
Syn-CB:KP (2:3)	1.13	6.62	-	0.48	2.41	0.36	1.00
Syn-CB:KP (1:4)	1.27	6.67	-	0.56	2.46	0.35	0.82

*Anaerobic condition: 0.2 vvm N_2 supply for 3 min; RG: Residual glycerol; -: not detectable.

Effect of E. coli addition on mono-culture of CB and co-culture of CB and KP for 1,3-PDO production under non-N_2 aeration conditions

E. coli (EC), a microbe that does not produce 1,3-PDO but consumes oxygen, was inoculated to the mono-culture and co-culture cultivation to improve 1,3-PDO production under micro-aerobic conditions. To this end, the results obtained for 1,3-PDO by different synthetic microbial consortia are represented in Fig. 1.

The results showed that *E. coli* could significantly increase 1,3-PDO production in the CB:EC co-culture at different inoculation ratios when compared to the CB mono-culture without N_2 supply. Glycerol was almost depleted after 24 h of fermentation in the CB mono-culture and the CB:EC co-culture with 0.2 vvm N_2 supply. In the CB:EC co-culture with a 1:1 inoculation ratio and no N_2 supply, 16.38 g/L glycerol remained in the fermentation media, and only 1.72 g/L 1,3-PDO was produced within 24 h. Glycerol was nearly depleted in the CB:EC co-culture after 48 h of fermentation in the absence of N_2. The results showed that the presence of *E. coli* causes *C. butyricum* to grow and produce 1,3-PDO slowly in the absence of N_2. The fermentation time for the CB:EC co-culture was extended under micro-aerobic conditions (no N_2 supply), and the yield of 1,3-PDO to glycerol decreased by 24% at the inoculation ratio of 1:1 when compared to anaerobic conditions (0.2 vvm N_2 supply). Once the anaerobic condition was established by *E. coli*, the CB:EC co-culture could efficiently produce 1,3-PDO regardless of the CB:EC inoculation ratios. At a CB:EC inoculation ratio of 3:2, the optimal 1,3-PDO concentration of 8.48 g/L with a yield to glycerol of 0.42 g/g was obtained. Furthermore, for both the CB mono-culture and the CB:EC co-culture, butyric acid was the main byproduct, followed by acetic acid.

The metabolic profile of the KP:EC co-culture was similar to that of the KP mono-culture. Byproducts included lactic acid, succinic acid, acetic acid, and ethanol. Among these byproducts, lactic acid had the highest concentration. When EC was inoculated to the CB:KP co-culture, butyric acid was produced as the main byproduct instead of lactic acid, indicating that anaerobic cultivation conditions were formed and *C. butyricum* could grow well in the CB:KP:EC co-culture. In comparison to the CB:KP co-culture, the CB:KP:EC co-culture demonstrated a greater ability to remove oxygen due to *E. coli* inoculation. As a result, the highest 1,3-PDO concentration of 7.02 g/L was obtained in the CB:KP:EC co-culture at a CB:KP:EC inoculation ratio of 13:1:1. The CB:KP:EC co-culture reduced the final 1,3-PDO concentration compared to the CB:EC co-culture because *C. butyricum* and *K. pneumoniae* competed for glycerol to produce 1,3-PDO. The ability of KP to produce 1,3-PDO is weaker than the ability of CB, and KP is more competitive than CB under micro-aerobic conditions, making KP to become the dominant in the CB:KP:EC co-culture.

Metabolic profile and dynamic abundance of synthetic microbial consortia in batch fermentation under non-strictly anaerobic conditions

In our previous study, natural microbial consortium DUT08 isolated from wastewater samples could efficiently covert crude glycerol to 1,3-propanediol (1,3-PDO) under anaerobic and micro-aerobic conditions. Because of the coexistence of facultative *K. pneumoniae* and strictly anaerobic *C. butyricum*,

the organism compositions in DUT08 changed during the long-term preservation process. Furthermore, it was difficult to investigate and determine the function of other unidentified organisms in consortium DUT08, which had a low abundance of 2.20%. As a result, construction and maintaining a stable, controlled and robust microbial consortium is critical for efficient 1,3-PDO production. In this case, we constructed and investigated various synthetic microbial consortia composed of strictly anaerobic *C. butyricum*, facultative *K. pneumoniae*, and facultative *E. coli* with or without N_2 supply to efficiently produce 1,3-PDO. the co-production of 1,3-PDO, butyric acid and lactic acid by the synthetic co-culture under batch fermentation at micro-aerobic and anaerobic conditions was investigated, and the results are shown in Fig. 2.

C. butyricum grew well, and no substrate inhibition was observed. At an initial glycerol concentration of 106.01 g/L and 0.20 vvm N_2 supply, an excellent glycerol consumption rate of 3.55 g/(L.h) was obtained (Fig. 2A). As a result, 47.43 g/L 1,3-PDO with 7.45 g/L butyric acid, 5.73 g/L acetic acid, and 2.48 g/L lactic acid were produced by the CB mono-culture under 0.2 vvm N_2 supply. The yield and productivity of 1,3-PDO was 0.45 g/g and 1.61 g/(L.h), respectively. Furthermore, *C. butyricum* did not grow under 0.02 vvm N_2 supply for 3 h at the same initial glycerol concentration, implying that a strictly anaerobic environment did not developed. It has been reported that *C. butyricum* exhibits substrate inhibition at initial glycerol concentration greater than 70 g/L [14]. The same inhibition was also observed by Dietz et al. when mixed cultures were used [8]. In our previous study, obvious substrate inhibition was observed by *C. butyricum* at the initial glycerol of 125.18 g/L [4]. *C. butyricum* grew well in this study, with no substrate inhibition occurred at an initial glycerol of 106.01 g/L and a supply of 0.2 vvm N_2. However, the cells ceased to grow when supplied with 0.02 vvm N_2. The inhibition of dissolved oxygen was relieved once KP and EC were induced into the mono-culture system. Therefore, to obtain a high 1,3-PDO yield and productivity by *C. butyricum*, it is safe to keep the glycerol concentration below 110 g/L and no dissolved oxygen in medium.

The performance of the KP mono-culture was also investigated and the results are presented in Fig. 2B. Although the final 1,3-PDO concentration obtained by the KP mono-culture with an initial glycerol of 110.09 g/L was comparable to *C. butyricum*, *K. pneumoniae* demonstrated weaker glycerol consumption ability and lower 1,3-PDO productivity than *C. butyricum*. The fermentation lasted for 46 h with the KP mono-culture. The final 1,3-PDO concentration, yield, productivity, and glycerol consumption rate were 45.03 g/L, 0.41 g/g, 0.98 g/(L.h), and 2.37 g/(L.h), respectively. When compared to *C. butyricum*, 1,3-PDO yield, productivity, and glycerol consumption rate by *K. pneumoniae* decreased about 9%, 39%, and 33%, respectively. Lactic acid was obtained at a concentration of 25.92 g/L as the main byproduct by *K. pneumoniae*. The critical concentration of lactic acid for inhibiting cell growth of *K. pneumoniae* was determined to be 19 g/L under anaerobic conditions and 26 g/L under aerobic conditions, respectively [15]. Obvious cell growth inhibition was also observed at 12.59 g/L lactic acid after fermenting for 22 h by *K. pneumoniae* DUT2. Most *K. pneumoniae* produce 2,3-butanediol as a byproduct, and the similarity of 2,3-butanediol and 1,3-pandediol causes the separation barriers [16]. *K. pneumoniae* DUT2 shows unique characters from other *K. pneumoniae* strains in that no 2,3-butanediol is produced during the

fermentation process. In a 5L bioreactor, the dynamic dissolved oxygen and oxidation-reduction potential (ORP) variation of the KP mono-culture during the fermentation process was studied. In the KP mono-culture, dissolved oxygen was quickly depleted within 0.5 h. ORP decreased from -250 mv to -580 mv in 8 h, then remained at -580 mv for 15 h before increasing to -500 mv at the end of fermentation. According to the findings, *K. pneumoniae* scavenged oxygen and could create an anaerobic environment for *C. butyricum* growth in order to produce 1,3-PDO.

When KP and CB are co-cultured, KP and CB compete for glycerol consumption to produce 1, 3-PDO, while KP provides an anaerobic environment for CB growth. However, in this study, KP's ability to produce 1,3-PDO from glycerol is weaker than CB's. As a result, if the KP abundance in mix culture is too high, the final 1, 3-PDO concentration and yield to glycerol will be low. In order for CB to grow and produce 1,3-PDO efficiently in the CB: KP co-culture, KP abundance should be kept to a low level. In our pervious study, natural microbial consortium DUT08 showed efficient conversion of crude glycerol to 1,3-PDO at an initial abundance of 85.25% *Clostridium*, 12.54% *Escherichia*, and 0.007% *Klebsiella* [4]. Therefore, the initial abundance of CB in the synthetic microbial consortia was guaranteed to be at least 85%. In the CB:KP co-culture with initial inoculation ratio of 92.5:7.5, 52.08 g/L 1,3-PDO with a yield of 0.49 g/g, a productivity of 1.80 g/(L.h) was obtained at an initial glycerol of 108.79 g/L with 0.02 vvm N_2 supply. Furthermore, byproducts such as 7.14 g/L butyric acid, 2.93 g/L lactic acid, and 6.39 g/L acetic acid were generated (Fig. 2C). No CB growth was observed under the same cultivation conditions as the CB mono-culture. CB accounted for 92.50% of the initial abundance, while KP accounted for 7.50% (Fig. 3). Within 5 h, the KP ratio increased to 28.81%, while the CB ratio decreased to 71.19%. Following that, the abundance of CB increased to 87.17% within 10 h and remained at a high level of 93.41% at 14 h.

The EC mono-dynamic culture's dissolved oxygen and oxidation-reduction potential (ORP) variation was also investigated. Under 0.02 vvm N_2 supply, dissolved oxygen was quickly exhausted in the EC mono-culture within 10 min. ORP decreased from -280 mv to -590 mv in 4 h, then remained at -590 mv for 5 h, increasing to -400 mv at 12.5 h (20 g/L glucose used). The results indicated that *E. coli* scavenged oxygen more quickly than *K. pneumoniae* and provided a anaerobic environment for *C. butyricum* growth to produce 1,3-PDO faster. In the CB:EC co-culture with initial inoculation ratio of 92.5:7.5, 51.33 g/L 1,3-PDO with a yield of 0.49 g/g, a productivity of 1.47 g/(L.h) was obtained at an initial glycerol of 107.18 g/L with 0.02 vvm N_2 supply (Fig. 2D). Compared to the CB:EC co-culture, KP inoculation in the CB mono-culture can enhance glycerol utilization rate and improve 1,3-PDO productivity. The abundance of synthetic CB:EC co-culture was also investigated and the results indicated that the abundance of EC declined from 7.50–3.23% within 5 h, and only 0.83% at 15 h. The abundance of CB increased from 92.5–99.17% within 15 h (Fig. 3).

In the synthetic CB:KP:EC co-culture with initial inoculation ratio of 85:7.5:7.5, 51.43 g/L 1,3-PDO with a yield of 0.49 g/g, a productivity of 1.77 g/(L.h) was obtained at an initial glycerol of 107.18 g/L with 0.02 vvm N_2 supply (Fig. 2E). Furthermore, the main byproducts were 7.77 g/L butyric acid, 6.03 g/L acetic acid, and 3.29 g/L lactic acid, resulting in a total product yield to glycerol of 0.66 g/g. As shown in Fig. 3,

the abundance of synthetic CB:KP:EC indicated that CB was always the dominant bacterium, accounting for over 80% of the total abundance for the whole fermentation.

The metabolic flux distribution of the products under different cultures was examined as shown in Fig. 4. For the KP mono-culture, the metabolic flow was mainly distributed to 1,3-PDO and lactic acid pathway which occupied about 65.06% of the whole glycerol metabolism. For the CB mono-culture under strictly anaerobic condition, 52.14% of glycerol metabolic flow was distributed to 1,3-PDO and butyric acid pathway. The results of the synthetic CB:KP, CB:EC, and CB:KP:EC co-cultures showed that strictly anaerobic and facultative bacteria can coexist under micro-aerobic conditions. The highest 1,3-PDO flux of 49.17% was obtained by the synthetic CB:KP:EC co-culture while 7.43%, 5.77%, 3.15% 4.24% and 2.13% of flux was distributed to butyric acid, acetic acid, lactic acid, ethanol and succinic acid pathway. Synthetic microbial consortia can all efficiently convert crude glycerol to produce 1,3-PDO and achieve high 1,3-PDO yield. The ability to produce 1,3-PDO is dependent on the abundance of 1,3-PDO producing bacteria that have a high 1,3-PDO production capacity as well as a high glycerol utilization capacity. In this study, a maximum 1,3-PDO concentration of 52.08 g/L was obtained by the synthetic CB:KP co-culture at an initial abundance ratio of 92.5:7.5, with a yield of 0.49 g/g and a productivity of 1.80 g/(L.h). Compared to the CB mono-culture under strictly anaerobic condition, the final 1,3-PDO concentration, yield, and productivity by the synthetic CB:KP consortia increased by 10%, 9%, and 12%, respectively. Compared to the KP mono-culture, the final 1,3-PDO concentration, yield, and productivity by the synthetic CB:KP consortia increased by 16%, 19%, and 84%, respectively. The results indicated that synthetic co-culture established in this study improved 1,3-PDO production from crude glycerol significantly.

Several efforts were developed to improve 1,3-PDO production using co-cultivation. A waste lard utilizing *Pseudomonas alcaligenes* PA-3 and a 1,3-PDO producing *Klebsiella pneumoniae* AA405 were co-cultivated by using waste lard as the sole carbon source [17]. In bioreactor, the co-cultivation system produced 5.98 g/L 1,3-PDO and 4.29 g/L 2,3-butanediol (2,3-BD) from 100 g/L waste lard within 72 h, and the conversion rate of 1,3-PDO and 2,3-BD from waste lard were 62.95% and 0.75%, respectively. Moreover, the control of redox balances in co-culture fermentation of *Geobacter sulfurreducens* and *Clostridium pasteurianum* was studied (Moscoviz et al., 2017). As a result, it was demonstrated that *G. sulfurreducens* could grow with *C. pasteurianum* as sole electron acceptor. The metabolic pattern of *C. pasteurianum* was significantly altered in favor of improved 1,3-PDO and butyric acid production (+37% and +38% resp.) at the expense of butanol and ethanol production (-16% and -20% resp.). A novel mixed culture comprising *C. butyricum* and a methane bacterium, *Methanosarcina mazei*, to relieve the inhibition and to utilize the by-products for energy production was investigated by metabolic modelling [18]. The calculations revealed that the presence of methanol can increase methane production by 130%. *M. mazei* can consume more than 70% of the acetate secreted by *C. butyricum*.

Performance and dynamic abundance of synthetic microbial consortia in fed-batch fermentation under non-strictly anaerobic conditions

As previously stated, a high initial glycerol concentration inhibits cell growth and reduces glycerol conversion yield. As a result, fed-batch cultivation by various synthetic consortia was developed and compared to evaluate consortia performance. As shown in Fig. 5, obvious cell growth inhibition was observed in the CB mono-culture with 0.02 vvm N_2 supply, which lasted for at least 12 h. The synthetic CB:KP:EC co-culture exhibited high cell density due to the inoculation of KP and EC. When compared to the synthetic consortia, the natural consortium DUT08 showed similar cell growth to the KP mono-culture and a weak 1,3-PDO production capability. There was only 44.29 g/L 1,3-PDO produced, with a yield of 0.36 g/g and a productivity of 0.94 g/(L.h). The results were very similar to those of the KP mono-culture indicating that *K. pneumoniae* replaced *C. butyricum* as the dominant bacterium in natural consortium DUT08. The final butyric acid concentration of 1.75 g/L and lactic acid concentration of 22.91 g/L obtained by DUT08 also verified this deduction. The results presented here are consistent with those presented in the previous section, indicating that the CB inoculation ratio was an important parameter in the development of synthetic consortia capable of efficiently producing 1,3-PDO from crude glycerol. In our previous report, *C. butyricum* was the dominant bacterium accounting for 85.25% of the proportion in DUT08 [4]. As a result, a higher 1,3-PDO concentration of 58.33 g/L was produced, with a yield of 0.52 g/g and total productivity of 1.94 g/(L.h) at an initial glycerol concentration of 117.01 g/L. Due to the coexistence of facultative bacteria and strictly anaerobic bacteria, the preservation conditions for DUT08 are very strict in order to maintain the bacteria ratio. The varied composition of consortium DUT08 makes the 1,3-PDO production unstable. Therefore, maintaining a stable and controlled composition of organisms within a co-culture is essential and important for efficient 1,3-PDO production.

The stronger 1,3-PDO formation capability was obtained by synthetic consortia of CB:KP, CB:EC, and CB:KP:EC co-cultures. Among the three constructed synthetic consortia, synthetic CB:KP:EC showed the maximum 1,3-PDO concentration of 77.68 g/L which is 30% higher than the production by the CB mono-culture at 0.02 vvm N_2 supply. A higher 1,3-PDO concentration of 81.39 g/L was obtained by the synthetic CB:KP:EC consortium under 0.2 vvm N_2 supply. However, the 1,3-PDO yield decreased from 0.51 g/g to 0.49 g/g when N_2 supply increased from 0.02 vvm to 0.2 vvm. When synthetic consortia were used, butyric acid was the main byproduct. Synthetic CB:KP:EC co-culture yielded over 17 g/L butyric acid. Moreover, the synthetic CB:EC co-culture and CB:KP co-culture produced 14.57 g/L and 15.60 g/L butyric acid, respectively. Because of the high concentration of butyric acid produced, the synthetic consortia are primarily composed of *C. butyricum*, which produces butyric acid. Lactic acid was produced as the primary byproduct by using the natural consortium DUT08 and the KP mono-culture. Lactic acid concentrations in the natural consortium DUT08 and the KP mono-culture are 22.91 g/L and 20.81 g/L, respectively.

As shown in Fig. 6, the abundance of CB in synthetic CB:EC, CB:KP, and CB:KP:EC co-culture accounted for 92.5%, 92.5%, and 85.0% at the beginning, respectively. The abundance of KP increased to 23.61% and 20.10% within 4 h in synthetic CB:KP and CB:KP:EC co-culture under 0.02 vvm N_2 supply, respectively. The results indicated that CB growth was inhibited for the first 4 h in above two synthetic consortia. Subsequently, the abundance of CB started to increase to 91.82% and 89.71% within 8 h, and remained

high at 89.27% and 90.27% at 12 h, respectively. Within 10 min, *E. coli* created an anaerobic condition for the CB:EC co-culture under 0.02 vvm N_2 supply. As a result, the abundance of CB has always been greater than 85%.

Conclusions

In this study, the synthetic microbial consortia were constructed by adding facultative microbe of *K. pneumoniae* DUT2 and/or *E. coli* DUT3 into strict anaerobic microbe of *C. butyricum* DUT1 culture under micro-aerobic conditions. The interaction of commensalism and competition resulted in synthetic microbial consortia that could efficiently convert crude glycerol to 1,3-PDO even under micro-aerobic conditions. In a batch fermentation, the maximum 1,3-PDO titer of 52.08 g/L, with a yield of 0.49 g/g and a productivity of 1.80 g/(L.h) were obtained by synthetic CB:KP co-culture at an initial abundance ratio of 92.5:7.5. Compared to the CB mono-culture under strictly anaerobic condition, the final 1,3-PDO concentration, yield, and productivity by the synthetic CB:KP consortia increased 10%, 9%, and 12%, respectively. The highest 1,3-PDO flux of 49.17% was obtained by the synthetic CB:KP:EC co-culture at an initial abundance ratio of 85:7.5:7.5 while 7.43%, 5.77%, 3.15% 4.24% and 2.13% of flux was distributed to butyric acid, acetic acid, lactic acid, ethanol and succinic acid pathway. In a fed-batch fermentation, synthetic CB:KP:EC co-culture demonstrated maximum 1,3-PDO concentration of 77.68 g/L with a yield of 0.51 g/g which is 30% and 13% higher than the production by the CB mono-culture at 0.02 vvm N_2 supply. The results presented here indicating that the CB inoculation ratio was an important parameter in the development of synthetic consortia capable of efficiently producing 1,3-PDO from crude glycerol.

Materials And Methods

Strains and medium

C. butyricum DTU1, *K. pneumoniae* DTU2 and *E. coli* DTU3 were all isolated from natural microbial consortium DUT08, which was enriched and isolated from wastewater in sewage treatment of petrochemical company [4]. *C. butyricum* DTU1 was anaerobically pre-cultured in a serum bottle containing 100 mL nitrogen-gassed sterilized seed medium at 200 rpm and 37 ^{o}C for 12 h. *K. pneumoniae* DTU2 and *E. coli* DTU3 were aerobically pre-cultured in a serum bottle containing 100 mL sterilized seed medium at 200 rpm and 37 ^{o}C for 12 h, respectively. Seed and fermentation media, as well as crude glycerol used were described as our previous report [4]. Crude glycerol concentration given in this study indicated the absolute glycerol content of the solution regardless of the impurities.

Culture conditions

The synthetic microbial consortia were inoculated to the fermentation medium to determined their ability to convert crude glycerol to 1,3-PDO. The volume ratio was used in all of the inoculation ratios. Before inoculation, the seed cultures were diluted to a similar OD value to maintain a constant inoculation volume. Shake flask culture was carried out in 250 mL serum bottles with media volume of 200 mL. The

batch and fed-batch fermentations were carried out in a 5 L bioreactor with the working volume of 2 L at 37^{o}C and 200 rpm. The inoculum volume for synthetic consortia was 10% (v/v). The pH was controlled at 7.0 automatically adding 5 mol/L NaOH. Continuous feeding was started in fed-batch fermentations when the residue glycerol concentration was less than 20 g/L, and the feeding rate was adjusted to keep the glycerol concentration between 20 and 30 g/L. Anaerobic fermentation was performed with 0.2 vvm N_2 supply for 1 h before and 2 h after inoculation. Micro-aerobic fermentation was achieved with or without 0.02 vvm N_2 supply for 1 h before and 2 h after inoculation. Samples were taken at regular intervals to analyze biomass, glycerol and products concentration. The metabolic flux distributions of the products were calculated by dividing the product formation rates (qp) by the glycerol uptake rate (qs) and multiplying by 100.

qPCR analysis

The bacterial compositions in synthetic microbial consortia were analyzed by quantitative Real-time PCR. Genomic DNA was extracted according to the manufacturer's instructions (TaKaRa MiniBest Bacterial Genome DNA Extraction Kit Ver. 3.0). The purity of DNA was checked by using a NanoDrop ND-2000 spectrophotometer (NanoDrop technologies, Wilmington, DE) and electrophoresis. Primers were designed according to 16S rRNA sequence difference among *C. butyricum* DUT1, *K. pneumoniae* DUT2 and *E. coli* DUT3 listed in Table 2. The reaction mixture for each assay contained 10 μL of SYBR Premic EX Taq (TaKaRa Bio technology Co., Dalian, China), 0.8 μL of each primer, 0.4 μL of ROX Reference Dye II, and 2 μL (20 ng) of genomic DNA. The PCR was performed using the following protocol: 95 ^{o}C for 30 s, following by 40 cycles at 95 ^{o}C for 3 s, and 60°C for 30 s. The assay was performed at least three times for each sample. To generate standard curves for quantification, three standard plasmids were used. Standard plasmid was constructed by pESI-T vector ligation with PCR product of 16S rRNA, and then transformed to *E. coli* DH5α. Copy number was calculated as follows.

$$\text{Copy number} = \frac{\text{DNA amount}}{\text{DNA length} \times 660 \times 1 \times 10^{9}} \times 6.022 \times 10^{23}$$

Table 2
Primers designed for *C. butyricum* DUT1, *K. pneumoniae* DUT2 and *E. coli* DUT3

Primer	Sequences(5'- 3')	Targeted strain
HgF	AAGAAGCTTTAGAAGATCCTAA	*C. butyricum* DUT1
HgR	GGACAACATGAGGTAAACATTG	*C. butyricum* DUT1
phoEF	TGCCCAGACCGATAACTTTA	*K. pneumoniae* DUT2
phoER	CTGTTTCTTCGCTTCACGG	*K. pneumoniae* DUT2
ybbWF	TGATTGGCAAAATCTGGCCG	*E. coli* DUT3
ybbWR	GAAATCGCCCAAATCGCCAT	*E. coli* DUT3

qPCR reactions were run on serial dilutions of each standard plasmid to relate threshold cycle number (Ct value) to copy numbers of the target sequence and to generate standard curves for quantification in unknown samples. Standard curves were linear across five orders of magnitude of 10^2-10^7 copies with R^2 of 0.99-1.

Analytical methods

Biomass concentration was determined by the measurement of optical density at 650 nm (OD_{650}). As previously described, glycerol and its products (1,3-PDO, butyric acid, acetic acid, lactic acid, succinic acid, and ethanol) were analyzed using HPLC (Waters 600E) [4]. Sample solutions were diluted to suitable concentrations and filtered through a 0.22 μm membrane filter before injection.

Abbreviations

1,3-PDO: 1,3-propanediol; CB: *Clostridium butyricum* DUT1; KP: *Klebsiella pneumoniae* DUT2; EC: *Escherichia coli* DUT3; PTT: Polytrimethylene terephthalate; RG: Residual glycerol;

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

All authors approved the consent for publishing the manuscript to Biotechnology for Biofuels.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YQS advised on the experiments design, analyzed results and drafted this manuscript. LYL, YFZ and JDH designed and carried out the experiments. XZL revised the manuscript. All authors read and approved the final manuscript.

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Figures

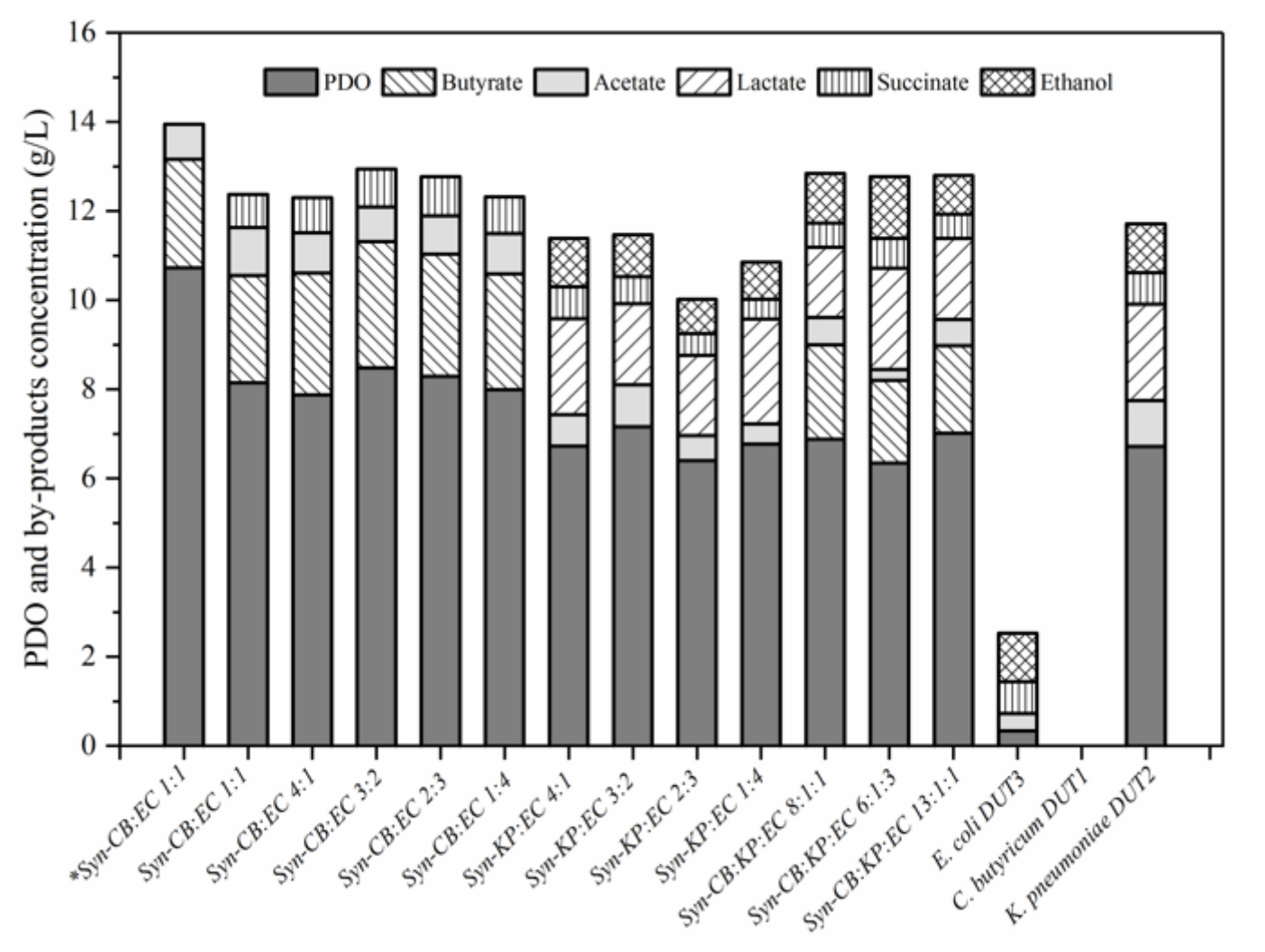

Figure 1

Comparison of 1,3-PDO and byproducts production by the mono-culture and the co-culture at different inoculation ratios without N2 supply. (*Anaerobic condition: 0.2 vvm N2 supply for 3 min)

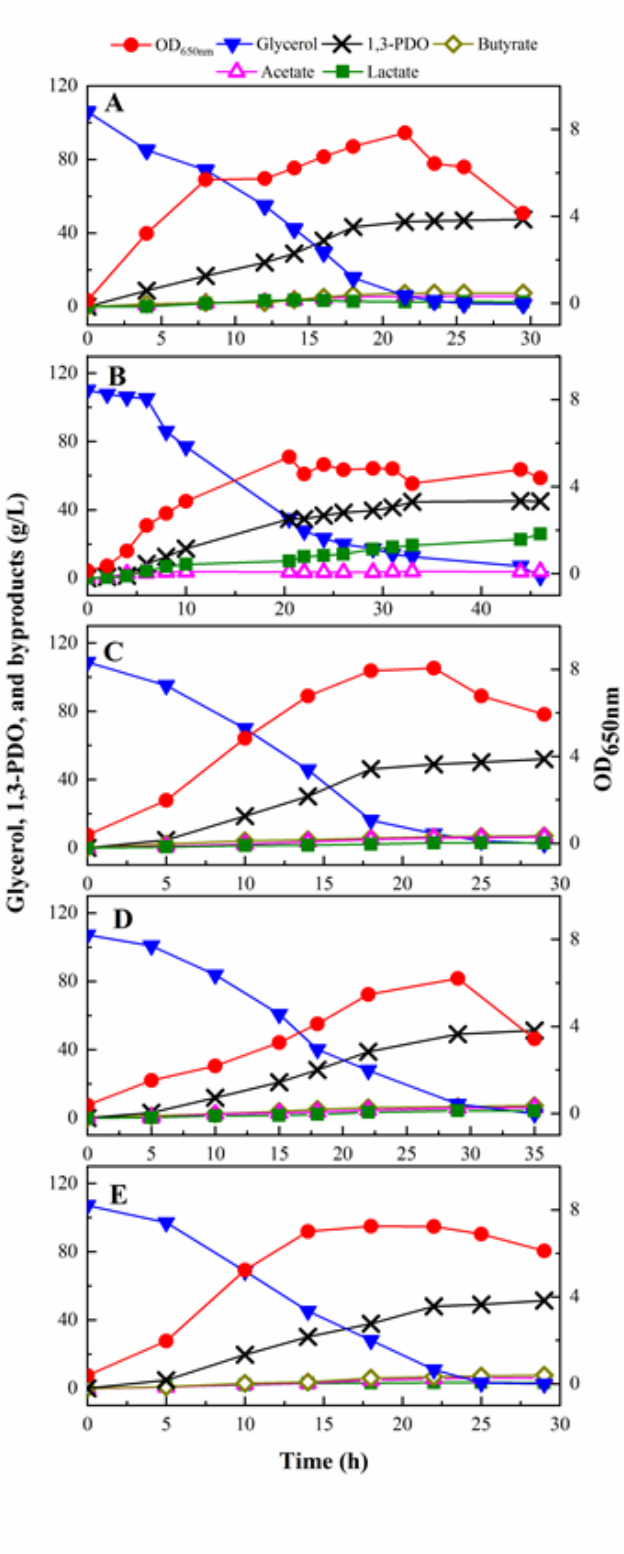

Figure 2

Fermentation performance of mono-culture and synthetic microbial consortia in batch fermentation. (A) The CB mono-culture with N2 supply at 0.2 vvm for 3 h; (B) The KP mono-culture without N2 supply; (C) The CB:KP co-culture with N2 supply at 0.02 vvm for 3 h; (D) The CB:EC co-culture with N2 supply at 0.02 vvm for 3 h; (E) The CB:KP:EC co-culture with N2 supply at 0.02 vvm for 3 h. Fermentation was carried out at 37 oC, pH 7.0 and 200 rpm in 5 L bioreactor containing 2 L medium.

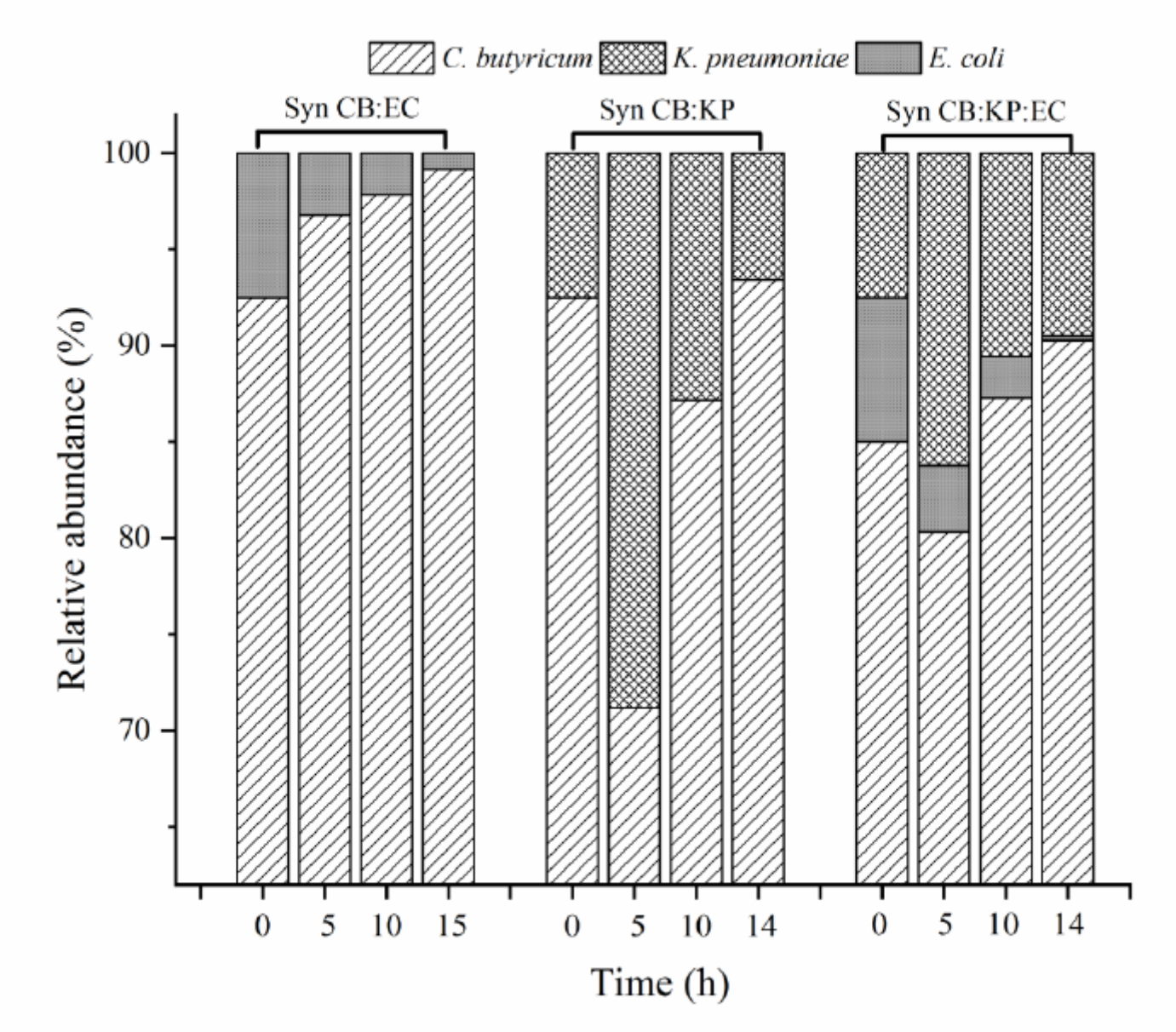

Figure 3

Abundance of synthetic microbial consortia during the batch fermentation at 0.02 vvm N2 supply for 3 h

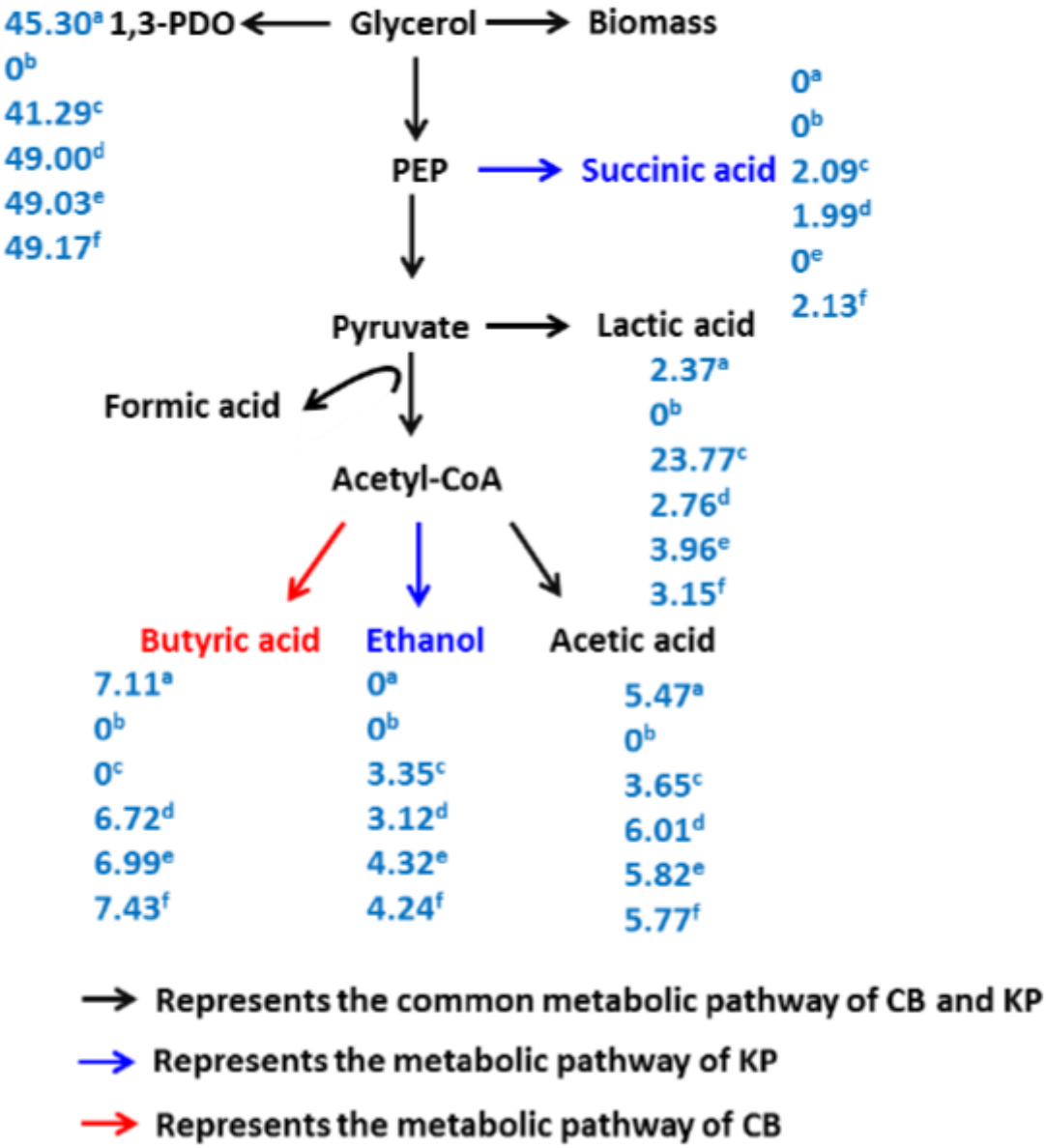

Figure 4

The flux distribution of glycerol metabolism at different mono-culture and synthetic co-culture in batch fermentation. aThe CB mono-culture with N2 supply at 0.2 vvm for 3 h; bThe CB mono-culture with N2 supply at 0.02 vvm for 3 h; cThe KP mono-culture without N2 supply; dThe CB:KP co-culture with N2 supply at 0.02 vvm for 3 h; eThe CB:EC co-culture with N2 supply at 0.02 vvm for 3 h; fThe CB:KP:EC co-culture with N2 supply at 0.02 vvm for 3 h.

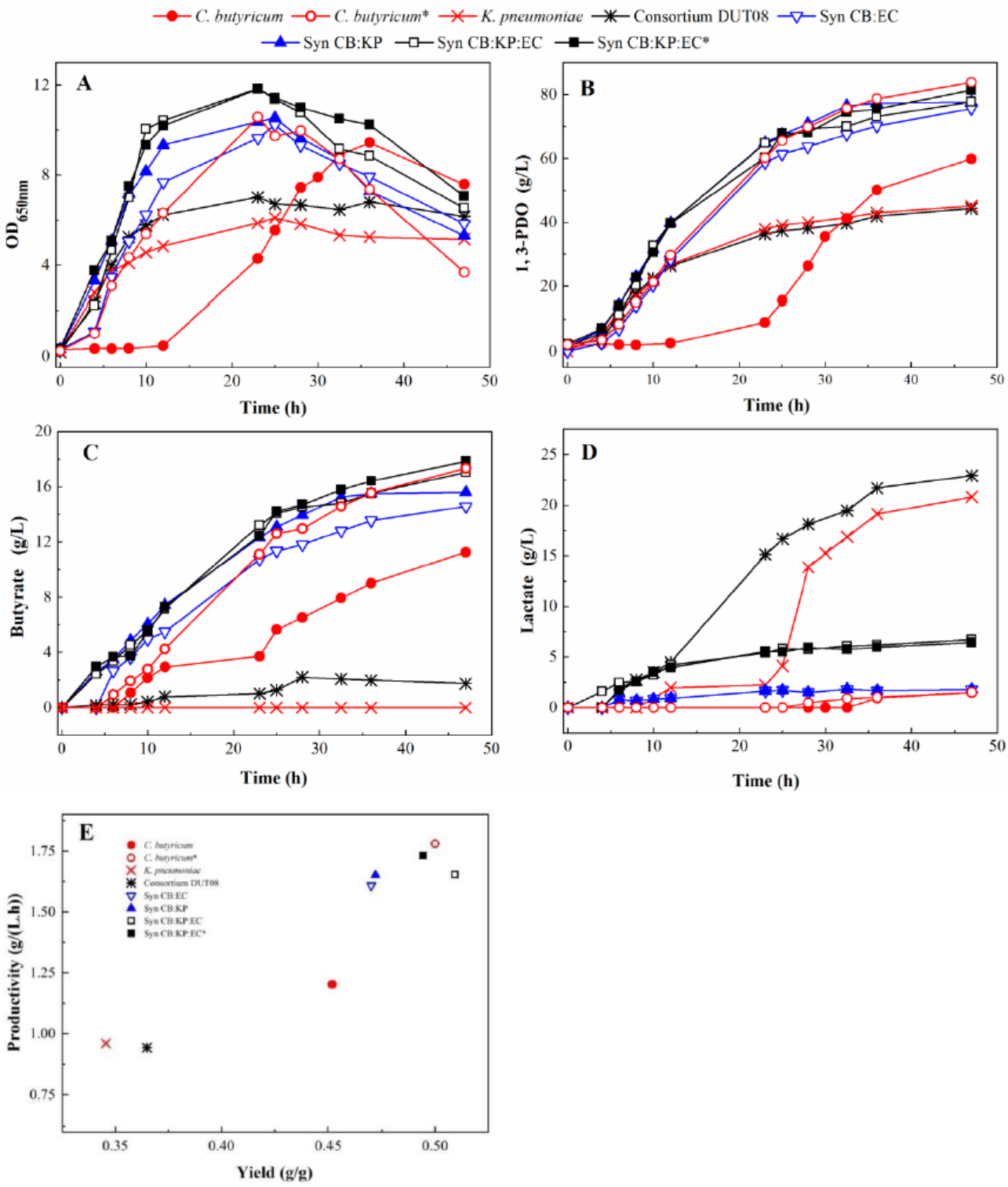

Figure 5

Fermentation performance of the mono-culture and the synthetic microbial consortia at 0.02 vvm N2 supply for 3 h under fed-batch fermentation (A)OD650nm; (B) 1,3-PDO concentration; (C) Butyrate concentration; (D) Lactate concentration; (E) Yield and productivity. Fermentation was carried out at 37 oC, pH 7.0 and 200 rpm in 5 L bioreactor containing 2 L medium. Residual glycerol controlled around 40 g/L. * represented 0.2 vvm N2 supply for 3 h.

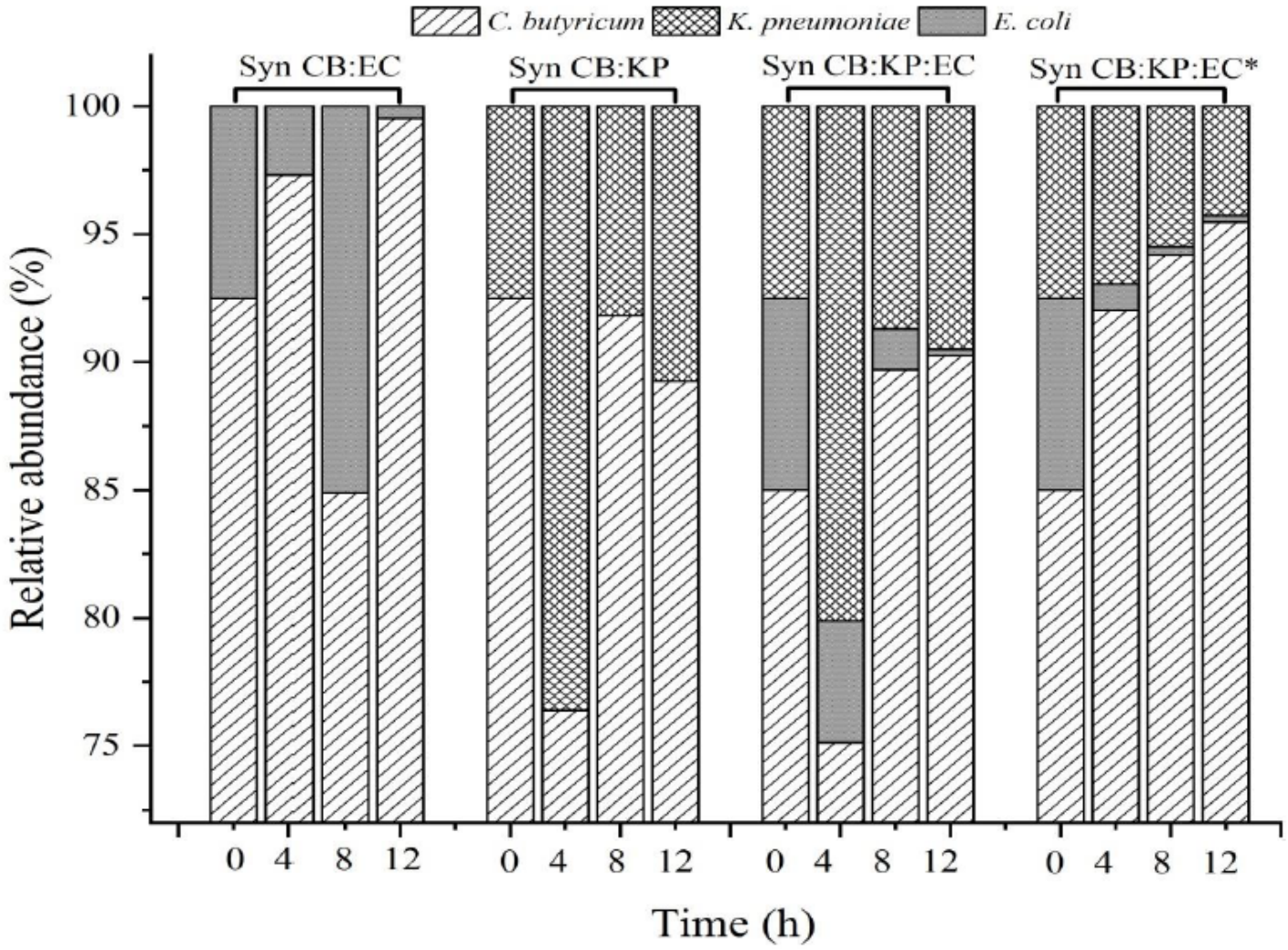

Figure 6

Abundance of synthetic microbial consortia during the fed-batch fermentation process at 0.02 vvm N2 supply for 3 h