Comprehensive evaluation for the one-pot biosynthesis of butyl-acetate by using microbial mono and co-cultures

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Abstract:

Butyl acetate has shown wide attention in food, cosmetics, medicine and biofuel sectors. These short chain fatty acid esters can be produced by either chemical or biological synthetic process with corresponding alcohols and acids. Currently, biosynthesis of short chain fatty acid esters, such as butyl butyrate through microbial fermentation systems has been achieved; however, few studies regarding biosynthesis of butyl acetate were reported. In this study, three proof-of-principle strategies for the one-pot butyl acetate production from glucose by microbial fermentation was designed and evaluated. (1) 7.3 g/L of butyl acetate was synthesized by butanol producing *Clostridium acetobutylicum* NJ4 with the supplementation of exogenous acetic acid; (2) With the addition of butanol, 5.76 g/L of butyl acetate can be synthesized by acetate producing *Actinobacillus succinogenes*130z (Δ*pflA*) with the supplementation of exogenous butanol; (3) Microbial co-culture of *C. acetobutylicum* NJ4 and *A. succinogenes*130z (Δ*pflA*) can directly produce 2.2 g/L of butyl acetate from glucose, representing the first study in terms of butyl acetate production by using microbial co-culture system. Through the immobilization of *A. succinogenes*130z (Δ*pflA*), butyl acetate production was further improved to 2.86 g/L. These strategies may be extended to the biosynthesis of a wide range of esters, especially to some longer chain ones.

Keywords: Butyl acetate; mono- microbial system; artificial co-culture system; immobilized

1. Introduction

Short chain fatty acid esters are a group of high value-added chemicals derived from alcohols and carboxylic acids (Cui, He, Yang, & Zhou, 2020; Guo, Zhu, Deng, & Liu, 2014; Leblanc et al., 1998). These esters naturally exist in some flowers and plant fruits, which have been widely applied in food, cosmetics, and medicine industries (Contino, Foucher, Mounaim-Rousselle, & Jeanmart, 2011; Gupta, Rehman, & Sarviya, 2010; Kojima & Moriga, 1993). Butyl acetate with a sweet smell of banana is a typical short chain fatty acid ester, which can not only be used as fruit flavoring in foods such as candy, ice cream, and baked goods, but also a high-boiling solvent with moderate polarity (Fang et al., 2020; Li, Li, Li, Fang, & Dong, 2019; Wang et al., 2020). In addition, it can also be used as a potential biodiesel additive (Jenkins, Munro, Nash, & Chuck, 2013). When butyl acetate is mixed with biodiesel, the combustion heat and cetane number of biodiesel is not affected. Furthermore, the emissions of soot and greenhouse gases are significantly reduced (Chen et al., 2017). Meanwhile, owning to its lower freezing point, the addition of butyl acetate will improve the fluidity of biodiesel at low temperature, which shows promising potential in aviation sectors (Mangili & Prata, 2019).

Traditionally, butyl acetate can be synthesized by the Fischer esterification of acetic acid and butanol with the presence of catalytic sulfuric acid under high temperature (Loning, Horst, & Hoffmann, 2000; Tian, Zhao, Zheng, & Huang, 2015; Z. T. Zhang, Taylor, & Wang, 2017). However, some disadvantages occurred in this chemical conversion process, such as strong corrosiveness of catalysts, by-products

generation, and environmental pollution et al. (Jermy & Pandurangan, 2005). Alternatively, biological conversion of butanol and acetic acid to butyl acetate under the catalysis of lipases attract more attentions owning to its mild reaction conditions and environmentally friendly properties, which has provided an energy-saving route for the production of esters (Sinumvayo, Zhao, Liu, Li, & Zhang, 2021)$^{-20}$.

Actually, the biological synthesis of some short-chain fatty acid esters, such as butyl butyrate has been achieved through microbial fermentation process (Cui et al., 2020; van den Berg, Heeres, van der Wielen, & Straathof, 2013; Z. T. Zhang et al., 2017). Currently, two strategies were mainly adopted for short chain fatty acid esters synthesis: microbial monoculture and co-culture fermentation (Sinumvayo et al., 2021; Z. T. Zhang et al., 2017). For the microbial monoculture fermentation strategy, microbe can synthesize one precursor, such as acid, and the other precursor such as alcohol can be exogenously supplemented (Xin, Basu, Yang, & He, 2016; Z. T. Zhang et al., 2017). With the catalysis of exogenous lipases, acid and alcohol can be converted to its relevant short chain fatty acid esters (Cui et al., 2020). Under the guidance of this principle, 22.4 g/L of butyl butyrate can be directly synthesized by butanol producing *Clostridium* sp. strain BOH3 with the exogeneous supplementation of 7.9 g/L of butyrate in the fed-batch fermentation process (Xin et al., 2016). Furthermore, when 10 g/L of butanol was added into butyric acid producing fermentation broth by using *C. tyrobutyricum*, 34.7 g/L of butyl butyrate can be synthesized, representing the highest butyl butyrate production through microbial fermentation process (Z. T. Zhang et al., 2017). Although high titer of butyl butyrate

can be obtained through microbial mono-culture fermentation process, however, high amounts of precursors should be supplemented, which will cause the cost increase. Alternatively, microbial co-culture strategy offers one promising way, as strain members can be specifically designed to synthesize alcohol and acid, respectively with the elimination of exogenous addition of acid or alcohol (Cui et al., 2020; Sinumvayo et al., 2021). For example one clostridial consortium composed of butanol-producing *C. beijerinckii* and butyrate-producing *C. tyrobutyricum* has been designed, which could directly produce 5.1 g/L of butyl butyrate from glucose without the addition of any exogenous precursors (Cui et al., 2020). Furthermore, a cognate "diamond-shaped" *Escherichia coli* consortium was metabolically constructed, which was capable of producing 7.2 g/L of butyl butyrate, resenting the highest butyl butyrate production by using microbial co-culture system (Sinumvayo et al., 2021).

Although butyl butyrate production through microbial fermentation process has been comprehensively studied, there are only few reports on butyl acetate production (Noh, Lee, & Jang, 2019; Sinumvayo et al., 2021; Xin, Zhang, & Jiang, 2019). Previously, we have genetically constructed *Actinobacillus succinogenes*130z (Δ*pflA*), which could efficiently produce acetic acid from glucose (W. M. Zhang et al., 2019). Furthermore, one butanol hyper producer of *C. acetobutylicum* NJ4 was isolated and stored by our lab (Jiang, Lv, Michenfelder, et al., 2020; Jiang, Lv, Wu, et al., 2020). Accordingly, three proof-of-principle strategies for butyl acetate production were comprehensively evaluated including *A. succinogenes*130z (Δ*pflA*) fermentation with the exogenous supplementation of butanol, *C. acetobutylicum* NJ4 fermentation with

the exogenous supplementation of acetic acid, and microbial co-culture fermentation composed of *A. succinogenes*130z (Δ*pflA*) and *C. acetobutylicum* NJ4. The fermentation conditions of these three systems were optimized, and the relationship between strain members in the microbial co-culture system was analyzed. Finally, the final titer of butyl acetate was improved by immobilization technology.

2. Materials and methods

2.1 Strains and media

C. acetobutylicum NJ4 was isolated and stored by our lab (Jiang, Lv, Michenfelder, et al., 2020; Jiang, Lv, Wu, et al., 2020). *A. succinogenes* 130z (Δ*pflA*) was obtained by knocking out pyruvate formate-lyase-activating enzyme (*pflA*) from *A. succinogenes* 130z (ATCC 55618), which can efficiently produce acetic acid from glucose (W. M. Zhang et al., 2019). The P1 fermentation medium contains 0.75 g/L of KH_2PO_4, 0.75 g/L of K_2HPO_4, 4.585 g/L of 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino] ethanesulfonic acid, 5 g/L of yeast extract. Na_2SeO_3−Na_2WO_4 solution (1 mL) (Lu, Lv, et al., 2020), 1 mL of trace element solution (Xin et al., 2016), 10 mL of salt solution (Lu, Lv, et al., 2020), and 10 mg of resazurin (oxygen indicator) were added into 1 L medium, respectively. In addition, 0.024 g/L of L(+)−cysteine was added as reductants under N_2. The medium (36 mL) and 600 g/L glucose concentrate (4 mL) was dispensed into 100 mL of serum bottle with nitrogen purged and then autoclaved at 121 °C for 15 min (Jiang, Lv, Wu, et al., 2020).

C. acetobutylicum NJ4 was cultivated in P1 medium. *A. succinogenes* 130z

(Δ*pflA*) was cultivated in medium containing 10 g/L of yeast extract, 1.36 g/L of NaAc, 0.3 g/L of $Na_2HPO_4 \cdot 12H_2O$, 1.6 g/L of $Na_2HPO_4 \cdot 2H_2O$, 3 g/L of K_2HPO_4, 1 g/L of NaCl, 0.2 g/L of $MgCl_2 \cdot 6H_2O$, 0.2 g/L of $CaCl_2$, and 7.5 g/L of corn steep liquor. The microbial consortium was cultivated in P1 medium with 7.5 g/L of corn steep liquor [22, 23].

2.2 Serum bottle fermentation using microbial mono-culture and co-culture

For microbial mono-culture of *C. acetobutylicum* NJ4, 1 mL of inoculum was added into 40 mL of medium with 60 g/L of glucose. The fermentation batches were incubated at 37 °C with 200 rpm. During the fermentation process, pH was adjusted to 5.5 by using 3 M sodium hydroxide solution. Concentrations of glucose, acetic acid, butanol, and butyl acetate in the sample were determined every 24 h. Each experiment was performed in triplicates.

For microbial mono-culture of *A. succinogenes* 130z (Δ*pflA*), 5 mL of inoculum was added into 50 mL medium with 60 g/L of glucose. Besides, during the fermentation process, pH was adjusted to 5.5 by using 3 M sodium hydroxide solution. The fermentation batches were performed at 37 °C with 200 rpm. Concentrations of glucose, acetic acid, butanol, and butyl acetate in the sample were measured every 24 h during the fermentation process. Each experiment was performed in triplicates.

For the microbial co-culture fermentation process, *C. acetobutylicum* NJ4 was first inoculated with 60 g/L of glucose as the carbon source under anaerobic conditions at 37 °C, and medium pH was controlled at 5.5 by using 3 M NaOH solution. The seed inoculum of *A. succinogenes* 130z (Δ*pflA*) was then added. At the

same time, 100 U/mL of lipase and dodecane were added. During the fermentation process, concentrations of glucose, acetic acid, butanol, and butyl acetate in the sample were determined every 24 h.

2.3 Optimization of fermentation conditions

For microbial cultivation of *C. acetobutylicum* NJ4, three factors including pH, acetic acid supplemental concentration and acetic acid addition time were found to have great influence on butyl acetate production. To optimize the fermentation process, the response surface methodology (RSM) was further applied. Acetic acid supplemental concentration, pH and acetic acid addition time were independent variables, while butyl acetate concentration was the dependent variable (Table 1). A set of 17 experiments was designed by using the statistical software Design Expert 10. Each experiment was carried out in triplicates. The relationship between dependent and independent variables is explained through the following second order polynomial equation:

$$Y=\alpha_0+\alpha_1X_1+\alpha_2X_2+\alpha_3X_3+\alpha_{12}X_1X_2+\alpha_{13}X_1X_3+\alpha_{23}X_2X_3+\alpha_{11}X_1^2+\alpha_{22}X_2^2+\alpha_{33}X_3^2 \quad (1)$$

where Y is predicted response (butyl acetate concentration); X1, X2, and X3 are independent variables (pH, acetic acid supplemental concentration and acetic acid addition time); α_0 is offset term; α_1, α_2, and α_3 are linear effects; α_{12}, α_{13}, and α_{23} are squared effects; and α_{11}, α_{22}, and α_{33} are interaction terms. Analysis of variance (ANOVA) was used to perform statistical analysis of the model (Lu, Lv, et al., 2020). For microbial mono-culture of *A. succinogenes* 130z (Δ*pflA*), the influence of several single factors, such as extracting agent, lipase supplemental level, speed and butanol

concentration on the butyl acetate production was explored. Then, the optimized mono-culture conditions were used to produce butyl acetate by microbial co-culture. For the microbial co-culture system, the inoculation time of co-culture was optimized and the titer of butyl acetate was increased by immobilization.

2.4 Analysis of relative transcriptional levels

Total RNAs from different samples of co-culture were extracted with FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China). The DNA was removed and then the RNAs were reverse-transcribed to complementary DNA (cDNA) by using the 5× HiScript II qRT SuperMix II (Vazyme, Nanjing, China). The cDNA was used as a template, and the quantitative real-time polymerase chain reaction (qPCR) assay was performed by using ChamQTM SYBR® qPCR Master Mix (High ROX Premixed, Vazyme, Nanjing, China) in Applied Biosystems (StepOne Plus) to quantify the transcription levels of related genes. The primers used in this study were listed in Table 2. The expression level of target gene was calculated by the method of $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001). The 16S rRNA gene was used to standardize the mRNA levels. Since 16S Δ*pflA* (amplification fragment) and 16S NJ4 (amplification fragment) are expressed with 6 and 11 copies in the genomes of *A. succinogenes* 130z (Δ*pflA*) and *C. acetobutylicum* NJ4, respectively, the abundance of each strain in the co-culture system was determined by Equations (1) and (2) (Geng, He, Qian, Yan, & Zhou, 2010; Wen et al., 2014).

Abundance of Δ*pflA* =

$$\frac{16S\Delta pflA\ copy\ number/6}{16S\Delta pflA\ copy\ number/6+16S\ NJ4\ copy\ number/11} \quad (1)$$

199 Abundance of NJ4 =

200 $$\frac{16S\ \mathrm{NJ4}\ copy\ number/11}{16S\Delta pflA\ copy\ number/6+16S\ NJ4\ copy\ number/11} \quad (2)$$

201 **2.5 Sodium alginate immobilization**

202 2.0 g of sodium alginate was dissolved in 100 mL DI water, which was then
203 autoclaved at 120 °C for 15 min. *A. succinogenes* 130z (Δ *pflA*) in logarithmic growth
204 phase was evenly mixed with sodium alginate solution in the ratio of 1:10. The
205 mixture of sodium alginate (10 mL) and strain 130z Δ *pflA* (1mL) was aspirated with
206 syringe, 20 g/L of $CaCl_2$ solution (80 drops/min) was dripped by drip. Immediately,
207 smooth gel beads were formed, which was then hardened at ambient temperature for
208 30 min and filtered out of the gel beads. After washed with sterile water, the gel beads
209 were filtered out again. After sterilized medium was washed, the surface water was
210 dried with absorbent paper (Lu, Peng, et al., 2020).

211 **2.6 Analytical methods**

212 Concentrations of acetic acid and glucose were measured by high-performance
213 liquid chromatography (HPLC; UltiMate 3000 HPLC system; Dionex, Sunnyvale, CA)
214 using an ion-exchange chromatographic column (Bio-Rad Aminex HPX-87H column)
215 at a wavelength of 215 nm on a UVD 170U ultraviolet detector (Jiang, Lv,
216 Michenfelder, et al., 2020). Butanol and butyl acetate were detected by gas
217 chromatography (GC-2010, Shimadzu Scientific Instruments, Japan) equipped with
218 an InterCap WAX column (0.25 mm×30 m, GL Sciences Inc., Japan) and a flame
219 ionization detector (FID) (Fang et al., 2020). All samples were centrifuged at 12,000 g
220 for 5 min, then 50 μL HCl (2M) was added in 950 mL of samples. Isobutanol was

used as internal standard. The total volume of biogas production was measured on-line through a mass flow controller, a mass flow meter (CS200-A,C,D MFC/MFM, Sevenstar, China) and a gas flow accumulator (D08-8C, Sevenstar, China) (Jiang et al., 2019).

3. Results and discussion

3.1 Biosynthesis of butyl acetate by using microbial mono-culture of *C. acetobutylicum* NJ4 with the supplementation of exogenous acetic acid

As stated in our previous studies, *C. acetobutylicum* NJ4 is a hyper butanol producer, which shows great potential for butyl acetate synthesis through the supplementation of acetic acid (Jiang, Lv, Wu, et al., 2020; Luo et al., 2017; Trindade & dos Santos, 2017). Lipase can directly catalyze acetic acid and butanol to butyl acetate, and *in situ* extraction of butyl acetate could further improve the butyl acetate production and maintain catalytic activities rather than hydrolytic activities of lipase (Xin et al., 2016; Z. T. Zhang et al., 2017). To obtain high butyl acetate production by using microbial monoculture of *C. acetobutylicum* NJ4, pH, supplemented acetic acid concentration and acetic acid addition time were comprehensively investigated. The optimization process using “one time one factor” strategy was first adopted, and the optimized conditions were as follows: fermentation pH at 5.5, acetic acid concentration at 15 g/L, acetic acid addition time at 120 h (Supplementary Fig. 1, 2 and 3).

RSM was further performed with 17 groups of experiments including five replications of the central point, where Y is butyl acetate production (g/L), X_1 represents pH values, X_2 represents the supplemented acetic acid concentration (g/L),

and X_3 represents the acetic acid adding time. Table 1 shows the coded factor levels and reals values for the variables. According to the response values obtained from these experimental results, a second-order regression equation was generated for the response surface: $Y= -459.44+151.32*X_1+1.39*X_2+18.90*X_3+0.07*X_1X_2+0.22*X_1X_3 -0.09*X_2X_3-14.21*X_1^2 -0.05*X_2^2 -1.81*X_3^2$. F and P values indicated the significance of the regression coefficient. The model F value of 0.0134 and P value of 0.0012 indicated that this model was significant. Through the analysis of F value, the importance order of these variables on the butyl acetate production is as follows: pH > acetic acid addition time > supplemented acetic acid concentration. In addition, the quadratic coefficients of X_1^2 and X_3^2 were significant ($p < 0.05$), indicating that these variables had considerable effects on final butyl acetate production. However, the linear coefficient X_2, X_3 and the interaction coefficients of X_1X_2, X_1X_3 and X_2X_3 were not significant in the estimated model with larger P values, suggesting that the interaction of X_1X_2, X_1X_3 and X_2X_3 was slight. The three-dimensional plots of response surfaces demonstrated the interaction between these variables and the optimum condition of each variable for maximum butyl acetate production, which also supported that the interaction coefficients of X_1X_2, X_1X_3 and X_2X_3 were not significant for the final butyl acetate production. As seen in Fig. 1A, 1B and 1C, the predicted maximum butyl acetate production from the response surface model was 7.13 g/L when supplemented acetic acid concentration, acetic acid addition time, and pH were 15.00 g/L, 120 h, and 5.5, respectively.

In order to verify the influence of optimized conditions obtained from Design

Expert software for butyl acetate synthesis by using *C. acetobutylicum* NJ4, the batch fermentation of *C. acetobutylicum* NJ4 with conditions of 15 g/L of supplemented acetic acid, acetic acid addition time at 120 h and pH of 5.5 was carried out. As seen in Fig. 1D, the actual titer of butyl acetate reached 7.30 g/L with a yield of 0.34 g/g glucose, which was equivalent to the predicted level. Compared with 0.25 g/L of butyl acetate before the process optimization, the optimized titer of butyl acetate was increased by 29.2-fold. In details, when *C. acetobutylicum* NJ4 was first cultured under anaerobic conditions for 72 h, 7.82 g/L of butanol and 2.55 g/L of acetic acid were accumulated. When 15 g/L of acetic acid was exogenously supplemented at 72 h, butanol concentration was decreased significantly, and butyl acetate synthesis was onset. Under the optimal conditions, *C. acetobutylicum* NJ4 entered the butanol-producing stage at 24 h. The maximum titer of butyl acetate finally reached 7.30 g/L after 96 h, representing the highest butyl acetate production through microbial fermentation process. With the prolonging of fermentation duration, butyl acetate was slightly hydrolyzed (Table 3). After 144 h, 55.6 g/L of glucose was consumed, meanwhile, 16.12 g/L of acetic acid and 8.39 g/L of butanol were also retained in the fermentation medium.

3.2 Biosynthesis of butyl acetate by using microbial monoculture of *A. succinogenes* 130z (Δ *pflA*) with the supplementation of exogenous butanol

A. succinogenes 130z (Δ *pflA*) was genetically constructed in our laboratory, which can be used for acetic acid production due to the deletion of *pflA* (W. M. Zhang et al., 2019). As known, acetic acid is also one precursor for butyl acetate synthesis,

accordingly, butyl acetate synthesis capability was evaluated by using *A. succinogenes* 130z (Δ *pflA*) based on similar principles as solventgenic *Clostridium* fermentation process. During the fermentation process of *A. succinogenes* 130z (Δ *pflA*), butanol and lipase were exogenously supplemented for butyl acetate synthesis with produced acetic acid. Meanwhile, butyl acetate was also simultaneously extracted into the organic phase of dodecane to increase the final titer. It should be noticed that *A. succinogenes* 130z (Δ *pflA*) is a facultative strain, and its acetic acid production capability varies significantly under aerobic and anaerobic conditions. Hence, acetic acid production capability by using *A. succinogenes* 130z (Δ *pflA*) under different conditions was first investigated. As seen in Fig. 2A, *A. succinogenes* 130z (Δ *pflA*) was capable of producing 10.02 g/L of acetic acid in the presence of oxygen and $MgCO_3$, which can maintain the fermentation pH at 6.8 in the batch fermentation process. While only 6.43 g/L of acetic acid was produced by using *A. succinogenes* 130z (Δ *pflA*) under anaerobic conditions without $MgCO_3$ when pH was controlled at 5.5. However, under aerobic conditions, pH did not show any obvious effects on final acetic acid production by using *A. succinogenes* 130z (Δ *pflA*). For example, 9.95 g/L of acetic acid was still produced under aerobic conditions with pH of 5.5. Taken together, aerobic conditions with pH controlled at 5.5 were adopted for the subsequent fermentation. On the other hand, high butanol concentration would lyse cell membrane and cause toxicity to microbes (Fang et al., 2020). Accordingly, butanol toxicity of *A. succinogenes* 130z (Δ *pflA*) was also evaluated. As shown in Fig. 2B, when exogenous butanol (up to 15 g/L) was supplemented into the fermentation broth of *A.*

succinogenes 130z (Δ *pflA*), there was almost no difference in the cell growth compared to that without butanol supplementation, indicating that low butanol concentration almost had no effect on the growth of strain 130z Δ *pflA*.

The ratio of extractant to medium directly affects the final butyl acetate concentration. The higher of the extractant proportion, the more butyl acetate can be simultaneously extracted from the aqueous phase. As seen from Fig. 2C, less than 0.5 g/L of butyl acetate was detected in the aqueous phase of the fermentation system without adding extractant of dodecane. When 5% extractant was added into the fermentation broth, butyl acetate concentration in the organic phase was improved to 3 g/L. However, further improvement of extractant ratio to 50% did not enhance the final butyl acetate production. The butyl acetate concentration produced by the aqueous phase was further compared (concentration of butyl acetate in aqueous phase = concentration of butyl acetate in organic phase * ratio of extractant). When the extractant ratio was 50%, the butyl acetate concentration produced by the aqueous phase was the highest, which was 1.46 g/L.

Aeration can facilitate acetic acid production of *A. succinogenes* 130z (Δ *pflA*), which would affect the final butyl acetate production. As shown in Fig. 2D, 2.38 g/L of butyl acetate was synthesized at 60 rpm. While at 200 rpm, butyl acetate titer reached 2.92 g/L, which was increased by 22.7%. Furthermore, with the increase of butanol supplementation, the butyl acetate titer was also improved. When 5 g/L of exogenous butanol was supplemented, butyl acetate was only 1.79 g/L. When 20 g/L of exogenous butanol was supplemented, the butyl acetate titer could reach 3.97 g/L, which was

increased by 121.7%. In addition, feeding butanol twice or once with total concentration of 15 g/L had no effect on final butyl acetate production (Fig. 2E). Taken together, when 20 g/L of butanol was added at 24 h, 5.76 g/L of butyl acetate was synthesized by *A. succinogenes* 130z (Δ*pflA*) at 200 rpm with the yield of 0.35 g/g glucose, representing the first report on butyl acetate production through acetate production process (Fig 2F, Table 3).

3.3 Biosynthesis of butyl acetate by using microbial co-culture composed of *C. acetobutylicum* NJ4 and *A. succinogenes* 130z (Δ*pflA*)

Different from the above investigated two examples, microbial co-culture system can eliminate the supplementation of exogenous acid or alcohol during the butyl acetate synthesis process. Accordingly, the microbial co-culture system composed of *C. acetobutylicum* NJ4 and *A. succinogenes* 130z (Δ*pflA*) was evaluated for butyl acetate production without the supplementation of any acetic acid or butanol. Based on their growth and metabolic characteristics, this microbial co-culture system can be divided into two stages. In the first stage, butanol can be specifically synthesized by solventogenic *C. acetobutylicum* NJ4, while in the second stage, *A. succinogenes* 130z (Δ*pflA*) can be inoculated, which was mainly responsible for the synthesis of acetic acid. Both butanol and acetic acid can be simultaneously converted into butyl-acetate under the esterification of lipases. The inoculation time of *A. succinogenes* 130z (Δ*pflA*) showed significant effect on final butyl-acetate production. As shown in Fig. 3A, when *A. succinogenes* 130z (Δ*pflA*) was inoculated at 48 h, the butyl acetate titer was only 0.2 g/L. However, when *A. succinogenes* 130z (Δ*pflA*) was inoculated at 96 h,

the butyl acetate titer reached 2.2 g/L at 168 h, which was almost 10-fold higher than that at 48 h. When *A. succinogenes* 130z (Δ*pflA*) was inoculated at the late fermentation stage of *C. acetobutylicum* NJ4 (120 h), butyl acetate production was decreased.

As seen in Fig. 3B, before the inoculation of *A. succinogenes* 130z (Δ*pflA*), *C. acetobutylicum* NJ4 produced 2.84 g/L of acetic acid, 4.82 g/L of butanol with the consumption of 36.15 g/L of glucose. Once *A. succinogenes* 130z (Δ*pflA*) was inoculated, both acetic acid and butyl acetate production was onset. With the increase of butyl acetate, butanol concentration was decreased, indicating that butanol was simultaneously catalyzed into butyl-acetate. Conversely, acetic acid production was increased with the synthesis of butyl acetate. The proportion change of bacteria composition in this microbial co-culture system was also analyzed by qPCR (Fig. 3C). At 72 h after the co-cultivation of *A. succinogenes* 130z (Δ*pflA*), the percentage of *C. acetobutylicum* NJ4 in this microbial co-culture system was decreased from 72.26% to 2.74%. The percentage of *A. succinogenes* 130z (Δ*pflA*) was increased from 27.54% to 96.26%. *A. succinogenes* 130z (Δ*pflA*) became the dominant strain within this microbial co-culture system at the late stage fermentation for butyl acetate production. The proportion change of bacteria composition within this microbial co-culture system was also in accordance with the change of metabolic profiles, in which butanol was first synthesized followed by acetic acid synthesis (Fig. 3B).

3.4 Transcriptional analysis of key genes expression levels for butyl acetate synthesis in microbial co-culture system

To elaborate the interaction mechanism after these two strain members were co-cultivated, the transcription levels of key genes in this system were analyzed. As seen from Fig. 4, the functional modules of this co-culture system can be divided into two parts. The first is the acetic acid synthesis module within *A. succinogenes* 130z (Δ *pflA*), and the second is the butanol synthesis module within *C. acetobutylicum* NJ4. For the butanol producing strain of *C. acetobutylicum* NJ4, the expression levels of alcohol/aldehyde dehydrogenase (*adhE*) and CoA transferase B (*ctfB*) genes related to butanol synthesis and butyric acid re-utilization were decreased gradually with the increase of the microbial co-cultivation duration (Fig. 4). After co-cultured with *A. succinogenes* 130z (Δ *pflA*), the expression levels of *ctfB* and *adhE* in *C. acetobutylicum* NJ4 showed 5.21- and 3.2-fold increase, respectively at 24 h compared with those of the microbial mono-culture of strain NJ4. However, only 1.32- and 1-fold increase for the expression levels of *adhE* and *ctfB* was observed at 72 h. Different from those of butanol synthesis genes, the expression levels of CoA transferase A (*ctfA*) showed a trend of increase first and then decrease, which was mainly responsible for acetic acid re-utilization (Jiang, Lv, Wu, et al., 2020). In details, the expression levels of *ctfA* showed 4-fold increase at 24 h compared to that of microbial monoculture, and then its expression level was increased to 5.23-fold at 48 h. After 72 h, its expression level was decreased by 2-fold. The change of *ctfA* expression levels in this microbial co-culture system may be attributed to the high acetic acid production by *A. succinogenes* 130z (Δ *pflA*).

In terms of the acetic acid synthesis module, the transcription levels of key genes

pta and *ack* for acetic acid production within *A. succinogenes* 130z (Δ *pflA*) showed a similar profile with that of *ctfA* within *C. acetobutylicum* NJ4, both of which were increased first and then decreased (Fig. 4). In details, after the microbial co-culture was onset, the expression levels of *pta* and *ack* showed 3-fold and 3.42-fold increase at 24 h, respectively. With the increase of acetic acid production, the expression levels of *pta* and *ack* genes were also increased significantly. For example, the highest increase of 6.45 and 7.32-fold for *pta* and *ack* expression levels was observed at 48 h. As seen in Fig.3 B, the highest acetic acid production also occurred after *A. succinogenes* 130z (Δ *pflA*) was co-cultured with *C. acetobutylicum* NJ4 for 48 h. When the fermentation duration was extended to 72 h, their expression levels were slightly decreased to 5.32 and 4.43-fold (Fig. 4). This indicated that the acetic acid productivity is the highest at 48 h. Taken together, the similar expression profile of *ctfA* in *C. acetobutylicum* NJ4 and *pta* and *ack* in *A. succinogenes* 130z (Δ *pflA*) proved that the acetic acid synthesis module can promote the acetic acid complement pathway for butanol production.

3.5 Improved butyl acetate production by using microbial co-culture composed of *C. acetobutylicum* NJ4 and immobilized *A. succinogenes* 130z (Δ*pflA*)

Generally, the microbial co-culture system is unstable as the metabolic products in fermentation medium will affect the microbial growth and metabolic activity (Liu, Lv, Zhang, & Deng, 2014; Lu, Peng, et al., 2020; Zhu et al., 2015) (Fig. 3C). Especially, when *A. succinogenes* 130z (Δ *pflA*) was inoculated into *C. acetobutylicum* NJ4 fermentation medium, the butanol initially produced by strain NJ4 would affect the growth of strain 130z (Δ *pflA*), leading to the instability of strain composition of

this microbial co-culture system (Fig. 3B). Material intervened biological fermentation has been proved as an effective method to improve the microbial stability. Especially, sodium alginate embedding technology has been used in microbial co-culture systems, which could effectively improve the stability of the microbial co-culture system (Liu et al., 2014; Lu, Peng, et al., 2020). Sodium alginate and calcium ions can be crosslinked to form insoluble gel, and cells can be immobilized in gel beads. Furthermore, alginate gel beads can reduce solvent damage to cell membrane (Duarte, Rodrigues, Moran, Valenca, & Nunhez, 2013; Pathania, Sharma, & Handa, 2017). Accordingly, the embedded *A. succinogenes*130z (Δ*pflA*) in sodium alginate was inoculated into this microbial co-culture system to further improve the final butyl acetate production. As shown in Fig. 5A, the inoculation time of embedded cells of *A. succinogenes*130z (Δ *pflA*) will not affect the final butyl acetate production; however, the inoculation time of free cells of *A. succinogenes*130z (Δ *pflA*) showed significant effect on butyl acetate production. For instance, the final butyl acetate production was maintained at around 2.18 g/L, no matter when embedded cells of *A. succinogenes*130z (Δ *pflA*) was inoculated at 96, 120 or 144 h. Instead, comparable butyl acetate production only occurred at 96 h when free cells of *A. succinogenes*130z (Δ *pflA*) were inoculated. When free cells of *A. succinogenes*130z (Δ *pflA*) were inoculated at 120 or 144 h, butyl acetate production was below 0.6 g/L (Fig. 5A). The reason could be that higher amount of butanol (12.2 g/L and 14.5 g/L) was produced by strain NJ4 at 120 or 144 h (data not shown here), and this high initial butanol concentration will affect the bacterial growth and

441 metabolic activity of strain 130z (Δ *pflA*).

442 To enhance the final butyl acetate concentration by using this microbial
443 co-culture system with embedded cells of *A. succinogenes*130z (Δ *pflA*), 5 g/L of
444 acetic acid was further supplemented exogenously to drive the esterification process
445 towards synthetic rather than hydrolytic sides (Fig. 5B). In details, when *C.*
446 *acetobutylicum* NJ4 was cultured for 96 h, it produced 2.84 g/L of acetic acid and 4.98
447 g/L of butanol (Fig. 5B). At this time, embedded cells of *A. succinogenes*130z (Δ*pflA*)
448 was inoculated. 2.02 g/L of butyl acetate was synthesized within total 144 h. Further
449 extension of the fermentation duration cannot improve the butyl acetate production,
450 indicating the esterification reached equilibrium state. When 5 g/L of acetic acid was
451 fed, the maximum 2.86 g/L of butyl acetate was obtained, which was 30% higher than
452 that of the microbial co-culture system without acetic acid.

453 **3.6 Comparison of different fermentation strategies on butyl acetate production**

454 Three fermentation strategies for one-pot butyl acetate production have been
455 designed for the first time. Within these three systems, microbial mono-culture system
456 always gave higher butyl acetate titer than that of co-culture system no matter by
457 using butanol or acetic acid production system (Fig. 1D, 2E and 3B). These findings
458 were in consistency with those of the other short chain fatty acid ester, mainly butyl
459 butyrate production systems (Sinumvayo et al., 2021; Z. T. Zhang et al., 2017). For
460 example, the highest butyl butyrate production occurred in butyrate production system
461 (34.7 g/L), followed by butanol production system (22.4 g/L) and microbial co-culture
462 system (7.2 g/L) (Sinumvayo et al., 2021; Xin et al., 2016; Z. T. Zhang et al., 2017).

In terms of microbial mono-culture system, one of precursors should be exogenously supplemented (Xin et al., 2019). As known, lipases possess two activities including hydrolytic and synthetic ones (van den Berg et al., 2013; Z. T. Zhang et al., 2017). Higher concentrations of acids or alcohols will help facilitate lipases towards synthetic rather than hydrolytic sides (van den Berg et al., 2013). For instance, Xin et al. fed total 7.8 g/L of butyrate (at 0, 48 and 72 h) to improve butyl butyrate to 22.4 g/L of butyl butyrate by using solventogenic *Clostridium* sp. BOH3 (Xin et al., 2016). It should be noticed that strain BOH3 also indigenously generated some amount of butyrate (Xin et al., 2016). Zhang et al. maintained butanol at 10 g/L during 168 h fermentation process by using *C. tyrobutyricum*, and 34.7 g/L of butyl butyrate was produced (Z. T. Zhang et al., 2017). Similarly, improvement of acetic acid or butanol concentration in butanol or acetic acid production system also helped improve final butyl acetate production (Seo, Wang, Lu, Jin, & Blaschek, 2017). The lower concentration of butyl acetate compared to butyl butyrate production system could be attributed to the lower equilibrium constant of lipases in butyl acetate production system (Z. T. Zhang et al., 2017). Further studies to adopt more efficient butanol or acetic acid production system and improvement of lipases equilibrium constant towards synthetic sides should be carried out to improve the final butyl acetate production efficiency (van den Berg et al., 2013; Z. T. Zhang et al., 2017).

Recently, microbial co-culture system has been widely used to synthesize complex structure chemicals, such as plant derived natural products (Kim, Kim, & Kim, 2018; Suastes-Rivas et al., 2020). In terms of short chain fatty acid esters,

485 microbial co-culture system shows advantages in the elimination of alcohol or acid
486 supplementation (Cui et al., 2020; Sinumvayo et al., 2021). Cui et al. have designed a
487 *Clostridium-Clostridium* co-culture system for esters mainly butyl butyrate production,
488 which was the first study reporting utilizing the microbial co-culture to produce short
489 chain fatty acid esters [1]. By adopting this strategy, 5.1 g/L of butyl butyrate could be
490 produced without the addition of exogenous butyrate, representing a more cost
491 effective way to produce esters from the fermentation product of *Clostridium* [1].
492 Furthermore, Sinumvayo et al. constructed a cognate *E. coli* consortium to produce
493 7.2 g/L of butyl butyrate without the exogenous addition of butanol or butyrate
494 (Sinumvayo et al., 2021). To the best of our knowledge, this is the highest titer and
495 yield of butyl butyrate production by microbial co-culture system reported to date
496 (Sinumvayo et al., 2021). Inspired by the successes of these examples, we also
497 constructed a *Clostridium* and *Actinobacillus* co-culture system, which finally
498 produced 2.86 g/L of butyl acetate, representing the first study on butyl acetate
499 production by using microbial co-culture system. The success of this system further
500 proved and provided a new way for the biotechnological production of other short
501 chain fatty acid esters, such as acetyl acetate, butyl lactate et al. Further studies to
502 improve the strain members stability and optimize the fermentation conditions should
503 be investigated to improve the final esters production efficiency.
504

505 ## 5. Conclusion

506 In this study, microbial mono and co-culture systems for butyl acetate

biosynthesis were successfully constructed. The highest 7.30 g/L of butyl acetate with a yield of 0.34 g/g glucose can be produced by using microbial mono-culture of *C. acetobutylicum* NJ4 with the supplementation of exogenous acetic acid after process optimization. Moreover, the highest 2.86 g/L of butyl acetate was produced by using microbial co-culture system composed of *C. acetobutylicum* NJ4 and *A. succinogenes* 130z (Δ *pflA*) with the elimination of butanol and acetic acid supplementation. To the best of our knowledge, these represent the first studies regarding butyl acetate production through microbial mono and co-culture fermentation systems. During these processes, although lipases are the most widely used enzymes for the esterification of carboxylic acids with alcohols, their cost remains a problem. To tackle this obstacle, future studies should focus on overproducing recombinant lipases for selective ester biosynthesis. Moreover, the challenge of different oxygen requirements for acetate and butanol biosynthesis needs to be addressed to achieve a higher yield of butyl acetate.

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Table 1. The factors and levels of Box-Behnken experiment

Independent variable	Units	Coded variable levels		
		-1	0	1
pH		5	5.5	6
Acetic acid concentration	(g/L)	10	15	20
Acetic acid addition time	(h)	4	5	6

Table 2. Primers used in this study

Primer	Sequence
r16s 130z	GCTTTCCATGCTGACGAGTG/GTCGGCTTGGTAGGCCTTTA
r16s NJ4	GGCAGCAGTGGGGAATATTG/CGCCTACACATCCTTTACGC
pta	TATTGGTGTACGGCGACTGT/GCGATACGGGTTGCTTCTTT
ack	CAACCCTGCCCACTTAATCG/ACCTAAACGTTTTGCCGCTT
adhe	ACGGACTAGCACTAGAGGCAAT/CCATAGTTGAAGCGTGAGCCAT
ctfA	CGGATCTGGCTTAGGTGGTGTA/TGCTACATCGGCTGTAAGAGGT
ctfB	ATGCTCTCTGGTATGGGTGGAG/TTGCTTGAGACTTTGCCGTGAG

Table 3. Comparison of short chain fatty acid esters production through microbial fermentation process

Product	Stain	Substrate	Method	Titer	Conversion rate	References
butyl oleate	-	Oleic acid+butanol	Lipase	-	73%	(Ghamgui, Karra-Chaabouni, & Gargouri, 2004)
butyl lactate	-	Ethyl lactate +butanol	Novozyme 435	-	93.6%	(Wang et al., 2020)
butyl butyrate	*Clostridium acetobutylicum*	glucose+butyric acid	Novozyme 435	5 g/L	-	(van den Berg et al., 2013)
butyl butyrate	*Clostridium tyrobutyricum*	glucose+butanol	Novozyme 435	34.7 g/L	-	(Z. T. Zhang et al., 2017)
butyl butyrate	*Clostridium tyrobutyricum*+ *Clostridium beijerinckii*	glucose	Novozyme 435	5.1 g/L	-	(Cui et al., 2020)
butyl butyrate	*E. coli consortium*	glucose	lipase	7.2 g/L		(Sinumvayo et al., 2021)
butyl acetate	*C. acetobutylicum* NJ4	glucose+acetic acid	Novozyme 435	7.30 g/L	-	This study
butyl acetate	*A. succinogenes*130z(Δ*pflA*)	glucose+butanol	Novozyme 435	5.76 g/L	-	This study
butyl acetate	*C. acetobutylicum* NJ4+ *A.*	glucose	Novozyme 435	2.20 g/L	-	This study

	*succinogenes*130z(Δ*pflA*)					
butyl acetate	*C. acetobutylicum* NJ4+ *A. succinogenes*130z(Δ*pflA*)	glucose+acetic acid	Novozyme 435	2.86 g/L	-	This study

Figure legends:

Figure 1. 3D response surface curves of the interactive effects including pH, adding time and concentration of acetic acid on butyl acetate production. (A) Acetic acid addition time and pH of *C. acetobutylicum* NJ4 at fixed level of acetic acid concentration. (B) Acetic acid addition time and acetic acid concentration of *C. acetobutylicum* NJ4 at fixed level of pH. (C) Acetic acid concentration and pH of *C. acetobutylicum* NJ4 at fixed level of acetic acid addition time. (D) Fermentation profiles of *C. acetobutylicum* NJ4 under optimal conditions.

Figure 2．Biosynthesis of butyl acetate by using *A. succinogenes*130z (Δ*pflA*) (A) Acetic acid synthesis of *A. succinogenes*130z (Δ*pflA*) under different conditions. (B) Tolerance of *A. succinogenes*130z (Δ*pflA*) to butanol. (C) Effect of extractant ratio on final concentration of butyl acetate. (D) Effect of speed on synthesis of butyl acetate. (E) Effect of butanol supplementation concentration on final concentration of butyl acetate synthesis. (F) Fermentation profiles of *A. succinogenes*130z (Δ*pflA*) under optimal conditions.

Figure 3．Biosynthesis of butyl acetate by using microbial co-culture system (A) Effect of mixing time on butyl acetate biosynthesis by using microbial co-culture system. (B) Fermentation profiles of microbial co-culture system under optimal conditions. (C) Changes of community composition during synthesis of butyl acetate by microbial co-culture system under optimum conditions

Figure 4．Analysis of key genes expression levels for butyl acetate synthesis in

microbial co-culture system

The transcription levels of key genes for acid reassimilation (*ctfA* and *ctfB*) and butanol production (*adhE*) of *C. acetobutylicum* and key genes in acetic acid production (*pta* and *ack*) of *A. succinogenes*130z (Δ*pflA*).

Figure 5．Butyl acetate production by using microbial co-culture system composed of *C. acetobutylicum* NJ4 and immobilized *A. succinogenes*130z (Δ*pflA*)

(A) Comparison of butyl acetate synthesis by microbial co-culture systems with immobilized and non-immobilized *A. succinogenes*130z (Δ*pflA*). (B) Synthesis of butyl acetate by adding 5 g/L acetic acid in co-culture system.