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Effects of end products on fermentation profiles in *Clostridium carboxidivorans* P7 for syngas fermentation

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HIGHLIGHTS

- Fermentation profiles in *Clostridium carboxidivorans* P7 strain was changed with addition of end-products.
- Acids re-assimilation is more favorable at 37 °C compared to 25 °C.
- Lower temperature facilitates carbon chain elongation.
- Redirection of carbon flow can be achieved by controlling the culture conditions.

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ABSTRACT

Clostridium carboxidivorans P7 is a strict anaerobic bacterium capable of converting syngas to biofuels. However, its fermentation profiles is poorly understood. Here, various end-products, including acetic acid, butyric acid, hexanoic acid, ethanol and butanol were supplemented to evaluate their effects on fermentation profiles in *C. carboxidivorans* at two temperatures. At 37 °C, fatty acids addition likely led to more corresponding alcohols production. At 25 °C, C2 and C4 fatty acids supplementation resulted in more corresponding higher fatty acids, while supplemented hexanoic acid increased yields of C2 and C4 fatty acids and hexanol. Supplementation of ethanol or butanol caused increased production of C2 and C4 acids at both temperatures; however, long-chain alcohols were still more likely produced at lower temperature. In conclusion, fermentation profiles of *C. carboxidivorans* can be changed in respond to pre-added end-products and carbon flow may be redirected to desired products by controlling culture conditions.

1. Introduction

Recent years, due to the exhaustion of fossil fuels and the greenhouse gas emission from the conventional petrochemical processes, special attentions have been paid to explore routes for biofuel and bioenergy production from renewable resources (Jang et al., 2012; Zhang et al., 2011; Kumar and Gayen, 2011). Biofuels such as ethanol and butanol are considered to be promising alternative fuels which can obtained from renewable feedstocks (Ezeji et al., 2007; Chen et al., 2013; Kudahettige-Nilsson et al., 2015; Lin et al., 2015; Munasinghe and Khanal, 2010). Among the various feedstocks used for biofuel production including starch, lignocellulose, waste streams and gases, synthesis gas (syngas) has lately attracted special attentions due to the double benefits of syngas fermentation for carbon sequestration (pollution reduction) and meanwhile energy generation; and also, apparently, such an energy production route does not compete with the current food supply (Mohammadi et al., 2011; Abubackar et al., 2011; Bengelsdorf et al., 2013).

Syngas is a gas mixture that contains primarily CO, CO_2, and H_2, which is mainly derived from gasification of biomass or industrial waste gases. Syngas-consuming microorganisms usually utilize the "Wood-Ljungdahl" pathway to synthesize acetyl-CoA, conserve energy and produce valuable products such as fatty acids and alcohols (Wood et al., 1986). So far, only very few microorganisms, mainly Clostridia species, were identified to be capable of using syngas as substrate to produce medium chain acids and/or alcohols (such as butyric acid, butanol, hexanoic acid and 1-hexanol). Among them, *Butyribacterium methylotrophicum* (Grethlein et al., 1991; Henstra et al., 2007) and *Clostridium ragsdalei* (Babu et al., 2010) were reported to be able to produce butanol from syngas. Lately, a new syngas-consuming clostridial strain named *Clostrid-*

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ium carboxidivorans P7, was isolated from an agricultural settling lagoon (Liou et al., 2005), and characterized to be capable of butanol and hexanol production (Fernandez-Naveira et al., 2016b; Phillips et al., 2015). The overall reactions for butyric acid, butanol, hexanoic acid and hexanol production from H_2 and CO are suggested by following equations (the changes in Gibbs Free Energy at 298 °K and 100 kPa are listed in the corresponding parentheses) (Rajagopalan et al., 2002; Ukpong et al., 2012; Ramachandriya et al., 2013):

$$10CO + 4H_2O \rightarrow C_3H_7COOH + 6CO_2 \ (\Delta G^\circ = -421 \text{ kJ/mol}) \quad (1)$$

$$10H_2 + 4CO_2 \rightarrow C_3H_7COOH + 6H_2O \ (\Delta G^\circ = -220 \text{ kJ/mol}) \quad (2)$$

$$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2 \ (\Delta G^\circ = -486 \text{ kJ/mol}) \quad (3)$$

$$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O \ (\Delta G^\circ = -245 \text{ kJ/mol}) \quad (4)$$

$$16CO + 6H_2O \rightarrow C_5H_{11}COOH + 10CO_2 \ (\Delta G^\circ = -663 \text{ kJ/mol}) \quad (5)$$

$$16H_2 + 6CO_2 \rightarrow C_5H_{11}COOH + 10H_2O \ (\Delta G^\circ = -341 \text{ kJ/mol}) \quad (6)$$

$$18CO + 7H_2O \rightarrow C_6H_{13}OH + 12CO_2 \ (\Delta G^\circ = -759 \text{ kJ/mol}) \quad (7)$$

$$18H_2 + 6CO_2 \rightarrow C_6H_{13}OH + 11H_2O \ (\Delta G^\circ = -397 \text{ kJ/mol}) \quad (8)$$

Although the Gibbs Free Energy calculation suggested favorable reactions for all the above reactions for C4 and C6 products, however, acetate and ethanol were usually the major metabolic products of *C. carboxidivorans* P7 strain when using syngas or CO as substrate (Bruant et al., 2010; Ukpong et al., 2012). Many attempts have been carried out to increase the production of alcohols (especially butanol and hexanol) in P7 by various researchers. Continuous CO supply along with pH regulation has been demonstrated as an efficient way in which the concentrations of ethanol and butanol reached 5.55 and 2.66 g/L, respectively, after 240 h of fermentation in a bioreactor (Fernandez-Naveira et al., 2016b). Phillips and co-workers found that some particular trace metals had significant effects on higher alcohol production in *C. carboxidivorans* P7 (Phillips et al., 2015). When using the modified medium with 10-fold of Mo with elimination of Cu, strain P7 produced 1.09 g/L butanol and 0.94 g/L hexanol. Incubation temperature is another key factor that can enhance the alcohol production (Ramio-Pujol et al., 2015). When *C. carboxidivorans* P7 was cultivated at 37 °C, the pH value of the culture decreased below 4.8 and then the "acid crash" effect was triggered which resulted in reduced solvent production. While the "acid crash" effect can be avoided by lowering the incubation temperature to 25 °C which led to increased production of ethanol and butanol, as well as hexanoic acid and hexanol (1.05 g/L and 0.84 g/L, respectively).

Recently, the whole genome of *C. carboxidivorans* P7 has been sequenced allowing one to gain insights into the solvent-producing metabolic pathways from the molecular level (Bruant et al., 2010; Paul et al., 2010). Genomic analysis revealed that P7 strain possessed the gene cluster of a complete Wood-Ljungdahl pathway as well as all the genetic determinants for ABE production with the exception of acetone formation pathway. However, the production pathways for hexanoic acid and hexanol are still unclear and the distribution of the end-products varies depending on the growth conditions. From a biofuel production viewpoint, fermentations are expected to be conducted in the presence of high concentrations of desired end-products. Many studies have been previously carried out to investigate the growth profiles and end-products production in the presence of end-products or intermediate metabolites. Fernández-Naveira et al. evaluated the inhibition effect of butanol and ethanol on the growth kinetics and bioconversion of CO by *C. carboxidivorans* (Fernandez-Naveira et al., 2016a). The impact of formate on the growth and biofuel productivity of P7 strain was also studied (Ramio-Pujol et al., 2014). The supplementation of high concentration of end-products into the fermentation will inhibit the relevant production pathways, followed by the change of the metabolic fluxes, which will help to understand the biosynthesis pathways and metabolic reprogramming. In order to increase the yield of desired end-products, it is necessary to elucidate the fermentation profiles in *C. carboxidivorans* P7 under controlled conditions. Therefore, in this study, elevated concentrations of acetic acid, butyric acid, hexanoic acid, ethanol, and butanol were supplemented into the fermentation broth to determine their influence on cell growth and end-product production in P7 strain grown on model syngas ($CO:H_2:CO_2$ [70:20:10]) at 25 °C and 37 °C. The redirection of carbon fluxes towards different fatty acids or alcohols under different end-product pressure and incubation temperatures were inspected with the aim to obtain a better understanding of the fermentative metabolism of *C. carboxidivorans* P7.

2. Materials and methods

2.1. Microorganism, medium and cultivation conditions

C. carboxidivorans P7 (DSM 15243) was obtained from DSMZ and was routinely cultivated anaerobically at 37 °C in a modified PETC 1754 medium (American Type Culture Collection [ATCC]) containing (per liter): NH_4Cl, 1.0 g; KCl, 0.1 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.8 g; KH_2PO_4, 0.1 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; $NaHCO_3$, 2.0 g; L-cysteine·HCl, 1.0 g; Yeast extract, 1.0 g; Trace elements (ATCC), 10 mL; Wolfe's vitamin solution (ATCC), 10 ml; Resazurin, 1.0 mg; 2-(N-morpholino)ethanesulfonic acid (MES), 10 g. The initial pH of the medium was adjusted to 6.0 with 1 M NaOH. Syngas was used as the sole carbon source for the fermentation. To cultivate the P7 strain in syngas, headspace gas of bottles was replaced by synthetic syngas mix ($CO:H_2:CO_2$ [70:20:10]) of high purity (Airgas Inc, USA) (Phillips et al., 2015).

2.2. Fermentation

Fermentations were carried out in 250 mL serum bottles with 30 mL reaction volume. Medium-filled bottles were thoroughly flushed with syngas mixture for 2 min and sealed with butyl rubber stoppers, and afterwards autoclaved. Various acids (acetic, butyric or hexanoic acids) and alcohols (ethanol or butanol) at pH 6.0 (pre-adjusted) were aseptically added into the bottles using sterilized syringes to reach the various designated concentrations. The concentrations of acids used in the fermentation were: 2, 4, 6, or 8 g/L acetic acid; 2, 4, 6, or 8 g/L butyric acid; 0.5, 1, 2, or 3 g/L hexanoic acid. The concentrations of alcohols were: 4, 8, 12, or 16 g/L ethanol; 4, 8, 12, or 16 g/L butanol. A 10% inoculum of exponential growth phase culture of *C. carboxidivorans* P7 was used for all fermentations. Fermentation experiments were conducted in duplicates at 25 °C and 37 °C with mild agitation (100 rpm) in shaking incubators. During the fermentation process, the headspace of each bottle was flushed with fresh syngas mixture for 1 min every two days to re-supplement the carbon source.

2.3. Cell growth, pH, and end-product analyses

Liquid samples (0.5–0.7 mL) was taken every one or two days until the end of the fermentation (15 days) to measure the OD at 600 nm using a cell density meter (Ultrospec 10, Biochrom Ltd, Cambridge, England). In order to estimate the cell biomass concentration (g/L), a calibration curve between OD_{600} and the biomass concentration that was reported previously was employed

(Fernandez-Naveira et al., 2016b; Fernandez-Naveira et al., 2016a). pH was measured only at the end of fermentation using a pH meter (B10P, VWR Inc., USA). Additional 1 mL of samples were withdrawn at the end of the fermentation to analyze the metabolic products. Acids (acetic, butyric and hexanoic acid) and alcohols (ethanol, butanol and hexanol) were quantified using a high performance liquid chromatography (HPLC) (Agilent 1260 series, Agilent Technologies, Santa Clara, CA, USA) equipped with an automatic sampler/injector and a refractive index detector (RID) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). 5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL/min at 25 °C. Liquid samples were centrifuged at 15,000 rpm for 10 min and supernatant was taken for the analysis.

With the supplementation of various concentrations of acids and alcohols, the cell biomass concentrations differ very much from each other. Thus, the comparison of absolute titers of the end-products in different fermentations could be misleading for the purpose of elucidating the fermentation profiles in *C. carboxidivorans* P7. Therefore, cell biomass specific yield (BSY, g/g) of the end-product, defined as the end-product production (g/L) normalized to the maximum cell biomass (g/L), was employed to profile the fermentations under various conditions. It is worthwhile to notice though that BSY is, strictly speaking, not a defined yield; the end-product production is not solely from the supplemented carbon source as acids or alcohols, but also from the syngas. Hence, it is normal to see BSY > 1.

3. Results and discussion

3.1. Cell growth and final culture pH with fatty acids supplementation

Growth of *C. carboxidivorans* P7 in the fermentations at 25 °C and 37 °C was monitored in the presence of various concentrations

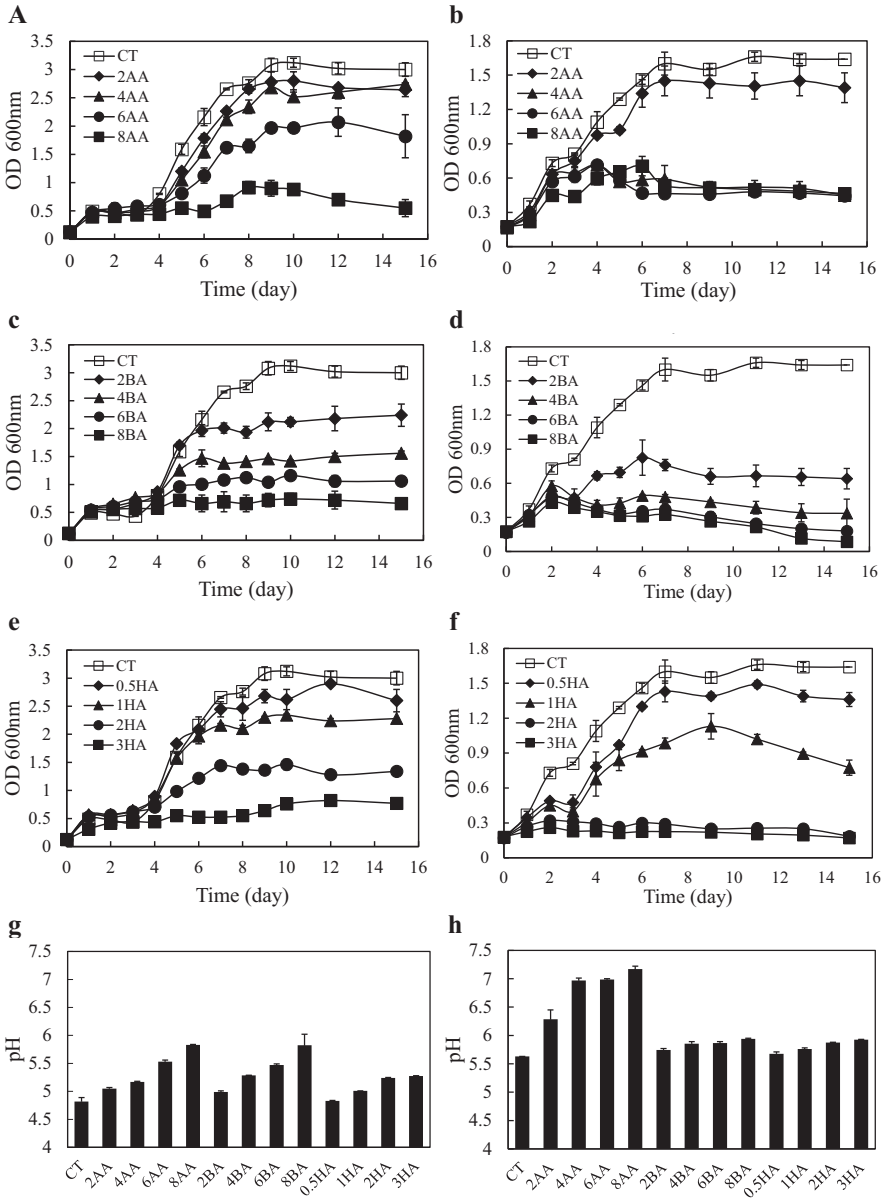

Fig. 1. Growth patterns (a, b, c, d, e, f) and final culture pH (g, h) of *C. carboxidivorans* P7 cultured at 25 °C (a, c, e, g) and 37 °C (b, d, f, h) in the presence of (a, b) 2, 4, 6, 8 g/L acetic acid (2AA, 4AA, 6AA, 8AA), (c, d) 2, 4, 6, 8 g/L butyric acid (2BA, 4BA, 6BA, 8BA) and (e, f) 0.5, 1, 2, 3 g/L hexanoic acid (0.5HA, 1HA, 2HA, 3HA). The fermentation without addition of acids was carried out as control (CT).

of individual acids (acetic, butyric or hexanoic acid). *C. carboxidivorans* P7 was able to grow under all individual acid supplementation conditions. However, the cell density at 25 °C was much higher than that of 37 °C (Fig. 1). The maximum OD_{600} of the control without addition of acids reached 3.12 at 25 °C, almost the double of that at 37 °C (maximum OD_{600} = 1.66). This is coincident with the result reported by Ramió-Pujol and co-workers that maximum OD_{600} of 0.55 was obtained at 37 °C and maximum OD_{600} of 1.2 was obtained at 25 °C (Ramio-Pujol et al., 2015). However, at the same temperature, the maximum cell density in our experiments was almost two times higher than that by Ramió-Pujol et al. This could be because different syngas substrates have been used in the different studies. Low cell biomass density is a major bottleneck for syngas based fermentations, therefore the culture condition used in our study is more favorable for biofuel production by P7 strain.

All three kinds of fatty acids added into cultures had negative effects on the cell growth. Generally, with the increase of supplemented acid concentration, the maximum OD_{600} of cell growth decreased. Under all conditions at 25 °C, a lag phase of ~4 days was observed; while for all conditions at 37 °C, a lag phase of <1 day was required. This indicated that the higher temperature facilitated the initiation of cell growth in the syngas fermentation. However, comparatively, the toxicity of acids on cell growth was lower at 25 °C than that at 37 °C (Fig. 1). When high concentrations of acetic acid ($\geqslant$4 g/L), butyric acid ($\geqslant$2 g/L) and hexanoic acid ($\geqslant$1 g/L) were added into the fermentation at 37 °C, the maximum cell density were decreased by >50% compared with the control. However, 50% inhibition on cell growth was observed at 25 °C when the concentration of the supplemented acetic acid, butyric acid and hexanoic acid reached 8 g/L, 4 g/L, and 2 g/L, respectively. When the same amount of individual acids was added in the culture at the same temperature, the inhibitory effect on cell growth was higher when the carbon chain of the added acid was longer. Fatty acids need to diffuse into the cell to induce cell growth inhibition. The longer the carbon-chain of the fatty acid, the higher lipophilicity of the acid, and thus the easier for the acid to diffuse into the cell and induce the inhibition (Ullah et al., 2012; Abbott et al., 2007). Among the three acids tested, hexanoic acid was the most toxic. No growth was observed when the concentration of added hexanoic acid was higher than 4 g/L (data not shown).

The final pH of the fermentation was dependent on the production and re-assimilation of acids in the fermentation process. The addition of acids in the medium had significant impact on final culture pH (Fig. 1). At both temperatures, generally the final pH values in the fermentation increased with increased concentrations of the supplemented individual acids; at 25 °C, the difference (or increment) of pH between the adjacent fermentation conditions was much more apparent than at 37 °C. Under all conditions tested, the final pH values were lower than the initial pH 6.0, except for the fermentation with acetic acid supplementation at 37 °C. With the increased concentrations of supplemented acetate at 37 °C, the final pH value in the fermentation increased from the starting pH 6.0 to as high as pH 7.2, as a result of a great amount of acetate re-assimilation (discussed below). Under the same condition, the final pH value at 25 °C was always lower than that of measured at 37 °C, which indicated that more acids were generated at lower temperature.

3.2. Effect of acetic acid on fermentation profiles

Final end-product production of *C. carboxidivorans* grown in the presence of four different acetate levels at 25 °C and 37 °C is shown in Fig. 2. With the increase of added acetic acid, the BSY of butyric acid and ethanol were increased dramatically, while the BSY of hexanoic acid was also slightly increased. However, no significant difference was observed for the BSY of butanol and hexanol (except that BSY of butanol was about twice of that of the control when 8 g/L acetic acid was added). The results indicated that high concentration of acetic acid in the culture inhibited the acetate production pathway, and led the metabolic flux mainly towards the adjacent pathways including reassimilation of acetate to produce ethanol and elongation of carbon chain to generate butyric acid. Meanwhile, the generation of butyric acid (and hexanoic acid) might serve as the main pathway for ATP production to support cell growth when the acetic acid production pathway was suppressed.

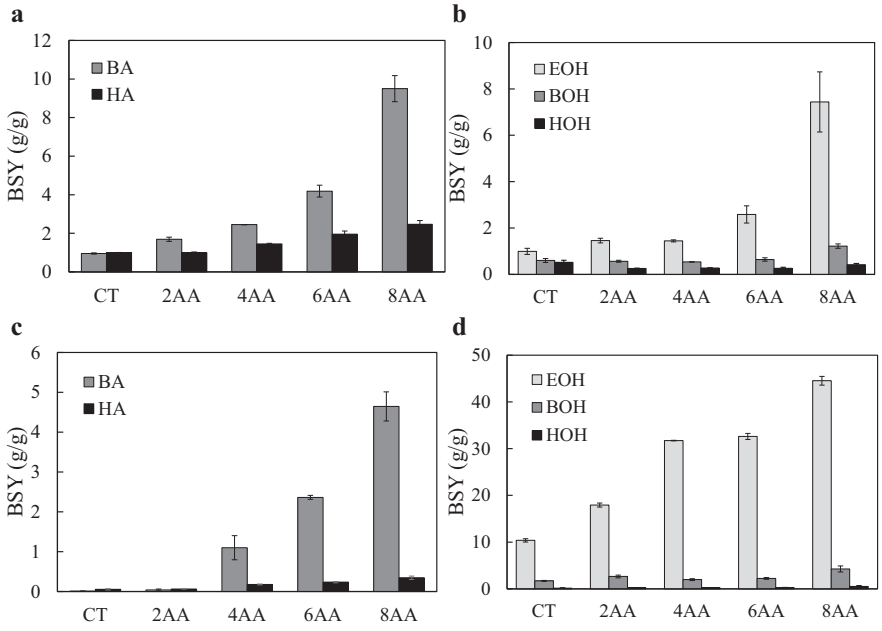

Fig. 2. The cell biomass specific yield (BSY) of butyric acid (BA), hexanoic acid (HA), ethanol (EOH), butanol (BOH) and hexanol (HOH) produced by *C. carboxidivorans* P7 with supplementation of 2, 4, 6, 8 g/L acetic acid (2AA, 4AA, 6AA, 8AA) at 25 °C (a, b) and 37 °C (c, d). The fermentation without addition of acetic acid was carried out as control (CT).

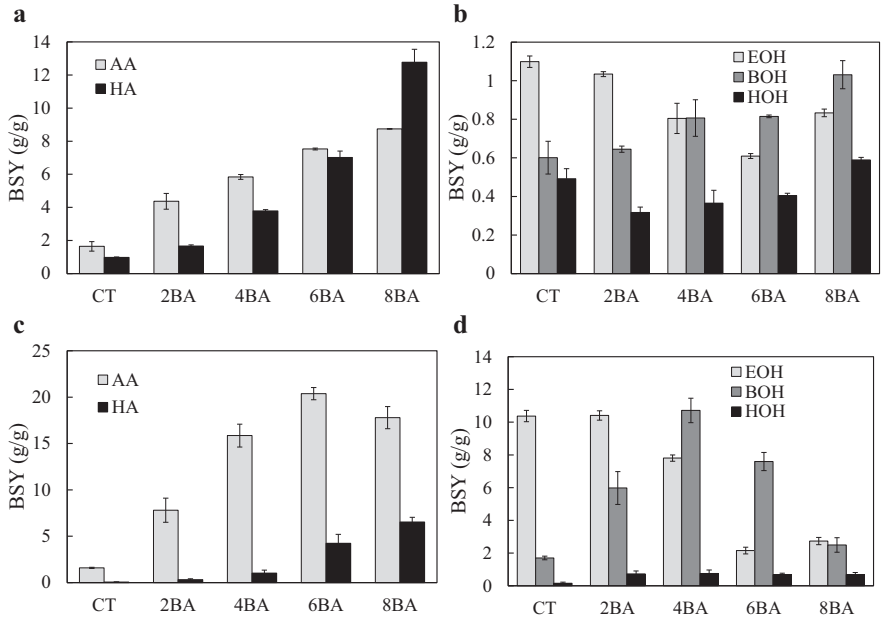

Fig. 3. The cell biomass specific yield (BSY) of acetic acid (AA), hexanoic acid (HA), ethanol (EOH), butanol (BOH) and hexanol (HOH) produced by *C. carboxidivorans* P7 with supplementation of 2, 4, 6, 8 g/L butyric acid (2BA, 4BA, 6BA, 8BA) at 25 °C (a, b) and 37 °C (c, d). The fermentation without addition of butyric acid was carried out as control (CT).

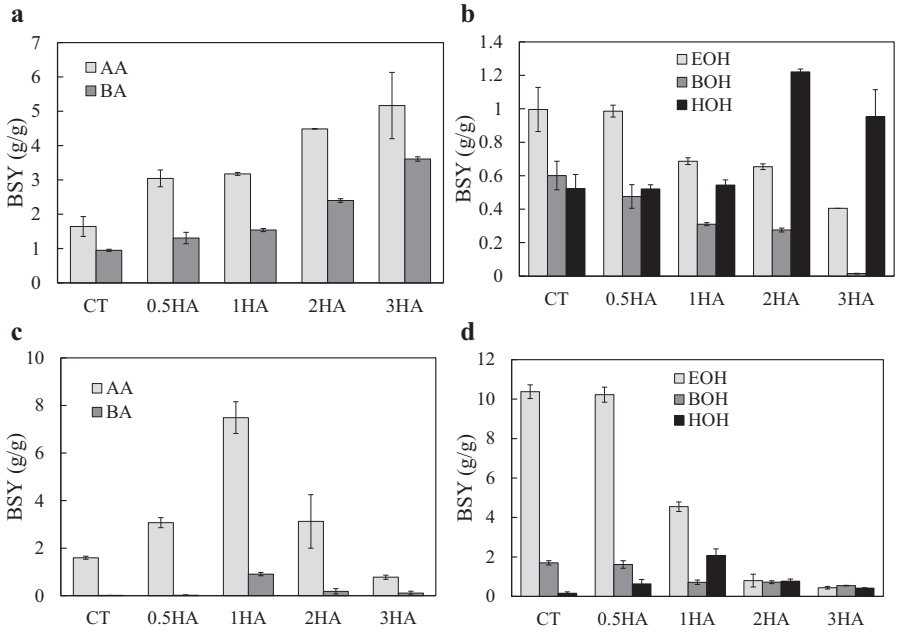

Fig. 4. The cell biomass specific yield (BSY) of acetic acid (AA), butyric acid (BA), ethanol (EOH), butanol (BOH) and hexanol (HOH) produced by *C. carboxidivorans* P7 with supplementation of 0.5, 1, 2, 3 g/L hexanoic acid (0.5HA, 1HA, 2HA, 3HA) at 25 °C (a, b) and 37 °C (c, d). The fermentation without addition of hexanoic acid was carried out as control (CT).

In the presence of 8 g/L acetic acid, the maximum BSY of ethanol and butyric acid at 25 °C were 7.44 g/g and 9.50 g/g, respectively, with a ratio of ethanol/butyrate as 0.78. However, in the fermentation with 8 g/L acetic acid supplemented at 37 °C, the maximum BSY of ethanol and butyric acid were 44.52 g/g and 4.64 g/g, respectively, leading to the ethanol/butyrate ratio as high as 9.59. These results indicated that lower temperature is beneficial to promote the elongation of carbon chain to produce butyric acid, while higher temperature is favorable to convert acetic acid to its corresponding alcohol, namely ethanol. The elevated ethanol production at 37 °C was also consistent with the increased final pH values (higher than initial pH 6.0) (Fig. 1h). A maximum butyric acid concentration of 2.03 g/L was achieved with the addition of 6 g/L acetic acid at 25 °C; a maximum ethanol concentration of 6.76 g/L was obtained with the addition of 2 g/L acetic acid at 37 °C. Both of them are the highest concentrations for the respective end-products ever reported for syngas fermentation with the P7 strain.

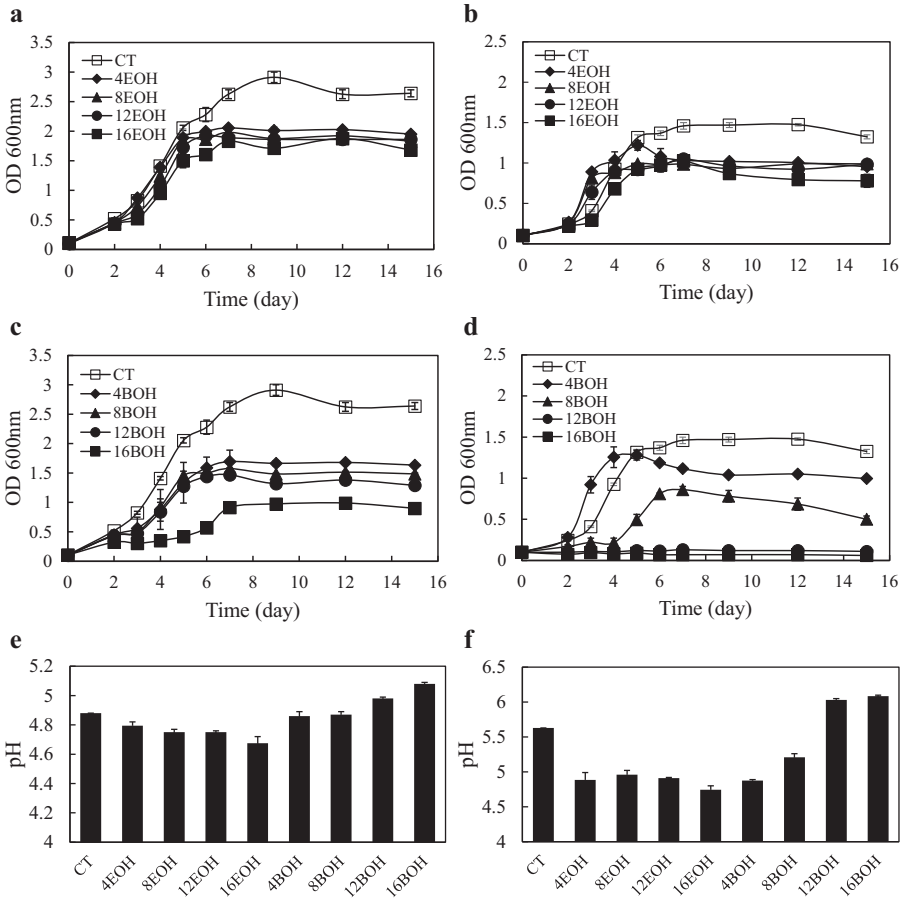

Fig. 5. Growth patterns (a, b, c, d) and final culture pH (e, f) of *C. carboxidivorans* P7 cultured in the presence of 4, 8, 12, 16 g/L ethanol (4EOH, 8EOH, 12EOH, 16EOH) or 4, 8, 12, 16 g/L butanol (4BOH, 8BOH, 12BOH, 16BOH) at 25 °C (a, c, e) and 37 °C (b, d, f). The fermentation without addition of alcohols was carried out as control (CT).

3.3. Effect of butyric acid on fermentation profiles

The effect of butyric acid at four different levels on the end-product formation in *C. carboxidivorans* was also investigated at 25 °C and 37 °C (Fig. 3). In the presence of elevated concentrations of butyric acid, *C. carboxidivorans* produced significantly more acetic acid, hexanoic acid and butanol at both temperatures, suggesting that the inhibition of butyrate formation pathway switched the carbon flow to generate substitute acids or re-assimilate butyrate into butanol. The maximum BSY of acetic acid, hexanoic acid and butanol at 25 °C reached 8.74 g/g, 12.77 g/g, and 1.03 g/g, respectively, compared with those of 20.38 g/g, 6.53 g/g, and 10.72 g/g, respectively, at 37 °C. The results indicated that higher temperature is advantageous to convert butyric acid to butanol or produce shorter chain acid (acetic acid), while lower temperature facilitates carbon chain elongation and longer chain acid (hexanoic acid) production. When 8 g/L butyric acid was added into the fermentation at 25 °C, the final titer of hexanoic acid reached 2.32 g/L, which was approximately 3-fold of that of the control. To the best of our knowledge, this is the highest hexanoic acid production that has been reported for the P7 strain. Due to the enhanced hexanoic acid production under this condition, the BSY of hexanol was also slighted increased (by 19.9%) compared to the control.

3.4. Effect of hexanoic acid on fermentation profiles

As shown in Fig. 4, the effect of hexanoic acid on fermentation profiles in *C. carboxidivorans* was further evaluated in this study. Because the toxicity of hexanoic acid is higher than acetic acid and butyric acid and no cell growth was observed when the hexanoic acid concentration exceeded 4 g/L (data not shown), the hexanoic acid levels tested in this experiment were set to below 3 g/L.

Generally, as expected, with the addition of hexanoic acid, the P7 strain produced more acetic acid, butyric acid and hexanol at both temperatures (Fig. 4). However, the tendency of fermentation profiles was different at two different temperatures. When the hexanoic acid production pathway was inhibited at 25 °C with the supplementation of hexanoic acid (Fig. 4a & b), the BSY of acetic acid and butyric acid were enhanced dramatically, while the BSY of hexanol was increased only in the presence of high concentration of hexanoic acid ($\geqslant$2 g/L), suggesting that the metabolic flux in this case was mainly drove towards alternative acid production at lower temperature. This observation was coincident with the results obtained with the addition of acetic acid and butyric acid at 25 °C. While at 37 °C, the increased BSY of acetic acid, butyric acid and hexanol were obtained only when lower levels ($\leqslant$1 g/L) of hexanoic acid were supplemented, indicating that more metabolic flux was turned towards acetic acid production, followed by hexanol production. However, the BSY of acetic acid, butyric acid and hexanol were decreased in the presence of high concentration of hexanoic acid ($\geqslant$2 g/L), mainly probably due to the high toxic effect of hexanoic acid on cellular metabolism at higher temperature (Fig. 1f).

3.5. Cell growth and final culture pH with alcohol supplementation

In the experiments with addition of individual alcohols (ethanol or butanol), *C. carboxidivorans* P7 was able to grow under all the conditions tested, with the exception of the fermentations in the

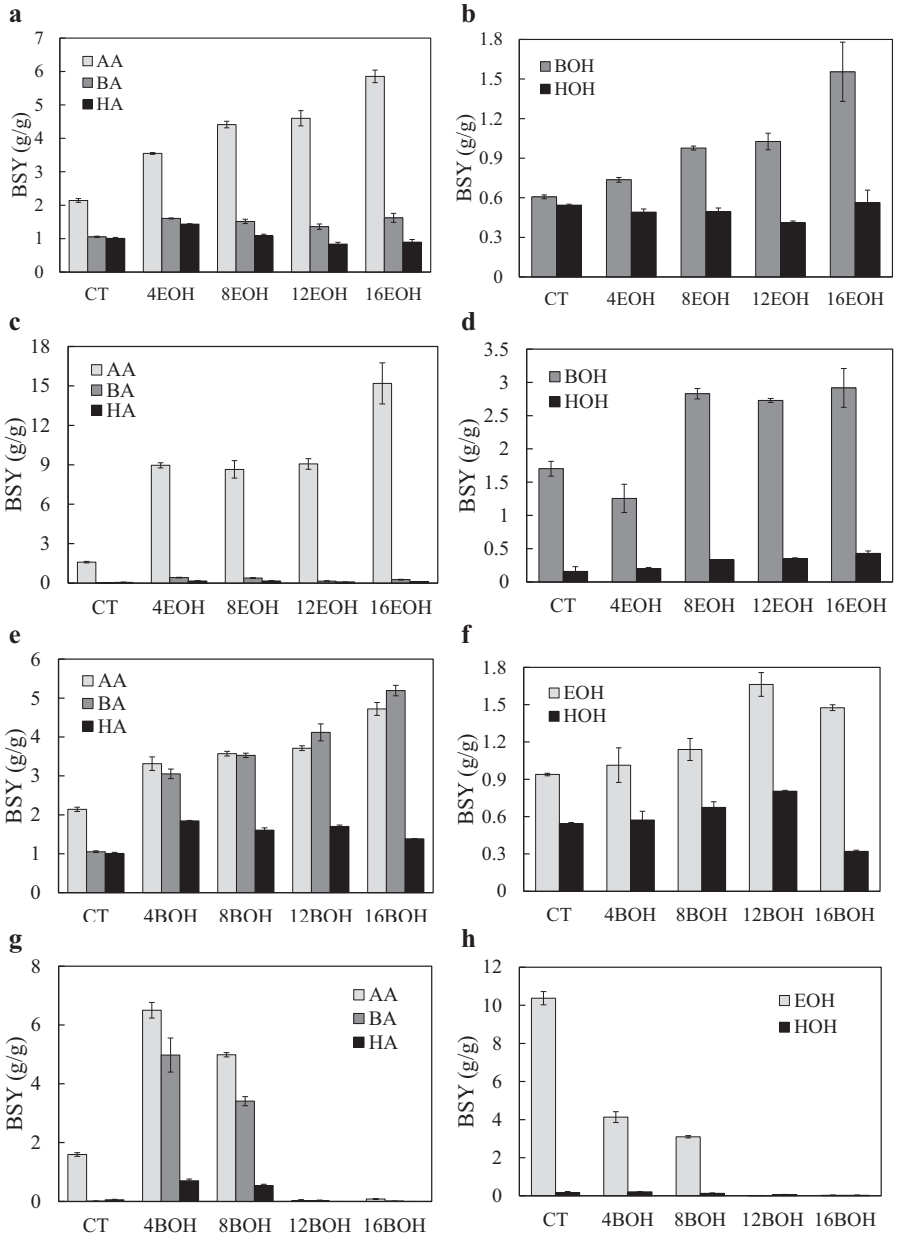

Fig. 6. The cell biomass specific yield (BSY) of acetic acid (AA), butyric acid (BA), hexanoic acid (HA), ethanol (EOH), butanol (BOH) and hexanol (HOH) produced by *C. carboxidivorans* P7 with supplementation of 4, 8, 12, 16 g/L ethanol (4EOH, 8EOH, 12EOH, 16EOH) or butanol (4BOH, 8BOH, 12BOH, 16BOH) at 25 °C (a, b, e, f) and 37 °C (c, d, g, h). The fermentation without addition of alcohols was carried out as control (CT).

presence of the high concentrations of butanol (12 g/L or 16 g/L) at 37 °C. These results suggested that butanol was more toxic to the cell than ethanol and also it was more toxic at higher temperature. When the same amount of individual alcohols were added into the culture, the maximum cell density at 25 °C was much higher (generally around 2x as high) than that at 37 °C (Fig. 5a, b, c and d). This is identical with the result obtained with acids addition and indicates that alcohols are also more toxic to the cell growth at higher temperatures. Similar phenomena were also reported for other microorganisms, including *Clostridium acetobutylicum* (Baer et al., 1987) and *Escherichia coli* (Knoshaug and Zhang, 2009). No growth was observed for *C. acetobutylicum* ATCC 824 grown in 1.5% (vol/vol) butanol at 42 °C, however slight growth appeared at 37 °C and 22 °C (Baer et al., 1987). *E. coli* demonstrated higher tolerance to butanol when grown at 30 °C than at 37 °C (Knoshaug and Zhang, 2009). Alcohol is toxic mainly due to its fluidization effects on the cell membrane which is much stronger at elevated temperature. At lower temperature, the membrane bilayer is tended to solidify which would offset the fluidizing effect of alcohol (Baer et al., 1987; Ingram, 1976). Roughly, the presence of ethanol and butanol in the medium had negative effects on cell growth similarly as for acids supplementation. However, at 37 °C, the fermentations with addition of low concentrations of ethanol (4 g/L, 8 g/L, or 12 g/L) or butanol (4 g/L) had a slightly higher growth rate (or shorter lag phage) than the control at the beginning of the fermentation; this observation was coincident with the results obtained by Fernández-Naveira et al. (Fernandez-Naveira et al., 2016a).

As shown in Fig. 5e and f, with increased concentrations of supplemented ethanol, the final pH values of the fermentation decreased slightly at both temperature conditions, while with the elevated concentrations of butanol, the final pH values in the fermentation increased gradually. The final pH values were lower

than the initial pH 6.0 under all tested conditions, except for the fermentations with no cell growth as described above.

3.6. Effect of ethanol on fermentation profiles

Fig. 6a, b, c and d showed the BSY of major end-products generated by the P7 strain with addition of four different concentrations of ethanol at 25 °C and 37 °C. At both temperatures, the BSY of acetic acid and butanol were increased significantly (except for a slight decrease in the BSY of butanol at 4 g/L ethanol supplementation at 37 °C), while no significant change was observed in the BSY of butyric acid, hexanoic acid and hexanol (there was a slight increase for hexanol at 37 °C when the supplemented ethanol >8 g/L). The results indicated that the metabolic flux was mainly drove towards adjacent pathways including production of acetate and generation of longer chain alcohol (namely, butanol) when the ethanol production pathway was inhibited.

With addition of 16 g/L ethanol, the BSY of acetic acid and butanol reached the maximum of 5.86 g/g and 1.56 g/g, respectively, at 25 °C, resulting in an acetate/butanol ratio of 3.76, compared to the corresponding 15.19 g/g and 2.92 g/g, respectively, at 37 °C with an acetate/butanol ratio of 5.20. Although the acetate formation is the predominant reaction with ethanol supplementation at both 25 °C and 37 °C, carbon chain elongation is more favorable at lower temperature (25 °C).

3.7. Effect of butanol on fermentation profiles

As shown in Fig. 6e, f, g and h, with addition of increased concentrations of butanol at 25 °C, the BSY of acetic acid, butyric acid and ethanol were enhanced dramatically, while there were also slight increase in the BSY of hexanoic acid and hexanol. The maximum BSY of acetic acid, butyric acid and ethanol at 25 °C reached 4.72 g/g, 5.19 g/g, and 1.66 g/g, respectively. When the fermentation was conducted at 37 °C, *C. carboxidivorans* produced significantly more acetic acid and butyric acid, though there were no cell growth and thus no products generated with addition of high concentration of butanol ($\geqslant$12 g/L) (Fig. 5d). The BSY of hexanoic acid were also enhanced slightly, while only negligible hexanol production was observed. The results indicated that short-chain acids formation is the major reaction at both temperatures under butanol pressure, whereas the carbon chain elongation of butanol for hexanol production is still favorable at lower temperature.

4. Conclusions

In this study, effects of end-products on fermentation profiles in *C. carboxidivorans* P7 were investigated. Results demonstrated that the addition of fatty acids or alcohols inhibited relevant production pathways, followed by the change of fermentation profiles. At 37 °C, more acids were re-assimilated into alcohols compared to 25 °C. However, lower temperature facilitates carbon chain elongation to produce long-chain acids and alcohols. Thus, redirecting carbon flow to desired product can be achieved via manipulation of culture conditions. Further elucidation of syngas fermentation mechanism by *C. carboxidivorans* P7 can be carried out by transcriptomics/proteomics and ^{13}C-based metabolic flux analyses.

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