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Optimization of the cell immobilization-based chain elongation process for efficient *n*-caproate production

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Abstract: Biosynthesis of *n*-caproate through chain elongation has attracted increasing attention in the past years. However, the low titers limited its wide application. To improve the production of *n*-caproate, the influence of process parameters on caproate fermentation was comprehensively investigated in the cell immobilized system. Batch tests showed that the caproate production was remarkably affected by the ethanol to acetate (E/A) ratio, the concentration of substrates, and pH. The corresponding optimum values of these three parameters were 8:3, 660 mmol/L, and 7.0-7.4, respectively. The immobilized cells preferred to use the mixture of acetate and butyrate as electron acceptors, obtaining the highest caproate production of 146.6 mmol/L (17.0 g/L) with the acetate/butyrate ratio of 1:1. Analysis of biomass weight, thermogravimetry (TG) / differential scanning calorimetry (DSC), and Fourier Transform Infrared (FTIR) Spectroscopy illustrated that the immobilized cells dominated the total amount of cells in the immobilization system. The extracellular polymeric substance (EPS) played an important role in improving the activity of biofilm. The feasibility of high caproate production from real wastewater was finally validated. This study provides an insight into the development of an efficient and cost-effective way for caproate production from waste biomass.

Keywords: Chain elongation; wheat straw; Extracellular polymeric substance; Electron acceptor; Biofilm; *Clostridium kluyveri*

Capsule: The optimized operating conditions for the cell immobilization-based chain elongation process were identified to improve *n*-caproate production.

INTRODUCTION

Medium-chain fatty acids (MCFAs) such as *n*-caproate, which can be used as platform chemicals as well as fuel precursors, exhibit a promising prospect in industrial applications.[1] Caproate can be used in the production of flavor additives, antibiotics, and energy carriers (e.g., hexanol).[2] However, it is mainly produced from the chemical process using fossil fuels as major feedstocks associated with serious environmental pollution, and thus, against the green and sustainable development of modern society.[3,4] Caproate can also be extracted from plant oils, while the low concentration and high cost of caproate limited the wide application of this technology.[5] In this regard, biological processes such as anaerobic fermentation that can convert wastes into bioproducts have gained much attention.[6-8]

Clostridium kluyveri is a typical bacteria capable of converting ethanol and acetate into *n*-caproate through chain elongation.[6,9,10] The reverse β-oxidation pathway (Figure S1) of *C. kluyveri* has been described in previous reports.[2, 4, 8] Briefly, the electron donor such as ethanol is firstly converted into acetyl-CoA which would take part in the reactions of the two cycles of reverse β oxidation (RBO). Most of the acetyl-CoA (4/5-5/6 of the total) is elongated into butyrate with the participation of acetate in the first cycle (RBO cycle I), and another acetyl-CoA reacted with butyrate for further chain elongation to produce caproate in the second cycle (RBO cycle II).[2] Theoretically, the biochemical reaction of ethanol with acetate is thermodynamically feasible because the Gibbs free energy ($\Delta G_r^{o'}$ of Eq. (1)) is far lower than the theoretical (ΔG_{thr}) value of -23.3 kJ/mol.[11] In this context, the balance of ethanol to acetate (E/A) ratio is vital for the efficient conversion of substrates to caproate.[12,13]

$$6\ C_2H_5OH + 3\ CH_3COOH \rightarrow 3\ C_3H_7COOH + C_5H_{11}COOH + 2H_2 + H^+ + 4\ H_2O;$$

$$\Delta G_r^{0'} = -183\ kJ/mol \quad (1)$$

Caproate biosynthesis can be influenced by various factors including nutrition (e.g., electron donor and acceptor and yeast extract), environmental factors (e.g., pH and gas pressure), inhibitors (e.g., ammonia), and the bioreactor configuration (e.g., anaerobic filter and anaerobic sequencing batch reactor).[4] In the past decades, considerable efforts have been devoted to improving the production of caproate. The highest caproate production of 21.4 g/L (by *C. kluyveri*) was reported in a recent study, using ethanol and acetate as substrates.[8] Evidently, high concentrations of the substrates should be supplied in the fermentation system to obtain high production of caproate. However, the inhibitory effect may emerge when the concentrations of substrates exceeded the threshold concentration. Weimer and Stevenson found the highest caproate production was obtained with 700 mmol/L ethanol and 120 mmol/L acetate.[14] Further increase in the substrate concentration would hinder the microbial growth and caproate production. Apart from the substrate concentration, both the E/A ratio and the pH were key factors influencing caproate production.[13] It was reported that the neutral pH (6.5 ~ 7.5) was suitable for the chain elongation than the mildly acidic condition (around 5.5).[15,16] Nevertheless, there was no report on what range of the pH should be controlled to conduct caproate fermentation without of inhibitory effect.

Several waste resources such as waste industrial syngas and glycerol have been tested for the low-cost production of caproate.[12,17] Theoretically, any wastewater containing high concentrations of ethanol and acetate should be suitable for being used as substrates for caproate production.[13] However, the

high concentrations of butyrate and other undesired chemical compounds (i.e., ammonia) were usually detected in the wastewater such as the effluent of anaerobic digestion. These toxic compounds can not be ignored because they will negatively influence the activity of *C. kluyveri*.[18] To date, few studies have been conducted to focus on the caproate production from the real wastewater, and it is still unclear how would the butyrate in the presence of high concentration affect the chain elongation process.

In the cell suspended system, the activity of microbial cells is easily inhibited and the cell loss usually occurred in the continuous operation mode. Immobilization is an effective way to overcome this barrier, where cells can be protected from harsh environments such as the presence of inhibitors, drastic changes in substrate, products, pH, and temperature.[19,20] A cell immobilized system was previously developed to tolerate high concentrations of ammonia,[7] yet the capacity of the biofilm to produce caproate was not fully explored and it is still unknown how would the operating parameters affect this novel chain elongation process. Thus, in this work, the influence of four key operating parameters (E/A ratio, total substrate concentration, pH, and type of electron acceptor) on the cell immobilization based chain elongation was evaluated. Thereafter, the performance of caproate production from the real bioethanol wastewater was examined.

EXPERIMENTAL SECTION

Microorganism and cultivation. *C. kluyveri* was used as inoculum as previously described.[7] Before fermentation, the bacteria were cultivated for 3-4 generations to improve the activity of microorganisms, until the optical density (OD_{600}) was stable at 0.9 ± 0.05. The medium of DSMZ-52 was used for

microbial cultivation, and the 100-mL serum bottle with an effective working volume of 55 mL was used for chain elongation. Before sterilization, all bottles were sealed with a rubber stopper and aluminum cap to make an anaerobic environment. Thereafter they were sterilized at 121 ^{o}C for 20 min. The pH of the medium was adjusted before adding 10% (inocula to medium, *v*/*v*) inocula. Then all the bottles were put into a shaker with 108 rpm and 37 ^{o}C for fermentation.

Batch Tests. To improve the caproate production, the environmental factors including E/A ratio, total substrate concentration, initial pH, and type of electron acceptor were investigated. Five different E/A ratios were firstly designed to investigate their effects on caproate production. For each group, the initial total substrate concentration was controlled at 440 mmol/L. And then the effect of total substrate concentration (220, 440, 660, 880, and 1100 mmol/L) on caproate was conducted with the E/A ratio of 8:3. Thereafter five different pH values were tested to investigate the influence of initial pH on caproate production. The optimum E/A ratio (8:3) and total substrate concentration (660 mmol/L) were applied. Finally, the influence of different acetate/butyrate (A/B) ratios on the caproate production was investigated with fixed total concentrations of electron acceptor (acetate and butyrate) and ethanol (180 and 480 mmol/L, respectively). For each group, a fermentation time of 156 h was chosen and all the experiments were conducted in duplicate.

Caproate Production from Bioethanol Wastewater. The raw bioethanol wastewater with an ethanol concentration of 1304 mmol/L was diluted into a solution with 480 mmol/L of ethanol. The diluted bioethanol wastewater (Table S1) was used as the electron donor, and the potassium acetate was used as

the electron acceptor for chain elongation. Moreover, the optimum E/A ratio of 8:3 and initial pH of 7.4 were adopted.

Analysis Methods. The concentrations of ethanol, short-chain fatty acids (SCFAs), and MCFAs of the liquid samples were detected by a gas chromatograph (GC-2010 plus, SHIMADZU, Japan), equipped with a flame ionization detector (FID) and capillary column (30 m×0.250 mm, HP-INNOWAX, Agilent). The temperatures of the injector, detector were 240 and 260 ^{o}C, respectively. The initial temperature in the column room was 90 ^{o}C for 3 min and raised to 150 ^{o}C for 2 min at a speed of 20 ^{o}C /min, and then raised to 230 ^{o}C for 2 min at a speed of 15 ^{o}C/ min. Nitrogen was used as the carrier gas and the flow rates of nitrogen, hydrogen, and air were 30, 40, and 400 mL/min, respectively. Before GC detection, all the liquid samples were pretreated using the following procedures: (1) mix 0.75 ml samples with 0.05 ml ortho-phosphorus (45%, *w*/*w*); (2) centrifuge at 10000 rpm for 10min; (3) take supernatant liquid for detection. Hydrogen was determined by a gas chromatograph (Agilent 7890B, Agilent USA), equipped with a TCD detector and a stainless steel packed column (TDX-01, Zhonghuida, China). The temperature of the injector, column, and detector was set as 200, 180, and 200 ^{o}C, respectively. Argon was used as a carrier gas with a flow rate of 30 mL/min. The obtained hydrogen volume was corrected and expressed at the standard temperature (273.15 K) and pressure (100 KPa). The Fourier Transform Infrared (FTIR) Spectroscopy (WQF-510A, Ruili, China) was applied to detect the surface functional groups using KBr pellet method. The spectra were recorded from 4000 to 400 cm^{-1} at room temperature.

The total polysaccharide of extracellular polymeric substance (EPS) was

detected by the phenol-sulfuric acid method using glucose as a standard.[21] The protein was determined by the Bradford method with bovine serum albumin as a standard. The pH of the liquid was measured by a pH meter (pHB-1, Sanxin, China).

The concentration of undissociated caproic acid was calculated based on the *Henderson-Hasselbalch* equation, as described in Eq. (2).

$$pH = pKa + \lg(\frac{A^-}{HA}) \quad (2)$$

where pK_a presents the dissociation constant under certain pH; A^+ is the concentration of dissociated caproic acid, and *HA* is the concentration of undissociated caproic acid.

RESULTS AND DISCUSSION

Effect of E/A Ratio on Caproate Production. The effect of E/A ratio on caproate production is shown in Figure 1. The caproate production increased from 13.8 to 88.5 mmol/L with the increase of the E/A ratio from 1:10 to 8:3 (Figure 1a). However, it sharply decreased to 34.9 mmol/L when the E/A ratio further increased to 10:1. Meanwhile, the variation in pH showed different trends (Figure 1b). Our previous studies found that the decrease of the pH was an indicator of the production of caproate.[4] There was no remarkable decrease of pH in the group with the E/A ratio of 1:10, indicating the failure of caproate production. Thus, the optimum E/A ratio for caproate production by the immobilized cells was 8:3.

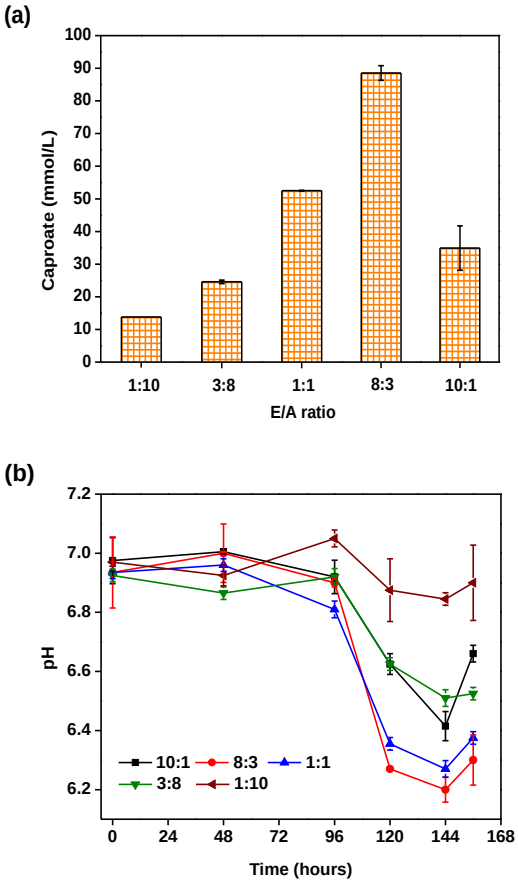


Figure 1. Effect of the E/A ratio on caproate production. (a: caproate production; b: variation of pH)

Leng et al. reported that the optimum ratio was 9:3[12] and Yin et al. found that a ratio of 7:3 was helpful to improve the caproate production,[13] implying that the optimum E/A ratio was in the range of 7:3-9:3. The results of this study were in strong agreement with that of the previous studies. Theoretically, the E/A ratio was 2:1 in the case that the ethanol and acetate were completely converted into caproate, as shown in Eq. (1), yet it should be higher than 2:1 in practice. This phenomenon can be explained by the pathway followed by *C. kluyveri*, where the chain elongation started with the oxidation of ethanol and the production of acetyl-CoA. Approximate 1/6 of the produced acetyl-CoA was converted into acetate for energy (ATP) supply, while another 5/6 of the acetyl-CoA entered the cyclic reverse β oxidation pathway to produce butyrate

in the first cycle and then to produce caproate in the second cycle with a series of enzymatic reactions.[2,22] It was supposed that there was not enough energy released to motivate the chain elongation reactions with a low E/A ratio of 1:10, which might be the main reason for the failure of caproate production. In a study reported by Leng et al.,[12] there was not enough free energy release for caproate production with the E/A ratio increased from the optimum value of 3 to 4 or 5. In this study, there was not enough acetate to support the reactions of the first cycle with the high E/A ratio of 10:1, which led to the failure of the reactions in the first cycle as well as the production of butyrate, explaining the low caproate production.

Effect of Total Substrate Concentration on Caproate Production. The effect of the total concentration of ethanol and acetate on the chain elongation process was investigated with the optimum E/A ratio of 8:3. As shown in Figure 2, the caproate production increased with the total substrate concentration, i.e., it increased from 83.1 to 93.6 mmol/L with the increasing of total substrate concentration from 220 to 660 mmol/L. However, caproate production failed when the total substrate concentration exceeded 880 mmol/L. These results indicated that caproate production was inhibited by the high concentrations of ethanol and acetate. Yin et al. found that caproate biosynthesis would be inhibited when the ethanol concentration exceeded 700 mmol/L.[13] Similarly, in a previous study,[14] at a fixed acetate concentration of 120 mmol/L, the caproate production increased with the increase of ethanol concentration to 700 mmol/L. In this study, the corresponding ethanol concentration was 640 mmol/L when the total substrate concentration was 880 mmol/L. Hence, the inhibitory effect of ethanol was originally unexpected.

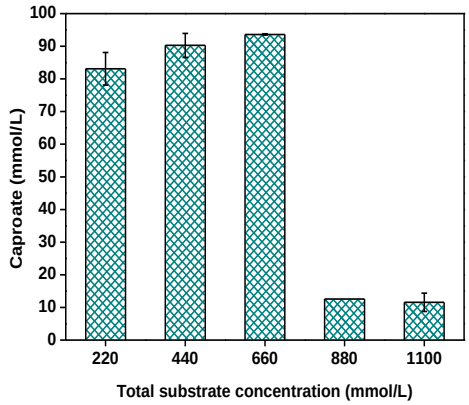
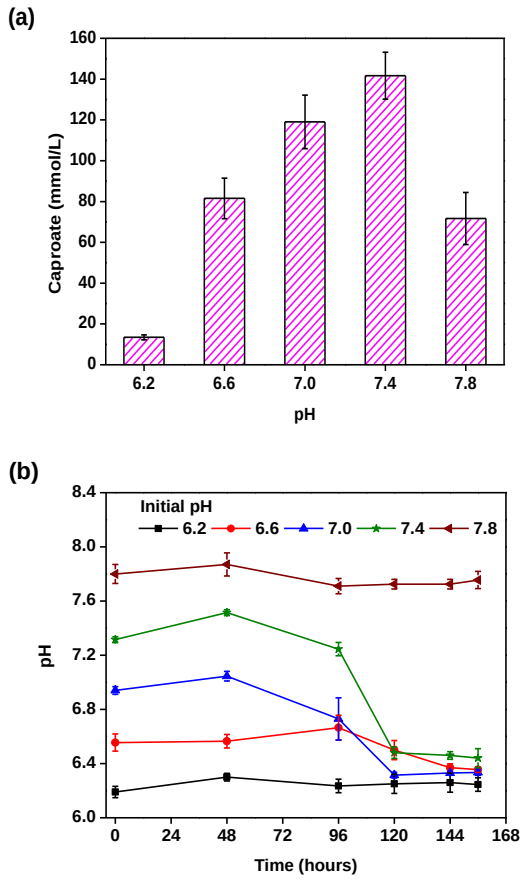


Figure 2. Caproate production after fermentation of 156 h with different total substrate concentrations.

Compared with alcohols, clostridia were easy to be inhibited by high concentrations of short-chain fatty acids and MCFAs (C_3-C_{16}), especially of the undissociated acids.[8,23] For instance, inhibition was observed when the concentration of undissociated caproic acid exceeded 0.2 g/L, and a high concentration of 0.87 g/L was regarded to be the up toxic limit.[24,25] Further studies of this study showed that caproate production was remarkably affected by the acetate concentration (Figure S2a). With a fixed ethanol concentration of 480 mmol/L, the caproate was 65.8 mmol/L when the acetate concentration increased to 240 mmol/L. While the caproate production failed with the total substrate concentration increased to 880 mmol/L (640 and 240 mmol/L of ethanol and acetate, respectively). Besides, it was noteworthy that the initial caproate concentration was about 13.3 mmol/L at hour 0, coming from the inoculum. Thus, it was calculated that ethanol and acetate contributed to 65.7% and 34.3% of the total inhibition, respectively. Moreover, Figure S2a also showed that the threshold of acetate was 300 mmol/L (18 g/L) where the caproate production was completely inhibited, illustrating that acetate exhibited a stronger inhibitory effect to *C. kluyveri* than ethanol.

Effect of the Initial pH on Caproate Production. As shown in Figure 3a, caproate production could be remarkably influenced by the initial pH. The caproate production failed with the initial pH of 6.2, while it increased sharply with the increasing of the initial pH increased from 6.2 to 7.4. The highest caproate production of 141.7 mmol/L (16.5 g/L) was obtained at an initial pH of 7.4. However, the caproate production decreased to 71.7 mmol/L by further increasing the initial pH to 7.8. This phenomenon might be attributed to the low activity of enzymes in the cells at pH 7.8. These results demonstrated that the pH was one of the most important parameters influencing caproate biosynthesis.



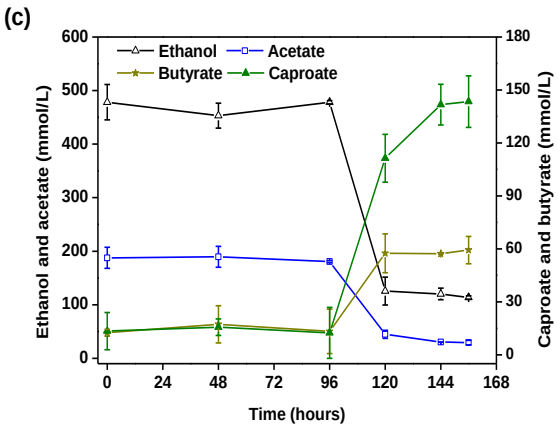


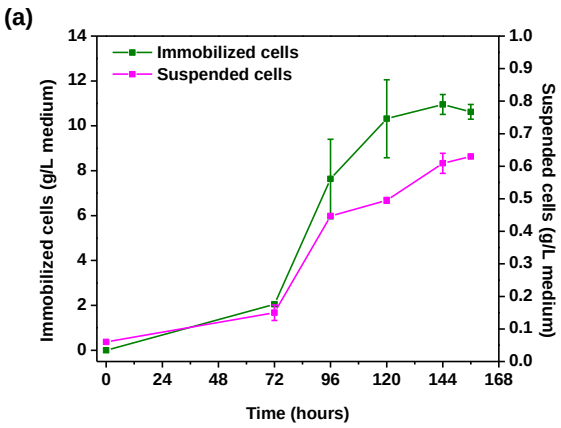
Figure 3. Influence of the initial pH on caproate production. (a: caproate production; b: variation of pH; c: caproate production with the initial pH of 7.4)

During caproate production, the pH of the system decreased (Figure 3b) due to the accumulation of hydrogen, as described in Eq. (1). However, no remarkable pH variation was observed in the groups with an initial pH of 6.2 and 7.8. This phenomenon indicated that a little amount of hydrogen was produced from ethanol when the initial pH was beyond the pH spectrum of 6.6 ~ 7.4. In this case, the electron capable of driving the chain elongation process can not be generated, resulting in the low production of caproate.

With the initial pH of 7.4, the consumption of ethanol and acetate, and the production of caproate and butyrate are shown in Figure 3c. Results showed that the lag phase was 96 h, and caproate was produced with the consumption of ethanol and acetate after the lag phase. After 156 h fermentation, the concentrations of caproate and butyrate became stable. The corresponding conversion efficiency of ethanol and acetate was 76.2% and 84.3%, respectively. It was noteworthy that the pH decreased to the value below 6.5 after 120 h fermentation (Figure 3b), which was beyond the suggested range of 6.6 ~ 7.4. It was deduced that the increased concentration of undissociated acids (2.61 mmol/L at pH 6.5) resulted in the inhibition of chain elongation. The

inhibitory effect was validated by the caproate production rate as well. The caproate production rate was 98.9 mmol/L/d from 96 to 120 h, while it sharply decreased to 30.5 mmol/L/d after 120 h. These results indicated that it was of utmost importance to regulate the pH in a neutral spectrum for high caproate production.

With the initial pH of 7.4, the biomass of immobilized and suspended cells was investigated to reveal the cell distribution in the immobilized system (Figure 4). A sharp increase in the cells was observed after fermentation of 72 hours and the highest concentration of immobilized cells was 10.62 g/L at hour 156 when the caproate production was almost finished (Figure 3c). Comparatively, the concentration of immobilized cells was higher than that of suspended cells, and the highest ratio of immobilized to suspended cells (IC/SC) was 20.8 after 120 hours of fermentation (Figure 4b). Thus it was supposed that the immobilized cells played the key role in catalyzing the chain elongation reaction in the cell immobilized system.



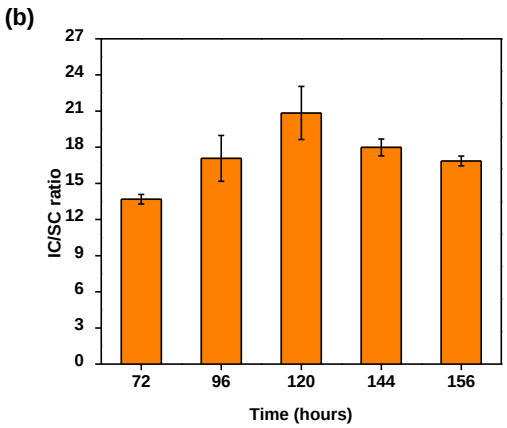


Figure 4. Cell growth of the immobilized system. (a: weight of cells; b: IC/SC ratio)

The TG, derivative thermogravimetry (DTG), and DSC curves are presented in Figure S3. Two stages on the curves were mainly observed for the wheat straw, cells of *C. kluyveri*, and carrier with cells. The first stage appeared before 120 °C due to the evaporation of the adsorbed bound water.[26] For the wheat straw, the maximum weight loss occurred between 260 and 400 °C (Figure S3a), and the peak of the derivative curve was observed at 370 °C (Figure S3b). It has been extensively reported that the wheat straw was comprised of cellulose, hemicellulose, and lignin and their thermal decomposition occurred at 160-240, 240-360, and 160-627 °C, respectively.[27] The mass loss of wheat straw at the second stage was ascribed to the degradation of these cellulosic materials. Different from pure wheat straw, the mass loss of wheat straw immobilized with cells mainly occurred between 160 and 500 °C. For the free cell sample, a wide spectrum of 110-500 °C was observed for biomass loss. The acetate and butyrate would evaporate before 160 °C due to their low boiling points. The different characteristics of suspended cells and wheat straw immobilized with cells in the range of 110-160 °C might be ascribed to the existence of more residual acetate in the

suspended cells because less acetate was converted into caproate in the cell suspended system. The other organics such as lipid and protein would decompose which mainly occurred at around 280 °C. With further heating, they were completely degraded at around 500 °C. It was noteworthy that the peaks of derivative curves of the pure cells and wheat straw immobilized with cells were both observed at 280 °C (Figure S3b). Similar trends of TG and DTG were observed on the carrier, which were remarkably different from the pure wheat straw. This phenomenon indicated that the wheat straw in the fermentation system carried a considerable amount of cells of *C. kluyveri*, explaining the high caproate production of the immobilized system.

The FTIR spectra of wheat straw, cells of *C. kluyveri,* and carrier with cells are shown in Figure 5. The three different materials performed strong OH transmission peaks at 3350 cm^{-1} and fluctuated at 2920 cm^{-1} due to the stretching vibrations of the CH group. The main differences among the three materials were observed at 1660 and 1565 - 1538 cm^{-1} representing the amide in proteins.[28] Compared with pure wheat straw, strong transmission peaks were observed in the samples of cells and wheat straw immobilized with cells, owing to the protein existing in the cells. Moreover, the peaks at 1200 - 1000 cm^{-1} represented stretching C-O-C group, which existed in cellulosic materials of wheat straw and polysaccharides on the membrane of cells.[29,30] More strong transmission peaks were observed with the carrier, indicating considerable cells were attached to the carrier.

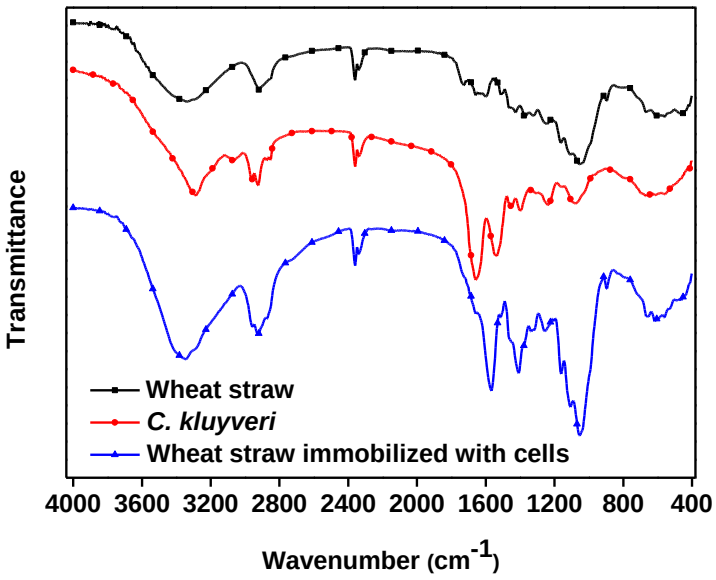


Figure 5. The infrared spectrum of wheat straw, cells of *C. kluyveri,* and carrier with cells.

Caproate Production Using the Mixed Electron Acceptors of Acetate and Butyrate. As shown in Figure 6a, the highest caproate production was obtained at the A/B ratio 1:1. The corresponding caproate production was 146.6 mmol/L (17.0 g/L), which was the highest production in this study. It can be observed that both the acetate and butyrate decreased with the production of caproate during the fermentation process (Figure 6b). These results indicated that acetate and butyrate can be utilized simultaneously by *C. kluyveri* for caproate biosynthesis. The strain did not show its preference to utilize either acetate or butyrate individually. Conversely, it showed a preference to use the mixture of acetate and butyrate to carry out the chain elongation process, which was in agreement with the previous studies.[18]

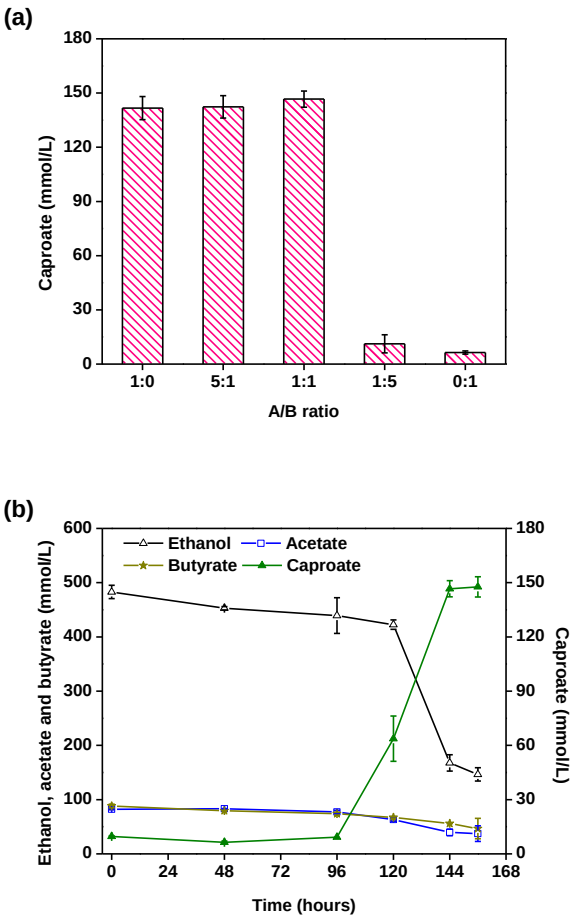


Figure 6. Profile of caproate production with the mixed electron acceptors. (a: caproate production; b: caproate production with the A/B ratio of 1:1)

It seems that the lag phase was prolonged using the mixture of acetate and butyrate as electron acceptors. For example, the caproate production was 63.7 mmol/L (Figure 6b) using the mixed electron acceptors, while it was 111.3 mmol/L (Figure 3c) when the acetate was used as the mono electron acceptor. With the addition of a high concentration of extra butyrate, the enzymatic reactions of the first cycle might have been inhibited, which might be the main reason for the prolongation of the lag phase. Moreover, the inhibitory effect of butyrate on caproate production was also observed in Figure 6a, i.e., the production of caproate failed with the A/B ratio of 1:5. These results implied that the butyrate performed a much higher inhibitory effect on *C. kluyveri* than

the acetate.

The high inhibition capacity of butyrate to *C. kluyveri* was verified by further studies, where the butyrate was used as the mono electron acceptor, as shown in Figure S2b. The caproate achieved the highest production of 23.5 mmol/L with the addition of 30 mmol/L butyrate and 480 mmol/L ethanol, while caproate production sharply decreased when the butyrate exceeded 60 mmol/L. This phenomenon can be explained by the pathway of chain elongation, where considerable acetate was required to produce butyrate in the first cycle.[2] Without enough supply of acetate, the reactions of the first cycle were not sustainable even though a little amount of acetate can be produced via the oxidation of ethanol, resulting in the failure of chain elongation as well as caproate production. It was noteworthy that the production of caproate was 146.6 mmol/L using 90 mmol/L of butyrate and 90 mmol/L of acetate as electron acceptors (Figure 6a). This phenomenon illustrated that the existence of acetate favored the conversion of butyrate to caproate.

Apart from drawbacks, advantages of the existence of butyrate were observed as well. After fermentation of 156 h, 69.7% of ethanol, 54.7% of acetate, and 47.2% of butyrate were consumed (Figure 6b). With an equal total concentration of electron acceptor, the conversion efficiency of electron acceptor decreased by 35.1% for acetate and 44.0% for butyrate, respectively, compared with those using acetate as the exclusive acceptor (Figure 3c). However, a slight increase rather than a decrease in the caproate production was observed when the mixed acceptors were used (Figure 6a). On the one hand, it could be due to the difference in the carbon chain length between

acetate and butyrate, where two moles of ethanol was required by one mole of acetate, while only one mole of ethanol was required by butyrate to produce one mole of caproate. On the other hand, butyrate was the intermediate product of the first cycle of reversed β oxidation, which was produced from acetyl-CoA and acetate through a series of enzymatic reactions.[2] Lots of energy (ATP) can be saved if a part of acetate was replaced by butyrate. More energy would be required in the second cycle where caproate was produced through the reactions of acetyl-CoA with butyrate, resulting in a higher caproate production with the A/B ratio of 1:1 (Figure 6a). The above findings cast an insight that the acidic wastewater can be utilized for caproate production even if the acetate and butyrate coexist in the wastewater.

EPS are important substrates participating in biofilm formation. The dominant components of EPS are polysaccharide and protein, which account for about 75%-90% of the total. In this study, the EPS of the optimum groups determined in section 3.1-3.4 were detected to observe the influence of different fermentative conditions on the growth of *C. kluyveri* and biofilm formation. Results showed that the concentration of EPS can be affected by environmental factors, as shown in Figure 7a. With a pH of 6.9, the EPS decreased by 12.1% when the total substrate concentration increased from 440 (R1) to 660 (R2) mmol/L. And then the EPS increased from 531.7 to 610.0 mg/L when the pH increased from 6.9 to 7.4 with the fixed substrate concentration of 660 mmol/L. The change of EPS was remarkable with the variation of fermentative conditions. With the equivalent amount of electron acceptor of R3, the EPS increased to 632.4 mg/L in the group of R4 where half of the acetate was replaced by butyrate. High EPS concentration was

associated with the high production of caproate, e.g., the caproate production of R2 and R3 were 93.6 and 141.7 mmol/L, corresponding to the EPS of 531.7 and 610.0 mg/L, respectively. This implied that the activity of biofilm was in a positive correlation with EPS concentration.

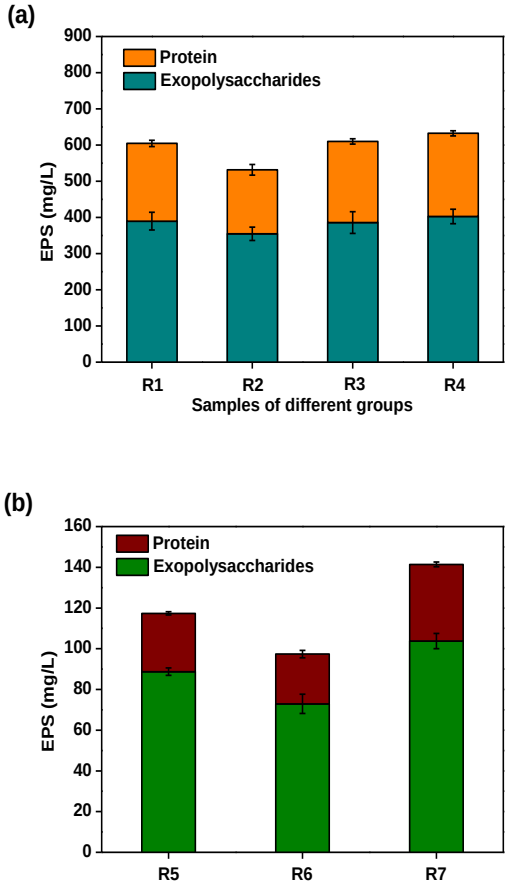


Figure 7. EPS of the optimum groups (a) and EPS adsorption by wheat straw (b) (R1: initial pH 6.9, 440 mmol/L substrates; R2: initial pH 6.9 and 660 mmol/L substrates; R3: initial pH 7.4, 660 mmol/L substrates; R4: initial pH 7.4, 660 mmol/L substrates, A/B ratio 1:1; R5: EPS of the mixture of medium and inoculum; R6: EPS of raw wheat straw; R7: EPS on the wheat straw one hour later after the wheat straw was added into the mixture of medium and inoculum).

Further studies showed that the wheat straw exhibits strong absorptivity on

EPS (Figure 7b). The EPS concentration of the mixture of medium and inoculum was 117.3 mg/L and the raw wheat straw hold an EPS concentration of 97.3 mg/L. The wheat straw absorbed 37.6% of the total EPS of the liquid fraction one hour later after the wheat straw was added into the mixture of medium and inoculum. Various functions of EPS have been reported in previous studies, such as cell to cell recognition, aggregations of bacterial cells, and adhesion to surfaces,[31] which benefited for biofilm formation. The process of biofilm formation in this study was described in Figure S4. At the initial of fermentation, pure wheat straw and free cells were mixed in the immobilization system. Part of the EPS was adsorbed on the wheat straw (Figure 7b). Then the viscosity of cells and wheat straw would increase due to the attachment of more EPS, resulting in strong adhesion between wheat straw and cells and finally formed biofilm. Thereafter, the cells propagated on the surface of wheat straw, resulting in more EPS and biofilm formed. As shown in Figure 4a, about 144 hours were used to achieve the mature state of biofilm where the number of immobilized cells was constant. As observed by SEM (Figures 8a and 8b), the surface of the cross-section and lateral surface were significantly different, where coarse surface and porous structure were observed on the cross-section it was smooth on the sidewall. The cells and EPS were easier to adhere to the coarse surface and part of the cells can grow in the hole of wheat straw because the diameter of the hole (30-70 μm) (Figure 8a) was remarkably higher than the length of the cells (2-10 μm) (Figures 8c and 8d). The porous structure of the wheat straw could provide a large specific surface area for the attachment of biofilm, which benefits caproate production. It was noteworthy that the polymeric substances with incompact shape were observed on the

surface of cells and carrier, which was supposed to be EPS. The adhesion of cell-cell and cell-carrier was observed with the connection of EPS, implying that EPS played an important role in biofilm formation.

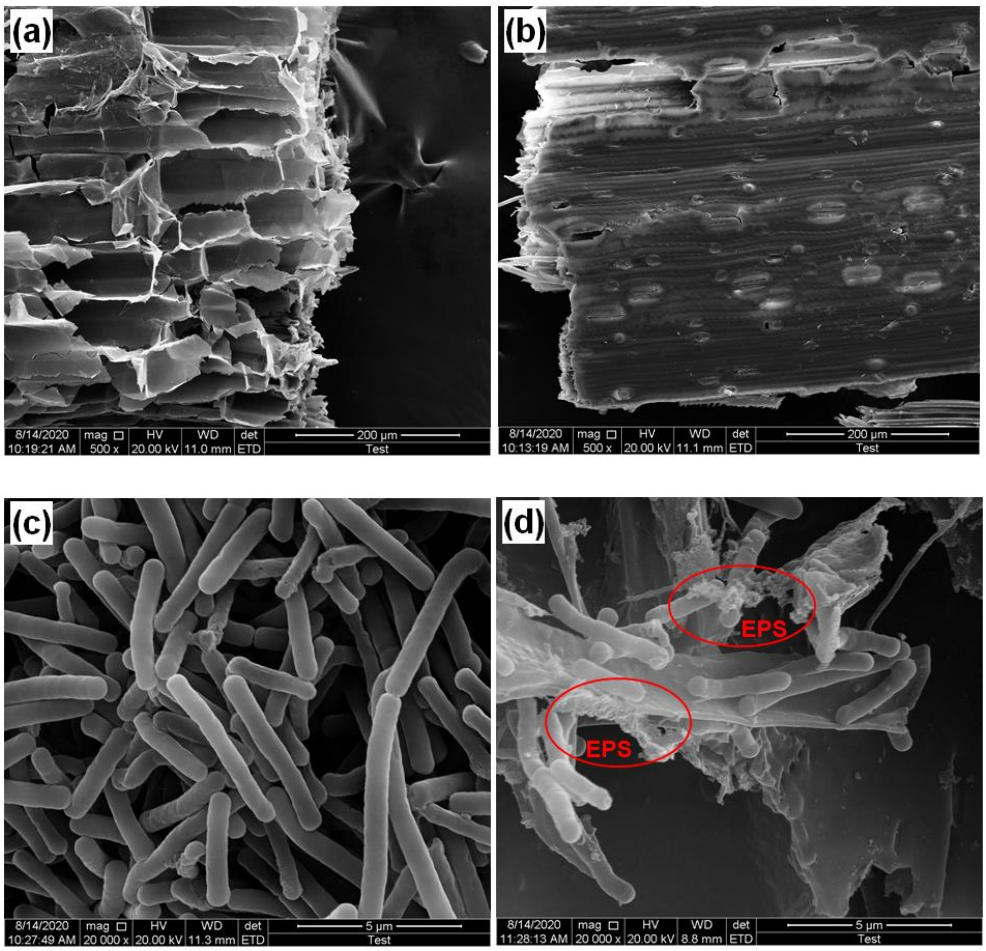


Figure 8. Structure of the cross section (a) and lateral surface (b) of wheat straw, free cells of *C. kluyveri* (c), and biofilm (d) formed on the wheat straw.

Caproate Production Using Bioethanol Wastewater. As shown in Table S1, ethanol was the main composition of the wastewater. Besides, a few amounts of organic substrates such as glucose and protein were also observed, and the ammonia concentration was 139.6 mg/L which was not toxic to *the immobilized C. kluyveri*.[7] Figure 9a shows the consumption of ethanol and acetate for the production of butyrate and caproate using the diluted bioethanol wastewater. After fermentation of 156 h, the cell immobilized system achieved

the maximum caproate production of 131.8 mmol/L (15.3 g/L), and the corresponding butyrate and hydrogen production were 59.7 and 72.4 mmol/L (Figure 9b), respectively. These results demonstrated that the wastewater rich in ethanol can be applied as substrate for caproate production. even in the presence of glucose and protein.

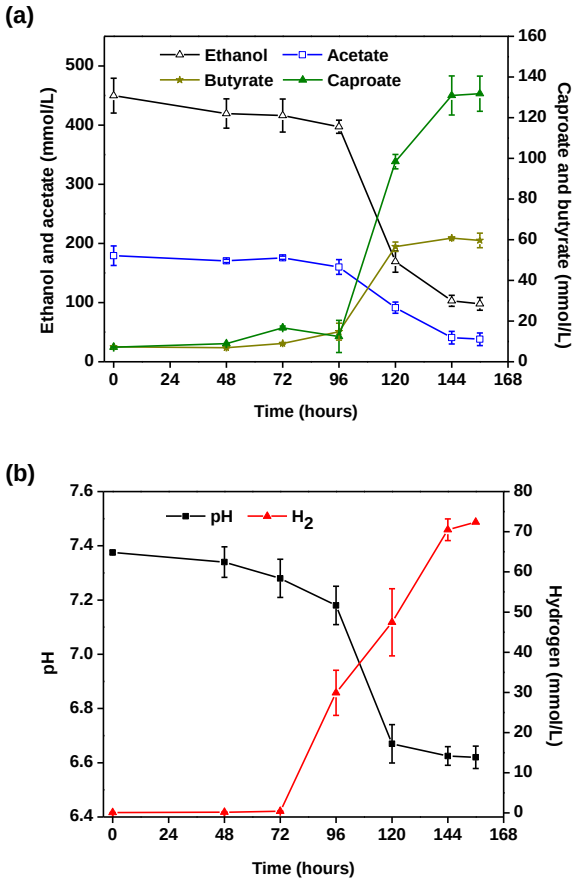


Figure 9. Anaerobic fermentation of ethanol wastewater for caproate production. (a: caproate production during fermentation; b: variation of pH and production of hydrogen)

The caproic acid in fermentation broth existed in two forms, i.e. dissociated and undissociated acids. Compared with dissociated acid, the undissociated acid was more harmful to microorganisms because it can easily pass through the cell membrane.[32] Our calculation showed that the concentration of

undissociated caproic acid was 2.29 mmol/L (0.266 g/L) after fermentation of 156 h (Figure 9), which was higher than the inhibitory concentration (0.2 g/L).[24] Hence, it was concluded that the caproate was inhibited by the undissociated caproic acid with the pH decreased from 7.4 to 6.6. Calculation base on Figure 9a showed that the consumed ethanol and acetate accounted for 78.2% and 78.8% (Figure 9a) of their initial concentrations, respectively. These results implied that more than 20% of the substrates was probably converted into caproate in the case that the inhibitory effect was addressed. To quantify the relationship between the inhibitory effect and pH, the operable pH range was suggested in Table S2. Results showed that a narrow pH range of 7.0 -7.4 was required to avoid the inhibitory effect caused by butyrate and caproate. To maintain the pH stable, continuous addition of buffer solution (e.g., bicarbonate) can be employed during the fermentation process.[8]

The caproate production of this study was compared with some of the data reported previously, as listed in Table S3. Using the pure culture of *C. kluyveri*, San-Valero et al. obtained a caproate production of 181.6 mmol/L in a 2-L BIOFLO 110 vessels through regulating the pH be constant at 6.8 by using bicarbonate.[8] The corporate production was higher than the highest value (110 mmol/L) reported by Weimer and Stevenson.[14] Although the highest caproate production (146.6 mmol/L) of this study was lower than the production (181.6 mmol/L) reported by San-Valero et al.,[8] the fermentation conditions between the two studies were significantly different. Particularly, there was no regulation of pH during the chain elongation process in this study. The difference in the caproate production further proved the importance of pH for chain elongation. Nevertheless, there was an ultimate value for caproate

production in batch fermentation even if the optimum E/A ratio and constant neutral pH was employed. Based on Eq. (2), the ultimate caproate production would be 224 mmol/L (26 g/L), considering the inhibition concentration (0.2 g/L) of undissociated caproic acid as a boundary value. Consequently, the separation of caproate would also be essential for high caproate production in future research.

High purity of caproate was essential to save the cost of purification in the downstream process. In this study, the impurities of the broth mainly comprise ethanol, acetate, and butyrate. It was found that there was a little amount (< 3 mmol/L) of *n*-octylic acid in the broth. However, its concentration was far lower than those of other chemicals, which can be ignored. According to the concentration of each chemical shown in Figure 3c, it was calculated that the purity of caproate in the broth was 41.5% (mol/mol), where the ethanol and acetate were used as substrates. Many previous studies focused on the co-culture and open-culture systems to convert the waste biomass into caproate.[33,34] Compared with the co-culture system, the advantages of the mono-culture system in this study was that there was no substrate competition between *C. kluyveri* and other bacteria such as methanogen. In this regard, high purity and production of caproate could be obtained, which benefits for the separation of caproate from the broth. Moreover, it is easy to achieve the highest activity of *C. kluyveri* through controlling the fermentative parameters such as pH and substrate concentration, without consideration of other bacteria in the monoculture system.

The application of caproate has been broadened from a relatively small market to a large platform in the chemical industry.[22] It will be a promising trend

to utilize wasted biomass to produce caproate because these substrates are sustainable resources without competition with food crops and land.[4] Currently, several chemicals such as ethanol, lactic acid, and glycerol can be used as the electron donor, and acetate and butyrate can be used as the electron acceptor,[4,18,35] which implied the wastewater containing these chemicals can be used for caproate production. However, the component of the wastewater will be significantly different on the amount and type of alcohol and organic acids. Thus, it will be essential to balance the nutrition of wastewater before fermentation. In addition, continuous fermentation would be required for the long-term production of caproate, where the biofilm will be extremely important due to its high resistant ability to inhibitors such as ammonia and undissociated carboxylic acids.[7,23] Our research provided a new avenue for the long-term production of a high concentration of caproate with less cell loss.

CONCLUSIONS

The biofilm performed high performance in caproate production, obtaining a high production of 146.6 mmol/L with the optimized conditions. The balance of the concentration of electron donor and acceptor was important for the carbon chain elongation reactions. Excessive supply of substrates led to strong inhibition to caproate production. Moreover, the caproate biosynthesis can be remarkably influenced by the pH where a neutral pH was more preferable. Besides, the strain preferred to use the co-electron acceptors of acetate and butyrate to produce caproate. However, butyrate presented high toxicity to biofilm when its concentration achieved 150 mmol/L. The high caproate production from the bioethanol wastewater implied that low-cost caproate production can be achieved using wasted organic substrates.

SUPPORTING INFORMATION

Supplementary material and methods; pathway of *Clostridium kluyveri*; influence of acetate and butyrate on caproate production; thermal characteristics of pure wheat straw, *C. kluyveri*, and wheat straw immobilized with cells; the process of biofilm formation in the immobilization system; characteristics of ethanol wastewater; the suggested pH range to avoid inhibition caused by butyrate and caproate; caproate production obtained from different studies.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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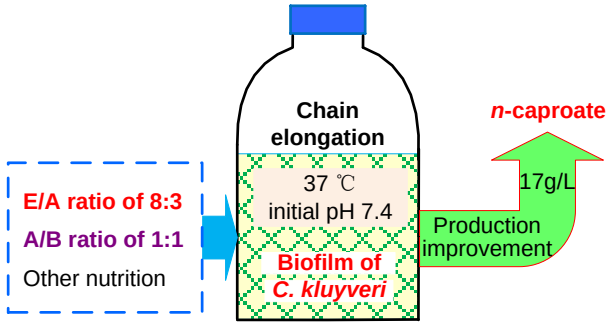

Turning waste biomass into caproate through optimization of a cell-immobilized chain elongation process