Insight Into Effects of Long-chain Fatty Acids on Propionic Acid Production in Anaerobic Fermentation: A Case Study of Oleic Acid and Palmitic Acid

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Abstract

This study revealed long-chain fatty acids(LCFAs) on propionic acid production by lipid anaerobic acidogenesis. Oleic acid and palmitic acid, the two most common acids in LCFAs, were added into the acid production system to evaluate LCFAs type and concentration on the propionic acid production capacity of the acid production system. The results showed that when the concentration of oleic acid was 4g/L, the content of volatile fatty acids(VFAs) was 1.3 times higher than that of the blank group(propionic acid was the main product, > 70%). In contrast, palmitic acid and excess oleic acid inhibited propionic acid production(butyric acid was the main product). Propionic acid-producing bacteria were the dominant bacteria in an oleic acid production system. According to response surface analysis, the optimal concentration of oleic acid favoring for propionic acid production was 2.3 g/L and fermentation time was 7.4 days in this study. The results showed that a proper concentration of oleic acid has a promotion to propionic acid production. This study provides a theoretical basis for oleic acid to promote propionic acid production.

1. Introduction

Propionic acid is one of the most common odd-carbon-number volatile fatty acids(OCFAs) in anaerobic fermentation. More and more studies have shown that the extracted and recovered propionic acid plays a vital role in the downstream application. For example, propionic acid can be used as feed [1], to produce biofuels [2], for nitrogen and phosphorus removal improvement in sewage disposal [3], for polyhydroxyalkanoates(PHAs) monomers adjustment in microbial PHAs production [4], and so on.

In recent years, some researchers have found that lipids-rich wastes tend to produce propionic acid during anaerobic fermentation [5–7] or propionic acid enrichment in the lipids-rich methanogenic system [8, 9]. However, it is worth noting that the accumulation of long-chain fatty acids (LCFAs), the hydrolysates of lipids, often exists in the process of anaerobic fermentation, on account of the β -degradation of LCFAs is a rate-limiting step [10].

Because of the molecular structure, the accumulation of LCFAs is often considered to have an inhibitory effect on microorganisms in the anaerobic system. The composite structure of the hydrocarbon chain of LCFAs makes it have a polar hydrophilic end and non-polar hydrophobic end similar to the bacterial cell membrane [10]. Due to the similarity between LCFAs and cell membrane, LCFAs accumulated on the surface of bacteria will dissolve the lipid layer of the cell membrane, causing irreversible damage to it, thus destroying the life activities of anaerobic bacteria [11]. In addition, due to the hydrophobicity of LCFAs molecular structure, under the interaction of van der Waals force and static electricity, LCFAs attached to the surface of anaerobic bacteria may form a hydrophobic physical barrier, which can change the permeability of cell membrane and hinder the intracellular and extracellular material transportation [12].

The inhibitory effect of LCFAs in the anaerobic system is related to their species and concentration. Articles that mention LCFAs' impact on the anaerobic system are mostly focused on the methanogenic system [13]. In some articles about the effect of LCFAs on the acid production system, it is only briefly mentioned that the inhibitory effect of LCFAs is related to their type and concentration [14–16]. And the studies about producing propionic acid from lipid shown in the previous paragraph mainly evaluated the factors affecting propionic acid production from substrate type, pH, temperature, etc. Although these articles mentioned the accumulation of LCFAs in the propionic acid production system, they did not profoundly discuss the influence of specific factors such as the concentration and type of LCFAs on the propionic acid production system. There are still many problems debating the impact of LCFAs accumulation on the propionic acid production system for propionic acid production using lipids. For example, “Are the effects of different types of LCFAs on propionic acid production systems consistent?”, “Except inhibition, is it possible for LCFAs to promote propionic acid production in the acidogenic system?” and so on. It is essential to verify the specific impact of LCFAs on the propionic acid production system. Only then can we determine whether the inhibitory LCFAs need to be removed from the acid production system by adding mineralizer. The additional mineralizer will undoubtedly increase the production cost of propionic acid. This is of great significance to improving propionic acid production and the adjustment of propionic acid production cost.

To further explore the effect of LCFAs accumulation on propionic acid production in the process of lipid fermentation, this experiment was carried out based on our previous research: used waste hogwash fat for anaerobic fermentation to produce propionic acid [7, 17–19]. The two LCFAs with the highest content in the hogwash waste oil fermentation, oleic acid and palmitic acid, were added to the anaerobic acid production system separately for exploring the impact of LCFAs type and concentration on the propionic acid production capacity. The changes of VFAs concentration, propionic acid content, pH, and oxidation-reduction potential (ORP) in the propionic acid production system with different kinds and concentrations of LCFAs were investigated. In addition, the differences in microbial community structure between the oleic acid system and palmitic acid system were investigated, and the effects of LCFAs on propionic acid production were evaluated from the perspective of microbial ecology. This study provides valuable information for the actual process of propionic acid production by lipid anaerobic fermentation.

2. Materials And Methods

2.1 Substrates and inoculum

This study aims to reveal the effect of LCFAs on the acid production system in the fermentation process of hogwash waste oil. Therefore, in this study, the fermentation substrate was prepared by simulating the hydrolysate composition of hogwash waste oil according to our previous research. In our earlier research, waste hogwash oil was used as the fermentation substrate of the propionic acid production system, and the main component of the hydrolysate was glycerol ($10 \pm 1.5\%$)—LCFAs ($88 \pm 0.3\%$), sugar ($2 \pm 0.5\%$), protein ($6 \pm 0.5\%$), and other substances ($3 \pm 0.8\%$). Oleic acid ($50 \pm 2\%$) and palmitic acid ($35 \pm 5\%$) was the main products of LCFAs [7, 17, 18]. Due to its amphiphilic molecular structure, LCFAs can promote the

solubilization of proteins and polysaccharides (proteins and polysaccharides are also substrates used by microorganisms to produce VFAs)[10]. Therefore, to ensure a single variable in the test process, the ratio of simulated hydrolysate was simplified (i.e., the sugar and protein content was ignored when prepared the simulated hydrolysate). In this experiment, analytical pure glycerol, analytical pure palmitic acid, and analytical pure oleic acid were used for simulated hydrolysate preparation. These chemicals were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. The inoculated sludge was taken from the Secondary sedimentation tank of the local sewage treatment plant. The sludge retention time (SRT) was 12 days (Guangzhou, China). The characteristics of inoculated sludge are shown in Table.1.

Table 1
Basic characteristics of the inoculum.

^a TS(g/L)	^b VS(g/L)	VS/TS(%)	pH
20.53	6.9	33.36	6.48
^a TS, total solids; ^b VS, volatile solids.			

2.2 Experimental procedures

In this experiment, the parallel batch test was used, and three kinds of test groups were set up: (1) blank test group Q₁ for control effect; (2) The oleic acid experimental groups Q₂, Q₃, and Q₄ were used to study the effect of oleic acid concentration on the propionic acid production system; (3) Palmitic acid test groups Q₅, Q₆ and Q₇ were used to study the effect of palmitic acid concentration on the propionic acid production system.

All the experimental groups used a 1L glass fermentor as the testing device. The fermentor was equipped with a sealed top cover fitted with a sampling port and a stirring paddle port. According to the hydrolysate composition obtained in our previous research and the calculation for this test, 3.5g glycerol and 167.8mL seed sludge were added to each glass fermentor in this experiment. Then, according to the proportion of oleic acid and palmitic acid in hogwash waste oil hydrolysate in the previous study, 4g/L oleic acid and 5g/L palmitic acid were selected as the initial concentration of LCFAs dosage in this experiment. The corresponding concentration gradient was set by equal difference, and the calculated volume or mass of oleic acid and palmitic acid were added to the corresponding fermentation tank, respectively. Added no LCFAs to the blank group for comparison. So the concentration of oleic acid in the single fermentor of the oleic acid group was 4g/L, 8 g/L and 12 g/L, respectively. The concentration of palmitic acid in the single fermentor of the palmitic acid test group was 5g/L, 10 g/L and 15 g/L, respectively. The effective volume of each fermentor was 600 mL. To ensure the strict anaerobic condition, blew off the oxygen in the fermentation system with nitrogen for 10 min after all the feedstock was added. Then, each fermentor was placed at 35 ± 1°C in a constant temperature water bath, and the magnetic stirrer was used to stir at the speed of 700 ± 50 rpm mechanically. During anaerobic acid production, VFAs content and composition, propionic acid production, pH, and ORP in each fermentor were monitored and analyzed daily. The fermentation time was adopted as 12 days.

2.3 Analytical methods

During the fermentation time, a 10 mL syringe was used to sample each group every day. After sampling, centrifuged the samples at 4000r/min for 10min. Then the supernatant was filtered with 0.45 µm pinhole membrane for sample analysis. The pH and ORP of each sample were measured by acidimeter (PHS-3C, Tsushima, Japan). The content of propionic acid was determined by gas chromatography [19]. Besides, other VFAs in the fermentation broth, such as acetic acid, isobutyric acid, n-butyric acid, isovaleric acid, and n-valeric acid, were also determined by gas chromatography. Use the reference materials (analytical purity) of the substances mentioned above to prepare the corresponding standard solution respectively, and draw the standard curve. The samples were analyzed by GC7900 gas chromatograph (Shanghai Tianmei Scientific Instrument Co., Ltd., China), FID detector, and CNW CD-WAX column with an inner diameter of 0.32mm. The injection volume was 1.0µL sample, the injection port temperature was 220°C, the FID temperature was 250°C, the carrier gas was nitrogen, and the flow rate was about 25mL/ min. The analysis temperature started from the initial temperature of 80°C, rose to 110°C at the heating rate of 20°C/ min, stabilized for 2 min, and then gradually increased to 180°C at the programmed temperature of 10°C/ min, and the analyzed were terminated after 12 min. To ensure accuracy, added 0.1mL formic acid to the sample for acidification. The error range between the results and the average value was controlled within 5%. Repeat the determination of VFAs concentration and components twice, take the average value, record the peak area of various VFAs, and calculate the content of various VFAs (mg/L) through standard curve conversion.

2.4 Microbial community structure analysis

The reactor with the strongest acid production capacity would be selected respectively from the blank, oleic acid, and palmitic acid experimental group for microbial structure analysis in this test. Because this experiment was carried out in the form of the short-term batch, when each experimental group reached the peak of VFAs production, the acid production system at this moment was defined as operating stable and could give full play to the acid production capacity. The microbial community composition and density of the selected samples were analyzed for horizontal comparison. The specific method was as follows: during the test, biological samples were extracted from each test group every day (i.e., a total of 84 biological samples were extracted during the fermentation time). Used E.Z.N.A™ Mag-Bind Soil DNA Kit (OMEGA, America) to extract biological samples' total genomic DNA. Using F341 (5'-CCTACGGGNGGCWGCAG-3' and R805 (5'-GACTACHVGGGTATCTAATCC-3') as primers, the V3-V4 variable region of 16s rDNA gene was amplified by polymerase chain reaction (PCR), and the library was sequenced on Illumina MiSeq platform by Shanghai Sheng Gong Bioengineering Co., Ltd.

3. Results And Discussion

3.1. Effect of the concentration gradient of oleic acid and palmitic acid on propionic acid production

The VFAs yield is an important index to measure the performance of an acid production system. As shown in Fig. 1 (a), the VFAs yield of group Q₂ (2382.22 mg/L) and group Q₆ (2167.88 mg/L) were higher than the blank control group Q₁ (2083.74 mg/L), and they were 14.3% and 4% higher than that of the blank control group, respectively. This indicated that LCFAs could promote acid production when the concentration of LCFAs was at an appropriate value, and the promotion effect of oleic acid was stronger than that of palmitic acid. The promotion was led by the β -oxidation of the oleic acid or palmitic acid, which contained VFAs as oxidation products that raising the total yield of VFAs [20]. The acid production of the other experimental groups was lower than that of the blank control group, indicating that LCFAs inhibited the acid production capacity of the acid production system at the corresponding concentration of LCFAs.

In addition, it can be observed from Fig. 1 (a) that the time of VFAs production peak in each experimental group had a certain trend. The peak value of VFAs production in the blank control group was on the 6th day, that in oleic acid group was on the 6th, 11th and 5th day, and that in palmitic acid group was on the 9th, 10th and 11th day. Except for group Q₂ and Q₄ in the oleic acid group, acid production's peak value in other experimental groups appeared lag compared with that in the blank control group. The lag period of the palmitic acid test group was longer than that of oleic acid. Besides, the lag period increased with the increase of palmitic acid concentration, which showed its delaying effect on the acid production system. The reason for the delayed effect is that when oleic acid/palmitic acid was attached to the surface of acid-producing bacteria, undegraded LCFAs affect the interaction of internal and external substance exchange and metabolism of acid-producing bacteria. The reason why the lag time of the palmitic acid test group was longer is that the degradation rate of palmitic acid as saturated acid is much slower than that of oleic acid (unsaturated acid) [20–22]. Therefore, it can be concluded that the palmitic acid would accumulate on the surface of acidogenic bacteria for a longer time. When the concentration of attached LCFAs was so much that bacteria could not degrade it and beyond the tolerance range of bacteria, it showed the inhibitory effect of LCFAs on the acid production system.

As shown in Fig. 1 (b), palmitic acid negatively affected propionic acid production in this study. However, the inhibition of palmitic acid on propionic acid production capacity in the acid production system slowed down with the increase of palmitic acid dosage, and the propionic acid production curve in the palmitic acid test group showed a similar trend. The yield curve of propionic acid in the oleic acid group was divided into two movements. When the dosage of oleic acid was 4g/L in group Q₂, the peak concentration of propionic acid was 2027.57mg/L, and that in the blank control group Q₁ was 1533.76mg/L. Compared with group Q₁, the production of propionic acid in group Q₂ was 1.3 times higher than that in group Q₁, showing that propionic acid production was significantly improved. When the dosage of oleic acid was 8g/L and 12g/L, it offered an inhibitory effect on propionic acid production, and the propionic acid production curve was similar to that of the palmitic acid group. According to Sousa et al., when oleic acid was the only carbon source in the anaerobic system, no other VFAs were found as degradation products except acetic acid[23]; therefore, the possibility of conversion of oleic acid to propionic acid was ruled out. The reasons for the above results can be explained from the following two aspects: a) In the process of

oleic acid decomposition, some factors can promote the production of propionic acid existed [17]; b) According to some studies, oleic acid and palmitic acid had an inhibitory effect on propionic acid consuming bacteria, but oleic acid had a stronger inhibitory effect on propionic acid consumption[24, 25]. Hence, it can be considered that within the concentration range that acid-producing bacteria can tolerate, oleic acid could promote the production of propionic acid. And on the other hand, oleic acid reduced the consumption of propionic acid, thus forming the phenomenon of propionic acid accumulation.

3.2. Composition of VFAs in each experimental group

In this short-term batch test, when the production of VFAs in each acid-producing system reached its peak, it was indicated that at this moment, the key microbial community in the system came to a state that exerting the strongest acid-producing capacity, and the dominant acid-producing bacteria showed strong vitality. Table.2 showed the composition of VFAs in the system when each test group reached the peak production of VFAs.

Table.2 The VFAs composition under the maximum concentration in each group

Items	No.	Content (g/L)	^a VFAs (mg/L)	^b H _{AC} (%)	^b H _{Pr} (%)	^b H _{Isobu} (%)	^b H _{Bu} (%)	^b H _{Isova} (%)	^b H _{N-va} (%)
None	Q ₁	0	2198.21	20.98 ± 0.3	64.50 ± 0.9	0.75 ± 0.2	6.73 ± 0.8	1.77 ± 0.4	5.28 ± 1.1
oleic acid	Q ₂	4	2382.22	6.42 ± 0.2	76.50 ± 0.5	0.56 ± 0.2	5.99 ± 0.3	1.92 ± 0.2	8.61 ± 0.6
	Q ₃	8	1303.08	27.12 ± 1.1	11.31 ± 0.9	0.45 ± 0.1	41.90 ± 0.4	0.90 ± 0.2	18.31 ± 0.7
	Q ₄	12	1510.66	10.60 ± 0.8	15.80 ± 0.4	1.04 ± 0.6	48.54 ± 0.7	1.31 ± 0.5	22.71 ± 0.3
palmitic acid	Q ₅	5	1806.22	21.06 ± 1.2	31.88 ± 0.6	0.30 ± 0.1	35.73 ± 0.6	1.54 ± 0.3	9.50 ± 0.6
	Q ₆	10	2167.87	20.19 ± 1.1	22.27 ± 0.9	1.24 ± 0.2	39.94 ± 0.8	1.38 ± 0.3	14.98 ± 1.1
	Q ₇	15	1986.67	19.49 ± 0.5	26.72 ± 0.8	0.62 ± 0.2	41.66 ± 0.3	2.59 ± 0.6	8.92 ± 0.3
^a VFAs represents the maximum concentration in each experimental group.									
^b Ac, acetic acid; Pr, propionic acid; Isobu, Isobutyric acid; Bu, Butyric acid; Isova, Isovaleric acid; N-va, N-valeric acid.									

The composition of VFAs was closely related to the type and concentration of LCFAs during fermentation. It can be observed from Table.2 that acetic acid, propionic acid, n-butyric acid, and n-valeric acid are the

main components of VFAs in the acid production system. Since the REDOX degrees of glycerol and propionic acid are numerically equal ($\gamma = 4.67$ for both)[26], in the blank test group Q₁, glycerol was the main carbon source, propionic acid was the main VFAS component. Therefore, group Q₁ showed propionic acid-type fermentation. While with the dosage of LCFAs, the other groups showed a great difference. In the oleic acid experimental group, when the dosage of oleic acid was 4g/L, propionic acid production accounted for the absolute advantage. But it dramatically reduced as the concentration of oleic acid increased; instead, n-butyric acid and n-valeric acid content increased, and the acid production system changed from propionic acid-type fermentation to butyrate-type fermentation. In the palmitic acid test group, butyric acid was the main VFAs product no matter the concentration of palmitic acid. And all the palmitic acid experimental groups showed butyrate-type fermentation. This phenomenon was attributed to the fact that acid-producing bacteria were the main undertakers of VFAs production. The addition of LCFAs changed the original living environment of the microbial community and brought a change shock. The corresponding microbial community was formed dominant flora through transformation and reproduction to adapt to the lash of the living environment. Thus, different acid-production fermentation types emerged.

3.3. Effects of oleic acid and palmitic acid on pH and ORP in the acid production system

PH and ORP are the key parameters reflecting the acid production performance of the acid production system because they affect the microbial community and metabolic pathway and determine the production efficiency as well as the composition of VFAs in the process of anaerobic fermentation[27, 28]. Figure 2 shows the changes of pH and ORP of each experimental group during fermentation.

It can be seen from Fig. 2(a) that in the early stage of fermentation, the substrate was hydrolyzed and acidified rapidly under the action of microorganisms to produce a large number of organic acids, and the pH of all experimental groups showed a downward trend. With the increase of fermentation time, the pH of each experimental group showed a different state. In group Q₁, the pH trend downward at first but increased on the 8th day and then gradually stabilized. During the fermentation period, the pH range of group Q₁ remained at 4.0-4.7. As for the oleic acid group, the pH fluctuated greatly during the fermentation period, ranging from 3.5–4.9. In group Q₂, the pH showed a continuous downward trend from day 3 to day 8, and then reached the minimum pH value of 3.53 on the 8th day, finally rose in a broken line, showing that the degree of acidification was higher when the concentration of oleic acid was 4 g/L. And for the palmitic acid group, the pH ranging from 4.3–4.8 tended to be stable during the fermentation period. The phenomenon mentioned above indicated that oleic acid had more effect on pH than palmitic acid. In this way, the suitable pH values in the oleic acid favored the natural selection of propionic acid-producing bacteria over other microorganisms [29].

As for ORP, it can be found from Fig. 2 (b) that there was no significant difference in ORP range of the same type of LCFA: The ORP range of the blank control group was -7-131mV, 108–297 mV in the oleic acid group, and -166 – 139 mV in the palmitic acid group. Thus, it can be seen that the increase of ORP in the acid production system was mainly affected by LCFAs components but had little relationship with

its concentration. Besides, the results showed that the ORP of the oleic acid test group was higher than that of the palmitic acid test group, indicating that the oxidation of the oleic acid test group was stronger than that of the palmitic acid test group.

To sum up the above phenomena, the level of ORP was not related to the concentration of LCFAs, but more related to the types of LCFAs. And compared with palmitic acid, oleic acid had a greater impact on the pH of the acid production system, and the ORP level was higher, showing stronger oxidation. Due to the lack of an electron transport system in the acid-producing system of anaerobic fermentation, a large amount of NAD^+ is usually produced during the oxidative dehydrogenation of the substrate. Therefore, NAD^+/NADH is a standard REDOX pair in the intracellular metabolism of acid-producing bacteria[30]. ORP is the main index of electron transfer and REDOX balance of acid-producing bacteria in the reaction process [31]. Hence, according to the experimental phenomenon of higher ORP levels in the oleic acid test group, it can be concluded that the ratio of NAD^+/NADH in the oleic acid test group was higher than that in the palmitic acid test group. This phenomenon was related to the β -oxidative degradation of LCFAs. As an unsaturated fatty acid, Oleic acid degrades faster than palmitic acid and releases NAD^+ during the β -oxidative degradation[22]. The main substrate of each test group was glycerol in this experiment. With microbial-related enzymes, the glycerol was successively decomposed into dihydroxyacetone phosphate(DHAP), phosphoenolpyruvate(PEP), pyruvate and a large amount of NADH(Fig. 3). Thus, with the appropriate dosage of oleic acid (in this test, the dosage of oleic acid is 4g/L), the β -degradation of oleic acid facilitated the conversion of glycerol to the production of large amounts of NADH. To maintain the balance of NAD^+/NADH ratio in the acid-producing system, acid-producing bacteria would relieve the accumulated pressure of NADH through propionic acid production. This is consistent with Ren et al.[30].

3.4. Microbial community structure in oleic acid and palmitic acid systems

The final samples selected for microbial detection in this experiment were: the microbiological samples of blank control group Q_1 , group Q_2 , and group Q_6 when they reached acid production peak. The analysis data were shown as A_1 , A_2 and A_3 , respectively.

3.4.1. Microbial diversity and richness

Table.3 reflects the microbial community structure of the selected experimental group when the acid production was stable(i.e., when the VFAs production was the highest). Among them, OTUs, Chao, and Shannon indices are commonly used to measure microbial richness. The higher the index values, the higher the species richness of the samples. The high biodiversity of the acidogenic system is conducive to the stability of the acidogenic system and has a positive effect on the degradation and transformation of substrates in the acidification stage[33]. It can be seen from Table.3 that the richness of microbial community in the Q_2 and Q_6 was not far different from that in Q_1 . That means the microbial community in the acid-producing system was not significantly affected at the corresponding LCFAs concentration, which is the main reason for the similar total VFAs yields of the two experimental groups and the blank

control experimental group. Besides, the Chao value showed that LCFAs promoted the diversity of microbial communities in the system.

Table 3
Diversity indices of bacterial gene sequences from samples taken

Sample	OTUs	Shannon	Chao	Ace	Simpson	Coverage
A1	1396	4.07	1613.32	1609.89	0.06	0.99
A2	1495	4.00	1695.31	1678.44	0.09	1.00
A3	1522	4.36	1685.78	1681.00	0.07	1.00

3.4.2. Microbial community structure

Sequence distribution of bacterial communities with relatively high abundance in the three groups of samples at the Genus level is shown in Fig. 4. Among them, the species whose abundance ratio was less than 1% in the sample were classified as Other. The results showed that the three groups of samples had high similarities in bacterial composition. However, the dominant microbial community of each group had a great difference. When the acid-producing system was stable, the main bacterial community in group Q₁ were *Clostridium_sensu_stricto*, *Acetobacter*, *Anaerosinus*, and *Propionispira*. Their relative abundance was 31.81%, 10.84%, 10.33%, and 7.49%, respectively. The main bacterial populations in the oleic acid test group Q₂ were *Propionispira*, *Anaerosinus*, *Lactobacillus*, *Clostridium_Sensu_Stricto*, and their relative abundance was 25.43%, 11.94%, 9.18%, and 6.84%, respectively. Although relatively abundant, some bacterial genera, such as *Megasphaera*, still be involved in the crucial reaction of oxidizing glycerol to lactic acid and then converting it to propionic acid[34]. The main bacterial populations in the palmitic acid test group Q₆ were *Clostridium_sensu_stricto* and *Anaerosinus*, whose relative abundance was 38.43% and 11.76%, respectively.

The results showed that the types of LCFAs had important effects on the key propionic acid bacteria. For example, *Propionispira* is a typical propionic acid producer [12], usually found in anaerobic fermentation reactors that produce propionic acid using organic substances. It can be seen from Fig. 4 that the *Propionispira* was dominant in group Q₂, but the relative abundance of *Propionispira* in group Q₆ was only 1.47%. Besides, *Lactobacillus* is also a propionic acid producer [35]; it had a high abundance in group Q₂, a small amount in group Q₁, but a very low quantity in group Q₆. Combined with the acid production results, the rank of propionic acid production from high to low in the three experimental groups was Q₂ > Q₁ > Q₆. This phenomenon indicated that propionic acid-producing bacteria had good adaptability in the oleic acid environment and could give full play to its propionic acid production capacity; however, it was seriously inhibited by palmitic acid.

Another interesting phenomenon was that the dominant bacteria in group Q₁ and group Q₆ were both *Clostridium*, a kind of butyric acid-producing bacteria[36]. Theoretically, butyric acid should be the main

component of VFAs in group Q₁ and Q₆, because butyric acid bacteria dominate in abundance. However, in contrast to group Q₆, the main VFAs component in group Q₁ was propionic acid and acetic acid, indicating that there existed some kinds of bacterial communities competing or antagonizing with *Clostridium* in group Q₁. From the comparison of relative abundance, it can be inferred that *Acetobacte* is the competitive flora of *Clostridium*. The above analysis shows that the same seed sludge evolves different dominant community structures under different LCFA species, resulting from microbial communities adapting to the environment, and there was the interaction between microbial communities.

3.5. Analysis and prediction of optimal concentration of oleic acid in propionic acid production by Response Surface Method

From the above analysis, it can be determined that the appropriate concentration of oleic acid was conducive to propionic acid production. Design Expert 10 was used to perform response surface analysis to define further the oleic acid concentration that could promote propionic acid production. Set up propionic acid yield(Y) as the dependent variable, oleic acid concentration(A) and fermentation time(B) as independent variables. The analysis results were shown in Table. 4 and Fig. 5.

Table 4
Fitting and ANOVA result for the oleic acid test group model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1.63E + 07	9	1.81E + 06	44.84	0.0001
A-LCFA concentration	1.02E + 07	1	1.02E + 07	252.21	0.0001
B-fermentation time	6.43E + 04	1	6.43E + 04	1.59	0.2146
AB	5.24E + 05	1	5.24E + 05	12.97	0.0009
A ²	3.39E + 06	1	3.39E + 06	84.04	0.0001
B ²	3.32E + 05	1	3.32E + 05	8.21	0.0067
A ² B	2.19E + 05	1	2.19E + 05	5.43	0.0252
AB ²	3.42E + 05	1	3.42E + 05	8.46	0.0060
A ³	5.68E + 06	1	5.68E + 06	140.61	0.0001
B ³	1.14E + 05	1	1.14E + 05	2.83	0.1009
Residual	1.53E + 06	38	40373.04		
Cor Total	1.78E + 07	47			
Note: R-Squared = 0.9139, Adj R-Squared = 0.8936, coefficient of variation(C.V.) = 22.63%, Adeq Precisor = 21.319					

Evaluate the multiple regression fitting model according to the analysis of variance (Table. 4), the F value of the model was 44.84, and the value of $P_{\text{model}} > F$ was less than 0.0001. The fitting results of the software showed that the response of the model to propionic acid concentration was highly significant. In addition, the coefficient of variation (C.V.) of the model was 22.63%, and the Adeq precision was 21.319, which was more than 4, indicating that the model had high reliability and accuracy. According to the corresponding F value obtained by ANOVA, the concentration of oleic acid ($F = 252.21$) was higher than that of fermentation time ($F = 1.59$), indicating that the concentration of oleic acid was a more important factor affecting the production of propionic acid. The test data were fitted by multiple regression, and the mathematical regression formula generated by the coding factor (Table. 4) was shown in Eq. (1).

$$Y = 524.51 - 2482.10A + 175.99B - 409.65AB + 1830.03A^2 - 332.30B^2 - 413.10A^2B + 482.90AB^2 + 379.31A^3 + 338.98B^3 \quad (1)$$

The "optimization" tool in software Design Expert 10 was used to analyze and predict the optimal conditions for propionic acid production (Fig. 5): When the concentration of oleic acid was 2.3g/L and the fermentation time was on the 7.4th day, the expected yield of propionic acid was 2028.16mg/L.

4. Conclusion

In this study, the concentration of oleic acid and palmitic acid significantly differs in propionic acid production performance. When the concentration of oleic acid was 4 g/L, the yield of propionic acid was the highest in all the experimental groups, 1822.51 mg/L, which was 1.3 times higher than the blank control group. In addition, the propionic acid-producing producers were the dominant strain in the experimental group, while the butyric acid-producing strain was the dominant strain in the palmitic acid experimental group. The results showed that a suitable concentration of oleic acid could promote propionic acid production, while palmitic acid and excess oleic acid could inhibit propionic acid production. According to the regression model fitted by the response surface method, the optimal concentration of oleic acid was 2.3g/L and fermentation time was 7.4th day.

Declarations

Ethical Approval

The authors declare that the submitted manuscript has not been previously published, whole or in part, and will not be published elsewhere. And this manuscript is the original work of the authors. The authors abide by the rules of a specific discipline when obtaining, selecting, and processing data.

Consent to Participate

The authors declare that all authors have approved the content and authorship of the final version of the submitted manuscript's content and authorship and have mutually agreed that it should be submitted to

Consent to Publish

The authors declare that all authors mutually agree that Applied Biochemistry and Biotechnology publish this work.

Authors' contributions

T. W : Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing-original draft, Writing-reviewing and Editing;

Q. F : Conceptualization, Supervision, Funding acquisition, Writing-reviewing and Editing.

JXL : Conceptualization, Methodology, Validation, Data curation, Formal analysis.

YJ.C: Formal analysis, Data curation.

KQ.Z: Writing-original draft.

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Competing Interests

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

Availability of data and materials

Not applicable

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Figures

Figure 1

(a) VFAs production and (b) propionic acid concentration under different concentration gradients of oleic acid and palmitic acid.

Figure 4

Comparison of microbial community distribution of three groups of samples at genus level

Figure 5

The 3D structure and contour map of response surface

Supplementary Files

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