INTRODUCTION

As development has continued around the world, the energy crisis resulting from shortages of fossil fuels can no longer be ignored.1 Biodiesel has attracted much attention as an alternative to fossil fuels because of its renewability and sustainability.2 As a promising green biofuels, biodiesel can be generated by transesterification of triacylglycerol by various feedstocks, including vegetable oils, animal fats, and waste oils.3 However, the edible oils compete with food production and are responsible for up to 70%-85% of the total cost of biodiesel production.4

Microbial oils are considered to be promising feedstock for biodiesel production because of their numerous benefits, including a shorter fermentation time, increased productivity, reduced labor and land requirements, and the elimination of restrictions in terms of season and climate.5 Oleaginous yeasts, which have the ability to accumulate lipids to more...
than 20% of their biomass, have received a great deal of attention due to their high lipid content and ability to utilize large quantities of feedstocks for lipid synthesis. However, the carbon sources required for lipid production, including glucose, xylose, and glycerol, are expensive and responsible for more than 60% of the total necessary expenditures. Hence, studies that facilitate the discovery of cheaper carbon sources for lipid production are essential.

Recently, many studies have focused on the use of volatile fatty acids (VFAs) as a carbon source for lipid accumulation. The VFAs can economically derive from municipal solid waste (MSW), industrial effluents, waste-active sludge, and food waste by anaerobic fermentation. Then, VFAs can be biotransformed by oleaginous yeasts into high-valued microbial lipids to reduce lipid production cost. The metabolic pathways that utilize VFAs for lipid synthesis are much shorter compared with those using sugar as a substrate because VFAs can be converted directly to acyl-CoA by acyl-CoA synthetase, which can be used to increase cell growth and lipid accumulation through a series of reactions. The dissociated and undissociated form of VFAs coexist because of the dissolution equilibrium when VFAs are used as substrates for lipid production, and most of VFAs appear in a dissociated form and could be utilized for lipid production only at neutral or weak faintly acidic pH. However, the undissociated form of the VFA molecule has an adverse effect on yeast cells. The undissociated acid molecule preferentially permeates yeast cells by passive diffusion. Once an undissociated acid molecule invades a yeast cell, it dissociates intra-cellularly and simultaneously leads to cytosolic acidification, which may impose stress on the cell metabolism and affect cell division and lipid production. Liu et al calculated the undissociated acetic acid content and found that it decreased immediately from 227 to 22.8 mg/L as the pH increased from 7 to 8 in medium with 40 g/L of acetic acid, which meant the pH decreased the undissociated acetic acid molecule content in the medium and then lessened inhibition induced by acidification of the cytoplasm. Fei et al investigated the effect of pH ranging from 2.5 to 8.5 on C. albicans and showed that the greatest lipid titer was obtained at pH 6.0 (a weak faintly acidic pH) and that obvious inhibition was observed when the pH was higher than 7.0. Therefore, pH is a key factor that affects lipid production that utilizes VFAs as the sole carbon source.

To date, several oleaginous yeast strains, such as Cryptococcus curvatus, Yarrowia lipolytica, Cryptococcus albicans, and Rhodosporidium toruloides, have been reported to produce lipids using VFAs as the sole carbon source. Chi et al investigated the pH-stat fed-batch fermentation of C. curvatus ATCC 20 509 and obtained a maximum lipid titer of 37 g/L at pH 7.0. Extensive studies have been conducted on Rhodotorula glutinis, an oleaginous yeast, that have revealed its high lipid productivity when it utilizes glucose as the carbon source; however, reports regarding lipid synthesis using VFAs are limited.

This work investigated lipid synthesis by R. glutinis when using VFAs as the substrate. pH clearly affects lipid synthesis, and the effects of pH on lipid synthesis by R. glutinis in the presence of VFAs were systematically investigated. Lipid production under different pH conditions was compared and a culture strategy that utilizing two-stage pH regulation was developed in order to increase both cell growth and lipid production. The constituents of the lipids were also analyzed and compared with those of lipids produced using sugar as a substrate. This work is beneficial for increasing the comprehensive understanding of the mechanisms underlying lipid synthesis using VFAs. This work also provided a novel strategy that can be used to enhance lipid production using VFAs.

2 | MATERIALS AND METHODS

2.1 | Microorganisms and medium

The oleaginous yeast R. glutinis (CGMCC 2.703) was purchased from Institute of Microbiology Chinese Academy of Science and maintained in our laboratory. The strain was storage at −80°C. Before fermentation, it was maintained at 4°C on the agar slants. The agar slants medium contained 20 g/L glucose, 10 g/L yeast extract (YE), 20 g/L peptone, and 20 g/L agar. The seed medium contained 15 g/L glucose, 2 g/L (NH₄)₂SO₄, 1 g/L YE, 6 g/L KH₂PO₄, 2 g/L Na₂HPO₄, and 1 g/L MgSO₄ and had an initial pH that was adjusted to 6.0. The fermentation medium contained 23 g/L acetate, 8.68 g/L yeast extract, 6 g/L KH₂PO₄, 2 g/L Na₂HPO₄, 2 g/L MgSO₄, 0.1 g/L CaCl₂, and 0.07 g/L FeCl₃, and had an initial pH that was adjusted to 7.0 unless otherwise noted. All the chemical reactants were purchased from Sinopharm Chemical Reagent Co. Ltd (SCRC) unless otherwise noted.

2.2 | Culture methods

2.2.1 | Shake flask fermentation

For the seed culture, the strain was incubated in a 500-mL Erlenmeyer flask containing 100 mL seed medium at 30°C and 180 rpm for 24 hours. During batch fermentation, the cells were cultured as described above. The inoculation rate used for fermentation was 10% (v/v).

2.2.2 | pH-stat batch culture in 1-L fermenter

The pH-stat batch culture was grown in a 1-L bioreactor (Sartorius, Germany) containing 500 mL fermentation medium at 30°C. The agitation speed and aeration were maintained at 500 rpm and 1vvm, respectively. The different pH
levels were maintained by the automatic addition of 4 mol/L hydrochloric acid solution (HCl) during fermentation.

2.2.3 Fed-batch culture in 5-L fermenter

The fed-batch culture was grown in a 5-L bioreactor (Baoxing, China). The working volume was 2 L at 30°C, and the initial acetate concentration was 23 g/L. The agitation speed and aeration were 500 rpm and 3vvm, respectively. Concentrated acetic acid (30% v/v) and yeast extract (3.4 g/L; C/N ratio was 350) were automatically added by a pH controller to maintain a constant pH. With the consumption of acetic acid, pH in the medium would increase, and then, acetic acid and yeast extract were fed into the medium. To maintain a constant pH, almost equivalent acetic acid was fed into the medium, so the concentration of acetic acid and C/N ratio in the medium fluctuated slightly. For the single-stage fed-batch culture, the pH was maintained at a constant level (pH 6.6 or pH 7.0) during the whole fermentation. During two-stage pH regulation, which was implemented as part of the fermentation strategy, the pH was maintained at 6.6 during the first 72 hours and was then automatically increased to 7.0 during 72–240 hours.

2.3 Analytical methods

2.3.1 Determination of cell growth

Cell growth was determined via measurement of the OD_{600} using a spectrophotometer (NanoDrop 2000C, Thermo Fisher). For biomass determination, 1 mL of broth was centrifuged at 10 000 g for 5 minutes, and the resulting pellet was washed twice and dried at 40°C until it reached a constant weight.

2.3.2 Determination of acetate and glucose concentrations

The acetate and glucose concentrations were quantified using a HPLC apparatus (10AVP HPLC system, Shimadzu) equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad) and a refractive index detector (RID). Five millimolar H_{2}SO_{4} was used as the mobile phase and was pumped at a rate of 0.8 mL/min. The column was maintained at 65°C.

2.3.3 Lipid analysis

The lipid analysis was conducted by a gas chromatography apparatus (GC-2010) equipped with a flame ionization detector (FID) and a Varian capillary column (CP-FFAP CB; 25 m, 0.32 mm, 0.3 μm; # CP 7485). A mixed standard (purchased from Sigma) including C8-C24 fatty acid methyl esters was used for identification. The retention time of sample is consistent with that of the standard. The method used was based on a modification of a direct transesterification protocol described by Griffiths et al^{23}.

A sample containing approximately 1 mg dry cells was harvested and centrifuged at 10 000 g for 10 minutes to obtain a pellet (room temperature). Subsequently, 100 μL of 2 mg/mL glyceryl tridecanoate (purchased from Sigma) dissolved in hexane was added to each sample as an internal standard for the purposes of transesterification efficiency correction. Meanwhile, the transesterification reaction that produced the fatty acid methyl esters (FAMEs) was started by adding 500 μL of 20 g/L sodium methoxide (2 g sodium hydroxide in 100 mL anhydrous methanol) and incubated in a vortex mixer for 60 minutes. After the reaction was completed, 40 μL of concentrated H_{2}SO_{4} was added for the purposes of pH neutralization. Five hundred milliliters of hexane was added, and the mixture was vortexed for 30 minutes for FAME extraction. After centrifugation at 6000 g for 1 minutes, the supernatant was used to obtain the lipid titer and for compositional analysis.

The injector and FID detector temperatures were set to 250°C and 260°C, respectively. A 1 μL hexane layer was injected into the GC for analysis. The column temperature was maintained at 180°C for 0.5 minutes and then raised to 250°C at a rate of 10°C/min, after which it was maintained at 250°C for 6 minutes. Nitrogen was used as the carrier gas, and the split ratio was 50. The methyl esters were identified and quantified via comparison with the standard curve. All experiments were performed in triplicate, and the mean values were presented as the results of the data analysis.

3 RESULTS AND DISCUSSION

3.1 Effect of the initial acetate concentration on cell growth and lipid accumulation in R. glutinis

The initial acetate concentration affected cell growth and lipid accumulation in R. glutinis. However, the acetic acid cannot be directly utilized by the yeast because of the low pH. Therefore, sodium acetate which would be dissociated to acetic acid in the broth companied with the neutral pH was employed as the initial carbon source. The initial concentrations of acetate (acetate ions) that were used were 20, 23, 26, and 31 g/L, and 50 g/L glucose was used as the control. The pH was maintained at 7.0, and the results are shown in Figure 1.

As shown in Figure 1, when acetate was used as the substrate, the cell growth rate gradually decreased as the acetate concentration increased from 20 to 26 g/L; the cells did not grow at all when the acetate concentration was 31 g/L, which demonstrated the total inhibition of cell growth that was induced at high concentrations of acetate. Rodrigues$^{24}$...
found that the inhibition of organic acids on cell growth was due to chemical interference with the membrane transport of phosphate. Then, more energy (ATP) would be expended for cell growth; hence, lipid synthesis would be inhibited simultaneously. In addition, the highest lipid production rate was obtained when 23 g/L acetate was utilized, and the lipid production rate sharply decreased when the acetate concentration was more than 26 g/L. Although the greatest biomass (6.8 g/L) was obtained using 20 g/L acetate, the highest lipid titer (1.22 g/L) and lipid content (26.5%) were achieved using 23 g/L acetate. Compared with the control, the biomass yield was obviously decreased when acetate was used as a substrate; however, the lipid content and lipid yield were greater than that obtained using glucose (18.3% and 6.5%) when 23 g/L acetate was utilized (26.5% and 12.1%). The results indicated that acetate can be efficiently utilized to produce lipid accumulation in *R. glutinis*.

The tolerance of oleaginous yeasts, including *C. curvatus* MUCL 29819,9 *C. albidus* ATCC 10672,10 and *Y. lipolytica* MUCL 28849,11 for VFAs was usually below 5 g/L, except for in the case of *C. curvatus* ATCC 20509,12,13 which could tolerate 30 g/L acetate. Therefore, the *R. glutinis* CGMCC 2.703 strain used in this work was demonstrated to have a high tolerance for acetate25 and to be capable of accumulating lipid efficiently in the presence of acetate.

### 3.2 Effect of the C/N ratio on cell growth and lipid accumulation in *R. glutinis*

In general, there are two pathways that can lead to lipid accumulation: de novo and ex novo.26 Ex novo lipid production utilizes hydrophobic substrates, while the de novo process utilizes hydrophilic substrates, such as acetic acid and other VFAs. The C/N ratio is a significant factor that affects lipid content when glucose is used by oleaginous yeasts as a substrate to synthesize lipids. A high C/N ratio is often required for lipid accumulation via the de novo pathway.27 However, an extremely high C/N ratio would result in the depletion of nitrogen sources, which would slow cell growth and result in correspondingly poor lipid production.28 Therefore, the effect of the initial C/N ratio on lipid accumulation when acetate was utilized as a carbon source by *R. glutinis* was investigated in this work.

In our previous work, the effect of inorganic nitrogen (nitrate of potash and ammonium sulfate) and organic nitrogen (yeast extract) has been investigated. The results showed that yeast extract was a better nitrogen source for cell growth and lipid production. Therefore, the initial acetate concentration was 23 g/L, and different C/N ratios (10-500) were produced by increasing the yeast extract concentration from 0.18 to 9.14 g/L. The results are shown in Figure 2. In Figure 2, we can see that, when the C/N ratio was increased from 10 to
110, the biomass yield decreased from 7.7 to 6.0 g/L, while the lipid titer and lipid content continued to increase from 0.39 to 1.16 g/L and 5.1% to 19.7%, respectively, which may because the increase of C/N ratio changed the intracellular carbon flow and led to an accumulation of citric acid (Figure 3), that might promote lipid production and slightly decreased the cell growth.29 Nevertheless, the lipid titer and lipid content were still maintained at high levels and the biomass yield was stable at high C/N ratios that ranged from 220 to 500, and this was possibly due to the production of citric acid at proper C/N ratios. The highest lipid titer and lipid content (1.98 g/L and 25.4%, respectively) were obtained at a C/N ratio of 350; the lipid titer was decreased when the C/N ratio was higher than 350. Béligon et al also demonstrated that a higher C/N ratio (>300) was beneficial for lipid production.19 Therefore, the optimal C/N ratio was determined to be 350.

An appropriate increase in the C/N ratio is advantageous in enhancing lipid synthesis by increasing the production of citric acid due to a decrease in the activity of the Krebs cycle (Figure 3). Then, the citric acid can be utilized for lipid synthesis. However, if the C/N ratio is further increased, the excess citric acid which cannot be totally dissociated by ATP-citrate lyase (ACL) would be secreted and inhibits cell growth, and this process results in a rapidly decrease in lipid yield.11,30 In this work, no citric acid was observed in the broth. However, the pH increased due to the consumption of acetate during fermentation and was able to greatly influence the activity of acetyl-CoA synthetase (ACS), which is one of the key enzymes in the lipid metabolic pathway. Therefore, the pH has been demonstrated to be an important factor contributing to VFAs metabolism by R. glutinis.

### 3.3 Effect of pH on cell growth and lipid accumulation in R. glutinis

Different with using glucose as a substrate which often employs an acidic pH for lipid production, a neutral or slightly acidic pH is employed for lipid accumulation with VFAs as substrates because of the existence of dissolution equilibrium. pH has been shown to be a critical factor affecting lipid accumulation when VFAs are used as a carbon source.12,14,17,18

The effect of pH on cell growth and lipid accumulation in R. glutinis was investigated. Lipid production by R. glutinis in 1-L bioreactors in the presence of an initial acetate concentration of 23 g/L was measured at different pH levels ranging from 6.0 to 7.4. The results are shown in Figure 4.

In these pH conditions, no obvious inhibition during fermentation could be observed. However, cell growth and lipid production were quite different at various pH levels. The highest biomass of 5.9 g/L was obtained at pH 6.6 and was 1.37-fold higher than the lowest biomass, which was obtained at pH 7.4. The maximum lipid titer of 1.22 g/L and lipid content of 26.5% were obtained at pH 7.0 and were 3.08-fold and 2.92-fold greater, respectively, than those obtained at pH 7.4. An alkaline pH was not suitable for lipid accumulation.10,12,18

In general, an acidic pH (5.0-6.0) is beneficial for lipid accumulation when glucose is utilized as a carbon source by oleaginous yeasts. However, the pKa of acetic acid is 4.75, which would result in the presence of a large amount of undissociated molecular acetic acid under acidic conditions. In this study, the amount of undissociated acetic acid was 1243, 325, and 131 mg/L when the pH was 6.0, 6.6, and 7.0, respectively. As the pH increased, the amount of undissociated acetic acid decreased, which reduced the intracellular dissociation of undissociated acetic acid. Therefore, the inhibition induced by cytosolic acidification would be reduced.19

![Figure 3](image)

**Figure 3** Effect of citric acid on cell growth and lipid production in R. glutinis. Abbreviations: ACL, ATP-citrate lyase; TAG, triacylglycerol

![Figure 4](image)

**Figure 4** Effect of pH on cell growth and lipid accumulation in R. glutinis
3.4 Batch fermentation of *R. glutinis* in a 5-L bioreactor using acetate as the sole carbon source

As demonstrated by the results given above, *R. glutinis* exhibited a high tolerance to acetate. Subsequently, batch fermentation of lipids in a 5-L bioreactor was performed. The initial acetate concentration and C/N ratio were 23 g/L and 350, respectively. As shown in Figure 5, a biomass yield, lipid titer, and lipid content of 4.1 g/L, 1.78 g/L, and 43.4% were obtained after 42 hours of fermentation, respectively.

The cells grew rapidly after the 8-hours lag phase and lipid accumulated simultaneously. The lag phase of *R. glutinis* was observed to be shorter than that of *R. toruloides* AS 2.1389 after a 3-day two-stage batch cultivation using an initial acetate concentration of 20 g/L.29 The acetate was consumed rapidly after 8 hours and was eventually completely consumed. The lipid productivity of *R. glutinis* was 1.018 g L⁻¹ d⁻¹, which was much higher than that of *R. toruloides* AS 2.1389.29 Christophe et al used a two-stage batch culture strategy, in which glucose was used as a substrate during the first stage and acetate was utilized during the second stage, to obtain a lipid productivity of 0.505 g L⁻¹ d⁻¹.9

3.5 Fed-batch culture of *R. glutinis* using acetate as a substrate in pH-stat conditions

Fed-batch fermentation was carried out in a 5-L fermenter to increase cell growth and lipid production using an initial sodium acetate concentration of 30 g/L (23 g/L of ionic acetate). During fermentation, 30% acetic acid was fed in order to maintain a constant pH in the medium. Since a pH of 6.6 was found to be optimal for cell growth and a pH of 7.0 was more suitable for lipid accumulation, fermentation at pH 7.0 and pH 6.6 was compared. The results are shown in Figure 6. At pH 6.6, the highest biomass yield and lipid titer of 67.2 and 26.3 g/L, respectively, were obtained at 192 hours. Compared with that of batch fermentation, the biomass yield and lipid titer were increased 15.4-fold and 13.8-fold, respectively. At pH 7.0, the highest biomass yield and lipid titer were also obtained at 192 hours and reached 59.7 and 29.3 g/L, respectively, which were 13.6-fold and 15.5-fold higher, respectively, than that obtained from the batch culture. As shown in Figure 6, a biomass yield of 46.3 g/L was obtained during the first 72 hours at pH 6.6, while 33.2 g/L was obtained at pH 7.0, which indicated that a pH of 6.6 was more suitable for cell growth than a pH of 7.0 for fed-batch culture. However, for the purpose of lipid production, pH 7.0 was better than pH 6.6.

3.6 Fed-batch culture of *R. glutinis* using a two-stage pH regulation strategy for lipid production

Since pH 6.6 was optimal for cell growth and pH 7.0 was suitable for lipid accumulation, a culture strategy that

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**FIGURE 5** Batch fermentation of *R. glutinis* in a 5-L fermenter using acetate as a substrate

**FIGURE 6** Fed-batch fermentation of *R. glutinis* in pH-stat conditions. A, Fermentation at pH 7.0. B, Fermentation at pH 6.6
utilizing two-stage pH regulation was developed to enhance both cell growth and lipid synthesis. During the first stage (0-72 hours), the pH was maintained at 6.6 to increase the biomass, and during the second stage (72-240 hours), the pH was increased to 7.0 to enhance lipid production.

As shown in Figure 7, during the first 72 hours, the cells grew rapidly until the biomass reached 41.2 g/L, which corresponds to a high cell growth rate of 0.572 g L⁻¹ h⁻¹; however, the lipid titer reached only 4.5 g/L, which corresponds to a low lipid productivity of 0.062 g L⁻¹ h⁻¹. During the second stage, the cell growth rate decreased while the lipid content increased from 9.8% to 52.2%. The final lipid titer reached 35.8 g/L at 192 hours, while the biomass yield, lipid titer, and lipid content began to decrease after 192 hours. Compared with the pH-stat fermentations conducted at pH 6.6 and pH 7.0, the lipid titer increased by 36.1% and 22.2% and the lipid content increased by 34.3% and 6.7%, respectively, when the two-stage pH regulation strategy was employed (Table 1).

The biomass yield was also increased (14.2%) compared with that of the pH-stat (pH 7.0) culture and reached 68.2 g/L. Therefore, the two-stage pH regulation strategy was used to first obtain a high cell density, and then to enhance the rate of lipid accumulation (Table 1).

Compared with batch fermentation conducted in a 5-L fermenter, the biomass yield, lipid titer, lipid yield, and lipid productivity were increased by 15.6-fold, 19.1-fold, 16.9%, and 3.39-fold, respectively. These results demonstrated that the two-stage pH regulation strategy was successful in increasing both cell growth and lipid synthesis when using acetate as a substrate (Table 1).

The comparison of our results and those obtained using other strains that also utilize VFAs as a substrate is shown in Table 2. Christophe et al reported that, during a pH-stat fed-batch fermentation of C. curvatus MUCL 29819 that utilized acetate as a substrate, a relatively poor lipid titer of 6.87 g/L was obtained. Kolouchová et al investigated R. glutinis CCY 20-2-20 and was able to obtain a lipid titer and lipid content of 0.90 g/L and 28.4%, respectively, using a two-stage culture strategy. The results indicated that the R. glutinis CGMCC 2.703 strain that was used in this study exhibited better performance when using acetate as a substrate for lipid production. When using a two-stage pH regulation strategy for a fed-batch culture, both the biomass yield and lipid production were clearly enhanced. The results indicated the R. glutinis CGMCC 2.703 has a better performance in lipid production with a two-stage pH regulation strategy when VFAs as the substrate.

3.7 Analysis of lipid composition

The lipid composition was analyzed using GC. As shown in Table 3, the main fatty acids that were identified different pH values were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). As the pH increased, the amount of linoleic acid (C18:2) decreased, which might due to its being transformed into linolenic acid (C18:3). At pH 7.4, the amount of linolenic (C18:3) was fairly high, while at an acidic or neutral pH, it was decreased. The total amount of unsaturated fatty acids reached a high level at all of the different pH values. Compared with glucose-derived lipids, the amounts of C16:0 and C18:1 fatty acids were decreased; C18:3 fatty acids were present when acetate was utilized for lipid production. The amount of unsaturated fatty acids in both the

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**FIGURE 7** Time course of cell growth and lipid production by *R. glutinis* when cultured using a two-stage pH regulation strategy in a 5-L fermenter

**TABLE 1** Lipid production by *R. glutinis* in 5-L fermenter resulting from different culture strategies

| Cultivation strategy                      | Biomass yield (g/L) | Lipid titer (g/L) | Lipid content (%)<sup>a</sup> | Lipid yield (%)<sup>b</sup> | Lipid productivity (g L⁻¹ h⁻¹) |
|-------------------------------------------|---------------------|-------------------|-----------------------------|--------------------------|-------------------------------|
| Batch culture (pH 7.0)                    | 4.1 ± 0.2           | 1.78 ± 0.10       | 43.4 ± 2.3                  | 7.7 ± 0.4                | 0.0424 ± 0.0056               |
| pH-stat fed-batch culture (pH 6.6)        | 67.2 ± 1.1          | 26.3 ± 0.5        | 39.1 ± 0.6                  | 7.6 ± 0.4                | 0.137 ± 0.036                 |
| pH-stat fed-batch culture (pH 7.0)        | 59.7 ± 0.9          | 29.3 ± 0.4        | 49.1 ± 0.7                  | 8.9 ± 0.4                | 0.153 ± 0.058                 |
| Two-stage pH regulation fed-batch culture | 68.2 ± 0.8          | 35.8 ± 0.5        | 52.5 ± 0.6                  | 10.2 ± 0.5               | 0.186 ± 0.046                 |

<sup>a</sup>Lipid content: g lipid/ g biomass.

<sup>b</sup>Lipid yield: g lipid/ g acetic acid.
acetate-derived and glucose-derived lipids was similar to that found in palm oil. In general, lipids containing greater amounts of unsaturated fatty acids exhibit increased quality when used as fuels at low temperatures. The results indicated that lipids produced by R. glutinis show promise for use as feedstocks for biodiesel production.

### TABLE 2 Lipid production by different strains

| Yeast strain     | Culture strategy                      | Carbon source | Lipid titer (g/L) | Lipid content (%) | Lipid productivity (g L⁻¹ h⁻¹) | Reference |
|------------------|--------------------------------------|---------------|-------------------|-------------------|---------------------------------|-----------|
| C. curvatus MUCL 29 819 | pH-stat two-stage fed-batch (fermenter) | Glucose + Acetate | 6.87              | 50.9              | 0.137                           | 11        |
| C. curvatus ATCC 20 509  | pH-stat fed-batch (fermenter)          | Acetate       | 37.0              | 53.8              | 0.510                           | 14        |
| C. albidus ATCC 10 672  | pH-stat two-stage fed-batch (fermenter) | Glucose + VFAs | 14.55             | 55.1              | 0.076                           | 6         |
| Y. lipolytica MUCL 28 849 | pH-stat two-stage fed-batch             | Glucose + VFAs | 16.50             | 40.22             |                                 | 13        |
| R. toruloides AS 2.1389  | Sequencing batch (flask)               | Acetate       | 1.62              | 38.6              | 0.024                           | 25        |
| R. glutinis CCY 20-2-20 | Batch (flask)                          | Acetate       | 0.55              | 19.5              | —                               | 26        |
|                    |                                      | Glucose + Acetate | 0.90             | 28.4              | —                               | 26        |
| R. glutinis CGMCC 2.703 | Batch (fermenter)                      | Acetate       | 1.78              | 43.4              | 0.042                           | This study|
|                    |                                      | Two-stage pH regulation fed-batch (fermenter) | Acetate | 35.8 | 52.5 | 0.186 | This study |

### TABLE 3 Lipid composition analysis

| Carbon source | pH   | Palmitic acid (C16:0) | Stearic acid (C18:0) | Oleic acid (C18:1) | Linoleic acid (C18:2) | Linolenic acid (C18:3) |
|---------------|------|-----------------------|----------------------|--------------------|-----------------------|------------------------|
| Acetate       | 6.0  | 15.7 ± 0.6            | 19.3 ± 0.4           | 40.0 ± 0.9         | 25.0 ± 0.3            |                        |
|               | 6.6  | 17.8 ± 0.4            | 14.3 ± 0.5           | 22.6 ± 0.3         | 19.9 ± 0.8            | 25.4 ± 0.7             |
|               | 7.0  | 18.2 ± 0.2            | 17.1 ± 0.7           | 49.4 ± 1.3         | 10.7 ± 0.6            |                        |
|               | 7.4  | 12.1 ± 0.2            | 21.6 ± 0.3           | 20.0 ± 0.1         | 46.3 ± 0.8            |                        |
| Glucose       | 6.0  | 26.6 ± 0.7            | 12.3 ± 0.6           | 54.6 ± 1.6         | 6.4 ± 1.1             |                        |
| Palm oil^27   | 6.0  | 20.4                  | 10.3                 | 47.9               | 7.3                   | 0.9                    |

### 4 CONCLUSIONS

This work demonstrated that R. glutinis has a high tolerance to acetate and that pH played a significant role in productivity when VFAs were used as a substrate. In the 1-L bioreactor containing an initial acetate concentration of 23 g/L, the most suitable pH for cell growth was 6.6, while pH 7.0 was optimal for lipid production. When using the two-stage pH regulation culture strategy, the biomass yield, lipid titer, and lipid content reached 68.2 g/L, 35.8 g/L, and 52.5%, respectively. Compared with the pH-stat fed-batch culture, both cell growth and lipid synthesis were clearly enhanced. The analysis of the fatty acids indicated that lipids produced by R. glutinis in the presence of acetate showed promise for use as a biodiesel feedstock.

### ACKNOWLEDGMENTS

This work was supported by National Nature Science Foundation of China (No. 21676159), the project of NSFC-NRCT (No. 51861145103), and the Subject Construction Foundation of Institute of Nuclear and New Energy Technology, Tsinghua University (No. 2017HYYXKJS)

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How to cite this article: Zhang W, Wu J, Zhou Y-J, Liu H-J, Zhang J-A. Enhanced lipid production by Rhodotorula glutinis CGMCC 2.703 using a two-stage pH regulation strategy with acetate as the substrate. Energy Sci Eng. 2019;7:2077–2085. https://doi.org/10.1002/ese3.413