

## Effects of Acetic Acid and pH on the Growth and Lipid Accumulation of the Oleaginous Yeast *Trichosporon fermentans*

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Acetic acid, one major inhibitor released during the hydrolysis of lignocellulosic biomass, can be utilized by the oleaginous yeast *Trichosporon fermentans* without glucose repression. The effect of acetic acid on the cell growth and lipid accumulation of *T. fermentans* under controlled pH conditions was investigated in a 5-L fermentor. Undissociated acetic acid with concentrations of 0.026, 0.052, and 0.096 g L<sup>-1</sup> in media contributed to approximately 12-, 24-, and 48-h lag phases, respectively, indicating that undissociated acetic acid is the inhibitory molecular form. The inhibition of cell growth was correlated with undissociated acetic acid concentration. However, acetic acid had little influence on the lipid accumulation of *T. fermentans* at different pH conditions. The specific glucose consumption rate decreased with increasing acetic acid concentration, but the impact of acetic acid on the specific xylose consumption rate was not pronounced. In addition, the variation of pH and acetic acid concentration had no significant influence on the fatty acid composition of the lipids. Acetic acid showed more severe inhibition under low pH conditions. The reduction of intracellular pH partly explains this inhibitory effect.

**Keywords:** Lignocellulosic biomass; Acetic acid; Lipid production; Intracellular pH; *Trichosporon fermentans*

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### INTRODUCTION

Biodiesel, as a renewable and green alternative to traditional fossil fuels, has attracted increasing attention in recent years. The most commonly used feedstocks in biodiesel production are waste oils and vegetable oils. However, widespread use of vegetable oils may contribute to a reduction of food supply, and the limited amount of waste oils cannot meet the increasing demand for biodiesel production (Adamczak *et al.* 2009). Microbial oils, namely single-cell oils (SCOs), produced by oleaginous microorganisms, have long been considered as substitutes for value-added oils with rare fatty acid composition or structure, such as cocoa-butter and those containing polyunsaturated fatty acids (Ratledge 1993; Papanikolaou and Aggelis 2010, 2011). However, in recent years attention has focused on adoption of SCOs as feedstocks for biodiesel production because of their similarity to vegetable oils in fatty acid composition (Li *et al.* 2008). Presently, although the high cost of the fermentation process (*e.g.*

aeration and agitation) makes SCOs less competitive compared with vegetable oils (Koutinas *et al.* 2014), their beneficial impacts in the production process, such as non-use of arable land, high productivity, and easy scaling-up, makes them a promising candidate for future utilization. Moreover, the use of renewable and low-cost lignocellulosic biomass for SCOs production seems to be a sustainable development strategy (Huang *et al.* 2009).

Up to now, the bioconversion of lignocellulosic sugars (mostly xylose, xylose/glucose blends, or lignocellulosic hydrolysates) into SCOs has been extensively studied with various oleaginous microorganisms (Fakas *et al.* 2009; Economou *et al.* 2011; Zikou *et al.* 2012 Ruan *et al.* 2012, 2015). However, the use of lignocellulosic biomass as feedstock for SCO production still presents many technical hurdles. One challenge is that oleaginous microorganisms should be capable of utilizing mixed sugars and tolerant to inhibitors (Hahn-Hägerdal *et al.* 2007) because lignocellulosic hydrolyzates contain a variety of sugars (mostly glucose and xylose) and inhibitory compounds.

Acetic acid is one of the major inhibitors released during the solubilization and hydrolysis of hemicellulose (Palmqvist and Hahn-Hägerdal 2000). Generally, the inhibitory effect of acetic acid is attributed to uncoupling (Baronofsky *et al.* 1984; Herrero *et al.* 1985; Luli and Strohl 1990) and intracellular anion accumulation (Russell 1991, 1992). The undissociated form of acetic acid (HAc), which is liposoluble, can diffuse across the plasma membrane and dissociate into the anion ( $\text{Ac}^-$ ) and a proton ( $\text{H}^+$ ) in the near-neutral cytosol. The drop in intracellular pH ( $\text{pH}_i$ ) caused by the dissociation can be neutralized by the action of the plasma membrane ATPase at the expense of ATP hydrolysis (Verduyn *et al.* 1992). At high concentrations of acetic acid, the proton pumping capacity of the cell is exhausted, resulting in depletion of the ATP content and acidification of the cytoplasm (Imai and Ohno 1995). In addition to a perturbation of  $\text{pH}_i$  homeostasis, intracellular accumulation of high levels of the acetate anion may give rise to organic acid toxicity (Pampulha *et al.* 1989).

In a previous study, this group reported that the oleaginous yeast *Trichosporon fermentans* can use mixed sugars in lignocellulosic hydrolyzates for lipid production. However, *T. fermentans* gave a poor lipid yield on non-detoxified dilute sulfuric acid-treated rice straw hydrolyzate containing a concentration of acetic acid ranging from 1 to 3 g L<sup>-1</sup> (Huang *et al.* 2009).

Although there have been many works investigating the inhibitory effect of acetic acid on ethanologenic yeasts under anaerobic conditions (Pampulha *et al.* 1989; Bellissimi *et al.* 2009; Casey *et al.* 2010), so far only a few reports have referred to the effect of acetic acid on oleaginous microorganisms under aerobic conditions (Chen *et al.* 2009; Hu *et al.* 2009; Huang *et al.* 2012). Therefore, little is known about this acid's inhibitory mechanism.

Acetic acid can be also used to synthesize acetyl-CoA (Vorapreeda *et al.* 2012), which is a two-carbon unit for the synthesis of fatty acid. Hence, in this study, the impacts of acetic acid on the cell growth and lipid accumulation of *T. fermentans* under controlled pH conditions were examined. To further understand the inhibitory mechanism of acetic acid, the influence of acetic acid on the  $\text{pH}_i$  of *T. fermentans* was investigated using the fluorescence probe 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM).

## EXPERIMENTAL

### Yeast Strain and Materials

The oleaginous yeast *Trichosporon fermentans* CICC 1368 was obtained from the China Center of Industrial Culture Collection and kept on wort agar (130 g L<sup>-1</sup> malt extract, 15 g L<sup>-1</sup> agar, and 0.1 g L<sup>-1</sup> chloramphenicol) at 4 °C. The fluorescent probe BCECF-AM was purchased from Beyotime Biotech. (China). Ionophore nigericin, which was used in the *in situ* calibration of intracellular pH, was purchased from Enzo Life Sciences Inc. (Switzerland). The 0.22 µm pore size polyether sulfone (PES) filter used for medium filtration sterilization was purchased from Ameritech Scientific Corp. (USA). Other materials and chemicals were purchased from commercial sources at the highest purity available.

### Cultivation and Media

#### Media

The precultivation medium (*i.e.* YEPD medium) was as follows (g L<sup>-1</sup>): glucose 10, xylose 10, peptone 10, and yeast extract 10. The fermentation medium contained (g L<sup>-1</sup>) glucose 20, xylose 20, yeast extract 0.2, peptone 0.7, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.4, KH<sub>2</sub>PO<sub>4</sub> 2.0, MnSO<sub>4</sub>·H<sub>2</sub>O 0.003, and CuSO<sub>4</sub>·5H<sub>2</sub>O 0.0001. To investigate the metabolism of acetic acid as sole carbon source by *T. fermentans*, 1.5 to 30 g L<sup>-1</sup> acetic acid were supplemented into the fermentation media instead of glucose and xylose before adjusting the pH to 6.5 by a pH meter (Sartorius, Germany) with 1 M NaOH or 1 M HCl. To avoid a pH change, the medium containing acetic acid was sterilized by filtration through a 0.22 µm pore size PES filter.

#### Flask cultivation

After 24 h of precultivation at 28 °C and 160 rpm in a rotary shaker, 2.5 mL of seed culture was used to inoculate 50 mL fermentation medium in a 250-mL conical flask. Then, cultivation was performed at 25 °C and 160 rpm (Zhu *et al.* 2008).

#### Batch fermentation in 5-L fermentor

The inoculum for the fermentor was prepared by precultivation of *T. fermentans* in a rotary shaker set at 28 °C and 160 rpm in 500-mL flasks containing 100 mL of precultivation medium. Once the cell density of seed culture reached approximately 4.5 g dry cells L<sup>-1</sup>, the culture was used to inoculate the fermentor to an initial cell density of OD<sub>600</sub> = 0.4 to 0.6. Batch fermentation was carried out at 25 °C in a 5-L laboratory fermentor (Biostat A Plus Sartorius, Germany) with a working volume of 1.5 L. The concentrations of acetic acid examined were 0 g L<sup>-1</sup>, 1.5 g L<sup>-1</sup> (25 mM), and 3 g L<sup>-1</sup> (50 mM). The pH was adjusted to pH 6.5 or 4.5 before inoculation by automatic addition of 1 M NaOH or HCl and kept constant during the fermentation process. Dissolved O<sub>2</sub> and pH were monitored with an autoclavable O<sub>2</sub> electrode and pH electrode (Hamilton, Switzerland). All fermentations were performed at least in duplicate.

### Determination of Biomass, Lipid Content, and Fatty Acid Composition of Lipid

Biomass, lipid content, and fatty acid composition of lipids were determined as described in this group's previous work (Zhu *et al.* 2008). Biomass was harvested by centrifugation, and the weight was measured in lyophilized form. Extraction of lipids was

carried out according to the modified method of Dyer and Bligh (1959). After extraction of the oil with a mixture of methanol and chloroform (1/2, v/v) for 60 min, the mixture was separated by centrifugation and the chloroform phase was collected and evaporated by a vacuum rotary evaporator (EYELA, Japan) at 100 rpm and 50 °C. The fatty acid profile of lipid was determined by gas chromatography. Lipids were converted to fatty acid methyl esters (FAMES) according to the method of Morrison and Smith (1964). Then, the FAMES were determined by gas chromatography (GC-2010) with an ionization detector and a DB-1 capillary column (0.25 cm × 30 m, Agilent Technologies Inc., USA). The column temperature was increased from 170 to 220 °C at a rate of 3 °C min<sup>-1</sup> and kept at 220 °C for 3 min. Nitrogen was used as the carrier gas at 0.80 mL min<sup>-1</sup>. The split ratio was 1:50 (v/v). The injector and the detector temperatures were set at 250 and 280 °C, respectively (Zhu *et al.* 2008).

### Metabolite Analysis

Cell optical density was recorded at 600 nm with a spectrophotometer (Shimadzu UV-2550, Japan). The supernatant obtained following centrifugation of culture sample was analyzed for glucose, xylose, xylitol, and acetic acid *via* HPLC with a Waters 2410 refractive-index detector and an Aminex HPX-87H column (Bio Rad Corp., USA) at 50 °C. The mobile phase was 0.005 M H<sub>2</sub>SO<sub>4</sub> at 0.5 mL min<sup>-1</sup> (Huang *et al.* 2009).

### Determination of Intracellular pH Change

#### *Cell loading*

Cells grown in YEPD were diluted to an OD<sub>600</sub> of approximately 0.8, then centrifuged at 1500 g for 5 min and resuspended in an equal volume of 100 mM citric/phosphate buffer (potassium dihydrogen orthophosphate and citric acid) at pH 4.5. BCECF-AM is a fluorescent substance which displays pH-dependent excitation, thus allowing the implementation of p*H*<sub>i</sub> measurement. Here, it was added at a final concentration of 5 μM, and then in the loading process yeast cells were incubated at 28 °C for 1 h.

#### *Calibration curve and determination of intracellular pH change*

Loaded cells were diluted with citric/phosphate buffer at pH values between 6.0 and 8.0 (increments of 0.5 pH units) to OD<sub>600</sub> of approximately 0.4. To equilibrate the p*H*<sub>i</sub> (intracellular pH) with the controlled extracellular media, H<sup>+</sup>/K<sup>+</sup> ionophore nigericin was added at a concentration of 30 μM in the presence of 100 mM potassium in citric/phosphate buffer, and incubated for 60 min.

Fluorescence measurements were acquired on a SpectraMax M5 (Molecular Devices, USA) fluorescence spectrophotometer using 96-well black microplates (Corning, USA) with a working volume of 200 μL. A calibration curve was established for BCECF-AM by plotting the ratio of fluorescence intensities (emission wavelength 530 nm) at the pH-dependent excitation wavelengths of 490 and 440 nm as a function of p*H*<sub>i</sub>. It was essential to determine the levels of fluorescence in the culture supernatant separately to remove the background fluorescence interference. Thus, for each sample, total fluorescence was examined, followed by a fluorescence determination of the supernatant only (cells were removed by filtering through 0.22-μm filters).

To examine the effect of acetic acid on the p*H*<sub>i</sub> of *T. fermentans*, acetic acid was added to the culture at a buffered pH of 4.5 and an OD<sub>600</sub> of 0.4 with a final concentration of acetic acid of 50 mM (3.0 g L<sup>-1</sup>). The control was made using the same procedure

without addition of acetic acid. Fluorescence determinations were made as described previously. The pH<sub>i</sub> change was calculated based on the ratio of fluorescence intensities and the calibration curve.

To confirm the distribution and the level of the loaded BCECF-AM within the cytosol of the yeast cells, loaded cells were examined with a confocal laser scanning microscope (CLSM). The cells were visualized using a Leica TCS SP5 CLSM (Leica, Germany) equipped with a 100-mW argon laser and objective magnification of 100× (Leica 100× oil). Split screen images were obtained using the dual-channel collection mode. One channel was an illumination phase-contrast image, and the other channel was an epi-fluorescent image of intracellular BCECF-AM (Bracey *et al.* 1998).

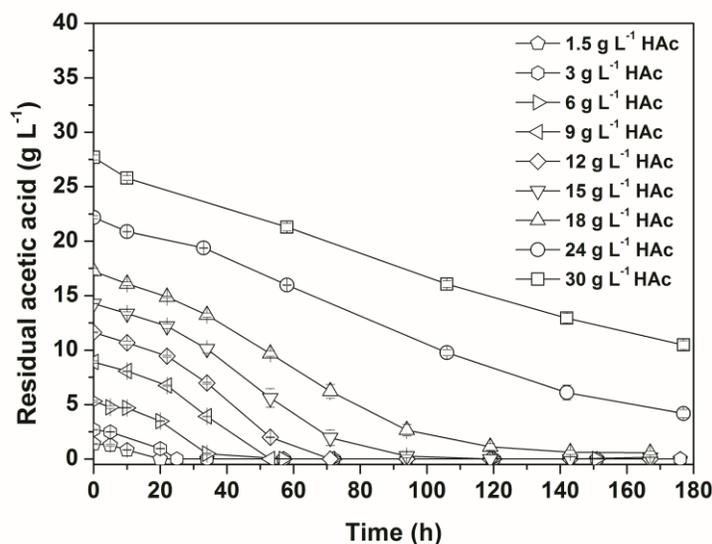
### Statistical Analysis

All the experiments were performed in duplicate, and their average values with standard deviations were used for statistical analysis. SPSS software (Version 17.0, Chicago, USA) were used to analyze the resulting data. One-way analysis of variance (ANOVA) and Turkey's Honestly Significant Differences (HSD) Test were used to determine the significant differences of data at a 95% confidence interval.

## RESULTS AND DISCUSSION

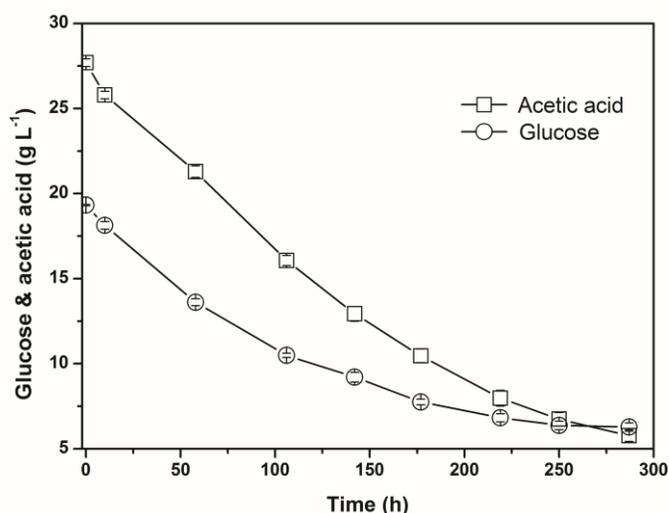
### Metabolism of Acetic Acid by *T. fermentans*

To understand the role of acetic acid in the fermentation by oleaginous yeast *T. fermentans*, acetic acid was used as the sole carbon source for a culture of *T. fermentans*. As shown in Fig. 1, in the concentration range of 1.5 g L<sup>-1</sup> to 30 g L<sup>-1</sup>, acetic acid could be assimilated by *T. fermentans*. When 20 g L<sup>-1</sup> acetic acid was used as the sole carbon source, the biomass and lipid content were 5.82 g L<sup>-1</sup> and 34.1%, respectively, after shake-flask cultivation for 60 h at an initial pH of 6.5.



**Fig. 1.** Utilization of various concentrations of acetic acid by *T. fermentans* in shake-flask cultivation. Cultures were incubated at initial pH 6.5, 25 °C, and 160 rpm. Data are the mean and standard error of two duplicate fermentations.

Interestingly, the initial utilization rate of acetic acid did not correlate with the concentration, remaining at approximately  $0.1 \text{ g L}^{-1} \text{ h}^{-1}$  within the concentration range tested. This result indicates that acetic acid can be metabolized by *T. fermentans* without substrate inhibition. It was also reported that *Cryptococcus curvatus* and *Yarrowia lipolytica* could use acetic acid as substrate (or co-substrate) for efficient SCO production (Christophe *et al.* 2012; Fontanille *et al.* 2012). As shown in Fig. 2, *T. fermentans* can metabolize acetic acid and glucose simultaneously at a pH of 6.5, suggesting that there is no glucose repression and that the enzymes responsible for acetic acid metabolism are non-inducible. This behavior is similar to *Zygosaccharomyces bailii* in the acetic acid metabolic pathway (Sousa *et al.* 1998).

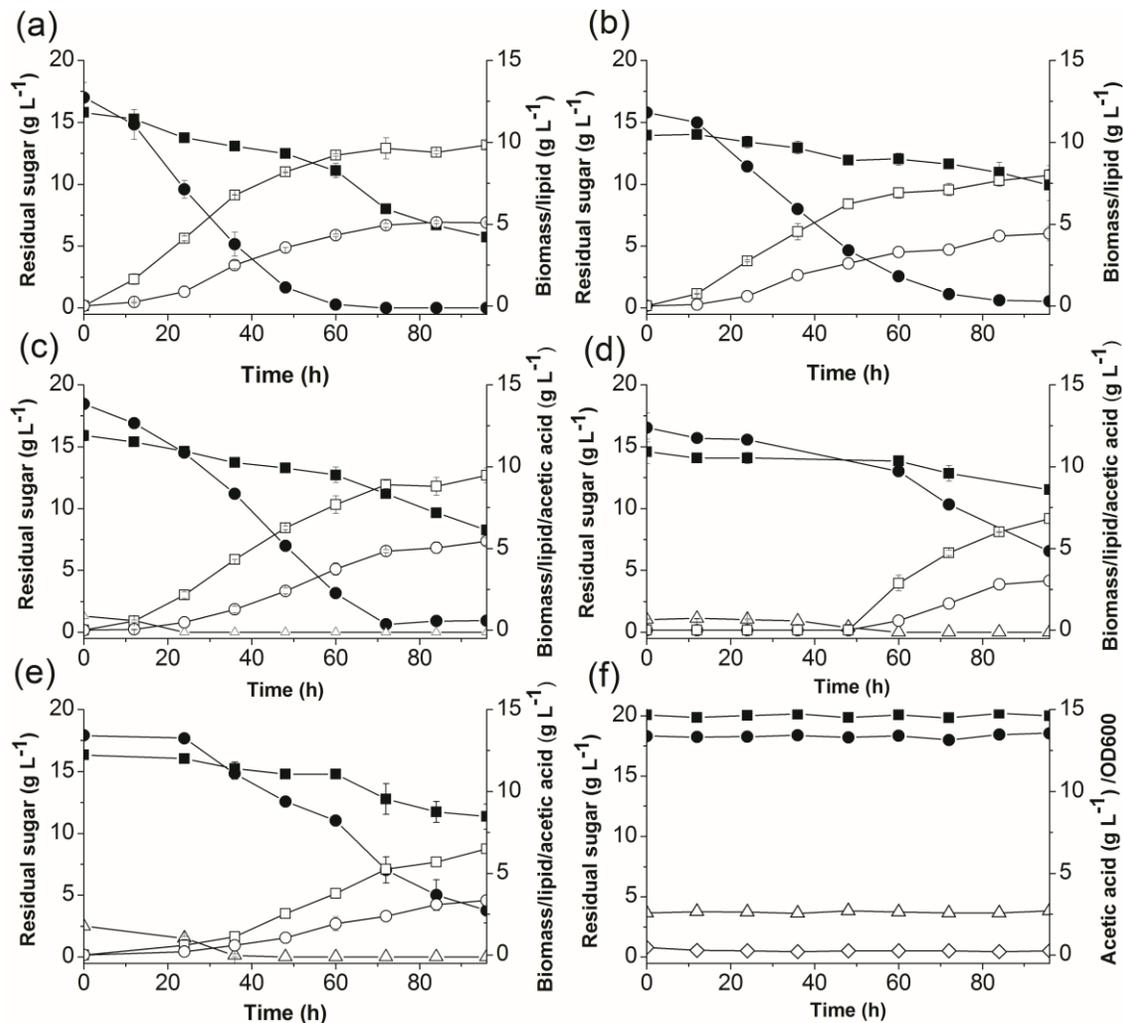


**Fig. 2.** Time course profile for the fermentation of glucose and acetic acid by *T. fermentans* in shake-flask cultivation. Cultures were incubated at initial pH 6.5, 25 °C, and 160 rpm. Data are the mean and standard error of two duplicate fermentations.

### Effect of Acetic Acid and pH on Cell Growth and Lipid Accumulation

Acetic acid showed more serious inhibition on ethanologenic yeast under low pH conditions (Pampulha *et al.* 1989), indicating that the inhibition of acetic acid is correlated with culture pH. To study the combined effects of acetic acid and pH on the cell growth and lipid accumulation of *T. fermentans*, three concentrations of acetic acid (0, 1.5, and 3  $\text{g L}^{-1}$ ) and two levels of pH (6.5 and 4.5) were tested for their influence on biomass, sugar utilization rate, and lipid yield. The time course profiles for the fermentation are shown in Fig. 3, and corresponding results are summarized in Table 1.

As can be seen in Fig. 3 and Table 1, the inhibition of acetic acid on the cell growth of *T. fermentans* is closely related to the concentration of undissociated acetic acid. The undissociated acetic acid concentration was related to the culture pH. Increasing the concentration of undissociated acid prolonged the lag phase, with 0.026, 0.052, and 0.096  $\text{g L}^{-1}$  in culture media contributing to approximately 12, 24, and 48 h lag phases, respectively. Less than 0.052  $\text{g L}^{-1}$  of undissociated acid did not affect the growth of *T. fermentans* heavily, allowing glucose and acetic acid to be consumed simultaneously. When the undissociated acetic acid concentration was as high as 1.92  $\text{g L}^{-1}$ , the growth of *T. fermentans* ceased, suggesting that the undissociated acetic acid is the inhibitory molecular form.



**Fig. 3.** Time course profiles for the co-fermentation of glucose and xylose by *T. fermentans* under different acetic acid concentrations and pH values. (a) 0 g L<sup>-1</sup> acetic acid, pH 6.5; (b) 0 g L<sup>-1</sup> acetic acid, pH 4.5; (c) 1.5 g L<sup>-1</sup> acetic acid, pH 6.5; (d) 1.5 g L<sup>-1</sup> acetic acid, pH 4.5; (e) 3 g L<sup>-1</sup> acetic acid, pH 6.5; (f) 3 g L<sup>-1</sup> acetic acid, pH 4.5. ● Glucose; ■ xylose; □ biomass; ○ lipid; △ acetic acid; ◇ OD<sub>600</sub>. Data are the mean and standard error of two duplicate fermentations.

Interestingly, varying the concentration of acetic acid and pH had little influence on the lipid accumulation of *T. fermentans*. Specifically, at pH 6.5 a greater lipid content of 57% in comparison to the control of 51% was obtained for *T. fermentans* in the presence of low concentrations of acetic acid (1.5 g L<sup>-1</sup>). Lipid yield (gram lipid per gram sugar) under these conditions (pH 6.5, 1.5 g L<sup>-1</sup> acetic acid) was also a little greater than that of the control (0.22 vs. 0.19). One possible reason is that the acetic acid may be involved in the lipid synthesis because acetate ions can be used in the formation of acetyl-CoA *via* the action of acetyl-CoA synthetase, which is subsequently applied in the fatty acid synthesis.

It was reported by Sousa *et al.* that the specific enzyme activity of acetyl-CoA synthetase increased when the spoilage yeast *Zygosaccharomyces bailii* was cultivated in media containing acetic acid (Sousa *et al.* 1998). Vorapreeda *et al.* also found that acetic acid is one of the acetyl-CoA sources for fatty acid synthesis (Vorapreeda *et al.* 2012).

**Table 1.** Summary of Results Obtained from Co-fermentation of Glucose and Xylose by *T. fermentans* under Varying pH Values and Acetic Acid Concentrations

| Acetic acid concentration (g L <sup>-1</sup> )  | pH 6.5       |              |              | pH 4.5       |              |       |
|---|--------------|--------------|--------------|--------------|--------------|-------|
|   | 0            | 1.5          | 3            | 0            | 1.5          | 3     |
| Amount of undissociated acetic acid (g L <sup>-1</sup> )                                | 0            | 0.026        | 0.052        | 0            | 0.096        | 1.920 |
| Specific growth rate (h <sup>-1</sup> )   | 0.15 ± 0.00  | 0.08 ± 0.01  | 0.05 ± 0.01  | 0.07 ± 0.01  | 0.06 ± 0.02  | N.D.  |
| Biomass yield (g biomass g <sup>-1</sup> sugar)   | 0.37 ± 0.01  | 0.37 ± 0.01  | 0.34 ± 0.01  | 0.40 ± 0.02  | 0.36 ± 0.01  | N.D.  |
| Lipid yield (g lipid g <sup>-1</sup> sugar)   | 0.19 ± 0.01  | 0.22 ± 0.00  | 0.18 ± 0.00  | 0.24 ± 0.00  | 0.22 ± 0.04  | N.D.  |
| Specific glucose consumption rate (g glucose g <sup>-1</sup> dry cell h <sup>-1</sup> ) | 0.16 ± 0.01  | 0.07 ± 0.00  | 0.06 ± 0.00  | 0.15 ± 0.00  | 0.13 ± 0.01  | N.D.  |
| Specific xylose consumption rate (g xylose g <sup>-1</sup> dry cell h <sup>-1</sup> )   | 0.026 ± 0.01 | 0.021 ± 0.00 | 0.021 ± 0.00 | 0.016 ± 0.00 | 0.016 ± 0.00 | N.D.  |

N.D.= Not Detected.

Values shown are the mean and standard error of two duplicate fermentations for each condition. Specific growth rate was calculated by dividing 0.693 by doubling time of the cell concentration. Specific sugar consumption rate was calculated by dividing the slope of steepest portion of sugar consumption curve by the average cell concentration over that period. The concentrations of undissociated acid were calculated according to Henderson–Hasselbalch equation, using a pKa of 4.75 for acetic acid, the pH value, and total acid concentration for each fermentation condition.

The fatty acid compositions of the lipids obtained under different pH and acetic acid concentrations are summarized in Table 2. The majority of the fatty acids present were oleic acid, palmitic acid, linoleic acid, and stearic acid, with oleic acid accounting for about 60% of the total fatty acids. The variation of pH and acetic acid concentrations had no significant influence on the fatty acid composition of lipids ( $p > 0.05$ ). The possible reason is that the activities of key enzymes responsible for lipid synthesis were not seriously affected during fermentation. However, the related mechanism needs to be further investigated in our ongoing research. It is worth noting that a low pH was beneficial for oleic acid synthesis, while the synthesis of palmitic acid was stimulated by acetic acid at both pH conditions.

### Effect of Acetic Acid and pH on Glucose and Xylose Consumption

Generally, under nitrogen-limited condition, glucose is metabolized by oleaginous microorganisms through the glycolysis pathway and tricarboxylic acid cycle to generate large amounts of acetyl-CoA, which is then used for subsequent fatty acid synthesis. The metabolism of xylose into acetyl-CoA was considered to have two pathways: the pentose phosphate pathway (1 molar xylose generates 1.67 molar acetyl-CoA) and the phosphoketolase reaction (1 molar xylose generates 2 molar acetyl-CoA) (Evans and

Ratledge 1984; Fakas *et al.* 2009). As depicted in Fig. 3, *T. fermentans* can assimilate glucose and xylose simultaneously. A similar phenomenon was also observed when glucose and xylose were used as co-substrate for lipid production by *Thamnidium elegans* (Zikou *et al.* 2012).

**Table 2.** Effect of Acetic Acid and pH on the Fatty Acid Composition of Lipid from *T. fermentans*

| pH value | Concentration of acetic acid (g L <sup>-1</sup> ) | C16:0 (%)    | C18:0 (%)   | C18:1 (%)    | C18:2 (%)    |
|----------|---|--------------|-------------|--------------|--------------|
| 6.5      | 0   | 18.85 ± 1.23 | 7.46 ± 0.78 | 56.57 ± 1.43 | 11.15 ± 0.43 |
| 6.5      | 1.5   | 21.47 ± 1.04 | 7.09 ± 0.83 | 58.76 ± 0.82 | 12.65 ± 0.78 |
| 6.5      | 3   | 19.33 ± 0.65 | 6.94 ± 1.02 | 59.89 ± 1.84 | 13.83 ± 1.13 |
| 4.5      | 0   | 17.91 ± 0.88 | 6.69 ± 0.64 | 62.85 ± 1.67 | 12.53 ± 0.84 |
| 4.5      | 1.5   | 18.27 ± 1.63 | 7.15 ± 0.43 | 61.48 ± 0.68 | 13.08 ± 1.23 |
| 4.5      | 3   | N.D.         | N.D.        | N.D.         | N.D.         |

N.D.= Not Detected

Values shown are the mean and standard error of two duplicate fermentations for each condition.

As shown in Table 1, at each pH condition the specific glucose consumption rate decreased with an increase in acetic acid concentration. No acceleration was observed in the glucose consumption rate at low acetic acid concentrations and pH 6.5. In contrast, under such conditions the glucose consumption rate of the ethanologenic yeast *Saccharomyces cerevisiae* increased (Pampulha *et al.* 1989). As for xylose, the specific xylose consumption rate declined slightly as the acetic acid concentration increased at pH 6.5. When the pH was maintained at 4.5, the specific xylose consumption rates in the presence of 0 g L<sup>-1</sup> and 1.5 g L<sup>-1</sup> acetic acid were similar. There was no xylose and glucose consumption at 3 g L<sup>-1</sup> acetic acid, which explains the cessation of cell growth under this condition. Interestingly, the lipid yield of SCO produced per mixture of sugars consumed at most of the conditions was quite high (0.19 to 0.24 g/g), which is higher than or comparable to the one achieved by other oleaginous yeast and fungi on blends of xylose and glucose (Yu *et al.* 2014; Fakas *et al.* 2009), indicating that *T. fermentans* is a robust strain for SCO production with lignocellulosic sugars as substrate.

Previous works on ethanologenic yeasts found that undissociated acid led to a more severe inhibition of xylose metabolism than glucose consumption (Casey *et al.* 2010; Hasunuma *et al.* 2011). For ethanol fermentation, the inhibition of xylose consumption by acetic acid may be linked to a lower capacity of ATP generation during xylose metabolism under anaerobic conditions (Bellissimi *et al.* 2009; Casey *et al.* 2010) that cannot meet the ATP requirement for expelling excess protons and anionic species. However, in this study, the specific xylose consumption rate was not reduced substantially by the addition of acetic acid at pH 6.5 or 4.5, probably because of the higher ATP regeneration rate in aerobic fermentation. It is worth noting that the concentration of xylitol detected in the fermentation broth was less than 0.01 g L<sup>-1</sup>, indicating that acetic acid did not influence the stoichiometry of the xylose metabolic

pathway. In contrast, some yeasts utilize xylose *via* xylose reductase (XR) and xylitol dehydrogenase (XDH), thus increasing the acetic acid concentration in the culture medium and favoring the production of xylitol (Felipe *et al.* 1995; Helle *et al.* 2004).

In addition, low concentrations of acetic acid have been shown to stimulate ethanogenic yeasts' glucose consumption rate to accelerate ATP regeneration under anaerobic conditions (Pampulha *et al.* 2006; Keating *et al.* 2006). However, no increase in the glucose consumption rate was observed with the addition of acetic acid in this study. This may be attributed to the different energy-producing route in aerobic fermentation, which does not merely rely on the glycolytic pathway.

### Effect of Acetic Acid on Intracellular pH

To better understand why 3 g L<sup>-1</sup> acetic acid caused serious inhibition on cell growth and lipid accumulation of *T. fermentans* at pH 4.5, the effect of acetic acid on the pH<sub>i</sub> of *T. fermentans* cells was investigated using fluorescent probe BCECF-AM. As described previously, the ratio value of fluorescence intensity between 490 and 440 nm was plotted as a function of pH<sub>i</sub> to establish a calibration curve,

$$\text{Ratio value } (x) = (tF_{490} - bF_{490}) / (tF_{440} - bF_{440}) \quad (1)$$

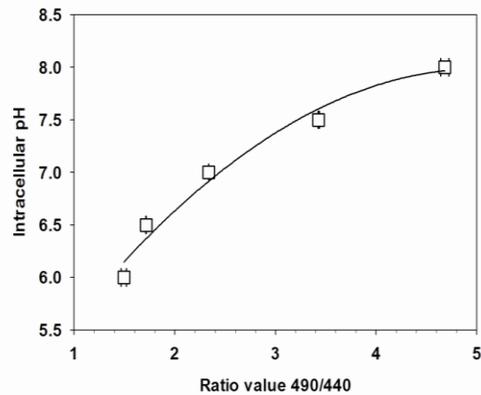
where  $tF_{490}$  and  $tF_{440}$  are the total fluorescence intensities at 490 and 440 nm, respectively, and  $bF_{490}$  and  $bF_{440}$  are the background fluorescence intensities at 490 and 440 nm, respectively.

As shown in Fig. 4, the calibration curve of the ratio values of fluorescence intensities of BCECF-AM against intracellular pH was established. A polynomial function can be fitted to calculate pH<sub>i</sub> ( $y$ ) from the ratio value ( $x$ ). The equation of best fit to the calibration curve was:

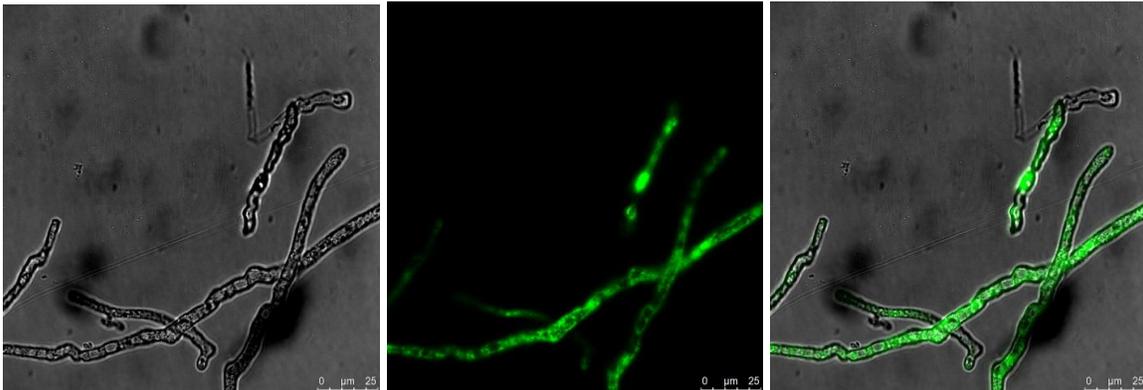
$$y = -0.1457 x^2 + 1.4714 x + 4.2734 \quad (2)$$

The regression coefficient ( $R^2$ ) for this equation was 0.9768.

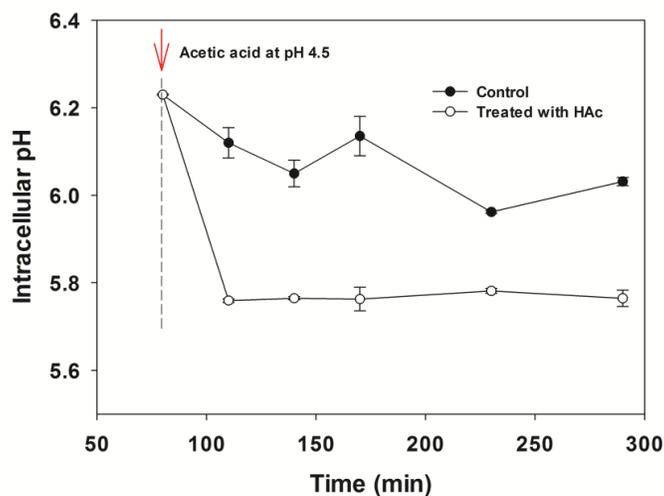
*T. fermentans* cells in the exponential phase were transferred to media containing no acetic acid (the control) and 50 mM (3 g L<sup>-1</sup>) acetic acid, respectively, in pH 4.5 citric/phosphate buffer. Unlike <sup>31</sup>P-NMR or the distribution of radio labelled weak acid technique, BCECF-AM analysis is a non-invasive *in vivo* method that is based on the pH-dependent fluorescence of BCECF-AM in the cytosol of the cells. It was reported that loading of the fungus *Neurospora crassa* with BCECF-AM resulted in accumulation of the fluorescent indicator in vacuoles instead of even distribution throughout the cytosol (Slayman *et al.* 1994). Hence, it is essential to confirm the level of fluorescent dye loading within the cells and the distribution of the loaded indicator within the cytosol of *T. fermentans* cells. As shown in Fig. 5, the loaded cells were visually analyzed using confocal laser scanning microscopy. Most cells showed high levels of intracellular fluorescence, which appeared to be in an even cytoplasmic distribution. As indicated in Fig. 6, the initial intracellular pH before addition of acetic acid was approximately 6.25, similar to the result of approximately 6.2 measured with the fluorescent probe carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Bracey *et al.* 1998). After adding acetic acid, the intracellular pH of *T. fermentans* cells declined to about 5.7. This pH<sub>i</sub> change is similar to the result determined by Roe *et al.* (1998) by approximately 0.4 pH units (Roe *et al.* 1998). The severe inhibition of acetic acid at low pH on lipid production is partly attributed to the reduction of the intracellular pH of *T. fermentans*.



**Fig. 4.** Calibration curve of BCECF-AM in citric/phosphate buffer with nigericin-treated cells of *T. fermentans*. Data are the mean and standard error of two duplicate fermentations.



**Fig. 5.** Images of *T. fermentans* cells loaded with BCECF-AM taken using confocal laser scanning microscopy: (left) an illumination phase contrast image; (middle) the corresponding fluorescent image; (right) the fluorescent image super-imposed onto a phase image.



**Fig. 6.** Intracellular pH of *T. fermentans* with or without acetic acid in 100 mM citric/phosphate buffer, pH 4.5. Solid circle, the control without acetic acid; open circle, addition of 50 mM ( $3 \text{ g L}^{-1}$ ) acetic acid. The dotted line and arrow show the time of addition. Data are the mean and standard error of two duplicate fermentations.

## CONCLUSIONS

1. Oleaginous yeast *T. fermentans* was able to metabolize acetic acid without glucose repression.
2. The effect of acetic acid on the cell growth and lipid accumulation of *T. fermentans* was related to the culture pH, and undissociated acetic acid is the inhibitory molecular form. The reduction of intracellular pH partly explains the inhibitory effect of acetic acid.
3. The change of pH and acetic acid concentration had no significant influence on the fatty acid composition of the lipids.

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