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Effect of Acetic Acid on Citric Acid Fermentation in an Integrated Citric Acid–Methane Fermentation Process

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Abstract An integrated citric acid–methane fermentation process was proposed to solve the problem of extraction wastewater in citric acid fermentation process. Extraction wastewater was treated by anaerobic digestion and then recycled for the next batch of citric acid fermentation to eliminate wastewater discharge and reduce water resource consumption. Acetic acid as an intermediate product of methane fermentation was present in anaerobic digestion effluent. In this study, the effect of acetic acid on citric acid fermentation was investigated and results showed that lower concentration of acetic acid could promote *Aspergillus niger* growth and citric acid production. 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) staining was used to quantify the activity of *A. niger* cells, and the results suggested that when acetic acid concentration was above 8 mM at initial pH 4.5, the morphology of *A. niger* became uneven and the part of the cells' activity was significantly reduced, thereby resulting in deceasing of citric acid production. Effects of acetic acid on citric acid fermentation, as influenced by initial pH and cell number in inocula, were also examined. The result indicated that inhibition by acetic acid increased as initial pH declined and was rarely influenced by cell number in inocula.

Keywords Citric acid · Acetic acid · Extraction wastewater · Anaerobic digestion · CTC staining · Cell number

Introduction

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid), one of the most important organic acids, is widely used in food, beverage, chemical, and metallurgical industries for many years [1]. Extraction wastewater, containing high concentration of chemical oxygen demand (COD) (15,000–20,000 mg/L) and low pH (4.5–4.8), has limited the development of cassava-based

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citric acid industry [2]. Biochemical and physical chemistry methods used to treat citric acid wastewater have been widely studied [3, 4]. Traditional process of biochemical methods to treat wastewater was anaerobic digestion followed by aerobic digestion [5–7]. However, capital investment and operating costs of the aerobic digestion are high. Recently, photosynthetic bacteria and *Chlorella vulgaris* have been developed for citric acid wastewater treatment by many scholars [3, 8], but the effluent could not meet the national discharge standard and still needs to further treatment. Physical chemistry methods include Fenton's reagent, emulsion liquid membrane, microwave radiation, and other methods [9–11]. These methods are operation complexity and costly and consume large amount of inorganic reagents, thereby being unable to apply on the industrial scale.

Directly recycling of citric acid wastewater has been studied by many researchers [12, 13]. However, metal ions and pigment substances could accumulate as recycling, causing inhibition to the growth of *Aspergillus niger* and reducing citric acid production. Acidic cation exchange resin and activated carbon adsorption were used to pretreat wastewater before recycling, and good results were obtained. Nevertheless, high costs and complicated regeneration of resin made these methods not quite suitable for industrial-scale production. Therefore, the disposal of wastewater is still a hard task to the citric acid industry.

To solve these problems, an integrated citric acid–methane fermentation process was proposed for wastewater reutilization. In this process (Fig. 1), cassava and corn starch are used to citric acid production while fiber, pectin, other unused materials, and metabolites of *A. niger* in fermentation are transformed to biogas through anaerobic digestion. The biogas can be used to produce electricity and heat while the anaerobic digestion effluent (ADE) was further treated and recycled as cooking water for the next citric acid fermentation. This process not only avoids the wastewater discharge but also decreases the water resource and energy consumption.

Acetic acid is one of the main precursor substances of CH_4 in anaerobic conditions. Anaerobic digestion of readily degradable organic compounds is a delicate balance between the rates of hydrolysis and methanogenesis. Methanogenic bacteria are more sensitive than hydrolytic and acidogenic bacteria to the fluctuation of organic loading rate (OLR). The increase of OLR would decline the vitality of methanogens and the rate of methanogenesis more rapidly than the rates of hydrolysis and acidogenesis, resulting in the inefficient utilization and accumulation of volatile fatty acids in the effluent [14, 15].

Previous experiment in our laboratory confirmed that acetic acid was the major component of the volatile fatty acids in ADE with the concentration of 0.15 g/L (Table 1), which could not influence the process. However, ADE sample from citric acid industry was detected, and

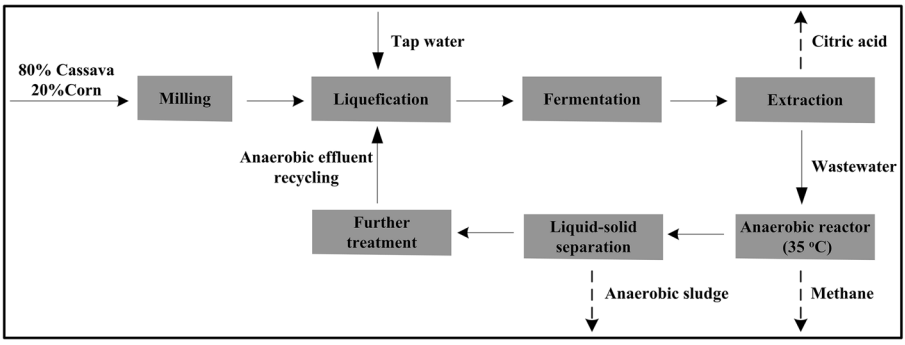

Fig. 1 Flow chart of the integrated citric acid–methane fermentation process

2.01 g/L acetic acid was found under acidification conditions. Complete inhibition was observed in this high concentration [16]. Consequently, it was necessary to systematically evaluate the effect of acetic acid on citric acid fermentation under different conditions to support and understand the application of this integrated process on the industrial scale.

In this study, the effect of acetic acid on citric acid fermentation was investigated in 5-L fermentor. Meanwhile, its influence in different pH and inoculation quantity conditions was also studied. Based on the results, corresponding responses were provided to avoid further deterioration of the coupled process with acidification conditions of the anaerobic digestion system.

Materials and Methods

Organism and Culture Maintenance

A. niger preserved in our lab and obtained from Anhui Fengyuan Co., Ltd., China, was used for citric acid fermentation in this study. Potato dextrose agar (PDA) slants maintained at 4 °C in a refrigerator were used for its preservation. Subculture of *A. niger* was performed every 2 months. Sand tube was used for its long-term preservation.

Seed Culture Conditions for Citric Acid Fermentation

Seed culture liquefied mash was prepared using cassava powder (starch content of 65–70 % (*w*/*w*), size of approximately 0.45 mm), which was provided by Henan Tianguan Co., Ltd., China. In mash preparation, cassava powder was mixed with cooking water at a ratio of 1:4 (*w*/*w*) and the slurry pH was adjusted to 6.0 with 30 % (*w*/*w*) H_2SO_4 or 10 % (*w*/*v*) NaOH. Ten units per gram thermostable α-amylase (20,000 U/mL, optimal temperature range of 95–105 °C, Genencor China Co., Ltd.) was then added, followed by heating of slurry to 100 °C for 2 h. Water loss during liquefaction was made up with sterile water. Subsequently, 0.1 % $(NH_4)_2SO_4$ (*w*/*v*) was added to the slurry as nitrogen source for spore germination. The slurry pH was adjusted to 5.5 and autoclaved at 115 °C for 20 min. Conidia from a 7-day-old potato dextrose agar slant were used for inoculation. Ten milliliters of a spore suspension in sterile water, which contained 6.0×10^6 conidia/mL, was added to the 80-mL sterile inoculum medium in a 1,000-mL shake flask and then incubated on a rotating shaker (200 rpm) at 36 ±1 °C for 20 h before the seed culture was incubated for citric acid fermentation.

Table 1 Organic acid in different ADE samples

ADE samples	Acetic acid (g/L)	Propanoic acid (g/L)	Butyric acid (g/L)	Pentanoic acid (g/L)	Lactic acid (g/L)
ADE[a]	0.15	0.001	N.D.	N.D.	N.D.
ADE[b]	0.24	0.03	0.04	0.18	N.D.
ADE[c]	2.01	1.50	0.94	0.30	0.13

ADE anaerobic digestion effluent, *N.D.* not determined

[a] ADE normal sample in our laboratory

[b] ADE normal sample from citric acid industrial

[c] ADE sample under acidification conditions from citric acid industry

Citric Acid Fermentation

To prepare the fermentation media, 80-g cassava powder and 20-g corn powder (starch content of 75–80 % (*w*/*w*), size of approximately 0.45 mm), which were provided by Henan Tianguan Co., Ltd., China, were added per 450-mL cooking water. The liquefaction operation was the same with that of inoculum medium. The pH was also adjusted to 5.5, and the concentration of initial total sugar was regulated to approximately 155 g/L. The liquefied mash was autoclaved at 115 °C for 20 min, and then seed broth at 15 % of fermentation volume (6/40 mL) was inoculated to a 500-mL shake flask. The fermentation was conducted at 260 rpm, 37.5±1 °C for 92 h. All the shake flask experiments were conducted in triplicate. Recycling experiments were employed in an agitator bioreactor (Korea Bioreactor Co., Ltd.) with 3-L working capacity (total capacity 5 L). Seed broth at 15 % of fermentation volume (450 mL/3 L) was inoculated to fermentor. Using a circulator bath, temperature throughout fermentation was maintained at 37.5±1 °C for 72 h. Dissolved oxygen during fermentation was controlled using three-bladed impellers operating at 400 rpm with an aeration rate of 2 vvm. Samples were collected in an interval of 12 h.

Effect of Acetic Acid in Different pH on the Citric Acid Fermentation

Acetic acid was added to the mash to the final concentrations of 1.0, 2.0, 4.0, 8.0, 12.0, 16.0, and 20.0 mM, respectively. The resulting slurry pH was adjusted to 4.1, 5.5, or 6.8 using 30 % (*w*/*w*) H_2SO_4 or 10 % (*w*/*v*) NaOH. After inoculated with seed broth, the fermentation pH was reduced to approximately 4.0, 4.5, and 5.0, respectively. Undissociated acetic acid was calculated using the Henderson–Hasselbalch equation, $pH = pKa + \log([A^-] / [HA])$, and acetic acid p*K*a of 4.75.

Effect of Acetic Acid in Different Inoculation Number on the Citric Acid Fermentation

Acetic acid was added to the mash to the final concentrations of 1.0, 2.0, 4.0, 8.0, 12.0, 16.0, and 20.0 mM, respectively. The resulting slurry pH was adjusted to 6.8 using 30 % (*w*/*w*) H_2SO_4 or 10 % (*w*/*v*) NaOH. Seed broth containing different cell numbers of 4.0×10^5, 7.0×10^5, and 1.2×10^6/mL was inoculated to the medium.

CTC Staining to Quantify the Activity of *A. niger*

CTC rapid staining kit (Dojindo Laboratories, Kumamoto, Japan) was used to quantify the activity of *A. niger* in culture medium in this study. *A. niger* cell collected from the culture medium was diluted by normal saline. One milliliter of cell suspension was mixed with 20 μL of CTC solution (with the concentration of 50 mM), followed by 5 μL of the enhancing reagent adding. The mixture was maintained at 37.5±1 °C for 30 min and then detected by laser scanning confocal microscope (Leica TCS SP8, Leica Microsystems GmbH, Wetzlar, Germany). The emission wavelength was 620–640 nm, and excitation wavelength was 488 nm.

Methane Fermentation Condition

Upflow anaerobic sludge blanket (UASB) reactor (Shanghai Daming, China) with a working volume of 5 L was used for methane fermentation. Circulator bath was used to maintain temperature at 35±1 °C. Thirty percent of the mesophilic anaerobic granular sludge, which

was provided by Yixing Xielian Biological Chemical Co., Ltd., China, was inoculated in the reactor. Every day, 1 L of wastewater was input into the reactor by an adjustable-speed peristaltic pump for methane fermentation. The ADE was centrifuged at 4,000 × *g* for 20 min, and the supernatant was stored at 4 °C before being used for citric acid fermentation. Water circulation within the reactor was performed by a peristaltic pump.

Analysis Methods

Residual reducing sugars and acetic and citric acids were analyzed by high-performance liquid chromatography (HPLC) (Dionex Co., USA), which was equipped with an ultraviolet and refraction index detector (UV/RID) and C18 3.9×300-mm column. The column was operated at 65 °C with 0.005 mol/L H_2SO_4 as the mobile phase at flow rate of 0.6 mL/min. The collected samples were centrifuged at 10,000 × *g* for 20 min, followed by filtering through a 0.45-μm membrane before analysis. Total sugars were detected by the biosensor (SBA-40B, Shandong Academy of Sciences, China) after the samples were acid-hydrolyzed (4 % hydrochloric acid, *w*/*w*) at 100 °C for 120 min. Cell number in the culture medium was evaluated by microscope count method after a suitable dilution. To obtain sufficiently precise measurements, cell counting was performed 20 times for each sample.

Results and Discussion

Effect of Acetic Acid on Citric Acid Fermentation in a 5-L Fermentor

Effect of acetic acid on citric acid fermentation was investigated in a 5-L fermentor at initial pH 4.5, and the result is shown in Fig. 2. When acetic acid concentration was below 8 mM, citric acid production was slightly higher than the control. However, citric acid production was significantly reduced as acetic acid concentration was above 8 mM (Fig. 2a). When acetic acid concentration was 4 and 8 mM, citric acid fermentation rarely had lag phase and only about 72 h was required for *A. niger* to consume all of the available sugar, while lag phase of 8 and 16 h was found as acetic acid concentration increased to 12 and 16 mM, respectively, and large amount of reducing sugar still contained in medium at the end of fermentation (Fig. 2b, c). Citric acid fermentation rate with different acetic acid concentrations was investigated (Fig. 2d), and the result suggested that when citric acid was not inhibited, highest fermentation rate was obtained at 24 h and indicated the highest activity of *A. niger* cells. Meanwhile, low concentration of acetic acid could slightly increase fermentation rate, and highest fermentation rate was achieved when 8 mM acetic acid was contained in medium, while high concentration of acetic acid significantly decreased fermentation rate. According to these results, it should be noted that acetic acid in the anaerobic effluent was not always harmful to citric acid fermentation, and regulating acetic acid concentration within a reasonable range was useful to promoting the integrated citric acid–methane fermentation process. Furthermore, it is reported that the effect of acetic acid on yeast could be influenced by pH of the culture medium [17]. Therefore, the effect of acetic acid on citric acid fermentation, as influenced by initial pH, was also examined.

Effect of Acetic Acid on Citric Acid Fermentation in Different Initial pH

As shown in Fig. 3a, *A. niger* exhibited an increasing resistance to acetic acid as the pH increased. Citric acid fermentation was inhibited as the concentration of acetic acid was above

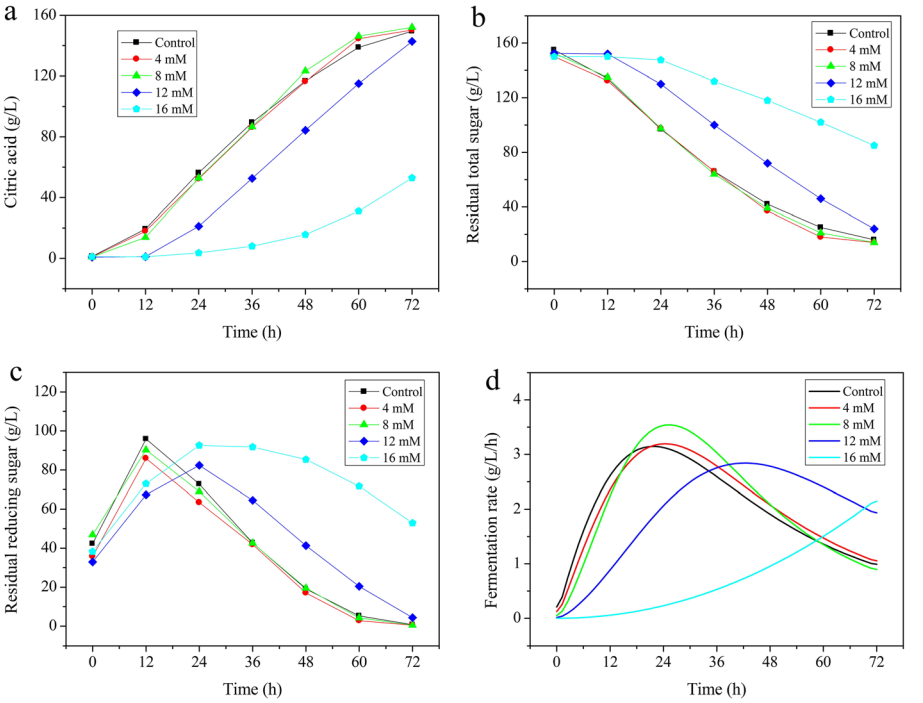

Fig. 2 Effect of acetic acid on **a** citric acid production, **b** residual total sugar, **c** residual reducing sugar, and **d** fermentation rate in 5-L fermentor

4, 8, and 12 mM at initial pH 4.0, 4.5, and 5.0, respectively. Below inhibitory concentration, citric acid production was hardly inhibited by acetic acid and even slightly higher than the control, while increasing acetic acid concentration caused citric acid production to drop rapidly. Compared with control of fermentation at initial pH 4.5 and 5.0, citric acid production was lower at initial pH 4.0 and residual total sugar was higher, while residual reducing sugar in medium was completely consumed (data not shown). It could be explained that the activity of glucoamylase in medium was inhibited by lower initial pH, thus decreased the available glucose, and reduced citric acid production.

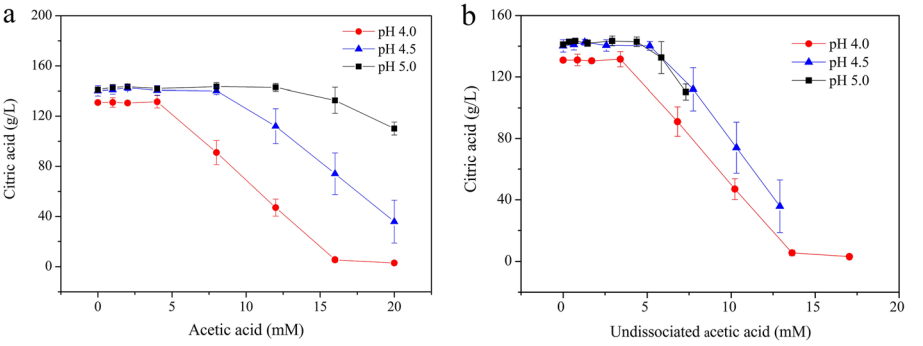

Fig. 3 Effect of acetic acid (**a**) and undissociated acetic acid (**b**) on citric acid fermentation at different initial pH

As to the yeast *Saccharomyces cerevisae*, acetic acid is assumed to diffuse freely into the cell in the undissociated form [18, 19]. The undissociated acid traverses the cell membrane and then dissociates in the higher pH environment of cytosol, causing both a cytoplasmic acidification and intracellular accumulation of acid anion, which could significantly inhibit yeast growth [20]. The influence of undissociated acetic acid on citric acid fermentation was investigated, and the results are shown in Fig. 3b. The effect of acetic acid on citric acid fermentation was most likely associated with undissociated acid, regardless of its total concentration at different pH. Therefore, it should be noted that the influence of acetic acid on citric acid production was through its undissociated form. As the concentration of undissociated acid was below 5.16 mM, citric acid production was not inhibited and even higher than the control, which was similar to widely reported results of ethanol fermentation by *S. cerevisiae* when acetic acid is added to the medium [17, 21, 22]. Thomas et al. [17] have reported that acetic acid stimulates ethanol production as much as 20 % with 167 mM acetic acid in pH 4.5 medium. Taherzadeh et al. [22] have reported a 21 % increase in ethanol yield that resulted from the addition of 3.5 g/L of acetic acid at pH 3.5.

Kornberg and Gotto [23] have used isotope labeling method to prove that *A. niger* involves induction of certain anabolic pathways, enzymes, and specific transport mechanism to use acetic acid as carbon sources for the synthesis of citric acid. These pathways and permeases are activated when glucose is exhausted. At the end of citric acid fermentation, available glucose in medium was completely consumed (data not shown); thereby, acetic acid could be converted to citric acid through glyoxylate cycle in our experiments. However, its conversion ratio was calculated (3 mol acetic acid could be converted to 1 mol citric acid), and theoretical citric acid production (10 mM acetic acid could produce 0.64 g/L citric acid) was much less than our achievement (approximately 2 g/L). Therefore, converted acetic acid might not be the major reason for citric acid production increase.

Thomas et al. [17] reported that acetic acid dissociation in the cytosol could cause acidification of cytoplasm in ethanol fermentation by *S. cerevisae*. To maintain the intracellular pH within a physiological range optimum for metabolism, yeast pumps out protons at the expense of metabolic energy (ATP). Increased diversion of ATP to pump out protons causes improved ethanol production. Similarly, undissociated acetic acid diffuses into *A. niger* cell and dissociates in the cytosol where common pH is 6.5–7.0 [24]. To maintain the intracellular pH within the normal range, ATP was consumed to pump out protons. Therefore, more glucose enters into tricarboxylate cycle, and the synthesis of ATP could result in overflow of citric acid. Furthermore, the buffer pairs formed from appropriate acetic acid could avoid a rapid decrease of medium pH which could be useful to maintain the activity of glucoamylase in medium, thus improving citric acid production.

When the concentration of undissociated acetic acid was above 5.16 mM, citric acid production decreased in a similar pattern among different pH conditions. The activity of *A. niger* cells harvested at 24 h at initial pH 4.5 was detected with CTC staining, and the result is shown in Fig. 4. Fermentation with tap water was used as control (Fig. 4a). When acetic acid concentration was below 8 mM, the activity and morphology of *A. niger* were rarely influenced and were similar to the control (Fig. 4b, c). However, the morphology of *A. niger* was uneven, and part of the cells' activity was significantly reduced as acetic acid concentration increased to 12 mM (Fig. 4d). When 16 mM acetic acid was contained in the culture medium, the morphology of *A. niger* was polarization. Part of cells became larger and tighter but still had high activity (Fig. 4e), while others were severely inhibited and the activity was very low (Fig. 4f). Therefore, it should be noted that high concentration of acetic acid could cause part of cells to seriously lose their activity. In our experiment, when the acetic acid concentration was excessive, undissociated acetic acid in medium entered

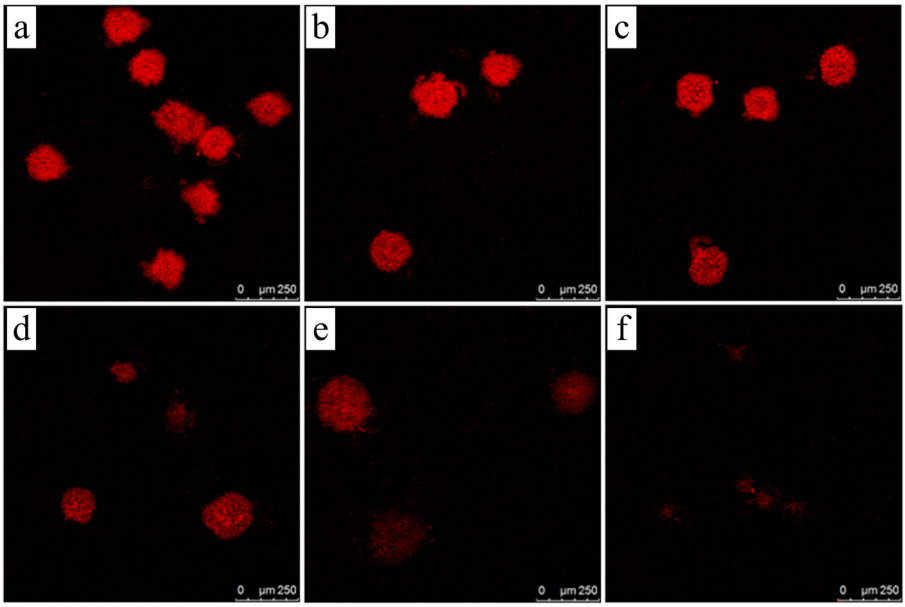

Fig. 4 Activity of *A. niger* cells in the culture medium **a** with initial pH 4.5 control which was fermented with tap water, **b** with 4 mM acetic acid in medium, **c** with 8 mM acetic acid in medium, **d** with 12 mM acetic acid in medium, and **e**, **f** activity of two morphology of *A. niger* with 16 mM acetic acid in medium

into cells and caused overacidification of the part of cells' cytoplasm, resulting in decreasing of citric acid production.

As shown in Table 2, pH at the end of fermentation in medium significantly rose as the concentration of acetic acid above 4, 8, and 12 mM at initial pH 4.0, 4.5, and 5.0, respectively. With low concentration of acetic acid, citric acid fermentation was not inhibited, and thus, pH hardly changed at the end of fermentation. In increased acetic acid concentration, citric acid production sharply decreased, resulting in increased pH in medium. Furthermore, the buffer pairs formed from acetic acid could be another reason that leads to higher pH. In normal citric acid fermentation, pH of medium could quickly decrease to below 3 at the initial growth stage [25], and in this condition, dissociated acid was nearly completely converted to undissociated acid (acetic acid p*K*a of 4.75). If citric acid fermentation was inhibited at fermentation stage, the magnitude of inhibition should depend on total acetic acid concentration, which was

Table 2 Effect of acetic acid on pH in medium at the end of fermentation

Acetic acid in medium (mM)	pH 4.0	pH 4.5	pH 5.0
0	1.72±0.01	1.72±0.01	1.71±0.01
1	1.72±0.01	1.73±0.01	1.71±0.01
2	1.72±0.01	1.74±0.01	1.72±0.00
4	1.73±0.02	1.75±0.01	1.74±0.01
8	1.94±0.04	1.75±0.01	1.74±0.01
12	2.60±0.39	1.82±0.05	1.76±0.01
16	3.49±0.74	1.96±0.08	1.83±0.03
20	3.98±0.02	2.12±0.05	1.91±0.01

opposite with the result in this study. Therefore, citric acid fermentation might be inhibited by acetic acid at growth stage.

Effect of Acetic Acid on Cell Number at Different Initial pH

In our experiments, larger and tighter cell pellet was observed when citric acid fermentation was inhibited. Increasing the concentration of acetic acid resulted in complete inhibition of cell pellet growth and even cell autolysis. Cell number in medium decreased sharply when the concentration of acetic acid was above 2, 4, and 8 mM at initial pH 4.0, 4.5, and 5.0, respectively (Fig. 5a). The influence of undissociated acetic acid on cell number was investigated, and the results are shown in Fig. 5b. Below 2.92 mM, cell number in medium rarely decreased, while increased undissociated acetic acid concentration could result in significant reduction of cell number. Similar to the citric acid production, the cell number was also most likely related to undissociated acetic acid. Besides, the citric acid production was inhibited when the cell number in the culture medium was lower than 6.0×10^4/mL (Fig. 3a).

Papagianni and Mattey [26] reported that cell number in medium could influence both cell structure and citric acid production. To better understand the effect of cell number on citric acid production, cell number in medium during citric acid fermentation and the performance of citric acid fermentation with different cell amount in inocula were studied.

As shown in Fig. 6a, cell number in medium hardly changed during citric acid fermentation and was stable at 1.0×10^5/mL which was close to the theoretical value (the inoculation amount was 15 % and contained 7.0×10^5/mL cell number in seed broth). Furthermore, a certain amount of small cell pellet was observed at the later fermentation stage. Papagianni et al. [27] reported that actual breakage of *A. niger* filaments occurred under intensive agitation conditions at early fermentation stage, and then, fragmentation of filaments could regrow. However, the degree and rate of regrowth were not evaluated. Our results have proved that although filaments could regrow during the citric acid fermentation, the regrowth rate was low and could not significantly influence the cell number in the culture medium. Therefore, cell amount in inocula could be a decisive factor for cell number in the culture medium.

As shown in Fig. 6b, cell number in medium could greatly influence the citric acid fermentation. When cell number in medium was below 3.5×10^4/mL, citric acid production decreased by 7.36 %. In the meantime, the structure of cell pellet became larger which might be caused by overnutrition. Increased cell number in medium resulted in smaller cell pellet.

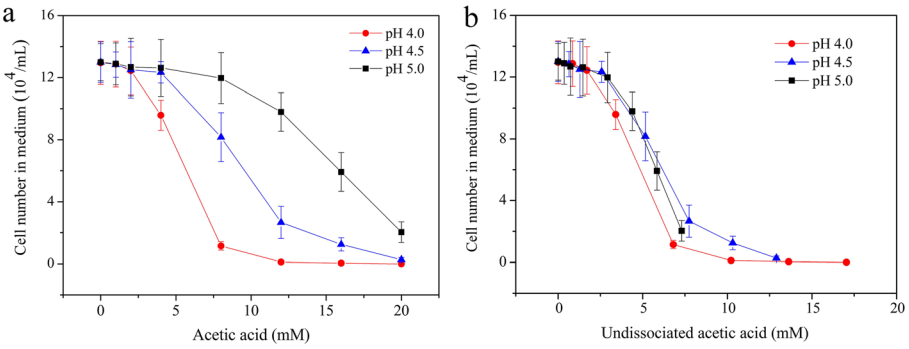

Fig. 5 Effect of acetic acid (**a**) and undissociated acetic acid (**b**) on cell number in medium at different initial pH

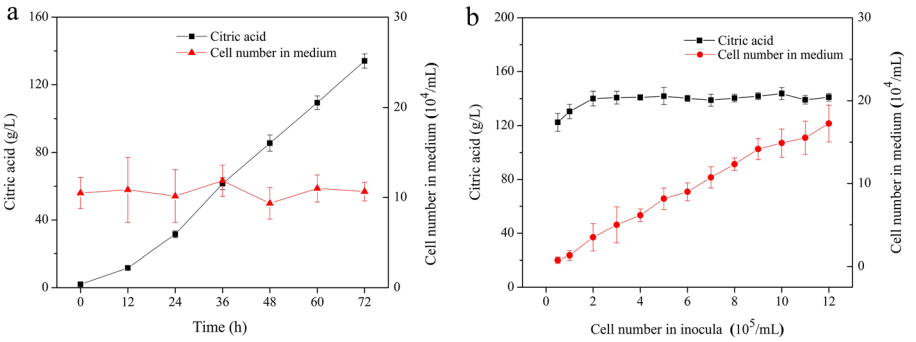

Fig. 6 Change of cell number in medium during citric acid fermentation (**a**) and the effect of citric acid fermentation with different cell amount in inocula (**b**)

And, normal citric acid production was obtained when the cell number increased to 1.7×10^5/mL. The result was supported by previous studies. Papagianni and Mattey [26] reported that pellets predominated when the inoculum was 10^4–10^5 spores/mL, while at much higher inoculum levels (10^8–10^9 spores/mL), the mycelium could develop in dispersed morphologies.

As shown in Fig. 5a, b, we could find that when acetic acid contained in medium, critical concentration of cell number (6.0×10^4/mL) was higher than the lower limit we have obtained (approximately 3.5×10^4/mL). Jernejc and Legiša [24] reported that one of the mechanisms stimulating citric acid accumulation by *A. niger* could be a slight cytoplasmic acidification. But, when the intracellular pH decreased below 6.5, concomitantly citric acid overflow was suppressed. In this study, when the acetic acid concentration was excessive, part of *A. niger* cells were not autolyzed, but their activity significantly decreased (Fig. 4). Therefore, the lower limit of cell number in the culture medium with acetic acid adding was higher than that of fermentation with tap water.

Effect of Acetic Acid on Citric Acid Fermentation with Different Cell Number in Medium

Previous experiments indicated that citric acid fermentation could be influenced by cell number in medium, and lower cell number could lead to decreasing citric acid production. Meanwhile, cell number in medium could be significantly influenced by acetic acid. Increased acetic acid concentration resulted in the decrease of cell number in medium and reduced citric acid production. Therefore, whether acetic acid influenced citric acid production by reducing the cell number in the culture medium or not should be studied.

As shown in Fig. 5a, increased acetic acid resulted in autolysis of part cell. That means that cell number in medium exhibited different resistance to high acetic acid concentration, and we could consider that the proportion was stable. If decreased citric acid production was caused by lower cell number, increased cell number in medium could enhance cell resistance of acetic acid and increase citric acid production when acetic acid was above the inhibition concentration. As shown in Fig. 7b, sharply decreased cell numbers in the culture medium were observed as the concentration of acetic acid was above 12 mM with different cell number in inocula at pH 5.0. With 4.0×10^5/mL cell number in inocula, cell number in the culture medium decreased from 7.0×10^4 to 4.1×10^4/mL as acetic acid concentration increased to 16 mM, while fermentation with 7.0×10^5 and 1.2×10^6/mL cell number in inocula decreased to 7.0×10^4 and 1.1×10^5/mL, respectively. In this concentration, cell number in medium was higher than the critical value (approximately 3.5×10^4/mL), but citric acid production was

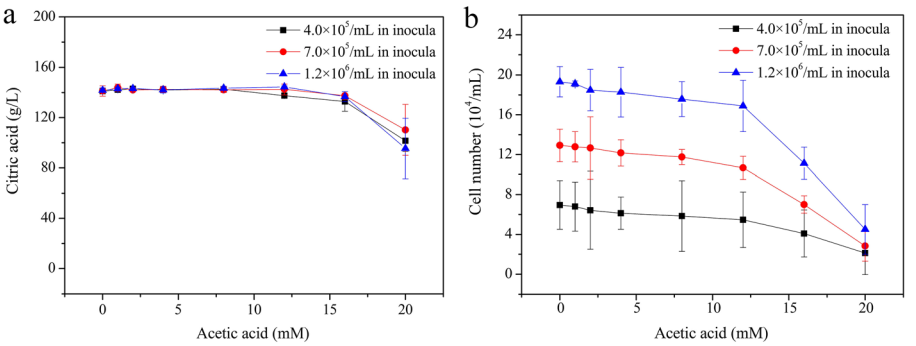

Fig. 7 Effect of acetic acid on citric acid fermentation (**a**) and cell number in medium (**b**) with different cell number in inocula (at initial pH 5.0)

significantly inhibited at the same time and the degree of depression was almost equal (Fig. 7a). It indicated that effect of acetic acid on citric acid fermentation was rarely influenced by cell number in medium, and the decreased production of citric acid caused by acetic acid was not due to the lower cell number in medium. In yeast cells, Pampulha and Loureiro-Dias [28] reported that the inhibition of fermentation by acetic acid can be explained by decreased hexokinase, phosphofructokinase, and enolase activities. This inhibition might also exist in *A. niger*, and related research is under way.

In fact, organic acid accumulation in the integrated citric acid–methane fermentation process would not occur in the normal methane fermentation. However, acidification of methane fermentation is the largest risk for our coupled process which could result in acetic acid accumulation in the anaerobic effluent. Therefore, measures should be taken to regulate the process. First, for citric acid fermentation, efficient treatment could be used to remove acetic acid from effluent before recycling when acetic acid concentration in ADE was excessive. Furthermore, initial fermentation pH could be adjusted to avoid or alleviate the potential inhibitory effect by acetic acid. On the other hand, for methane fermentation, part of effluent from UASB reactor should be mixed with influent to increase the influent pH and decrease the influent organic loading rate for the anaerobic digestion which may allow the regaining of normal methane fermentation as soon as possible.

Conclusions

An integrated citric acid–methane fermentation process was proposed by our laboratory to solve the problem of wastewater discharge in citric acid fermentation. Acetic acid in ADE could influence citric acid fermentation performance. Although high concentration of acetic acid exhibited great inhibitory effects on citric acid fermentation, reducing *A. niger* cells activity and citric acid production, low acetic acid concentration could promote *A. niger* growth and stimulate citric acid production. Inhibition by acetic acid increased as initial medium pH declined. Moreover, cell number in medium rarely changed during the fermentation, and citric acid production decreased when fermentation was carried out with low cell number in medium.

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