Full recycling of citric acid wastewater through anaerobic digestion, air-stripping and pH control

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ABSTRACT

Anaerobic digestion effluent (ADE) from the anaerobic digestion treatment of citric acid wastewater can be reused as a potential substitute for process water in the citric acid fermentation. However, excessive sodium contained in ADE significantly decreases citric acid production. In this paper, the inhibition mechanism of sodium on citric acid fermentation was investigated. We demonstrated that excessive sodium did not increase oxidative stress for *Aspergillus niger*, but reduced the pH of the medium significantly over the period 4–24 h, which led to lower activities of glucoamylase and isomaltase secreted by *A. niger*, with a decrease of available sugar concentration and citric acid production. ADE was pretreated by air-stripping prior to recycle and 18 g/L calcium carbonate was added at the start of fermentation to control the pH of the medium. The inhibition caused by ADE was completely alleviated and citric acid production substantially increased from 118.6 g/L to 141.4 g/L, comparable to the fermentation with deionized water (141.2 g/L). This novel process could decrease wastewater discharges and fresh water consumption in the citric acid industry, with benefit to the environment.

Key words | air-stripping, anaerobic digestion, calcium carbonate, citric acid wastewater, pH control, sodium

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INTRODUCTION

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is one of the most important intermediate compounds produced by fermentation and is widely used in the food and beverage industries because of its pleasant taste and palatability (Ciriminna *et al.* 2017). Except for a few factories in Mexico, Greece and South America where citric acid is still extracted from unripe citrus fruits, more than 99% of world citric acid production is via fermentation processes, primarily by submerged fermentation of starch-based or sucrose-based substrates with the filamentous fungus *Aspergillus niger* which has high citric acid productivity (Dhillon *et al.* 2011). In 2014, approximately 1.3 million tonnes of citric acid were manufactured in China, an increase of 6.6% compared with the previous year, and approaching 70% of world output (Zhang *et al.* 2017). Li *et al.* (2013) found that 50–60 tonnes of wastewater arise from each tonne of citric acid; thus above 65 million tonnes of citric acid wastewater are generated. This wastewater has a substantial chemical oxygen demand (COD, 15,000–20,000 mg/L) and low pH (4.5–4.8) and its management is a matter of great urgency for the citric acid industry (Zhi *et al.* 2010).

Conventionally, wastewater from citric acid production is treated initially by anaerobic and aerobic digestion (Figure 1), followed by further processing using physical or chemical methods to meet the national discharge standard (Svardal *et al.* 1993; Colleran *et al.* 1994). Anaerobic digestion is efficient in treating the high levels of organic matter in wastewater, and the end-products methane and granular anaerobic sludge can bring economic benefits. However, operation costs of the aerobic digestion and subsequent further treatment processes are high. Fenton's reagent, microwave radiation and emulsion liquid membrane

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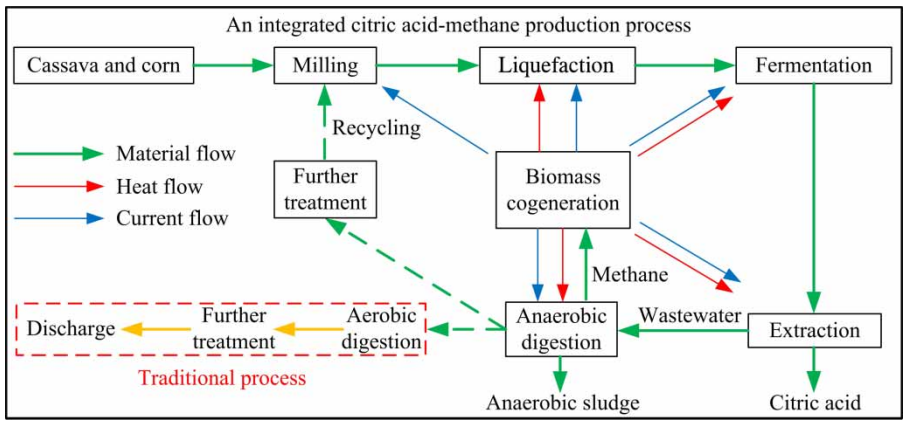

Figure 1 | Flow chart of the integrated citric acid–methane production process.

methods have been developed to treat citric acid wastewater and their COD removal rates are in the range 69–98% (Li *et al.* 2013), but these methods are complicated and consume large amounts of inorganic reagents; they are not suitable on a large industrial scale. In recent years photosynthetic bacteria and *Chlorella vulgaris* have been studied extensively for citric acid wastewater treatment (Kayombo *et al.* 2003; Li *et al.* 2013). These microorganisms can grow in the wastewater to produce proteins, vitamins and other compounds for animal feedstuff and the COD removal rates exceed 90%, but after such treatment the thalli were difficult to settle and the treated wastewater still could not achieve the national discharge standard. Thus the handling of wastewater remains a great challenge for the citric acid industry.

To solve this problem, an integrated citric acid–methane production process has been developed by our laboratory (Xu *et al.* 2015). In this process, citric acid wastewater is anaerobically digested to produce methane and the anaerobic digestion effluent (ADE) is further treated to remove inhibitors prior to recycle for the following citric acid fermentation, thereby minimizing wastewater discharge and reducing fresh water consumption (Figure 1). Previous experiments confirmed that ammonia and sodium contained in ADE are major inhibitors for the proposed process (Xu *et al.* 2015). Ammonia may be removed effectively through air-stripping, but sodium is difficult to separate and could accumulate to up to 1,000 mg/L in recycled effluent. Concentrations of sodium above 200 mg/L during fermentation result in an increase of residual total sugar and a decrease in citric acid production. Electrodialysis and nanofiltration have been used to remove excessive sodium from ADE and removal rates exceeded 90% (Xu *et al.* 2016; Zhang *et al.* 2017). However, pretreatment of ADE is needed and the proposed process is complicated and costly. In this paper, we investigated the mechanism of inhibition of citric acid fermentation by excessive sodium. Based on the results, an economical and simple approach was developed to alleviate the inhibition.

MATERIALS AND METHODS

Seed culture for citric acid fermentation

A. niger used for citric acid fermentation was provided by a citric acid plant in China. Seed culture medium for citric acid fermentation was prepared according to our previous study (Xu *et al.* 2015). Cassava powder (size approximately 0.45 mm, provided by the Henan Tianguan Co. Ltd, China) and deionized water were thoroughly mixed at 1:4 (w/v) and the pH adjusted to 6.0. The starch was then hydrolyzed with 10 U/g high-temperature amylase at 100 °C for 2 hours. Water lost during liquefaction was replaced with deionized water. The pH was adjusted to 5.5 and the mixture autoclaved at 115 °C for 20 min. After 0.1% (w/v) $(NH_4)_2SO_4$ was added to promote germination, the substrate was inoculated with 12.5% (v/v) spore suspension containing approximately 6×10^6/mL of conidia, and the seed culture medium cultivated at 200 rpm, 36 ± 1 °C, for 20–21 h.

Citric acid fermentation

The fermentation medium and operation conditions for citric acid production were as in our previous report (Xu *et al.* 2016). Aliquots of 80 g cassava powder and 20 g corn powder (size approximately 0.45 mm, provided by the Henan Tianguan Co. Ltd, China) were mixed with 450 mL process water (deionized water or ADE) and the liquefaction and autoclave operations were similar to the seed

culture medium preparation. The initial total sugar was regulated to 155–160 g/L and 15% (v/v) of the mature seed broth was inoculated. Calcium carbonate tests were conducted in 500 mL shake flask and the fermentation medium was cultivated at 260 rpm, 37.5 ± 1 °C for 92 h. Other experiments were processed in a 5 L agitator bioreactor and the fermentation medium was cultivated at 37.5 ± 1 °C for 72 h with a three-bladed impeller operating at 600 rpm and an aeration rate of 2 v/v/min. Samples were collected after 12 h and stored at −20 °C before analysis. In the pH control experiments, a peristaltic pump was used to introduce sulfuric acid or sodium hydroxide into the fermentation medium over the period 4–24 h with a constant speed of 7.5 mL/h (a total volume of 150 mL). Initial total sugar concentration was 170 g/L to ensure the normal process value of 155–160 g/L after pH adjustment.

Analytical methods

Citric acid, glucose, isomaltose and oxalic acid concentrations were determined using a Dionex U3000 high-performance liquid chromatography (HPLC) system as in our previous study (Xu *et al.* 2015). COD, volatile fatty acids (VFAs), ammonia, alkalinity, conductivity and color (410 nm) in ADE were measured according to standard methods (APHA/AWWA/WEF 1998), while metal ions were monitored using flame atomic absorption spectrometry. Activities of glucoamylase and isomaltase secreted by *A. niger* during citric acid fermentation were measured according to Xu *et al.* (2015), and superoxide dismutase (SOD) and catalase (CAT) activities of *A. niger* were measured according to Pokora *et al.* (2003) and Deng *et al.* (2010), respectively. Reactive oxygen species (ROS) generation of *A. niger* was analyzed according to Wei *et al.* (2011), while intracellular malondialdehyde (MDA) was measured according to Ohkawa *et al.* (1979). All data throughout the manuscript are shown as means or mean ± standard deviation. The data were analyzed using the one-way analysis of variance and the software package Statistica (SPSS Statistics 22.0, IBM, USA) and $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Influence of elevated sodium levels on oxidative stress in *A. niger* during citric acid fermentation

During respiratory metabolism, molecular oxygen is utilized as the terminal electron acceptor and large amounts of ROS are generated. Normally, ROS in microorganisms show a balance between generation and elimination, without accumulation. However, when microorganisms are exposed to high salinity/osmotic pressure, abundant ROS may be produced and exceed the homeostasis capacity of the cells, causing loss of function and cell aging. At the same time, levels of intracellular MDA (the end-product of membrane lipids oxidation) increase and large amounts of SOD, CAT and other antioxidant enzymes are induced (Grosicka-Maciąg *et al.* 2011).

To establish whether the increased osmotic pressure accompanying excess sodium was the reason for the inhibition of citric acid fermentation, we studied the influence of 1,000 mg/L Na^+ on oxidative stress levels in *A. niger*. The results are shown in Figure 2. Compared to the deionized water control, ROS accumulation and SOD levels were slightly elevated while no significant differences were observed for CAT. Intracellular MDA levels in *A. niger* were similar to the control for the first 48 h and then a significant increase was found, which may have been due to the complete consumption of glucose in the medium after 48 h (Xu *et al.* 2015), causing autolysis of the *A. niger* cells to begin. These results indicated that *A. niger* in citric acid fermentation was only minimally influenced by oxidative stress arising from addition of 1,000 mg/L Na^+.

Influence of elevated sodium on pH of the medium in citric acid fermentation

When cassava and corn starch are used as raw materials for citric acid fermentation, saccharification of starch occurs simultaneously with citric acid production and control of the pH is important. The optimal activity of *A. niger* glucoamylase is at pH 4.0–4.6, with a sharp decline at lower pH. The pH of citric acid fermentation is initially 5.0–6.0 to ensure the germination of *A. niger* spores, followed by a decrease to below 3.0 to reduce formation of the by-product oxalic acid (Wang & Jin 2000).

Figure 3(d) shows the influence of added sodium on pH of the medium during citric acid fermentation. In the control fermentation with deionized water, pH of the medium decreased sharply to below 3.0 during the first 24 h and then further declined slowly until the end of the fermentation. With 1,000 mg/L Na^+ added, pH declined at a rate even faster than in the control ($p < 0.05$). This might be caused by production of acidic metabolic by-products; Andersen *et al.* (2009) reported that *A. niger* could produce a range of organic acids, the type and amount of which were dependent on culture conditions. Further research is underway. As the fermentation continued, pH of the medium

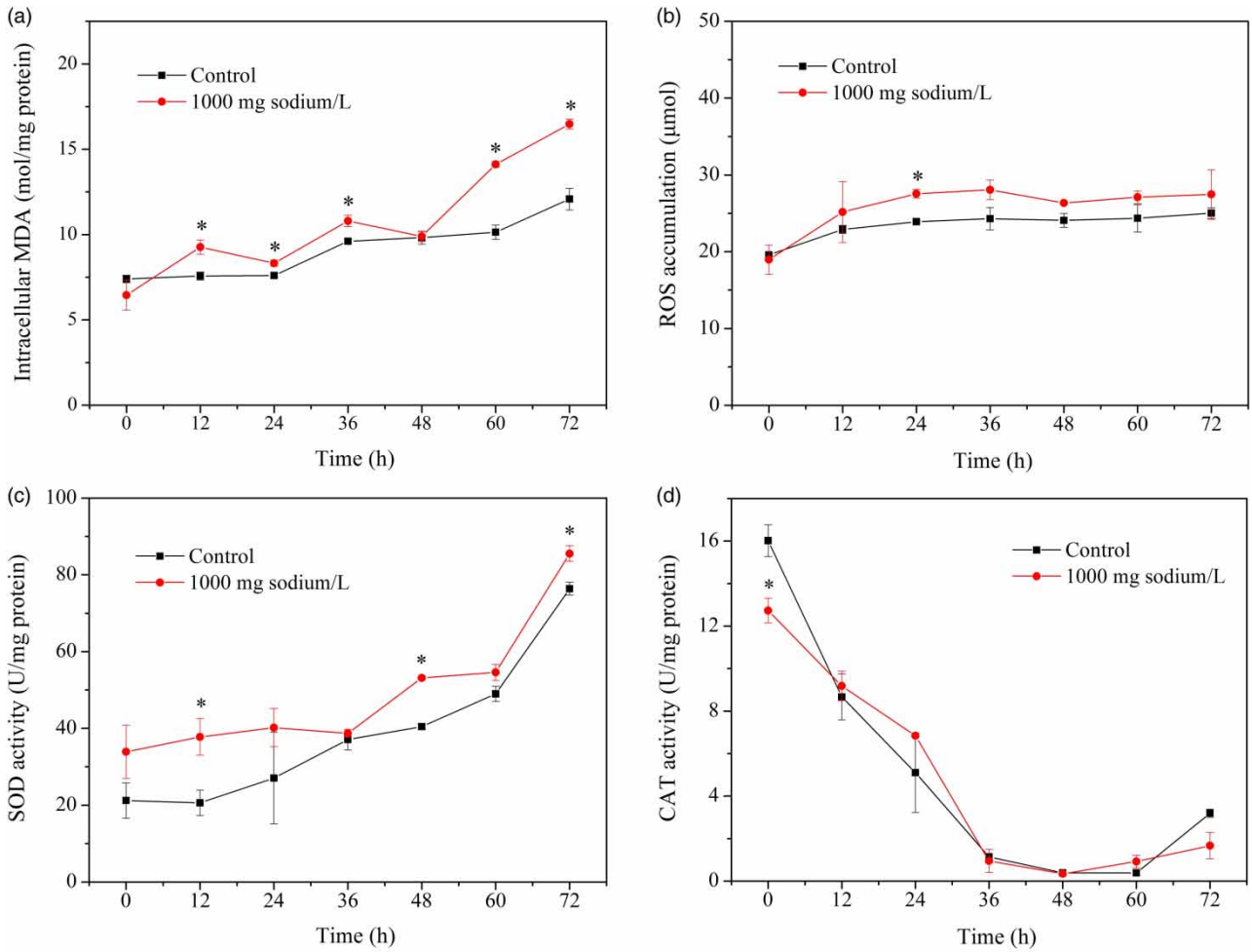

Figure 2 | Effect of added sodium on (a) intracellular MDA level, (b) ROS accumulation, (c) SOD and (d) CAT activities of *A. niger* in citric acid fermentation. Asterisk (*) indicates significant differences ($p < 0.05$) compared with the control.

declined slowly until 48 h, by which time glucose in the medium was completely consumed and little additional citric acid was produced. Previous experiments had confirmed that elevated sodium could inhibit the activities of *A. niger* glucoamylase and isomaltase, causing a reduction of the available sugar concentration and hence lower citric acid production (Xu *et al.* 2015). The influence of pH on the activities of these enzymes was investigated and the results indicated that the optimum pH values for glucoamylase and isomaltase were 5.0 and 5.5, respectively, with lower pH resulting in a sharp reduction in their activities (Figure 4). Therefore, the inhibition by excessive sodium of glucoamylase and isomaltase activities during citric acid fermentation might be due to its impact on the pH of the medium.

To test this hypothesis, deionized water was used for citric acid fermentation and sulfuric acid was fed into the system over the period 4–24 h to increase the rate of decline of the medium pH. As shown in Figure 3, citric acid production significantly decreased as the sulfuric acid concentration increased ($p < 0.05$). The fastest rate of decline of pH of the medium was achieved with addition of 50 g/L sulfuric acid, the highest level tested, when citric acid production decreased to 107.3 g/L, 24.2% less than the control (141.6 g/L). In addition, residual total sugar and residual isomaltose concentrations increased to 48.0 g/L and 18.8 g/L, respectively. These results indicated that the faster decline of medium pH in citric acid fermentation brought about by high levels of sodium substantially influences hydrolysis of isomaltose and other dextrins, giving rise to the observed decrease of citric acid production.

To further understand the inhibition of citric acid fermentation by excessive sodium, an alkaline solution was used to modify the pH during the period 4–24 h. Common

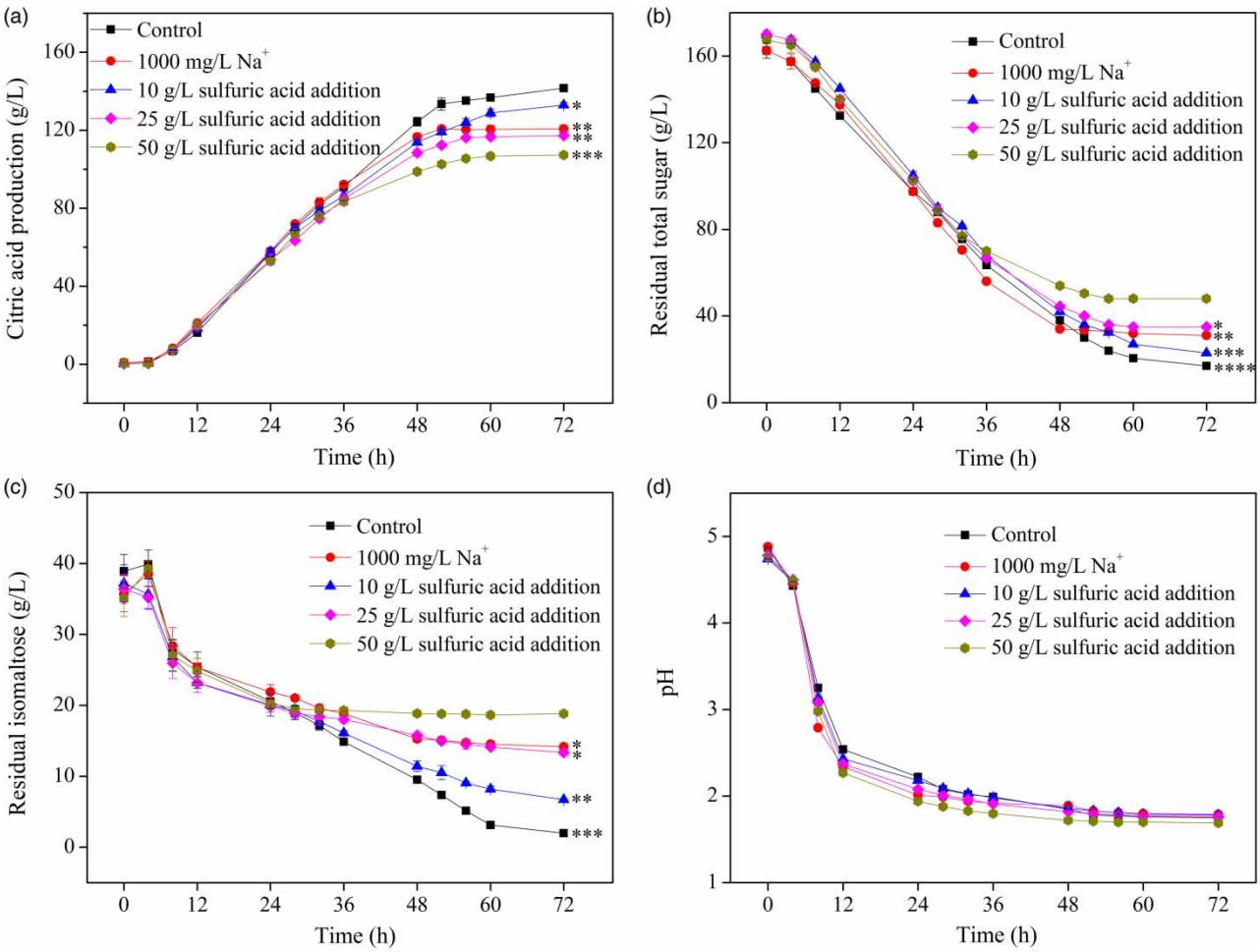

Figure 3 | Citric acid fermentation: influence of sulfuric acid addition on (a) citric acid production, (b) residual total sugar, (c) residual isomaltose and (d) pH of the medium. *, **, *** and **** are used to describe the statistical result and different symbols indicate significant difference.

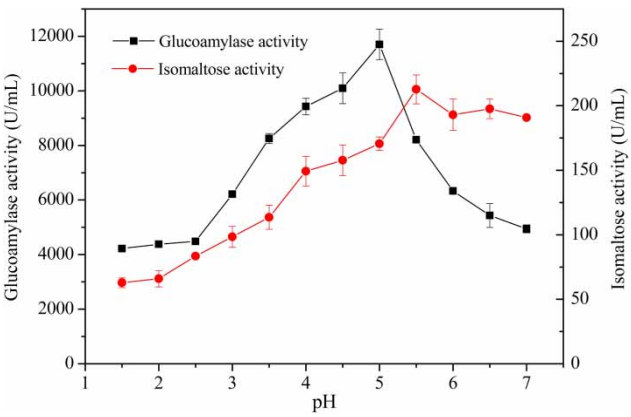

Figure 4 | Effect of pH on the activities of glucoamylase and isomaltase secreted by *A. niger*.

alkaline reagents used to adjust medium pH in the fermentation industry include ammonium hydroxide, sodium hydroxide and potassium hydroxide. Papagianni *et al.* (2005) reported that nitrogen concentrations in citric acid fermentation should be limited as excessive nitrogen could increase fungal growth and sugar consumption but decrease the amount of citric acid produced. This excluded the use of ammonium hydroxide, while potassium hydroxide is relatively expensive and additional positive ions may interfere with the result. Therefore, sodium hydroxide was chosen to adjust the pH and, if the impact of sodium on citric acid fermentation is due to factors other than the pH of the medium, the introduction of additional sodium would be expected to lead to enhanced inhibition. In fermentation tests incorporating an initial 1,000 mg/L Na^+, sodium hydroxide was constantly introduced into the system over the period 4–24 h to counter the normal drop in pH. The results are shown in Figure 5. With increasing concentration of added sodium hydroxide, the rate of decline of pH of the medium slowed down and citric acid production significantly improved, to a level comparable to the control.

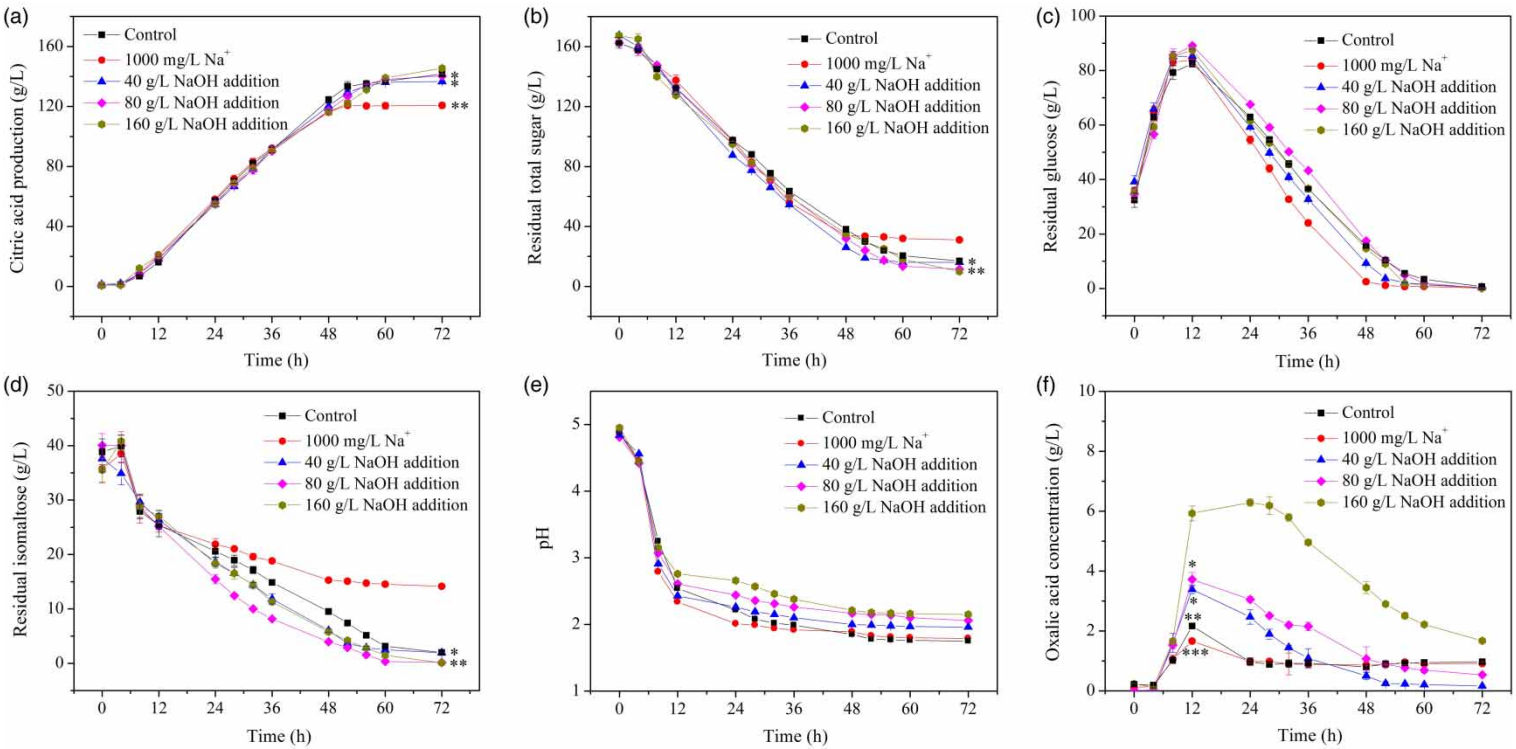

Figure 5 | Influence of sodium hydroxide addition on (a) citric acid production, (b) residual total sugar, (c) residual glucose, (d) residual isomaltose, (e) medium pH and (f) oxalic acid concentration in citric acid fermentations in the presence of 1,000 mg/L sodium. *, ** and *** are used to describe the statistical result and different symbols indicate significant difference.

In addition, marked decreases of residual total sugar and residual isomaltose concentrations were found. Thus pH control of the fermentation alleviates the inhibition and improves citric acid production. Moreover, these results also indicate that the additional inhibition caused by excessive sodium may be due to the sharper decline of medium pH during the productive stage of the fermentation. The consequent additional drop in glucoamylase and isomaltase activities would then act to reduce the hydrolysis of isomaltose and other dextrins, causing the decrease of available total sugar concentration and citric acid production.

In these sodium hydroxide feeding experiments, the residual total sugar concentration significantly decreased but the corresponding production of citric acid was not fully achieved. Dhillon *et al.* (2011) reported that oxalic acid was the main by-product in citric acid fermentation and higher initial pH resulted in its accumulation. Oxalic acid concentrations throughout the fermentation were monitored by HPLC and the results are shown in Figure 5(f). During fermentation with deionized water, oxalic acid concentration greatly increased over 12 h and then a slow decline occurred. During the initial stages of fermentation, the pH of the medium was above 3.0 and oxaloacetic acid hydrolysis had high activity, causing the accumulation of oxalic acid (Ruijter *et al.* 1999). Then the pH of the medium decreased rapidly to below 3.0 and activity of this enzyme declined sharply. Under these conditions, oxalate decarboxylase activity significantly increases and oxalic acid is converted to formic acid and carbon dioxide (Mäkelä *et al.* 2010), resulting in a reduced oxalic acid concentration. During citric acid fermentation with addition of 1,000 mg/L Na^+, pH of the medium and oxaloacetic acid hydrolysis activity declined faster than in the control. The highest oxalic acid concentration was 1.7 g/L, lower than in the fermentation with deionized water (2.2 g/L). With introduction of 40, 80 and 160 g/L sodium hydroxide, the decline of fermentation medium pH and of oxaloacetic acid hydrolysis activity was slowed down and the highest oxalic acid concentrations were 3.4, 3.7 and 6.3 g/L respectively. These results indicate that the activity of oxaloacetic acid hydrolysis was maintained by sodium hydroxide addition and as a consequence some available sugar was converted to oxalic acid by-product, causing the observed slight decrease of citric acid production.

Effects of calcium carbonate on citric acid fermentation with addition of elevated sodium

Control of pH using alkaline solution is cheap and simple in operation compared with electrodialysis and nanofiltration. However, this method consumes large amounts of inorganic reagents and also introduces high concentrations of cations, which might accumulate during recycling and influence the stability of the proposed process. Calcium carbonate is a common and low-cost raw material which may be used to adjust the fermentation medium pH. As precipitation with calcium is the normal industrial method for recovering the citric acid product (Pazouki & Panda 1998), the addition of calcium carbonate to eliminate the inhibition caused by excessive sodium should cause few problems.

As shown in Figure 6, with both 1,000 mg/L Na^+ and calcium carbonate added at the beginning of the fermentation, citric acid production significantly improved as the amount of calcium carbonate increased to 16–20 g/L, while residual total sugar and isomaltose declined. Final pH in the medium continually increased and oxalic acid concentration remained at low levels which may be because calcium oxalate precipitated (Bao 2017). With calcium carbonate addition at above 20 g/L, citric acid fermentation was reduced, with large amounts of total sugar and glucose remaining in the medium, while the morphology of *A. niger* changed and it began to autolyze. The highest citric acid production (139.7 ± 0.5 g/L) was achieved with 16–20 g/L calcium carbonate addition, an increase of 16.8% compared with fermentation with 1,000 mg/L Na^+ alone (119.6 g/L) and approaching the amount found after fermentation with deionized water alone (141.3 g/L). Meanwhile, residual total sugar decreased to 13.4 ± 0.9 g/L, which was well below the control (18.5 g/L). However, part of the available sugar may have been converted to oxalic acid because of the higher pH of the medium (Ruijter *et al.* 1999). These results indicate that pH control using appropriate levels of calcium carbonate is an effective method to counteract the inhibition caused by excessive sodium.

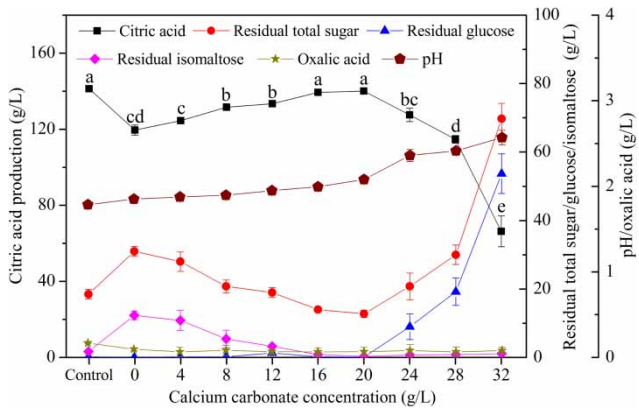

Figure 6 | Effect of calcium carbonate on citric acid fermentation with addition of 1,000 mg/L sodium. Different letters indicate significant differences ($p < 0.05$).

Effects of added calcium carbonate on citric acid fermentations employing recycled ADE

ADE from a commercial plant was recycled as process water for citric acid fermentation. Its chemical characteristics are shown in Table 1. Ammonia level was slightly above the critical level according to our previous work (Xu *et al.* 2015). Metal ions except Na^{+}, Mg^{2+} and Zn^{2+} were below the reported inhibitory concentrations and would not affect the fermentation (Xu *et al.* 2015, 2016; Zhang *et al.* 2017). Employing ADE (Figure 7), citric acid production was 118.6 g/L, reduced by 16.0% compared with the control (141.2 g/L). Glucose in the medium was completely consumed and residual total sugar and isomaltose concentrations were 33.0 g/L and 17.6 g/L respectively, which were much higher than after fermentation with deionized water (17.5 g/L and 1.9 g/L, respectively). After adding 18 g/L calcium carbonate to adjust the pH of the medium at the start of fermentation, citric acid production increased to 138.1 g/L, only slightly lower than the control, while glucose and isomaltose were completely consumed and residual total sugar significantly decreased to 14.0 g/L. In other experiments, ADE was air-stripped to eliminate the excessive ammonia, and Mg^{2+} and Zn^{2+} removed prior to recycle. Under these conditions citric acid production improved to 125.5 g/L. On addition of 18 g/L calcium carbonate, citric acid production increased markedly to 141.4 g/L, comparable to the fermentation with deionized water. Thus the combination of air-stripping and pH control using calcium carbonate effectively alleviated the inhibition caused by ADE, permitting unrestricted citric acid production and indicating the technical feasibility of the proposed process.

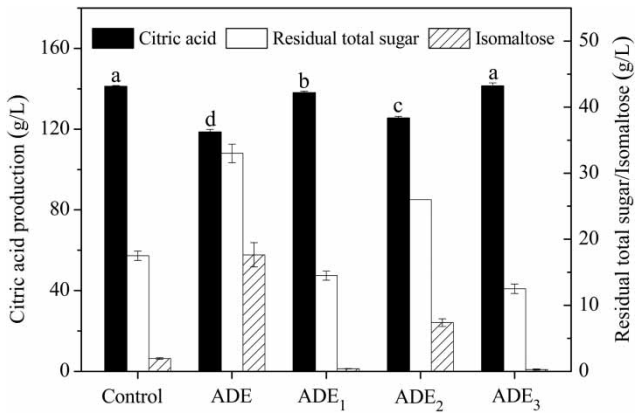

Figure 7 | Citric acid fermentation with recycled ADE as process water. ADE_1: 18 g/L calcium carbonate added at the start of fermentation; ADE_2: ADE treated by air-stripping; ADE_3: ADE treated by air-stripping and addition of 18 g/L calcium carbonate at the start of fermentation. Different letters indicate significant differences ($p < 0.05$).

Table 1 | Chemical characteristics of the ADE using for citric acid fermentation

Parameters	ADE	ADE[a]	Potential inhibitory concentration
pH	7.73	9.39	No data
COD (mg/L)	758.4	531.2	No data
VFAs (mg/L)	142.7	95.8	>480 mg/L (calculating as acetic acid)
Ammonia (mg/L)	139.6	45.2	>50 mg/L
Alkalinity (mg $CaCO_3$/L)	2,788	1,870	No data
Conductivity (μS/cm)	4,520	2,480	No data
Color (410 nm)	0.607	0.514	No data
Na (mg/L)	575.00	562.25	>200 mg/L
K (mg/L)	151.75	143.42	>300 mg/L
Mg (mg/L)	47.62	33.50	>40 mg/L
Ca (mg/L)	185.38	26.75	>250 mg/L
Zn (mg/L)	0.82	0.25	>0.6 mg/L
Fe (mg/L)	0.56	0.38	>3.0 mg/L
Cu (mg/L)	0.68	0.22	>8.0 mg/L
Mn (mg/L)	0.12	N.D.[b]	>0.5 mg/L

[a]ADE after air-stripping; [b]not detected.

CONCLUSIONS

In this study, a novel integrated citric acid–methane production process was improved. Citric acid wastewater was treated by anaerobic digestion and air-stripping prior to reuse as process water for the next batch of citric acid fermentation with calcium carbonate addition at the start to control the pH of the medium. The inhibition caused by ADE was effectively counteracted and citric acid production approached the level found for fermentation with deionized water. Application of the improved process could minimize wastewater discharge and reduce fresh water consumption in the citric acid industry.

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