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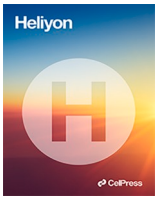

Research article

High-yield production of 5-keto-D-gluconic acid via regulated fermentation strategy of *Gluconobacter oxydans* and its conversion to L-(+)-tartaric acid

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ABSTRACT

Herein, we propose the production of 5-keto-D-gluconic acid (5KGA) by fermentation using *Gluconobacter oxydans* (*G. oxydans*) as the starting strain, from an initial concentration of 100 g/L glucose as substrate and the chemical conversion of 5KGA to L-(+)-tartaric acid (L-TA). The results show the efficacy and feasibility of two-stage pH (5.50→natural) linkage ventilation (0.5 vvm and 1.0 vvm, L/L/min) control of batch fermentation for 5KGA production. The final 5KGA yield of 100.2 g/L of 1.0 vvm is much higher than 0.5 vvm with an average productivity of 1.95 g/L/h. Changing the method of fermentation from batch to fed-batch can efficently prolong the high activity of *G. oxydans* for 5KGA production with an increased average productivity of 3.10 g/L/h, and the conversion rate of glucose to 5KGA is 92.50 %. The chemical conversion of 5KGA to L-TA catalyzed by metal ions *in vitro* indicates that the optimal catalyst is Cu^{2+} with a conversion rate of 35.09 % of 5KGA to L-TA. Our method can provide a practical and effective alternative for the industrial production of 5KGA and its conversion to L-TA.

1. Introduction

L-(+)-tartaric acid (L-TA), an organic acid found in plants like tamarinds and grapes, has numerous applications in the food, textile, chemical, pharmaceutical, and building industries [1]. Currently, the production methods of L-TA mainly include direct extraction [2], chemical resolution [3], enzymatic synthesis [4] and hyphenated biological fermentation-chemical conversion. In the past decades, enzymatic synthesis by the stereospecific hydrolase of *cis*-epoxysuccinate as substrate was the main way to produce L-TA due to the low efficiency of direct extraction, high cost of chemical resolution, and immature development state of hyphenated biological fermentation-chemical conversion. However, due to the lack of energy sources and the increasing severity of environmental problems in recent years, researchers have had to seek ways to utilize renewable resources. Fortunately, hyphenated biological fermentation-chemical conversion from glucose as a starting substrate has solved the dependence of enzymatic production on *cis*-epoxysuccinate derived from the petrochemical-based precursor maleic anhydride, thus making this production process again become a research hotspot for many scholars.

The synthesis of L-TA by the combination of hyphenated biological fermentation-chemical conversion can be carried out in two steps. Firstly, for biological fermentation, *G. oxydans* catalyzes the oxidation of glucose to gluconic acid and subsequently to 2-keto-D-

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gluconic acid (2KGA) or 5-keto-D-gluconic acid (5KGA), a promising precursor to L-TA. Secondly, for chemical conversion, a noble metal catalyzes the conversion of 5KGA to L-TA. In previous reports, scholars mainly focused on the genetic engineering modification of *G. oxydans* to enhance the 5KGA yield, like expressing gluconate:NADP 5-oxidoreductase, knocking out gluconate-2-dehydrogenase to inactive 2KGA production [5–8] and an efficient conversion route of 5KGA to L-TA involving nitric acid, ammonium vanadate and $CuSO_4{\cdot}5H_2O$ as catalysts [9–11], thus increasing the yield of L-TA. However, in addition to genetic modifications, the effective regulation of microbial fermentation strategy is also an important way to improve the production performance of microbial strains in industrial biotechnologies. Several experiments have been conducted to optimize the fermentation conditions of 5KGA production by *G. oxydans*, such as carbon and nitrogen sources, pH, dissolved oxygen (DO), or fed-batch; nonetheless, these parameters were studied separately and rarely in a systematic association [12–14]. Qazi et al. [15] found that pH 5.50 was the most conducive condition to the growth of *G. oxydans* in the culture process. This pH is also optimal for glucose dehydrogenase to oxidize glucose to gluconic acid [16]. In addition, Olijve and Kok [17] confirmed that the ability of *G. oxydans* to produce metabolites was closely related to dissolved oxygen (DO) in the medium. Therefore, in this study, instead of evaluating these conditions separately, two-stage pH linkage different ventilation (0.5 and 1.0 vvm) and fermentation ways (batch and fed-batch fermentation) were associated to investigate the effect of fermentation conditions on the production of 5KGA by *G. oxydans*. To set the two-stage pH conditions were implemented, in the first stage, the pH was set at 5.50 to ensure that *G. oxydans* can maximize the use of glucose for growth and oxidation to gluconic acid; in the second stage, the conversion of gluconic acid to 5KGA was allowed to control pH naturally. Meanwhile, the catalytic conversion of 5KGA to L-TA by different metal ions was investigated to determine the optimal catalyst.

2. Materials and methods

The *G. oxydans* CM1, a mutant which lacks gluconate-2-dehydrogenase, thus producing 5KGA but not 2KGA from glucose, was provided by Prof. Tan of Tianjin University of Science and Technology. Yeast powder and peptone were sourced from OXOID (UK). Potassium 5-keto-D-gluconate and L-TA were purchased from Sigma-Aldrich (USA). Glucose, mannitol, monopotassium phosphate (KH_2PO_4), ammonium chloride (NH_4Cl), magnesium sulfate pentahydrate ($MgSO_4{\cdot}5H_2O$), calcium carbonate ($CaCO_3$), copper sulfate ($CuSO_4$), zinc sulfate ($ZnSO_4$), ferric sulfate ($Fe_2(SO_4)_3$) and manganese sulfate pentahydrate ($MnSO_4{\cdot}5H_2O$) were all acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ammonium metavanadate was obtained from Shanghai Makclin Biochemical Co., Ltd. (China).

2.1. Seed culture and reactor operation

G. oxydans CM1 was routinely cultivated in the seed medium (mannitol 25 g/L, yeast extract 5 g/L, peptone 3 g/L) at 30 °C until the late exponential period. Subsequently, 300 mL of seed culture was inoculated into a 5 L automatic fermenter containing 3 L fermentation medium (glucose 100 g/L, corn powder 3 g/L, yeast extract 1.67 g/L, NH_4Cl 1.50 g/L, KH_2PO_4 0.10 g/L, $MgSO_4{\cdot}5H_2O$ 0.50 g/L, $CaCO_3$ 0.34 g/L) and incubated at 30 °C, 600 rpm. The experimental parameters including glucose concentration, 5KGA yield, DO, pH, dry cell weight (DCW), and productivity of 5KGA were recorded or analyzed to investigate the effects of different subsequent fermentation strategies on 5KGA production by *G. oxydans* (I: batch fermentation without pH control linkage ventilation of 0.5 vvm; II: batch fermentation with two-stage pH control linkage ventilation of 0.5 vvm; III: batch fermentation with two-stage pH control linkage ventilation of 1.0 vvm; IV: fed-batch fermentation with two-stage pH control linkage ventilation of 1.0 vvm).

2.1.1. Determination of glucose concentration

A volume of 5 mL fermentation broth was sampled and then centrifuged at 10,000 rpm for 10 min. The supernatant was diluted appropriately to determine the glucose concentration by a biosensor analyzer (SBA-40C, China).

2.1.2. Determination of 5KGA

The 5KGA was determined as described previously using an Agilent 1100 high-performance liquid chromatography system in conjunction with a Thermo BDS C18 (4.6 mm × 250 mm) reverse phase column with a mobile phase of 10 mmol/L $HClO_4$ at the flow rate of 0.5 mL/min [5]. The parameters were set to a column temperature of 25 °C, detection wavelength of 210 nm, and injection size of 20 μL.

2.2. Determination of pH and DO

The pH and DO were recorded online by the corresponding electrodes. DO for 100 % was fermented for 0 h after inoculation, and DO for 0 % was determined in saturated sodium sulfite before fermentation.

2.3. Determination of DCW

The DCW was determined by the drying weighing method. 5 mL of fermentation broth was sampled to filter with a piece of filter paper, then washed with distilled water twice, dried and weighed. DCW (g/L) = $(m_1\text{-}m_2)/v \times 1000$, where m_1 was the weight of drying filter paper after filtration (g), m_2 was the weight of drying filter paper before filtration (g), and v was the volume of sampled broth (mL).

2.4. Effect of different metal ions on the conversion of 5KGA to L-TA

A 500 mL triangulated flask was loaded with 50 mL fermentation broth containing 5KGA (100 g/L), 9 mL carbonate buffer solution (3 mol/L, pH = 10.0) and 0.2 mL sulfate catalyst solution (10 g/L) of different metal ions ($MgSO_4$, $CuSO_4$, $ZnSO_4$, $Fe_2(SO_4)_3$, $MnSO_4$). The mixture was reacted at 30 °C, 180 rpm for 72 h. During the process, K_2CO_3 (2 mol/L)-KOH (2 mol/L) solution was added to maintain pH 9.60 or above. After reaction, centrifugation was conducted at 10,000 rpm for 10 min and the precipitation was abandoned. The content of L-TA in the supernatant was determined by ammonium metavanadate colorimetry to select the optimal catalyst, and the catalyst was amplified in a 5-L bioreactor (liquid volume 2.5 L).

3. Results and discussion

3.1. Batch fermentation without pH control linkage ventilation of 0.5 vvm

The entire fermentation process occurred with natural pH control and ventilation control at 0.5 vvm, and timed samples were taken for the determination of the relevant parameters mentioned above. As shown in Fig. 1, the pH of the fermentation broth dropped from an initial 5.84 to 4.82 at 9 h and to 2.19 at 12 h, and then remained around 2.10. Meanwhile, the glucose concentration was dropped from an initial 100 g/L to 21 g/L at 12 h and remained at 10 g/L at 20 h without further decreasing. As for DO, it fell rapidly at first then rose sharply. This was due to the pH being suitable for the growth of *G. oxydans* at the early stage, with vigorous metabolism and large demand for oxygen consumption. At the later stage, the glucose was continuously converted into gluconic acid, resulting in pH decrease and stabilization at 2.10, which inhibited the growth of *G. oxydans* and lowered the demand for oxygen consumption. The DCW changed very little during the fermentation process, and the highest value was only 0.36 g/L at 18 h. The content of 5KGA was also determined but none was detected. The results indicated that the growth of *G. oxydans* was significantly inhibited in the acidic environment, with a lower pH even being harmful to *G. oxydans*, such that it could not continuously consume glucose to maintain growth for the synthesis of 5KGA.

3.2. Batch fermentation with two-stage pH control linkage ventilation of 0.5 vvm

In this process, the fermentation strategy was changed to two-stage pH control linkage ventilation of 0.5 vvm. As shown in Fig. 2a, the glucose concentration decreased continuously from an initial 100 g/L to 18 g/L at 32 h and it depleted at 35 h after an average consumption rate of 2.85 g/L/h. The initial pH of fermentation broth was 5.80, which dropped to 5.55 at 6 h; at this time, feeding alkali KOH was added automatically to keep the pH constant at 5.50 upon entering the first stage. At 35 h, the glucose concentration was exhausted to 0 g/L and KOH was no longer added for the pH to change naturally, which entered the second stage. Soon afterwards, the pH showed a rising trend, which was due to the fermentation broth no longer containing glucose, so that no gluconic acid was produced, and the existing gluconic acid was continuously converted into 5KGA. Additionally, as there was a large amount of potassium ion being fed into the solution, it gradually combined with 5KGA to form a strong base and weak acid salt, making the pH rise and finally stabilize between 7.50–8.00. With the consumption of glucose, the DO of fermentation broth decreased sharply and dropped to 2.1 % at 44 h, while the DCW increased rapidly, reaching a maximum of 3.0 g/L 5KGA was detected at 35 h and the final yield reached 101 g/L at the end of fermentation. The maximum and overall 5KGA productivity was 3.88 g/L/h at 42 h and 1.83 g/L/h, respectively

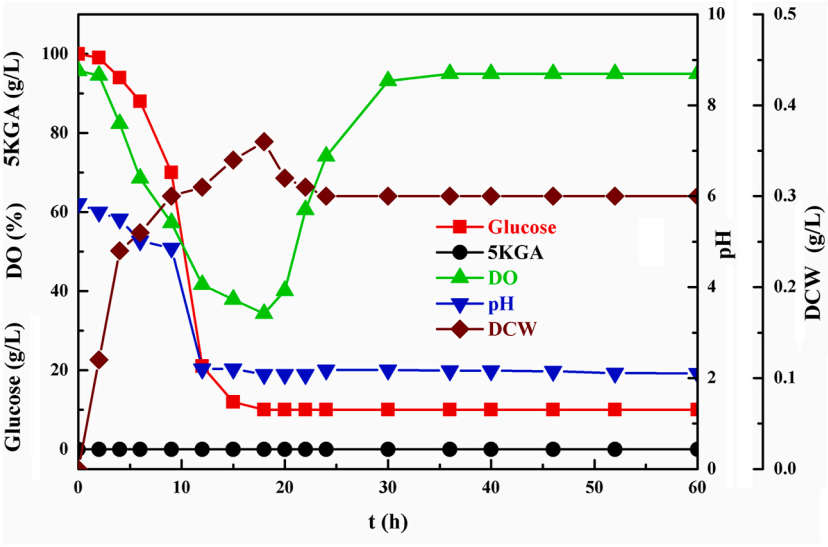

Fig. 1. Production of 5KGA by batch fermentation without pH control.

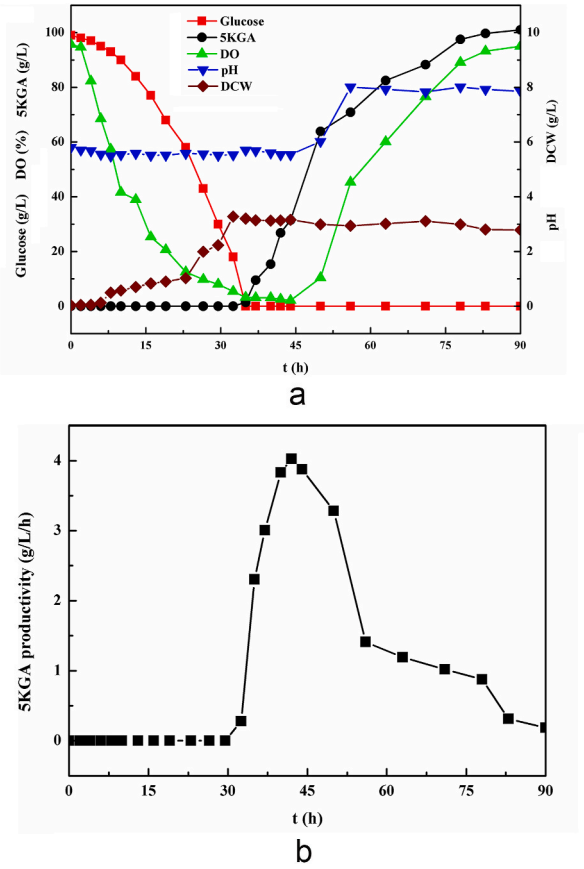

Fig. 2. Production of 5KGA by batch fermentation with two-stage pH linkage ventilation of 0.5 vvm (a) and curve of 5KGA productivity rate with fermentation time (b).

(as shown in Fig. 2b). Ano et al. [18] proved that the effect of pH on the formation of 5KGA was correlated with the pH dependency with glycerol dehydro genase, a membrane-bound quinoprotein, and also confirmed that pH 5.50, is a condition favorable to 5KGA production.

3.3. Batch fermentation with two-stage pH control linkage ventilation of 1.0 vvm

Olijve and Kok [15] confirmed that the ability of *G. oxydans* to produce metabolites is closely related to DO in the medium; therefore, ventilation was increased to 1.0 vvm. As shown in Fig. 3a, the glucose consumption, 5KGA production and growth of *G. oxydans* were all changed significantly. The glucose exhausted in just 11 h for entering the second stage of fermentation. At this moment, the pH was controlled naturally, then gradually rose to 7.90, and finally remained stable between 7.50 and 8.00, which was consistent with the results for 0.5 vvm but with significantly improved efficiency. It only took 9 h for 5KGA generation to begin, and the maximum productivity reached 8.6 g/L/h at 10.5 h (as shown in Fig. 3b). At 60 h, the yield of 5KGA exceeded 100 g/L and the overall average productivity was 1.95 g/L/h. Klasen et al. [19] pointed out that the optimal pH of gluconate oxidation was slightly alkaline, consistent with our result. Compared with 0.5 vvm, the glucose consumption rate was significantly accelerated and the growth and metabolism of *G. oxydans* were also enhanced, thus the fermentation time was significantly shortened and the average productivity was increased by 6.56 % after increasing the ventilation to 1.0 vvm. However, the final yield of 5KGA was not enhanced and subjected to a conservation of mass. Therefore, the oxidation of glucose to 5KGA in cells could be improved by two-stage pH and continuous high oxygen supply, which significantly increased the production efficiency of 5KGA. This was due to the metabolism of glucose in *G. oxydans* being a close-coupling bio-oxidation reaction of the dehydrogenation and the cellular respiration chain depending heavily on the oxygen supply. Glucose is first oxidized to be released and form gluconate, then converts to 5KGA by membrane-bound and soluble dehydrogenases. The electrons stored in the coenzyme (NADH/NADPH) are transferred forwards to the oxygen that was the terminal electron acceptor by a series of transporter complexes constituting the respiration chain. Specifically, oxygen is a final driving force running the serial oxidation reactions that maintain a continuous bioconversion process for catalyzing glucose to 5KGA [7,8]. Thus, it can be concluded that the efficient bio-oxidation of sugar depends on sufficient oxygen supply or a proper oxygen supply approach [5].

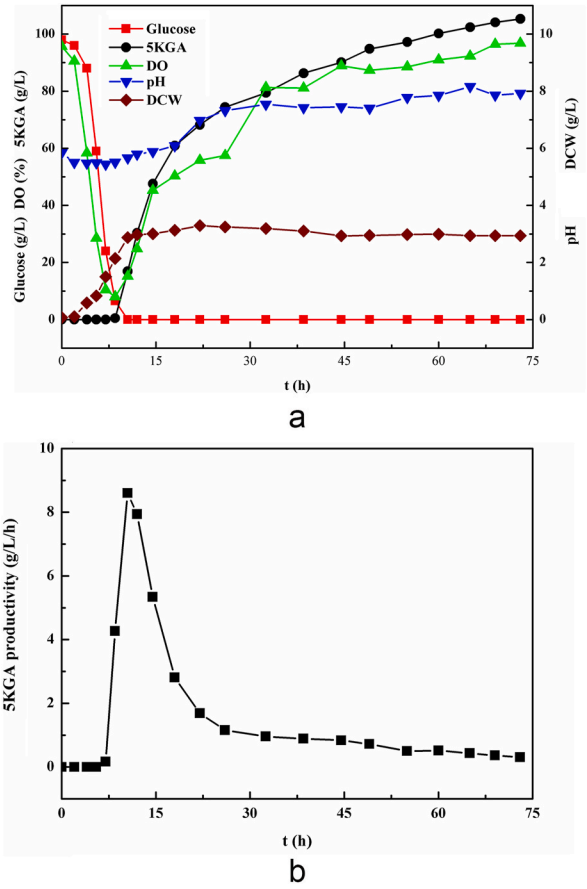

Fig. 3. Production of 5KGA by batch fermentation with two-stage pH linkage ventilation of 1.0 vvm (a) and curve of 5KGA productivity rate with fermentation time (b).

3.4. Fed-batch fermentation with two-stage pH control linkage ventilation of 1.0 vvm

By changing the fermentation method from batch to fed-batch, real-time monitoring of the parameters changes becomes possible. As shown in Fig. 4a, the glucose concentration decreased to 10 g/L at 9 h. At this time, 400 mL of solution containing 240 g glucose was added into the fermentation tank at a rate of 6 mL/min for feeding. Next, the glucose concentration was determined at 50 g/L at 11 h and depleted again at 19 h. Similarly, the pH was controlled at 5.50 in the first stage and KOH was no longer added to maintain the pH upon entering the second stage when glucose was exhausted. Ultimately, the pH also rose and remained stable between 7.50–8.00, and 5KGA was also generated after glucose depletion. The maximum productivity reached 6.84 g/L/h at 23 h, and the yield exceeded 100 g/L at 51 h. The overall average productivity was 3.1 g/L/h (as shown in Fig. 4b), which was 1.60 times that of batch fermentation. At the end of fermentation, the yield of 5KGA reached 179.4 g/L and the conversion rate of glucose reached 92.5 %. Elfari et al. [20] developed a strain of *G. oxydans* MF1 to generate 5KGA, and the glucose conversion rate was 84 %. The *G. oxydans* CM1 strain used in this study was 10.11 % more efficient than *G. oxydans* MF1 in converting glucose to 5KGA. In the fed-batch, it could achieve high density and make full use of the high vitality of cells, so as to maintain high production efficiency and reduce the fermentation cost.

3.5. Effects of different metal ions on the conversion of 5KGA to L-TA

As can be seen in Fig. 5, using different metal ion catalysts resulted in significant differences on the yield of L-TA in the shake-flask test. Among them, when $CuSO_4$ was the catalyst, the yield of L-TA was at a maximum of 20.5 g/L, followed by $MnSO_4$ and $Fe_2(SO_4)_3$. While $ZnSO_4$ and $MgSO_4$ were used as catalysts, the yield of L-TA was approximate without significant difference and also less than $CuSO_4$. Therefore, $CuSO_4$ was selected as the optimal catalyst for the next amplification reaction, in line with the data reported by Yuan et al. [11].

While using $CuSO_4$ as a catalyst, the chemical synthesis of L-TA from 5KGA was amplified in a 5 L bioreactor. As shown in Fig. 6, after initiating the reaction, the concentration of L-TA gradually increased and that of 5KGA decreased from 102.5 g/L to 0 g/L within 32 h; at the same time, the concentration of L-TA increased to 22.95 g/L and then not much further. The final concentration of L-TA was 27.81 g/L after 90 h. The conversion rate of 5KGA to L-TA was calculated as 35.09 %, and the conversion rate of glucose to L-TA was

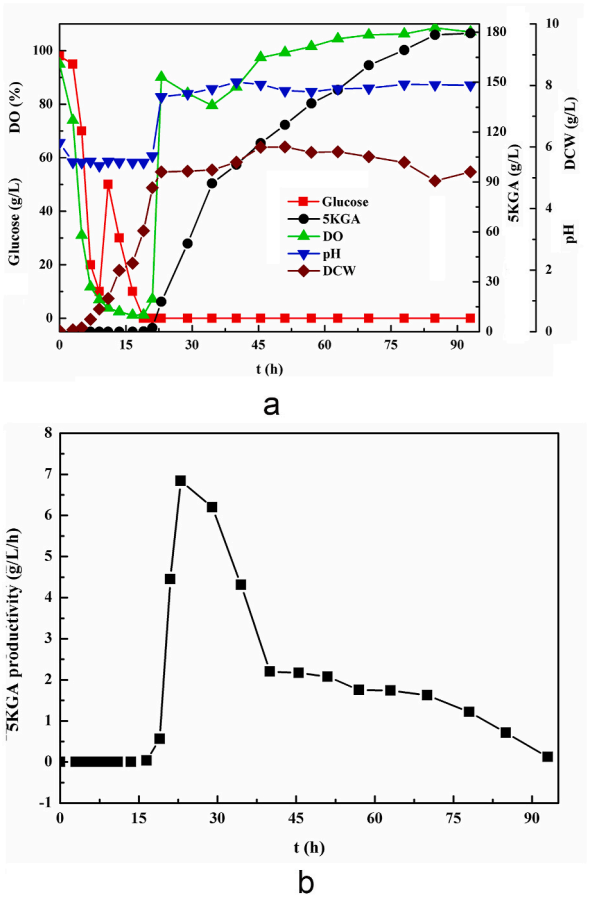

Fig. 4. Production of 5KGA by fed-batch fermentation with two-stage pH linkage ventilation of 1.0 vvm (a) and curve of 5KGA productivity rate with fermentation time (b).

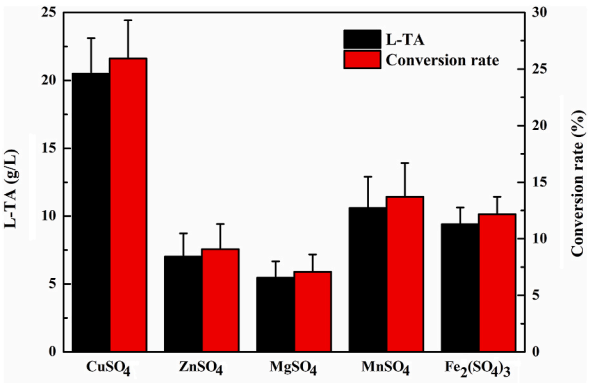

Fig. 5. Effect of different metal ion catalysts on the production of L-TA.

33.37 %. Yuan et al. [11] pointed out that oxygen plays an important role in the process of catalyzing 5KGA to L-TA. The assumed mechanism is that 5KGA in the carbonate buffer solution can be rapidly converted to dienols, after which a complex of Cu^{2+} as central ion and two 5KGA molecules as ligands were formed. This is followed by reaction with oxygen resulting in the formation of an oxygen-Cu^{2+}-5KGA complex to converted to L-TA in the same manner as oxidation by ascorbic acid. However, the conversion rate obtained with $CuSO_4$ was only 35.09 %, thus further study is required to explore this mechanism.

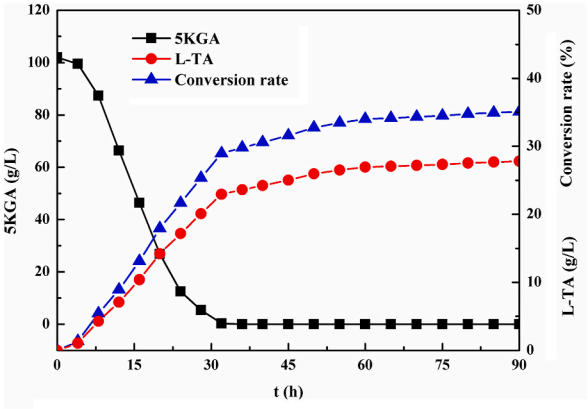

Fig. 6. Curve of L-TA yield with reaction time by $CuSO_4$ as catalyst.

4. Conclusions

In this paper, the biological fermentation strategy of 5KGA and its chemical conversion to L-TA was investigated. As expected, the fermentation yield of 5KGA by *G. oxydans* was closely related to fermentation pH, DO and the applied method. Our fermentation strategy of two-stage pH control linkage ventilation fermentation for the production of 5KGA was demonstrated to be effective and feasible, as it could significantly improve yield and shorten the fermentation time of 5KGA. Changing the fermentation method from batch to fed-batch could prolong and maintain a high production efficiency and reduce the fermentation cost. *In vitro*, the optimal conversion catalyst of 5KGA to L-TA by chemical metal ions was determined as Cu^{2+}. Further studies should focus on the low conversion rate of 5KGA to L-TA or screen microbial strains to explore the replacement of chemical catalysis by biological pathways, so as to realize the complete bio-manufacturing process of L-TA.

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Data availability statement

Data associated with our study has not been deposited into a publicly available repository and data will be made available on request.

CRediT authorship contribution statement

Zhicun Sheng: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Yanyan Li:** Writing – review & editing, Supervision, Project administration, Data curation. **Jing Wang:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

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

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