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Research article

Removing the by-products acetic acid and NH_4^+ from the L-tryptophan broth by vacuum thin film evaporation during L-tryptophan production

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ABSTRACT

Background: During L-tryptophan production by *Escherichia coli*, the by-products, acetic acid and NH_4^+, accumulate in the fermentation broth, resulting in inhibited cell growth and activity and decreased L-tryptophan production. To improve the L-tryptophan yield and glucose conversion rate, acetic acid and NH_4^+ were removed under low-temperature vacuum conditions by vacuum scraper concentrator evaporation; the fermentation broth after evaporation was pressed into another fermenter to continue fermentation. To increase the volatilisation rate of acetic acid and NH_4^+ and reduce damage to bacteria during evaporation, different vacuum evaporation conditions were studied.
Results: The optimum operating conditions were as follows: vacuum degree, 720 mm Hg; concentration ratio, 10%; temperature, 60°C; and feeding rate, 300 mL/min. The biomass yield of the control fermentation (CF) and fermentation by vacuum evaporation (VEF) broths was 55.1 g/L and 58.3 g/L at 38 h, respectively, (an increase of 5.8%); the living biomass yield increased from 8.9 (CF) to 10.2 pF (VEF; an increase of 14.6%). L-tryptophan production increased from 50.2 g/L (CF) to 60.2 g/L (VEF) (an increase of 19.9%), and glucose conversion increased from 18.2% (CF) to 19.5% (VEF; an increase of 7.1%). The acetic acid concentrations were 2.74 g/L and 6.70 g/L, and the NH_4^+ concentrations were 85.3 mmol/L and 130.9 mmol/L in VEF and CF broths, respectively.
Conclusions: The acetic acid and NH_4^+ in the fermentation broth were quickly removed using the vacuum scraper concentrator, which reduced bacterial inhibition, enhanced bacterial activity, and improved the production of L-tryptophan and glucose conversion rate.

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1. Introduction

L-Tryptophan is an essential amino acid for humans and animals and has been widely used in the food, medicine, and feed industries [1,2,3]. Presently, microbial fermentation is the first choice for the large-scale production of L-tryptophan, and L-tryptophan production by *Escherichia coli* fermentation has been studied in depth. Acetic acid is a primary inhibitory metabolite in *E. coli* cultivation and is detrimental to bacterial growth and formation of desired products [4,5]. The key to high production of L-tryptophan is controlling the production of acetic acid. The detailed mechanism underlying the effect of acetic acid on cell growth inhibition is not clear; it might inhibit the synthesis of DNA, RNA, proteins, or lipids. A high concentration of acetic acid (more than 5 g/L) will reduce the growth rate and L-tryptophan yield [6,7]. Two strategies have been applied to control acetic acid formation during L-tryptophan production by *E. coli* fermentation. One is through metabolic engineering to reduce carbon flow to the acetate biosynthesis pathway. The elimination of phosphotransacetylase, acetate kinase, and pyruvate oxidase B activities in *E. coli* has been found to result in a significant reduction in acetate accumulation [8,10,11]. The other strategy is through fermentation optimization to control the specific growth rate and residual glucose concentration. By adjusting the feeding rate of nutrition based on dissolved oxygen (DO) and pH value in fed-batch fermentation processes, the concentration of acetic acid can be maintained below a certain critical inhibitory value [4,12,13,14,15].

Ammonium hydroxide is commonly used as a nitrogen source and neutralising reagent in the industrial production of amino acids by

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bacterial fermentation. However, excessive accumulation of NH_4^+ has a negative effect on cell growth and L-tryptophan production [14]. Moreover, the concentrations of alanine, lactic acid, and acetic acid increase, and plasmid stability decreases with increasing NH_4^+ concentration [14]. Because the bacterial cell membrane is highly permeable to NH_4^+, the excessive uptake of NH_4^+ and the extrusion of protons may increase the specific rate of oxygen consumption, which results in energy deficiency and functional degeneration [16]. Therefore, it is critical to reduce the accumulation of NH_4^+ in L-tryptophan production.

In our previous studies, the by-products were reduced through genetic modification and fermentation process control [8,9,10,11,12,13,14]. However, we found that in the late stage of L-tryptophan production, the concentration of the by-products gradually increased and reached an inhibitory level, which resulted in lower L-tryptophan yield and productivity. To reduce the accumulation of metabolic by-products during fermentation and mitigate the inhibitory effects of the by-products on the synthesis of the target products, cell recycling technology was recently used in multi-batch fermentation [17,18,19]. With this cell recycling strategy, L-tryptophan production and the glucose conversion rate increased to 55.12 g/L and 19.75%, respectively, 17.55 and 10.77% higher than those without cell recycling [19]. However, cell recycling fermentation technology has a long cycling period, and a certain amount of damage to the fermentation strains is caused during the process. With the increase in shear force during centrifugation, cell activity gradually decreases [20,21]. Moreover, the disc centrifuge is required to be airtight, leading to large investment in equipment and high operating costs, which is a problem for industrial production of the target metabolite. To solve the aforementioned problems, the present study focuses on the use of vacuum thin film evaporation technology to reduce the acetic acid and NH_4^+ produced in L-tryptophan production. As acetic acid and NH_4^+ are volatile, they can easily be evaporated from the fermentation broth in a short time [22,23], which results in the alleviation of acetic acid- and NH_4^+-induced inhibition of L-tryptophan production and the improvement of L-tryptophan yield and productivity. The operating conditions of the vacuum thin film evaporation process were investigated. Meanwhile, the effect of vacuum evaporation treatment on L-tryptophan production was also studied.

2. Materials and methods

2.1. Microorganisms

E. coli TRTH [8] was obtained from the Center of Industrial Culture Collection of Tianjin University of Science and Technology.

2.2. Medium

As described in previous studies [10,11], the seed medium contained 20 g/L glucose, 15 g/L yeast extract, 10 g/L $(NH_4)_2SO_4$, 0.5 g/L sodium citrate, 5 g/L $MgSO_4 \cdot 7H_2O$, 1.5 g/L KH_2PO_4, 0.015 g/L $FeSO_4 \cdot 7H_2O$, and 0.1 g/L vitamin B_1. The fermentation medium contained 20 g/L glucose, 1 g/L yeast extract, 4 g/L $(NH_4)_2SO_4$, 2 g/L sodium citrate, 5 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L KH_2PO_4, and 0.1 g/L $FeSO_4 \cdot 7H_2O$.

2.3. Main instruments

The equipment used included a 5-L Automatic Fermenter and a 30-L Automatic Fermenter (Shanghai Baoxing Biological Equipment Engineering Co. Ltd), SBA-40C Biological Sensor (Shandong Academy of Sciences Institute of Biology), Agilent 1200 high-performance liquid chromatography apparatus (HPLC; Agilent Technologies), and a D30 Scraper Concentrator (Shanghai Gerui Environment Engineering Co. Ltd).

2.4. Culture conditions

As described previously [10,11], seed cultures were prepared by growing cells under the conditions of 36°C, 20–30% DO, and pH 7.0 in the 5-L automatic fermenter containing 2 L seed medium for 14 h. Batch fermentation was performed in the 30-L fermenter containing 14 L of medium. The temperature was maintained at 36°C, and the pH was adjusted to 7.0 with 25% ammonium hydroxide (*w*/w) during the cultivation period; the DO was maintained at 20% (0–20 h) and 30% (20–38 h). When the initial glucose was depleted, glucose solution (80% *w*/*v*) was added to the fermenter, according to the DO feedback strategy [24]. During L-tryptophan production, the on-line monitoring technology of living biomass was applied to detect the living biomass, and the feed rate of glucose could be quickly adjusted according to the detected amount of living biomass.

The vacuum evaporation process during L-tryptophan production is shown in Fig. 1. First, *E. coli* TRTH was cultured in fermenter A, and then the fermentation broth in fermenter A flowed into the vacuum scraper concentrator under a vacuum when fermentation had occurred for 20 h. The fermentation broth evaporated in the scraper concentrator together with volatile acetic acid and NH_4^+ when the level of the concentrated fermentation broth reached the top liquid level electrode. The fermentation broth was not fed into the vacuum scraper concentrator, and the vacuum was closed. The concentrated fermentation broth then flowed into fermenter B under sterile air pressure, and fermentation continued in fermenter B. The concentrator would automatically evaporate the broth the next time, repeatedly functioning until the fermentation broth in fermenter A was completely processed. The total volume of the fermentation broth in fermenter B was adjusted to the volume before concentration using aseptic water. Before and after the vacuum evaporation, the concentrations of acetic acid and NH_4^+ in the broth were detected respectively. The relative activity of bacteria was detected after fermenting 0.5 h in fermenter B.

2.5. Analysis method

The biomass yield was determined according to a method described previously [13]. The living biomass was detected indirectly using a living biomass on-line monitor, which measured the capacitance value (pF) to reflect the number of live cells in the fermenter [25]. The live cell on-line monitor was installed on the 30-L fermenter to clean and count the cells in turn. The glucose concentration was determined using a biological sensor. The L-tryptophan content and NH_4^+ concentration were determined, according to previously published methods [10]. The acetic acid concentration was determined by HPLC using an organic acid analysis column (Aminex HPX-87H). The mobile phase was 0.004 mol/L sulphuric acid, while the flow rate was 0.60 mL/min. The detection wavelength was 210 nm, and the column temperature was 35°C. The sample volume was 20 μL.

To measure the relative activity of the bacteria, methylene blue solution (1 mL, 0.1%) was pipetted into a test tube, and then 5 mL of the fermentation broth was pipetted into the test tube, which was shaken quickly. A timer was switched on, and the test tube was not shaken further. The timer was stopped when the mixture just became clear (no blue colour left). The vitality of the bacteria was higher when the time taken for the blue colour to disappear was shorter.

The relative activity was calculated according to the following formula:

$$K = \frac{T_{CF}}{T_{VEF}} \times \frac{OD_{CF}}{OD_{VEF}} \times 100\%$$

where K is the relative activity of bacteria in the broth subjected to fermentation by vacuum evaporation (VEF), and T_{VEF} and T_{CF} are the time taken for the blue colour to disappear in the VEF broth and CF

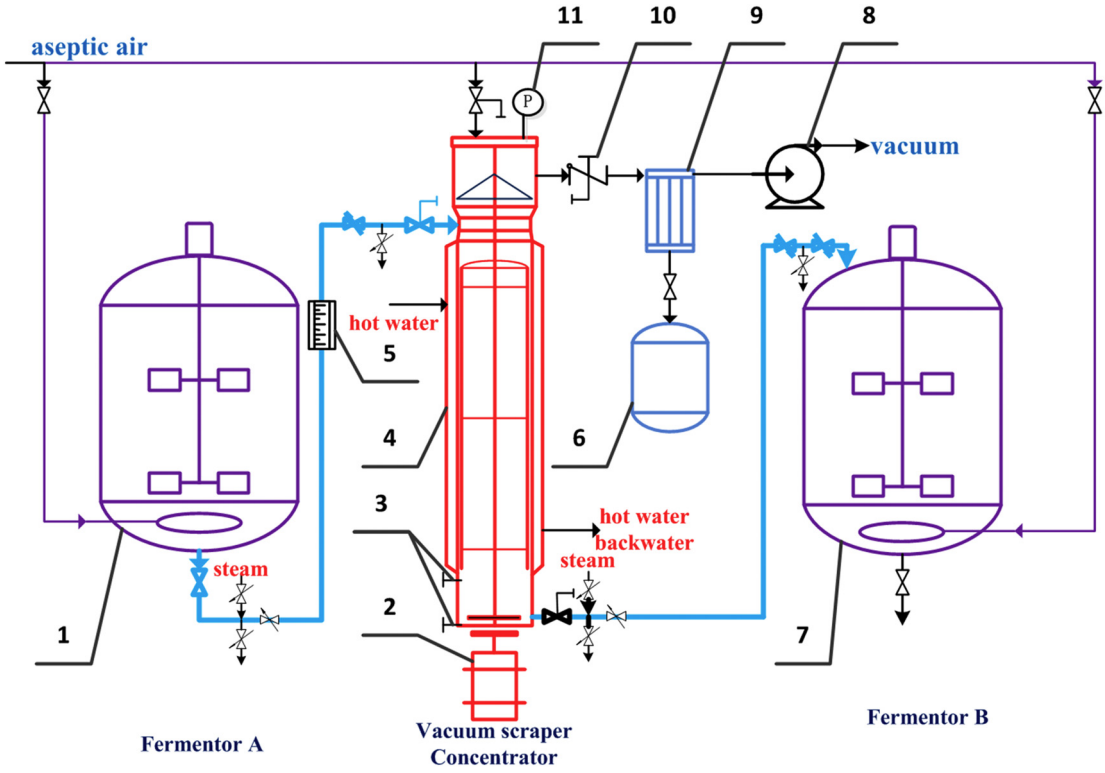

Fig. 1. L-Tryptophan production coupled with vacuum evaporation treatment. (1) Fermenter A; (2) electromagnetic stirring motor; (3) liquid level electrode; (4) automatic vacuum scraper concentrator; (5) rotor flow meter; (6) condensed liquid recovery tank; (7) fermenter B; (8) vacuum pump; (9) condenser; (10) non-return valve; (11) vacuum meter.

broth, respectively. OD_{VEF} and OD_{CF} are the bacterial concentrations of VEF and CF, respectively.

3. Results and discussion

3.1. Optimisation of the vacuum evaporation conditions for L-tryptophan production

The acetic acid and NH_4^+ produced in the L-tryptophan fermentation broth were rapidly removed by vacuum thin film evaporation. The effect of vacuum degree, concentration coefficient, heating water temperature, and feeding rate of the fermentation broth on vacuum evaporation were studied. Experiments were performed using three vacuum degrees of 600, 660, and 720 mm Hg (Fig. 2a). The results showed that as the vacuum degree increased from 600 to 720 mmHg, the acetic acid concentration in the fermentation broth reduced from 3.96 to 0.87 g/L, and the NH_4^+ concentration decreased from 76.2 to 20.2 mmol/L. The relative activity of bacteria in the broth was higher than 85% under different vacuum degrees, which indicated that the cell growth of *E. coli* was not affected by vacuum evaporation treatment.

Concentration coefficient is the ratio of the volume of the evaporated broth to the fermentation broth. We used different concentration coefficients of 5%, 10%, and 15% for the experiment (Fig. 2b). The results showed that the concentrations of acetic acid and NH_4^+ decreased with increasing concentration coefficient. The viability of bacteria remained almost unchanged because of the short process time. The concentrations of acetic acid and NH_4^+ did not change significantly for concentration coefficients of 10% and 15%; therefore, a concentration coefficient of 10% was chosen as the operating vacuum evaporation condition.

Evaporation requires a stable heat source. In general, steam is used to provide the heat; however, steam temperature will cause an excessive increase in the local temperature that will damage the bacteria. To reduce the damage caused by steam, we selected hot water at 40–70°C as the heat source for vacuum evaporation (Fig. 2c). The results showed that with increasing water temperature, the concentrations of acetic acid and NH_4^+ in the concentrated fermentation broth decreased. However, the bacterial activity showed a decreasing trend with increasing temperature. When the temperature reached 70°C, the acetic acid and NH_4^+ concentrations were 0.65 g/L and 17.5 mmol/L, respectively; however, the relative activity of the bacteria decreased to 90%. When the temperature was controlled at 60°C, the relative activity of bacteria was higher than 95%, and the concentrations of acetic acid and NH_4^+ were 0.76 g/L and 18.9 mmol/L, respectively. Therefore, 60°C was set as the optimal temperature.

In the process of vacuum evaporation, the feeding rate affects the retention time of the fermentation broth in the vacuum thin film evaporation equipment. Four feeding rate gradients were selected (Fig. 2d). The results showed that the concentrations of acetic acid and NH_4^+ and the cell activity increased with the feeding rate. When the feeding rate was 300 mL/min, the concentrations of acetic acid and NH_4^+ reached low levels of 0.74 g/L and 17.6 mmol/L, respectively, and the relative activity of bacteria was comparatively higher. Therefore, the feeding rate of the fermentation broth was set as 300 mL/min.

3.2. Effect of vacuum evaporation treatment on L-tryptophan production

When L-tryptophan was fermented for 20 h, vacuum evaporation was carried out using the optimised vacuum evaporation conditions. The bacterial biomass, living biomass, L-tryptophan production, L-tryptophan production rate, and glucose conversion rate are shown in Fig. 3. The amount of biomass and living biomass in the VEF and CF broth both increased rapidly and reached a maximum at 20 h. However, after 20 h, the amount of biomass and living biomass in the VEF broth decreased slower than that in the CF broth. The living biomass in the VEF and CF broth was 10.2 and 8.9 pF, respectively, at 38 h, and the biomass in the VEF and CF broth was 58.3 and 55.1 g/L, respectively. Thus, the living biomass and biomass in the VEF broth increased by 14.6% and 5.8%, respectively, compared with those in the CF broth, which indicated that

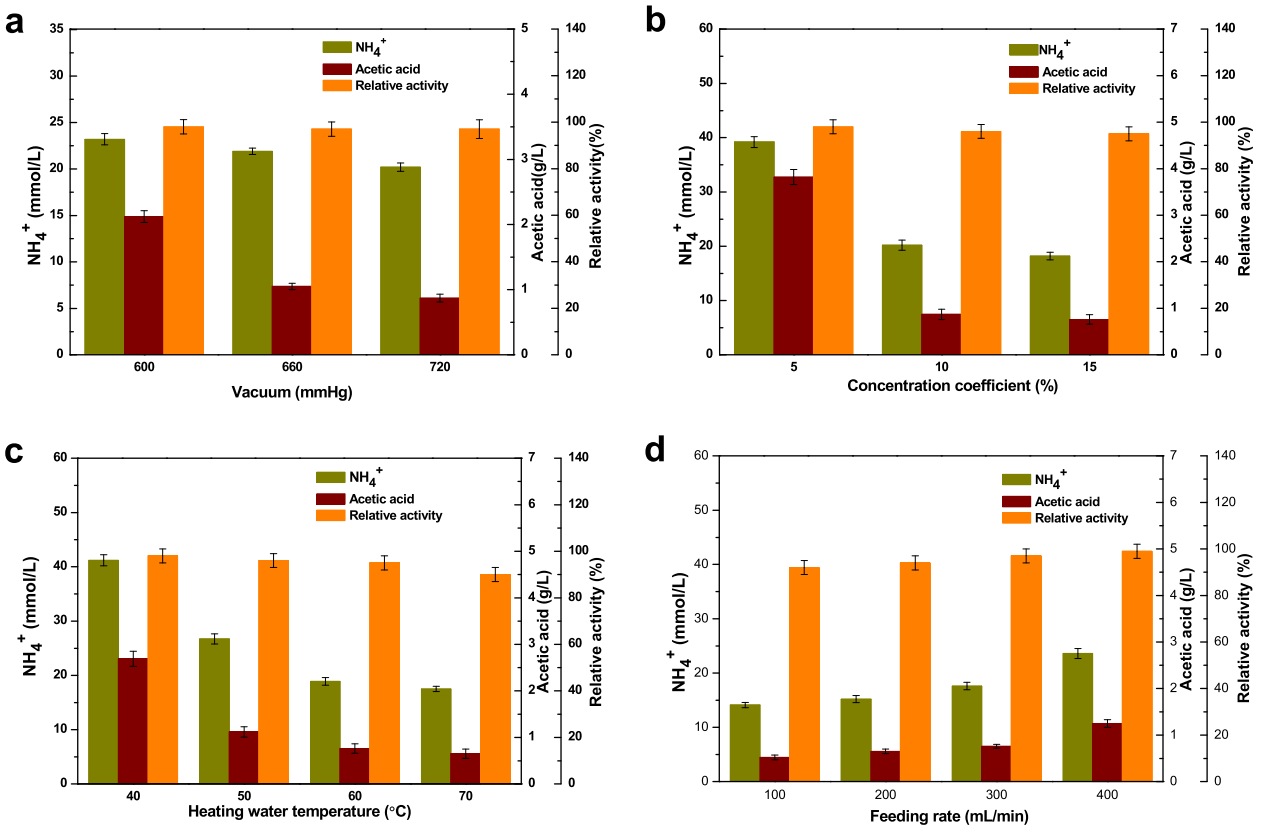

Fig. 2. Effect of different vacuum evaporation conditions on NH_4^+ and acetic acid concentration and the relative activity of the bacteria in the L-tryptophan fermentation broth. (a) Vacuum degree; (b) Concentration coefficient; (c) Heating water temperature; (d) Feeding rate.

the cell viability can be effectively improved by vacuum evaporation during L-tryptophan production.

L-tryptophan production by VEF and CF was 60.2 and 50.2 g/L, respectively, at 38 h, representing an increase of 19.9% (Fig. 3b). The L-tryptophan production rate of VEF was increased continuously, while the L-tryptophan production rate of CF decreased rapidly after 20 h. Toward the end of fermentation, the L-tryptophan production rate of CF dropped to 0.5 g/L·h, while that of VEF was maintained above 1.4 g/L·h. This was probably due to the released inhibition of cell growth and L-tryptophan production by acetic acid and NH_4^+. As shown in Fig. 3c, VEF maintained a higher glucose conversion rate than that of CF after 20 h. From 20–38 h, the average glucose conversion rates of VEF and CF were 20.2% and 18.8%, respectively, representing an increase of 7.4%. From 0–38 h, the average glucose conversion rates of VEF and CF were 19.5 and 18.2%, respectively, representing an increase of 7.1%.

3.3. Effect of vacuum evaporation treatment on the production of acetic acid and NH_4^+ by-products

Acetic acid and NH_4^+ existing in the fermentation broth can be effectively removed by vacuum evaporation. As shown in Fig. 4b, the concentration of acetic acid decreased from 3.96 to 0.76 g/L after vacuum evaporation. During the late stage of fermentation, the acetic acid concentration in the CF broth increased from 3.96 to 6.70 g/L, while that in the VEF broth increased from 0.76 to 2.74 g/L. From 20–38 h, the average acetic acid production rate of VEF and CF was 0.11 and 0.15 g/L·h, respectively. As shown in Fig. 4b, the concentration of NH_4^+ decreased from 76.2 to 20.2 mmol/L after vacuum evaporation. During the late stage of fermentation, the NH_4^+ concentration in the CF broth increased from 76.2 to 130.9 mmol/L, while that in the VEF broth increased from 20.2 to 85.3 mmol/L. The lower concentration of acetic acid and NH_4^+ during VEF resulted in reduced inhibition of cell growth, which was beneficial for L-tryptophan production. Moreover, because the acetic acid concentration in the VEF process was lower than that in the CF process, less NH_4^+ was needed to maintain acid–base balance.

4. Discussion

The continuous accumulation of acetic acid and NH_4^+ has become a bottleneck problem that obstructs L-tryptophan production. In this study, the acetic acid and NH_4^+ produced in the L-tryptophan fermentation broth were effectively removed by vacuum evaporation. Hot water was used instead of steam as the heat source to avoid damaging the cells by partial overheating of the evaporator. Compared with that of CF, the biomass yield of VEF was 58.3 g/L, showing an increase of 5.8%, and the living biomass yield was 10.2 pF, showing an increase of 14.6%. Thus, the bacteria in VEF had higher activity, and the glucose consumption rate was more than 8 g/L·h, while the glucose consumption rate of CF was lower than 7 g/L·h. The L-tryptophan yield and glucose conversion rate of VEF were 60.2 g/L and 20.2%, representing increases of 19.9% and 7.4%, respectively, compared with those of CF. Moreover, after vacuum evaporation, the acetic acid-generating rate was decreased significantly. Meanwhile, the concentration of other volatile by-products that inhibit the cell activity was also decreased. As a result, less NH_4^+ was needed to maintain acid–base balance, and the cell viability and L-tryptophan productivity were maintained at a high level at the late stage of fermentation. In a previous report [13], a combined feeding strategy of pseudo-exponential feeding and glucose-stat feeding was applied to reduce the acetic acid concentration (which reached a level of 0.9 g/L) and increase L-tryptophan production (which reached a level of 38.8 g/L). However, the NH_4^+ concentration was not controlled. Another pH feedback-controlled substrate feeding

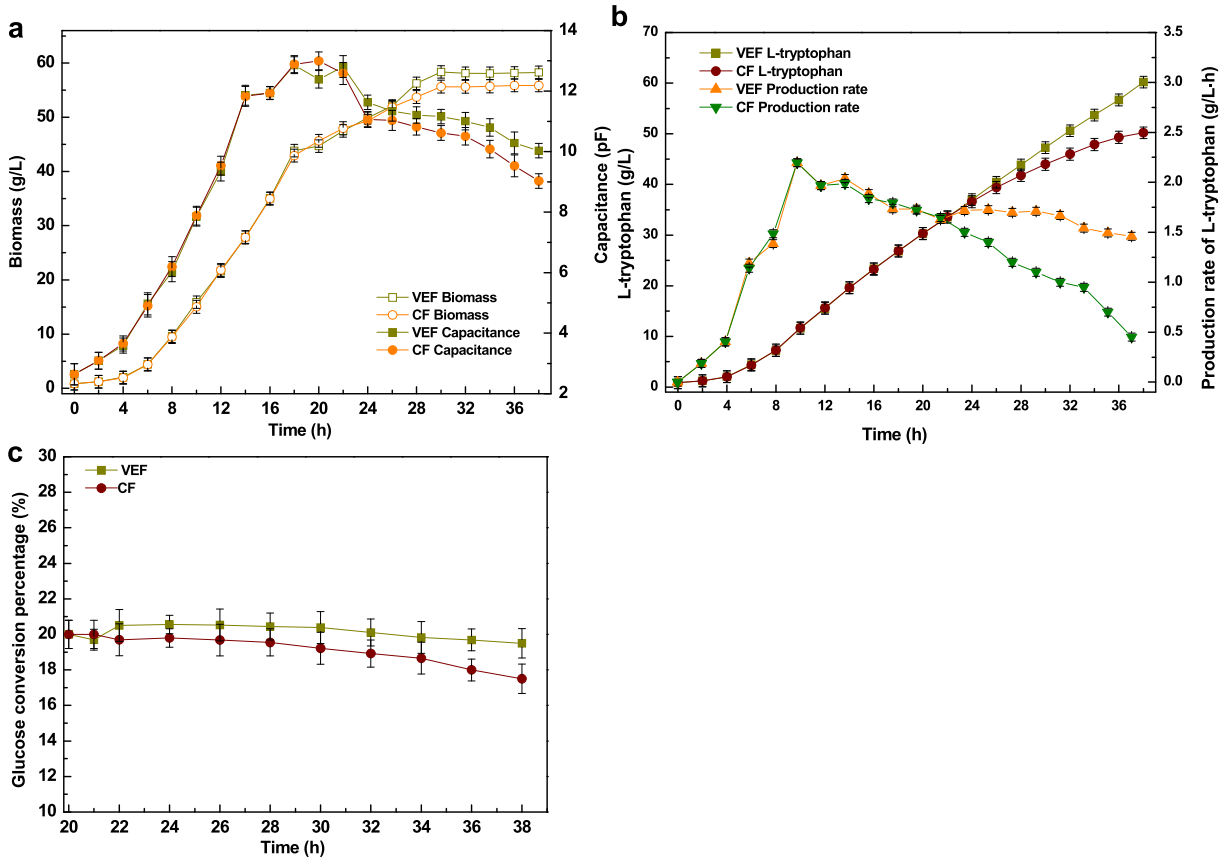

Fig. 3. Effect of vacuum evaporation treatment on L-tryptophan production. (a) Biomass; (b) L-tryptophan concentration and production rate; (c) Glucose conversion rate.

strategy was also applied to reduce the acetic acid (which reached a level of 1.15 g/L) and NH_4^+ concentration and increase L-tryptophan production (which reached a level of 43.65 g/L) [14]. However, the final NH_4^+ and K^+ concentration was controlled at 110 mM and 71 mM, respectively. Compared with that in the previous study, the acetic acid produced in VEF was a little higher, but the final NH_4^+ concentration was 85 mM, and K^+ was not needed. Therefore, the L-tryptophan production in this study was much higher (60.2 g/L). We previously used cell recycling technology based on a special disc centrifuge to separate L-tryptophan product and harmful intermediates [19]. The average glucose conversion rate and acetic acid concentration reached 55.12 g/L, 19.5%, and 1.50 g/L, respectively. Compared with that in the cell recycling fermentation, acetic acid production in VEF was higher, because the strain used in the cell recycling fermentation study was a TRTH mutant with deletion of the phosphotransacetylase-acetate kinase pathway. However, less cell damage was caused by the vacuum evaporation in VEF because of the shorter residence time in the concentrator (less than 30 s). Therefore, the relative activity of bacteria in VEF remained above 98%, and L-tryptophan production and the L-tryptophan production rate in VEF were much higher. Moreover, the equipment investment of a disc centrifuge is around 700,000 RMB, while the cost of vacuum evaporation equipment is only 80,000 RMB. Under the same working capacity of fermentation broth, the operation time of vacuum evaporation equipment is 75% shorter than that of disc centrifuge equipment. The total electricity consumption of vacuum evaporation equipment is 4.5 KW·h, 25% less than that of disc centrifuge equipment.

In summary, vacuum film evaporation technology has the advantages of shorter evaporation period, higher evaporation efficiency, and less

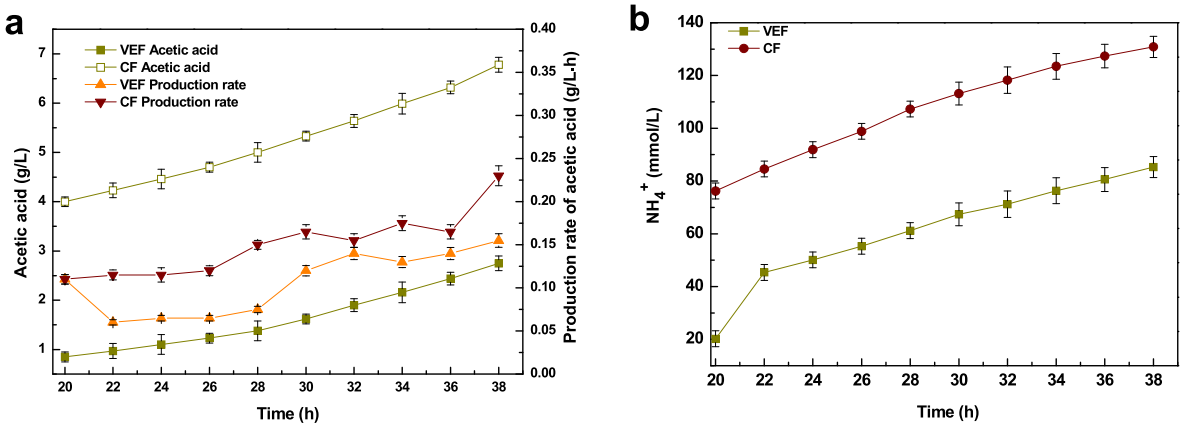

Fig. 4. Effect of vacuum evaporation treatment on acetic acid and NH_4^+ production during L-tryptophan production.

damage to the bacteria during L-tryptophan production. The acetic acid and NH_4^+ in the broth could be removed quickly and effectively by vacuum evaporation during the VEF process. Therefore, the vacuum film evaporation technology can significantly improve L-tryptophan production by *E. coli*.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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