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ORIGINAL PAPER

Enhanced acid tolerance of *Rhizopus oryzae* during fumaric acid production

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Abstract Ensuring a suitable pH in the culture broth is a major problem in microorganism-assisted industrial fermentation of organic acids. To address this issue, we investigated the physiological changes in *Rhizopus oryzae* at different extracellular pH levels and attempted to solve the issue of cell shortage under low pH conditions. We compared various parameters, such as membrane fatty acids' composition, intracellular pH, and adenosine triphosphate (ATP) concentration. It was found that the shortage of intracellular ATP might be the main reason for the low rate of fumaric acid production by *R. oryzae* under low pH conditions. When 1 g/l citrate was added to the culture medium at pH 3.0, the intracellular ATP concentration increased from 0.4 to 0.7 μmol/mg, and the fumaric acid titer was enhanced by 63 % compared with the control (pH 3.0 without citrate addition). The final fumaric acid concentration at pH 3.0 reached 21.9 g/l after 96 h of fermentation. This strategy is simple and feasible for industrial fumaric acid production under low pH conditions.

Keywords *Rhizopus oryzae* · Fumaric acid · Low pH fermentation · Acid tolerance · ATP

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Introduction

Fumaric acid is a four-carbon unsaturated dicarboxylic acid that is widely used in various industries, such as food acid and feed ingredient production, and is a promising material in the manufacture of synthetic resin [1]. The production of fumaric acid by fermentation has received increasing attention because of the continuing depletion of global resources. Fumaric acid production by fermentation is usually conducted with *Rhizopus oryzae* [2]. In the first 20 h of the fermentation process, the pH in the broth rapidly drops from 5.0 to 2.0 [3]. Neutralizing agents, such as calcium carbonate ($CaCO_3$), are used to maintain the optimum pH range [2, 4]. However, the use of such neutralizing agents leads to environmental waste and additional downstream process costs. This situation can be improved by conducting fermentation at a low pH level. Strategies to improve the production of fumaric acid under low pH conditions have been studied. Roa Engel et al. [5] showed that the fermentation of *R. oryzae* can be performed at pH 3.5 by regulating the volume percentage of CO_2 at 10 %, and the final fumaric acid concentration of 19.84 g/l was obtained, which was 30 % higher than that of the control. However, these studies have failed to describe the detailed mechanisms underlying *Rhizopus* sp. functions under low pH conditions. Moreover, the process control was complicated and required specific equipment.

A low pH does not only affect acid production, but also strongly affects cell metabolism and composition. Several acid-tolerance mechanisms have been identified in bacteria and yeast. Guan et al. investigated the intracellular pH, NAD+/NADH ratio, H+-ATPase activity, and intracellular amino acids of different *Propionibacterium acidipropionici* strains. They found that at low intracellular pH, a high amount of H+-ATPase could be biosynthesized, the

NAD+/NADH ratio could increase, and propionic acid production increased by 39.9 % when arginine and aspartic acid were added during fermentation [6]. Fozo et al. compared four oral bacterial strains, two of these oral bacterial strains were aciduric and the other two were acid sensitive. The concentration of long-chain monounsaturated fatty acids of the aciduric oral bacteria cell membrane increased under low pH conditions. However, the composition of the acid-sensitive strain's membrane remained the same [7]. In another study conducted by Zhou et al. [8], the enhancement of ATP supply was beneficial to yeast against acid stress. In the present study, we aimed to investigate the physiological characteristics of *R. oryzae* under different pH conditions and to determine a potential strategy to further improve fumaric acid production under low pH conditions.

Materials and methods

Strain and culture condition

Rhizopus oryzae S-1 was used as a material throughout the study. This strain was derived from a series of spontaneous mutations of *R. oryzae* ATCC20344 induced by ion injection. *Rhizopus oryzae* S-1 was cultured on potato-dextrose agar slant at 35 °C for 7 days. The agar slant with fungi spores was washed with sterile water at 4 °C. The spores were grown at 35 °C and under 200 rpm for 30 h in 50 ml of the seed culture medium containing 30 g/l glucose, 2 g/l urea, 0.6 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.11 g/l $ZnSO_4{\cdot}7H_2O$, and 0.0088 g/l $FeSO_4{\cdot}7H_2O$ (pH 2.5). Approximately, 10 % of the seed culture was inoculated into the fermentation medium containing 80 g/l glucose supplemented with other ingredients similar to those of the seed culture medium. Saturated Na_2CO_3 was selected as the neutralizing agent. The fermentation process was conducted in a 5 l fermentor at 35 °C and at 400 rpm.

Analytical methods

Fumaric acid and sugars

Fumaric acid was determined and quantified via high-performance liquid chromatography (HPLC), as previously reported [9]. An SBA-80C biosensor analyzer was used to measure glucose concentration (Institute of Biology, Shandong Academy of Sciences, China) [10].

Dry weight and osmolality

Broth samples (10 ml) were directly collected from the 5 l fermentor and washed thrice with demineralized water. Samples were dried in an oven at 60 °C until a constant weight was achieved. Osmolality was measured with a Roebling osmometer [11].

Intracellular pH (pHi)

The fluorescence method was used to determine the intracellular pH of *R. oryzae*. 2-7-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF AM) was used as the fluorescent probe. Cells grown at different extracellular pH levels (3.0, 4.0, and 5.0) were harvested in their exponential phase by centrifugation, washed with 50 mM HEPES-K buffer (pH 8.0) thrice, and re-suspended with the same buffer. Cells were incubated with BCECF AM for 50 min. The cells were washed thrice with 50 mM potassium phosphate buffer (pH 7.0) and re-suspended for determination. A fluorescence spectrophotometer with a wavelength range of 440 nm (pH insensitive) and an excitation spectrum of 490 nm (pH sensitive) was used to detect the fluorescence intensities. 525 nm was set as the emission wavelength. Excitation and emission slit widths were set at 5 nm. The ratios of the emission intensity at 490 and 440 nm of cell suspension (S) and filtrate (F) were calculated as follows: $R = (S490 - F490)/(S440 - F440)$ [6].

The calibration curve was determined using the following steps. Each strain culture was supplemented with 5 mM valinomycin and nigericin (Sigma) to maintain the equilibration of pHi with extracellular pH. The cultures were incubated for 20 min before washing and re-suspending in different buffers with pH values ranging from 3.0 to 8.0. BCECF AM was added at 1 µl. The remaining steps were the same as those mentioned above [6].

Intracellular ATP

To determine the intracellular ATP content, the culture medium containing *R. oryzae* cells grown for 36 h was collected and quenched by an equal volume of methanol at −40 °C. A reagent containing 50 % perchloric acid was used to extract ATP. HPLC with a UV detector at 254 nm was used to analyze the intracellular ATP. The mobile phase was 86 % (v/v) phosphate buffer solution comprising NaH_2PO_4 at 10.93 g/l, Na_2HPO_4 at 3.04 g/l, tetrabutylammonium bromide at 3.22 g/l (pH 6.5), and 4 % acetonitrile at a flow rate of 1 ml/min. A Sepax HP-C18 column (250 × 4.6 mm, 5 µm) was used at 35 °C.

Membrane fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by adding 0.2 ml of 4 M KOH–methanol and 3 ml of *N*-hexane to a 5 ml tube containing 0.1 g of dried cells. The solution was

mixed thoroughly using a vortex for 1 min, allowed to stand for 10 min, and centrifuged at 6,000 rpm for 3 min. The upper phase containing FAMEs was then transferred to a clean centrifuge tube and dried using anhydrous Na_2SO_4. The samples were then analyzed.

A GC–MS system (Thermo/Finnigan TraceGCMS, USA) equipped with a capillary column (DB-5MS, 30 × 0.32 mm) was used to analyze the FAMEs samples. The temperature of column was increased from 80 to 200 °C at a rate of 20 °C/min and further increased to 300 °C at a rate of 10 °C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Fatty acids were identified by comparison with external standards (Sigma, USA) and MS structure database [12].

Results and discussion

Effects of pH on glucose consumption and fumaric acid production

Experiments at pH values from 3.0 to 5.0 were conducted to determine the effect of pH on fumaric acid production by *R. oryzae*. The glucose consumption and fumaric acid production of all fermentation processes performed to determine pH effects are presented in Fig. 1. Glucose was rapidly consumed at pH 4.0, and the highest fumaric acid concentration of 26.3 g/l was achieved at the end of fermentation. This production was 96 and 16 % higher than those at pH 3.0 and 5.0, respectively. At pH 3.0, the glucose concentration slightly decreased from the beginning of fermentation. After 96 h of fermentation, a residue glucose concentration of 35 g/l was obtained. The lowest fumaric acid production of 13.4 g/l was observed. At pH 5.0, the glucose consumption rate during the first 24 h was close to that at pH 4.0. The glucose consumption rate gradually decreased and reached a final fumaric acid concentration of 22.6 g/l. This observation might be due to the addition of a high amount of Na_2CO_3 into the culture broth when the pH was controlled at 5.0 compared with those at pH 3.0 and 4.0. At pH 5.0, the fungi were exposed to an environment of high osmolality (as shown in Table 1), which probably resulted in a loss of water and subsequent interference of a variety of physiological processes, such as inhibition of protein translation and disruption of mitochondrial function [13]. Hence, fumaric acid production decreased.

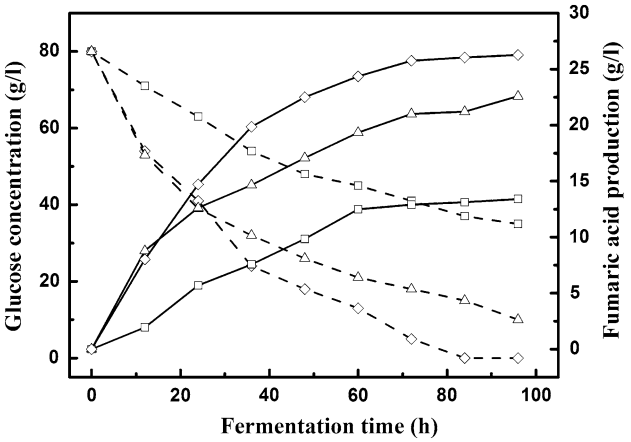

Fig. 1 Effect of different pH levels on glucose consumption and fumaric production. (1) *Open triangle* pH 5.0, *open diamond* pH 4.0, and *open square* pH 3.0. (2) *Dashed lines* represent glucose consumption, whereas *solid lines* are for fumaric acid production

Table 1 Changes in fatty acid proportion under different pH conditions

	pH 5	pH 4	pH 3
C16:0	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
C16:1	24.5 ± 0.9	24.9 ± 1.0	25.6 ± 1.0
C18:0	2.3 ± 0.3	2.1 ± 0.1	1.2 ± 0.3
Trans C18:2	4.3 ± 0.1	4.6 ± 0.1	5.5 ± 0.2
Cis C18:2	32.0 ± 0.7	32.4 ± 2.6	31.6 ± 1.2
C18:3n6	17.9 ± 0.6	18.5 ± 0.4	21.1 ± 0.6
C20:0	18.6 ± 0.2	17.0 ± 0.6	14.5 ± 0.6
TUFA	47.2 ± 0.4	51.5 ± 1.9	52.7 ± 0.4
TSFA	52.8 ± 0.4	48.6 ± 1.9	47.3 ± 0.4
U/S ratio	0.89 ± 0.01	1.05 ± 0.02	1.11 ± 0.02

TUF total unsaturated fatty, *TSF* total saturated fatty, *U/S* unsaturated/saturated

To elucidate the acid effects, the physiological characteristics of *R. oryzae* at different pH conditions were subsequently studied.

Effect of extracellular pH on the membrane fatty acids composition of *R. oryzae*

Biological membranes are the first barrier between the cells and environment. Thus, even small changes in the biological membranes can cause marked changes in the function of numerous essential membrane-attached proteins, such as transport proteins [14]. The physical properties of a membrane are largely determined by its lipid composition. One important factor is the degree of fatty acid unsaturation. To explore the relationship between membrane composition and extracellular pH, the membrane fatty acids of *R. oryzae* were detected. The results are summarized in Table 1. The unsaturated fatty acid and unsaturated-to-saturated fatty acid ratio (U/S) increased with decreasing pH, whereas the saturated fatty acid decreased. The concentration of saturated fatty acids C18:0

and C20:0 decreased with decreasing pH. The concentration of unsaturated fatty acids *trans* C18:2 and C18:3n6 at pH 3.0 was higher than those at pH 4.0 and 5.0. The concentration of the other fatty acids C16:0 and C16:1 was almost the same at different extracellular pH values.

The biochemical property of particular enzymes can possibly be affected by the acidic environment, thereby leading to the formation of monounsaturated fatty acids. The increase in monounsaturated fatty acids in the membrane could increase membrane fluidity, which is necessary for protecting the organism from environmental stress [14]. Alterations in the membrane fatty acid are common adaptation mechanisms of microorganisms in response to environmental stresses. *Streptococcus mutans* Ua159 shifts its membrane composition from a saturated fatty acid, short-chained profile at neutral pH to a monounsaturated, long-chained profile under low pH conditions [15]. In response to the increasing environmental temperature, *Bacillus stearothermophilus* showed a marked increase in saturated fatty acids and a decrease in unsaturated and branched chain fatty acid proportion [16]. In the present study, a similar adaptation mechanism was observed in *R. oryzae* under low pH conditions. Thus, the biological membrane composition might not be the most important limitation affecting the poor performance of *R. oryzae* under low pH conditions.

Effect of extracellular pH on the intracellular ATP concentration

The intracellular pH (pHi) and ATP concentration of *R. oryzae* at varying extracellular pH levels of 5.0, 4.0, and 3.0 were determined. As demonstrated in Table 2, pHi decreased with decreasing extracellular pH level. When the extracellular pH decreased to 3.0, the pHi dropped to 3.6, which was much lower than the pHi at an extracellular pH of 5.0. Low intracellular pH has numerous detrimental effects, such as interference of enzyme activity and inhibition of protein synthesis [17]. Therefore, maintaining the intracellular pH within a narrower range than the pH outside of the cell (a phenomenon known as "pH homeostasis") is very important for most organisms. The proton-translocation ATPase, which could pump protons out of the cells, serves an important function in maintaining pH homeostasis. A large amount of ATP is required in the proton transportation process. Thus, a high intracellular energy status is important for cells to cope with acidic conditions [6, 8]. In the present study, intracellular ATP concentration showed the same tendency as pHi. The lowest ATP level of 0.4 μmol/mg was observed at pH 3.0, and this level was 50 and 55 % lower than those at pH 4.0 and 5.0, respectively. Combined with the results of previous studies, the shortage of intracellular energy could be the most important reason for the low performance of fumaric acid accumulation by *R. oryzae* at pH 3.0.

Table 2 Osmotic pressure, intracellular pH, and ATP changes under different pH conditions

	Osmolality (osmol/g)	Intracellular pH (pHi)	Intracellular ATP concentration (umol/mg)
pH 3.0	677	3.6	0.4
pH 4.0	767	5.4	0.8
pH 5.0	832	6.1	0.9

Citrate protects *R. oryzae* at low pH

We know that a large quantity of ATP is required to maintain the high gradients between extracellular and intracellular pH levels. Increasing ATP supply can improve the cell's ability to cope under acidic conditions. Increasing the concentration of electron donors from the oxidative phosphorylation route can be an efficient strategy to enhance ATP concentration [18]. By adding an energy source, more NADH could be generated via the cytochrome respiration pathway and further converted to ATP, thereby increasing ATP concentration [8, 19]. The aim of the present study is to investigate the effect of citrate (one of the energy sources) addition on cell performance under low pH conditions.

The intracellular ATP concentration increased when citrate was added into the culture medium. When 1 g/l citrate was added into the culture medium, the intracellular ATP level increased to 0.7 μmol/mg (Table 3), which was 75 % higher than that of the control (pH 3.0, without citrate addition). The pHi was increased from 3.6 (control, pH 3.0, without citrate added) to 5.9 (Table 3), indicating that the intracellular pH status was successfully improved when citrate was added. The effects of citrate addition on glucose consumption and fumaric acid production are shown in Fig. 2. The average glucose consumption rate increased by 38 % compared with the control. The final fumaric acid concentration at pH 3.0 was to 21.9 g/l, which was much close to the fermentation performance at pH 4.0, and was higher than the value (19.84 g/l, pH 3.5) obtained in a

Table 3 Effect of citrate addition on intracellular ATP concentration and intracellular pH at pH 3.0

	Intracellular ATP (μmol/mg)	Intracellular pH (pHi)
pH 3.0, without citrate addition (control condition)	0.4	3.6
pH 3.0, 1 g/l citrate addition	0.7	5.9
pH 3.0, 2 g/l citrate addition	0.5	–

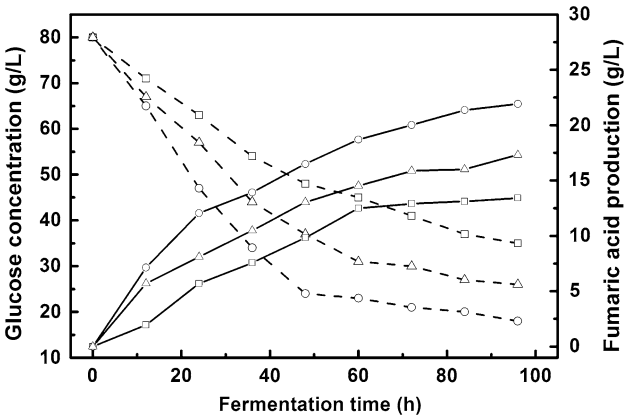

Fig. 2 Effect of citrate addition on glucose consumption and fumaric production. (1) *Open circle* 1 g/l citrate, *open triangle* 2 g/l citrate, and *open square* no citrate addition. (2) *Dashed lines* present glucose consumption, whereas *solid lines* present fumaric acid production

previous study [5]. Moreover, the amount of consumed Na_2CO_3 decreased by approximately 30 % compared with the consumed amount at pH 4.0. In the present study, we produced fumaric acid under low pH conditions by adding citrate into the culture medium. This strategy was much easier and the pH was lower than those reported previously (CO_2 regulation and pH shifting).

Conclusions

Understanding the acid-tolerance mechanism of *R. oryzae* is essential to produce fumaric acid under low pH conditions. However, few studies have been carried out to reveal how *Rhizopus* spp. respond to acid stresses. In the current study, the changes in *R. oryzae* physical characteristics at different pH stresses were comparatively studied to determine the factors that might improve the acid-tolerant capability of *R. oryzae*. In the analysis of the membrane fatty acids' composition, the intracellular pH and ATP concentration at different pH conditions indicated a shortage of ATP regeneration. Such limitation in ATP concentration could be the main reason for the poor performance of *R. oryzae* under low pH conditions. To the best of our knowledge, this study is the first to report on microenvironment changes in *Rhizopus* sp. under acidic stress. When 1 g/l citrate was added to the culture medium, the intracellular ATP supply significantly increased by 75 %, and fumaric acid production increased to 21.9 g/l at pH 3.0. The present study provided a simple and easy way to relieve the acidic stress of organic acid producers under low pH conditions, and this method is feasible for industrial organic acid production in the future.

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References

1. Xu Q, Li S, Huang H, Wen J (2012) Key technologies for the industrial production of fumaric acid by fermentation. Biotechnol Adv 30:1685–1696
2. Deng Y, Li S, Xu Q, Gao M, Huang H (2012) Production of fumaric acid by simultaneous saccharification and fermentation of starchy materials with 2-deoxyglucose-resistant mutant strains of *Rhizopus oryzae*. Bioresour Technol 107:363–367
3. Roa Engel CA (2010) Integration of fermentation and cooling crystallisation to produce organic acids[D]. Delft University of Technology, The Netherlands, pp 46–63
4. Zhou Z, Du G, Hua Z, Zhou J, Chen J (2011) Optimization of fumaric acid production by *Rhizopus delemar* based on the morphology formation. Bioresour Technol 102:9345–9349
5. Roa Engel CA, van Gulik WM, Marang L, van der Wielen LAM, Straathof AJJ (2011) Development of a low pH fermentation strategy for fumaric acid production by *Rhizopus oryzae*. Enzyme Microbial Technol 48:39–47
6. Guan N, Liu L, Shin H-D, Chen RR, Zhang J, Li J, Du G, Shi Z, Chen J (2013) Systems-level understanding of how *Propionibacterium acidipropionici* respond to propionic acid stress at the microenvironment levels: mechanism and application. J Biotechnol 167:56–63
7. Fozo EM, Kajfasz JK, Quivey RG (2004) Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol Lett 238:291–295
8. Zhou J, Liu L, Chen J (2011) Improved ATP supply enhances acid tolerance of *Candida glabrata* during pyruvic acid production. J Appl Microbiol 110:44–53
9. Xu Q, Li S, Fu Y, Tai C, Huang H (2010) Two-stage utilization of corn straw by *Rhizopus oryzae* for fumaric acid production. Bioresour Technol 101:6262–6264
10. Ding Y, Li S, Dou C, Yu Y, Huang H (2011) Production of fumaric acid by *Rhizopus oryzae*: role of carbon–nitrogen ratio. Appl Biochem Biotechnol 164:1461–1467
11. Varela C, Agosin E, Baez M, Klapa M, Stephanopoulos G (2003) Metabolic flux redistribution in *Corynebacterium glutamicum* in response to osmotic stress. Appl Microbiol Biotechnol 60:547–555
12. Ren L-J, Huang H, Xiao A-H, Lian M, Jin L-J, Ji X-J (2009) Enhanced docosahexaenoic acid production by reinforcing acetyl-CoA and NADPH supply in *Schizochytrium* sp. HX-308. Bioprocess Biosyst Eng 32:837–843
13. Xu S, Zhou J, Qin Y, Liu L, Chen J (2010) Water-forming NADH oxidase protects *Torulopsis glabrata* against hyperosmotic stress. Yeast 27:207–216
14. Rodríguez-Vargas S, Sánchez-García A, Martínez-Rivas JM, Prieto JA, Randez-Gil F (2007) Fluidization of membrane lipids enhances the tolerance of *Saccharomyces cerevisiae* to freezing and salt stress. Appl Environ Microbiol 73:110–116
15. Fozo EM, Quivey RG (2004) Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. Appl Environ Microbiol 70:929–936
16. McElhaney RN, Souza KA (1976) The relationship between environmental temperature, cell growth and the fluidity and physical state of the membrane lipids in *Bacillus*

stearothermophilus. Biochim Biophysica Acta (BBA) Biomembranes 443:348–359
17. Martínez-Muñoz GA, Kane P (2008) Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. J Biol Chem 283:20309–20319
18. Zhou J, Liu L, Shi Z, Du G, Chen J (2009) ATP in current biotechnology: regulation, applications and perspectives. Biotechnol Adv 27:94–101
19. Harris DM, van der Krogt ZA, van Gulik WM, van Dijken JP, Pronk JT (2007) Formate as an auxiliary substrate for glucose-limited cultivation of *Penicillium chrysogenum*: impact on penicillin G production and biomass yield. Appl Environ Microbiol 73:5020–5025