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Production of Fumaric Acid by *Rhizopus oryzae*: Role of Carbon–Nitrogen Ratio

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Abstract Cytosolic fumarase, a key enzyme for the accumulation of fumaric acid in *Rhizopus oryzae*, catalyzes the dehydration of L-malic acid to fumaric acid. The effects of carbon–nitrogen ratio on the acid production and activity of cytosolic fumarase were investigated. Under nitrogen limitation stress, the cytosolic fumarase could keep high activity. With the urea concentration decreased from 2.0 to 0.1 g l^{-1}, the cytosolic fumarase activity increased by 300% and the production of fumaric acid increased from 14.4 to 40.3 g l^{-1} and L-malic acid decreased from 2.1 to 0.3 g l^{-1}. Cytosolic fumarase could be inhibited by substrate analog 3-hydroxybutyric acid. With the addition of 3-hydroxybutyric acid (50 mM) in the fermentation culture, fumaric acid production decreased from 40.3 to 14.1 g l^{-1} and L-malic acid increased from 0.3 to 5.4 g l^{-1}.

Keywords Fumaric acid · Cytosolic fumarase · Substrate analog · *Rhizopus oryzae*

Introduction

Fumaric acid is a four-carbon unsaturated dicarboxylic acid that is widely used in food industry and is a valuable intermediate for preparing edible products such as L-malic and L-aspartic acid. Because of its double bond and two carboxylic groups, fumaric acid has many potential industrial applications, ranging from the manufacture of synthetic resins and biodegradable polymers to the production of intermediates for chemical syntheses [1]. The current US consumption of fumaric acid is 1.8×10^4 t per year [2]. Nowadays, it was derived exclusively from petroleum-based materials, but as the world's crude oil resources diminish, the production of chemicals from renewable resources was becoming more important.

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The glucose metabolism in *Rhizopus oryzae* had been demonstrated clearly by the method of ^{13}C nuclear magnetic and enzymatic activity studies [3]. Generally, fumaric acid was accumulated by the reductive carboxylation pathway in the cytosol [4]. In reductive carboxylation pathway, fumarase is a key enzyme, catalyzing the dehydration of L-malate to fumarate, which also exists in the mitochondria and catalyzed the reverse reaction. The role of fumarase in L-malic acid accumulation had been most thoroughly studied in yeast. *Saccharomyces cerevisiae* and *Aspergillus flavus* accumulate L-malic acid but not fumaric acid [5]. More attentions were attracted on the differences between these two kinds of fumarases, especially in yeast [6–8]. When people studied fumarase of *R. oryzae*, they found an interesting phenomenon: fumarase in the lysates of *R. oryzae* from growth medium (high urea concentration) had both activities when L-malic acid and fumaric acid as the substrate; while the fumarase from production medium (low urea concentration) was completely inhibited by 2 mM fumaric acid [9].

Up to date, there is still no clear evidence to explain this interesting question. Therefore, in the present work, it is the first time to clarify the fumarase in the production of fumaric acid by *R. oryzae*. In this paper, we discussed the effect of carbon–nitrogen ratio on the production of metabolites and the activity of cytosolic fumarase. In order to recognize the fumarase in the cytosol of *R. oryzae*, we investigated the inhibitions of cytosolic fumarase by fumaric acid and substrate analogs (such as succinate, L-aspartate, and 3-hydroxybutyric acid).

Materials and Methods

Microorganism and Culture Conditions

R. oryzae ME-F12, a mutant of *R. oryzae* ATCC 20344, was used in this study. The fungus was grown on potato dextrose agar slants at 35 °C for 7 days. For the experiments, the fungal spores were harvested from plants by using a platinum loop and suspended in sterilized water maintained at 4 °C. The spores (a final concentration of 10^7 spores/ml) were grown for 36 h in 50 ml seed culture medium containing glucose 30.0, urea 2.0, KH_2PO_4 0.6, $MgSO_4{\cdot}7H_2O$ 0.5, $ZnSO_4$ 0.11, and $FeSO_4{\cdot}7H_2O$ 0.0088 g l^{-1}, in 250 ml Erlenmeyer flasks at 35 °C with shaking at 200 rpm. This was inoculated at 10% (*v*/*v*) into the fermentation medium containing glucose 80, urea 0.1 (for the study of carbon–nitrogen ratio, the urea concentrations used were 0.1, 0.2, 0.4, 1, and 2 g l^{-1}), and $CaCO_3$ 50 g l^{-1} with other ingredients similar as the seed culture medium. Sterile exceed $CaCO_3$ was added to maintain the pH at 5.5. All the media were autoclaved at 115 °C for 30 min.

Enzyme Assay

Fumarase was assayed by the method of Kanarek and Hill [10] at 250 nm with L-malic acid as the substrate. The standard incubation mixture for fumarase activity, consisting of 50 mM potassium phosphate (pH 7.4), 50 mM sodium L-malate, was used. In the section of substrate analogs, the different concentrations of substrate analogs were added in the reaction system. To investigate the inhibition of fumaric acid on the cytosolic fumarase, reverse reaction was assayed in the same way except that 50 mM sodium fumarate was used as substrate, and the absorbance change at 300 nm was measured [11].

The specific enzyme activities were expressed as micromoles per minute per milligram (U/mg). The amount of protein was determined by the Bradford method [12] with bovine protein albumin as the standard.

Preparation of Cytosolic Fumarase

Cytosolic fumarase was prepared by the method of Daum et al. [13], with a little modification. Cells were harvested by centrifugation (5 min at 5,000×*g*), washed once with distilled water and washed once with 10 mM Tris–Cl, pH 7.4, 0.9% NaCl. Precipitates were harvested by centrifugation for 5 min at 3,500×*g* at 4 °C. After chilling on ice, spheroplasts were homogenized by many strokes in a tight-fitting Dounce homogenizer. After about 20–30 min, all the cells had been broken. From this point on, all operations were carried out at 4 °C. The supernatant was saved, and then they were centrifuged for 30 min at 40,000×*g*. Cytosolic fumarase was in the supernatant and saved at 4 °C for further study.

Analytical Methods

Fumaric acid was determined and quantified by HPLC (Dionex P680 pump, Chromeleon controller and Dionex UVD 170U Detector) with a Bio-Rad Aminex HPX-87H ion-exclusion column (300 by 7.8 mm). The column was eluted with diluted sulfuric acid (0.005 M) at a flow rate of 0.8 ml/min over a 15-min period. The concentration of glucose in the culture was measured with a biosensor with glucose oxidase electrode (Institute of Biology, Shandong Academy of Sciences SBA-40C). Biomass was determined by weighting the mycelia mass after drying at 60 °C overnight. The yield of fumaric acid was calculated by the amount of fumaric acid produced (g l^{-1}) divided by the amount of glucose consumed (g l^{-1}).

Results

Effect of Carbon–Nitrogen Ratio on the Production of Metabolites and the Activity of Cytosolic Fumarase

Five different concentrations (0.1, 0.2, 0.4, 1, and 2 g l^{-1}) of urea were employed in this study. The specific activities of cytosolic fumarase under different nitrogen concentrations were shown in Fig. 1. Obviously, under high carbon–nitrogen ratio, the activity of

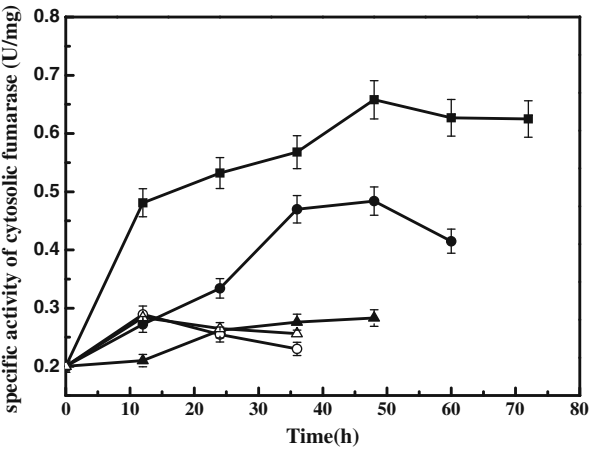

Fig. 1 Specific activities of cytosolic fumarase under different urea concentrations (L-malic acid as substrate). Concentrations of urea were 0.1 (*filled square*), 0.2 (*filled circle*), 0.4 (*filled triangle*), 1.0 (*circle*), and 2.0 g l^{-1} (*triangle*)

cytosolic fumarase was high, simultaneously specific production rate of fumaric acid reached the highest level. At 48 h of the fermentation process, the specific activity of cytosolic fumarase reached the highest point when the urea concentrations were 0.1, 0.2, and 0.4 g l^{-1}.

Table 1 showed the results of acid production, biomass, fermentation time and yield of fumaric acid under these different conditions. Biomass and L-malic acid increased with the increase of urea concentration. In reverse, fumaric acid production sharply decreased from 40.3 to 14.4 g l^{-1}. The yield of fumaric acid was higher under lower urea concentration. The highest yield reached 0.51 g/g at urea concentration of 0.1 g l^{-1}. Moreover, the glucose consumption rate decreased with the decrease of urea concentration.

As a result, lower urea concentration is beneficial for the cytosolic fumarase activity and accumulation of fumaric acid. Nitrogen limitation is a primary factor for the high production of fumaric acid by *R. oryzae*. In addition, cytosolic fumarase plays an important role in the cytosol pathway.

Inhibition of Cytosolic Fumarase by Fumaric Acid

Under all the urea concentrations media, the cytosolic fumarase was always not inhibited by fumaric acid in the process of fermentation. Figure 2 showed the specific activity of cytosolic fumarase, 50 mM fumaric acid as substrate, under different urea concentrations. The specific activity of cytosolic fumarase at lower urea concentration was higher than that under higher urea concentration.

Inhibitions of Cytosolic Fumarase by Substrate Analogs

Substrate analogs such as succinate, L-aspartate, and 3-hydroxybutyric acid (TCI, Japan) were investigated to characterize cytosolic fumarase. Among these substrate analogs (shown in Table 2), 3-hydroxybutyric acid (25 to 50 mM) inhibited the cytosolic fumarase significantly. The specific activity decreased more than 50% (data shown in Fig. 3). Table 3 showed the effect of 3-hydroxybutyric acid concentration on the metabolites of *R. oryzae*. With the increase of 3-hydroxybutyric acid concentration (20 to 50 mM), fumaric acid decreased from 40.3 to 14.1 gl^{-1} and the by-product L-malic acid increased from 0.3 to 5.4 gl^{-1}. However, oxalic acid, α-ketoglutarate, and fermentation time changed slightly. Because 3-hydroxybutyric acid inhibited the cytosolic fumarase, the reaction from L-malic acid to fumaric acid was partly inhibited. Consequently, more intermediate metabolite L-malic acid was accumulated.

Table 1 Effect of carbon–nitrogen ratio on the production of metabolites and biomass

Concentration of urea (g l^{-1})	Fumaric acid (g l^{-1})	L-malic acid (g l^{-1})	Biomass (g l^{-1})	Fermentation time (h)	Average glucose consumption rate (g/l/h)	Yield of fumaric acid (g g^{-1})
0.1	40.3±1.2	0.3±0.05	4.2±0.8	72±4	1.1	0.51
0.2	37.3±1.4	0.6±0.12	4.4±0.7	60±4	1.3	0.47
0.4	25.6±1.6	0.8±0.07	4.7±0.6	50±4	1.6	0.32
1.0	15.6±0.9	1.6±0.3	11.1±1.2	36±6	2.2	0.20
2.0	14.4±1.3	2.1±0.6	12.8±0.9	36±6	2.2	0.18

Results shown are average of three replicate experiments

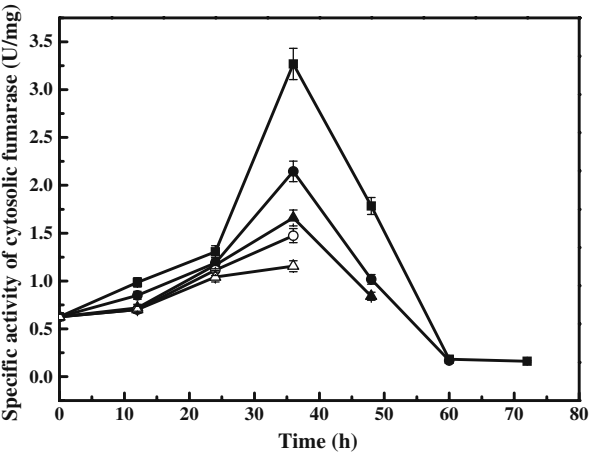

Fig. 2 Specific activities of cytosolic fumarase under different urea concentrations (fumaric acid as substrate). Concentrations of urea were 0.1 (*filled square*), 0.2 (*filled circle*), 0.4 (*filled triangle*), 1.0 (*circle*), and 2.0 gl^{-1} (*triangle*)

Discussion

In this study, the relationship among the carbon–nitrogen ratio, cytosolic fumarase, and yield of fumaric acid were investigated. We could conclude that cytosolic fumarase played a rather important role in the high accumulation of fumaric acid. Under lower urea concentration, both the activity of cytosolic fumarase and the yield of fumaric acid reached the highest, by-products such as L-malic acid were significantly reduced. High urea concentration was beneficial for biomass accumulation but inhibited the acid accumulation.

Stress appears to be a common requirement for the unusually high production and accumulation of organic acids by all producing organism. The most important factor is nitrogen limitation [14]; fungi starved of nitrogen produce mainly fumaric acid and/or L-malic acid, and not biomass, from glucose. In fermentations, only after full depletion of the limitation nitrogen source do the acids accumulate by secretion into the medium [9]. The definite relationship between cytosolic fumarase and production of fumaric acid can give efficient evident for the metabolic regulation of fumaric acid production by *R. oryzae*.

For many years, lots of efforts had been done on the research of fumarase of *R. oryzae*. Song et al. [15] cloned and characterized the FUMR from *R. oryzae* ATCC20344; the activity of FUMR catalyzing hydration of fumarate to L-malate was completely inhibited by 2 mM fumaric acid. While fumarase in the L-malic acid production organism, *A. flavus*, was not inhibited by fumaric acid, it catalyzed the hydration of fumaric acid to L-malic acid [16]. Until now, there were still no definite data to explain this strange phenomenon. In this study, we carried out a detailed study on the cytosolic fumarase of *R. oryzae*. The inhibitions of fumaric acid and substrate analogs on the cytosolic fumarase revealed the characters of cytosolic fumarase were different from other fumarase (such as from pig heart,

Table 2 Inhibitions of cytosolic fumarase by substrate analogs

Substrate analogs	Cytosolic fumarase
Succinate (20–200 mM)	Not inhibited
L-Aspartate (20–500 mM)	Not inhibited
3-Hydroxybutyric acid (25–50 mM)	Inhibited by at least 50%

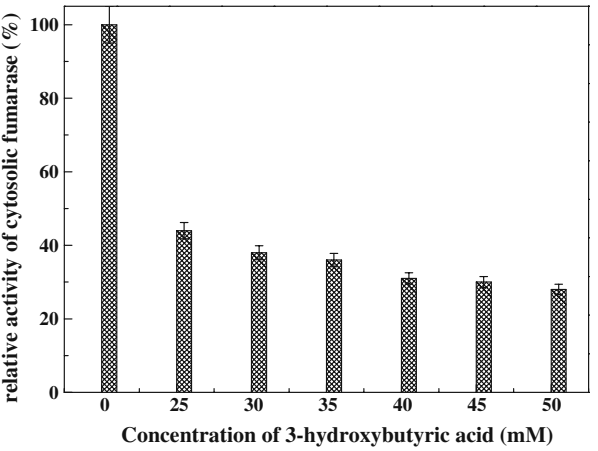

Fig. 3 Inhibitions of 3-hydroxybutyric acid (25 to 50 mM) on activity of cytosolic fumarase (the specific activity of cytosolic fumarase without addition of 3-hydroxybutyric acid as 100%)

Escherichia coli and *Corynebacterium glutamicum*). Usually, the additives may have toxicity on the cell [17]. However, substrate analogs 3-hydroxybutyric acid showed no toxicity on the cell. With the addition of 3-hydroxybutyric acid in fermentation, the glucose consumption rate did not change, other by-products, such as oxalic acid and α-ketoglutarate, changed slightly. As a result, 3-hydroxybutyric acid only affected the cytosolic pathway of *R. oryzae* and inhibited the cytosolic fumarase, led to the accumulation of L-malic acid.

Up to date, fumaric acid production by fermentation is still on the laboratory level [18]. With the increase of demand for biological fumaric acid, it is necessary to industrialize fumaric acid production by fermentation. Due to the high costs of pure materials, the process is less economic for industrial applications. The production of fumaric acid might be significantly reduced if cheap raw starch materials could be used [19]. However, there are many kinds of raw starchy materials. Different starchy materials have different compositions such as nitrogen and starch. For the relationship between carbon–nitrogen ratio and production of fumaric acid, this study provided a standard for the selection of raw materials.

In this study, we can conclude that the cytosolic fumarase plays an important role in the cytosolic pathway of *R. oryzae* for fumaric acid accumulation. Nitrogen source limitation is a significant factor for the activity of cytosolic fumarase and acid production. Substrate analogs 3-hydroxybutyric acid could inhibit the activity of cytosolic fumarase and result in

Table 3 Effects of 3-hydroxybutyric acid on the fermentation

Concentration of 3-hydroxybutyric acid (mM)	Fumaric acid ($g\ l^{-1}$)	L-malic acid ($g\ l^{-1}$)	Oxalic acid ($g\ l^{-1}$)	α-ketoglutarate ($g\ l^{-1}$)	Average Glucose consumption rate (g/l/h)	Biomass (g/l)	Fermentation time (h)
0	40.3±1.2	0.3±0.05	2.02±0.05	2.41±0.08	1.1	4.2±0.8	72±4
20	23.2±1.4	3.4±0.5	2.05±0.05	2.75±0.08	1.1	4.2±0.8	72±4
30	22.6±1.6	3.9±0.4	1.60±0.05	2.38±0.05	1.1	4.2±0.8	72±4
40	16.2±0.9	4.3±0.6	1.79±0.05	2.52±0.05	1.1	4.2±0.8	72±4
50	14.1±1.3	5.4±0.7	1.74±0.05	2.58±0.05	1.1	4.2±0.8	72±4

Results shown are average of three replicate experiments

the accumulation of L-malic acid. The selection of raw starch materials depends on the nitrogen content of each material.

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