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RAPID COMMUNICATION

Strain improvement of *Rhizopus oryzae* for over-production of fumaric acid by reducing ethanol synthesis pathway

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Abstract−*Rhizopus oryzae* mutants were isolated from the wild type strain ME-F01 after mutagenesis with UV coupled with nitrosoguanidine (NTG) and the following allyl alcohol resistance selection method. By analyzing the activities of alcohol dehydrogenase (ADH) involved in ethanol synthesis pathway and batch fermentation, one mutant, ME-UN-8, was isolated that produced 21.1% more fumaric acid than ME-F01 with the corresponding byproduct of ethanol decreased by 83.7%.

Key words: Alcohol Dehydrogenase, Allyl Alcohol, Fumaric Acid, Mutation, *Rhizopus oryzae*

INTRODUCTION

Fumaric acid is a four-carbon unsaturated dicarboxylic acid that is widely used as a food acidulant and beverage ingredient. 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]. Current US consumption of fumaric acid is about 40 million pounds per year [2]. Nowadays, it is derived exclusively from petroleum-based materials, but as the world's crude oil resources diminish, the production of chemicals from renewable resources is becoming more important.

Fumaric acid can be produced by fungal fermentation. Especially, the genus *Rhizopus* within the order *Mucorales* has been studied by many researchers [3-7]. The glucose metabolism was analyzed in *Rhizopus* spices by using the method of ^{13}C nuclear magnetic resonance, and enzymatic activity studies [5]. This proved that glucose was broken down into two molecules of pyruvate through the Embden-Meyerhof-Parnas (EMP) pathway, and then most pyruvate fluxes were transformed into fumaric acid via a carboxylation reaction yielding oxaloacetate, which was then converted to malic acid and further on to fumaric acid via the reductive reactions of the tricarboxylic acid cycle, while other fluxes flowed to ethanol, lactic acid, malic acid and other organic acids under limited nitrogen source [3,4].

The investigation of carbon metabolic products of *Rhizopus spices* showed that ethanol was a main metabolic by-product. The formation of ethanol would reduce the carbon flux conversion rate of pyruvate to fumaric acid during fumaric acid production [8-10]. Alcohol dehydrogenase (ADH) was the key enzyme in the ethanol synthesis pathway [11]. It was proposed that by screening the lower activity of alcohol dehydrogenase (ADH) mutant, thus decreasing the flux of pyruvic acid to ethanol may be a virtual method for increasing the conversion rate of glucose to fumaric acid. In the present work, mutagenesis of the wild type strain ME-F01 was conducted and the following an allyl alcohol resistance selection method [12,13], based on lethal effects of functional alcohol dehydrogenase (ADH) activity converting the allyl alcohol to toxic compound acrolein during production of ethanol, was developed to enhance fumaric acid production by selecting *Rhizopus oryzae* mutants carrying defects in the structural genes for the ADH involved in the ethanol synthesis pathway.

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EXPERIMENTAL

1. Microorganism and Culture Conditions

The strain used was *Rhizopus oryzae* ME-F01 obtained from American Type Culture Collection (ATCC). It was first grown on potato-dextrose agar (PDA) slants at 35 °C for 7 days. Spores were harvested from slants by using a platinum loop and suspended in sterilized water. The spores (a final concentration of 10^7 spores/ml) were grown for 36 h in 50 ml seed culture medium containing glucose 30 g/*l*, urea 2 g/*l*, KH_2PO_4 0.6 g/*l*, $MgSO_4 \cdot 7H_2O$ 0.5 g/*l*, $ZnSO_4$ 0.11 g/*l* and $FeSO_4 \cdot 7H_2O$ 0.0088 g/*l*, 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 100 g/*l*, urea 0.2 g/*l*, KH_2PO_4 0.6 g/*l*, $MgSO_4 \cdot 7H_2O$ 0.5 g/*l*, $ZnSO_4$ 0.11 g/*l*, $FeSO_4 \cdot 7H_2O$ 0.0088 g/*l*, $CaCO_3$ 50 g/*l*. The batch cultivations were conducted in a 5-*l* stirred bioreactor (NBS, New Brunswick, USA) containing 3 *l* fermentation medium at 35 °C, 0.5 vvm and 400 rpm for 96 h. Sterile exceed $CaCO_3$ was added whenever needed to maintain the pH at 5.5.

2. Mutagenesis and Selection of ADH Mutants

The wild type strain was mutagenized with UV coupled with nitrosoguanidine (NTG) based on the methods of Suntornsuk and Hang [14]. For UV mutagenesis, the single spore suspension was exposed to UV irradiation at 254 nm for 10 min at a distance of 30 cm. For NTG mutagenesis, 4 ml of the single spore suspension was treated with 100 μg NTG ml^{-1} of 100 mM phosphate buffer (pH 7) at 35 °C for 10 min. The coupling mutagenized spores were washed twice with sterile 100 mM phosphate buffer (pH 7) and once with sterile water;

the mutants were directly selected by plating onto PDA medium with the addition of allyl alcohol. This selective compound inhibits wild type cells, since cells with functional alcohol dehydrogenase (ADH) activity convert the allyl alcohol to toxic compound acrolein. Some of the survivors on the medium containing allyl alcohol were expected to produce less ethanol.

3. ADH Assays

To check the mutation in alcohol dehydrogenase (ADH), the wild type strain and its mutants were grown for 24 h in fermentation medium at 35 ºC. Biomass was filtered and washed twice with distilled water and once with the 100 mM phosphate buffer (pH 7). Samples were crushed to a powder form in a mortar under liquid nitrogen. The powder was suspended in the 100 mM phosphate buffer (pH 7) and cell debris was removed by centrifugation at 4 ºC for 10 min at 10,000 r/min. The activity of ADH was assayed by the method of Shory et al. [12]. One unit enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 μM NADH in 1 min.

4. Assay of Metabolic Products

For glucose and ethanol concentration determination, samples were centrifuged and the resulting supernatants were used. For fumaric acid, lactic and malic acid concentration determination, samples were diluted by addition of distilled water and hydrochloric acid, heated at 80 ºC until the broth was clear, then centrifuged and the resulting supernatants were used.

Glucose, fumaric acid, ethanol, lactic and malic acid concentrations were measured by high performance liquid chromatography (Summit P 680 HPLC, Dionex, USA; Shodex RI-101 Refractive Index Detector, Showa Denko, Japan; Aminex HPX-87 H Ion Exclusion Column 300 mm×7.8 mm, Bio-Rad, USA) under the following conditions: sample volume 20 μl; mobile phase 0.005 M H_2SO_4; flow rate 0.8 ml min^{-1}; column temperature 60 ºC.

RESULTS AND DISCUSSION

1. The Metabolite Profiles of *Rhizopus oryzae* ME-F01

The major metabolites of *Rhizopus oryzae* ME-F01 are given in Fig. 1. Among these products, ethanol was the main metabolic byproduct. The formation of ethanol competed with the biosynthesis of fumaric acid for the cofactor NADH and reduced the carbon flux conversion rate of pyruvate to fumaric acid, resulting in a decrease of fumaric acid yield [15]. It was deduced that by impairing ethanol fermentation, it might be possible to shuttle pyruvate into fumaric acid. Glycolysis should not be compromised because regeneration of NAD^+ occurs with the conversion of pyruvate to fumaric acid. So isolating the mutants affected in ethanol synthesis would be a straightforward method to improve the substrate availability and fumaric acid yield.

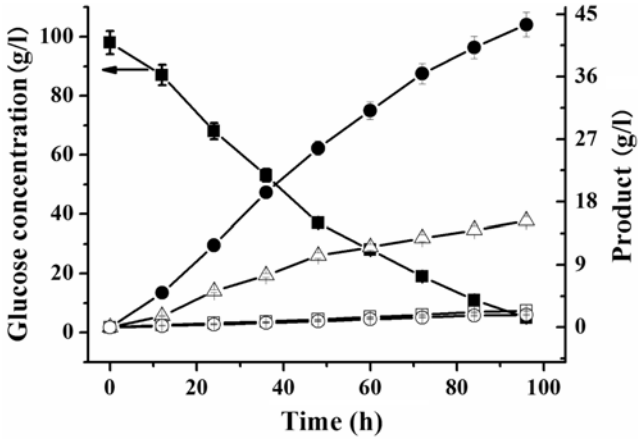

Fig. 1. Time-course of batch fermentation by *R. oryzae* ME-F01. fumaric acid (●); ethanol (△); lactic acid (○); malic acid (□); glucose (■).

2. Determination of Lethal Allyl Alcohol Concentrations

The effect of allyl alcohol on the growth of wild type strain was determined in order to find the suitable conditions for a lethal dose (Table 1). The spore survival was reduced by increasing the allyl alcohol concentration. Since low concentrations of allyl alcohol are better than high concentrations for avoiding osmotic inhibition of cell growth, the PDA medium containing 7 ml/*l* allyl alcohol was determined in here to be used for obtaining mutants in subsequent experiments.

3. Isolation of Mutants with Defects in Ethanol Synthesis Pathway

After dual mutagenization by UV and NTG, ten mutants were collected. These could have mutations in any gene for coding ADH catalyzing the ethanol synthesis, and thus might give increased fumaric acid production. All the obtained ten mutants numbered ME-UN-1, 2, …, 10 were transferred to the plate containing 7 ml/*l* allyl alcohol medium to investigate their hereditary stability for ten generations and all of them grew normally on this medium.

4. Analysis of ADH Activity Involved in the Ethanol Synthesis Pathway

To understand more clearly the changes in the mutants, the ADH activities of both wild type strain and its ten mutants were determined, respectively (Fig. 2). Six mutants were selected from the ten mutants obtained above (ME-UN-1, 3, 4, 6, 8, 9), and the ADH activities of which were smaller than half of the wild type strain.

Table 1. Effect of different allyl alcohol concentrations on growth of *Rhizopus oryzae*[a]

Allyl alcohol (ml/*l*)	0	1	2	3	4	5	6	7	8
Percentage survival[b] (%)	100	93.2	74.3	47.6	19.6	3.2	0.1	0	0

[a]Lethal concentrations of allyl alcohol on the growth of wild type strain were determined on the allyl alcohol-PDA medium containing different amounts of allyl alcohol. The single spore suspension was diluted in sterile physiological saline, and 1 ml spore suspension was spread onto the allyl alcohol-PDA medium to calculate the percentage survival

[b]Percentage survival (%)=(A×n_A/B×n_B)×100%; A, B indicate the average number of the colonies on the allyl alcohol-PDA and PDA medium plates of the spores, and n_A, n_B indicate the dilution times of the spores spread onto the allyl alcohol-PDA and PDA medium plates, respectively

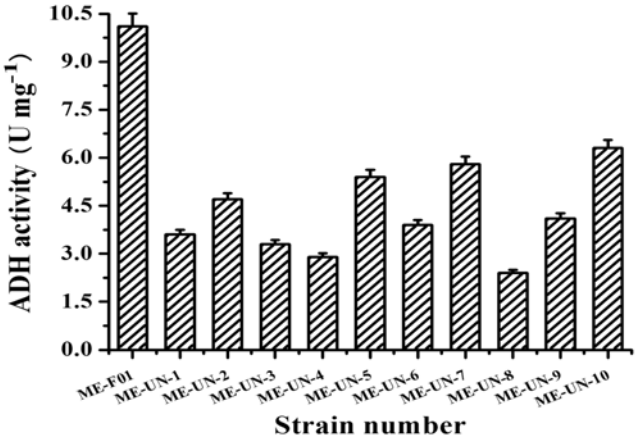

Fig. 2. Alcohol dehydrogenase activity of *R. oryzae* ME-F01 and its ten mutants.

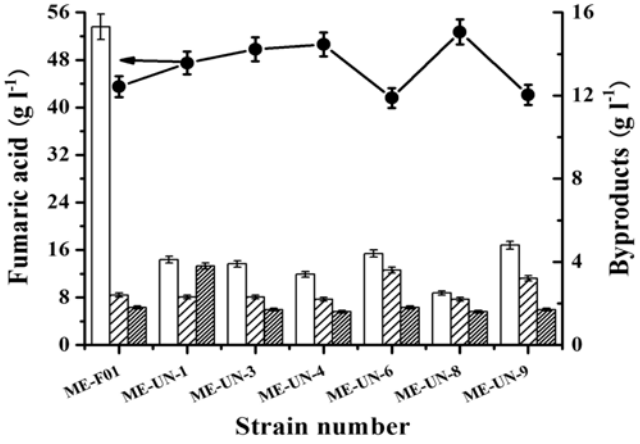

Fig. 3. Various metabolites produced by *R. oryzae* ME-F01 and its six mutants. Ethanol (blank bar), malic acid (wide cross-hatch bar), lactic acid (narrow cross-hatch bar), fumaric acid (●).

5. Analysis of Various Metabolites Formed by Different Mutants

Based on carbon metabolism network of *R. oryzae*, it is known that pyruvate can flow to malic and lactic acid in addition to fumaric acid and ethanol with the action of malate dehydrogenase and lactate dehydrogenase [5-7, 16, 17]. Thus, decreasing the pyruvate flux to ethanol may result in the increase of the flux to malic and lactic acid with the production of fumaric acid. So the levels of malic and lactic acid of six mutants (ME-UN-1, 3, 4, 6, 8, 9) and wild type strain in the bioreactor were also detected during fumaric acid fermentation.

Fig. 3 indicates that two mutants numbered ME-UN-6 and 9 showed a drop in production of ethanol, but fumaric acid production of which was also lowered in comparison with the wild type strain. On the contrary, malic acid production of the two mutants was enhanced unexpectedly. The problem arose in part from the inactivation of key enzymes such as pyruvate carboxylase and fumarase involved in the fumaric acid formation pathway [5,16]. On the other hand, in terms of the mutant numbered ME-UN-1, the production of lactic acid was enhanced during fumaric acid production; this might have been caused by the activation of lactate dehydrogenase (LDH) which catalyzed pyruvate to lactic acid [18,19]. However, the rest of the three mutants numbered ME-UN-3, 4 and 8 showed a higher fumaric acid production and high stability with little regression after ten generations (data not shown), while having no significant change in malic and lactic acid production with the ethanol production decreased sharply (Table 2).

Table 2. Comparison of the metabolite production of the wild type strain and the expected phenotype mutants

Strain no.	Fumaric acid (g/*l*)	Ethanol (g/*l*)	Malic acid (g/*l*)	Lactic acid (g/*l*)
ME-F01	43.5±1.7	15.3±0.6	2.4±0.1	1.8±0.1
ME-UN-3	49.8±2	3.9±0.2	2.3±0.1	1.7±0.1
ME-UN-4	50.6±2	3.4±0.1	2.2±0.1	1.6±0.1
ME-UN-8	52.7±2.1	2.5±0.1	2.2±0.1	1.6±0.1

SUMMARY

The ethanol synthesis pathway in *Rhizopus oryzae* ME-F01 was altered by the allyl alcohol resistance selection method. A mutant numbered ME-UN-8 that produced 21.1% more fumaric acid with the corresponding byproduct of ethanol decreased by 83.7% was isolated from the wide type strain, and it proved to be a promising strain for further fumaric acid production.

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