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ORIGINAL ARTICLE

High-throughput screening of high-yield colonies of *Rhizopus oryzae* for enhanced production of fumaric acid

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Abstract Fumaric acid is an important four-carbon dicarboxylic acid as a potential biorefinery target. A high-throughput screening method for fumaric acid overproduction strains was established. Nystatin (50 mg/L) was added into the production medium to restrict the spread of *Rhizopus oryzae* hyphae on agar plates. With bromocerol green as a pH indicator in the agar plates, the capability of fumaric acid biosynthesis by single colony was positively correlated with the diameter ratio of the colored ring and the colony. With this novel strategy, one high-yield mutant (*Rhizopus oryzae* ZJU11) was isolated from a large colony library of *Rhizopus oryzae* after UV irradiation. Starting with an optimized glucose concentration of 85 g/L, *Rhizopus oryzae* ZJU11 can produce 57.4 g/L fumaric acid in flask and 41.1 g/L in 5-L fermentor, which were 205% and 160% higher than that of the parent strain, respectively. Further studies showed that the production of fumaric acid by *Rhizopus oryzae* ZJU11 remained at the same level after three consecutive generations on the fermentation medium.

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Introduction

Fumaric acid is a natural four-carbon dicarboxylic acid with extensive applications in industries. Due to its non-toxic and non-hygroscopic properties, it is used as an acidulant in the beverages and food industry as well as in the pharmaceutical industry (Moresi et al. 1991). As it processes a double bond and two carboxylic groups, it is increasingly used as raw material in the etherification and polymer industry, such as the manufacture of synthetic resins and biodegradable polymers(Carta et al. 1999; Engel et al. 2008; Tao et al. 1993). In the present industry, fumaric acid is produced via the acid-catalyzed isomerization of maleic anhydride, which in turn is produced from butane. As petroleum prices are rising rather quickly and may be maintained at a very high level for a long time, maleic anhydride as a petroleum derivative has increased in price as well. This situation has caused a renewed interest in fumaric acid production by fermentation(Goldberg et al. 2006). This fermentation process is favorable because it can utilize a range of raw materials such as renewable carbohydrates to produce fumaric acid and simultaneously perform carbon dioxide fixation.

Fumaric acid can be produced by a variety of genera, including *Rhizopus*, *Mucor*, *Cunninghamella*, and *Circinella* species (Engel et al. 2008; Goldberg et al. 2006). Among these strains, *Rhizopus* species were the best-producing ones, yielding fumaric acid under aerobic and anaerobic conditions (Carta et al. 1999; Kenealy et al. 1986). Especially, *R. arrhizus* NRRL 2582 and *R. oryzae* ATCC 20344 gave the highest product titer, productivity and product yield values

(Cao et al. 1996b; Gangl et al. 1989). In order to improve the productivity of fumaric acid, physical and nutritional requirements were optimized for high accumulation of fumaric acid with *Rhizopus* species (Carta et al. 1999; Rhodes et al. 1959), and a comparison of fumaric acid production was studied by using different neutralizing agents (Petruccioli et al. 1996; Zhou et al. 2002). One of the difficulties of *Rhizopus* fermentation is that the mycelia tend to grow on the walls and on the stirrer of the reactor and sometimes form clumps. To reduce this oxygen transfer limitation, the formation of spherical cell pellets and rotary biofilm contactors were employed to produce fumaric acid and other organic acids, respectively, more efficiently (Cao et al. 1996a; Chen et al. 2009; Jiang et al. 2009a, b; Petruccioli et al. 1996; Liao et al. 2007; Xu and Yang 2007). However, few studies were focused on the genetic improvement of *Rhizopus* for enhancing the productivity of fumaric acid. The possible reason is that the mycelia of *Rhizopus oryzae* grow very quickly and spread everywhere on the surface of nutrient agar plates, and the picking of a single colony strain becomes ineffective. The development of some high-throughput screening methods is very beneficial to improve microbial genetics for the enhanced production of the targeted metabolites (Xu et al. 2005). In the present work, we provide an effective method to screen out high-yield mutants of *Rhizopus oryzae* by inhibiting the growth of mycelia in the nutrient plates and estimating the productivity of fumaric acid based on the diameter of the colored zone around a single colony. Furthermore, the fumaric acid productions of the mutant were investigated in the flask culture and batch fermentor.

Materials and methods

Microorganism

Rhizopus oryzae ZD-35 was used as the parent strain and preserved in the culture center at the Biological Engineering Institute in Zhejiang University (Hangzhou, PR China). It was cultivated and maintained on YMP agar plates. Spores from a 3-day-old plate at 35°C were suspended in sterile water for mutation.

Seed culture medium

All chemicals were purchased from Sangon Biotech (Shanghai) (PR China). The compositions of YMP medium used as seed medium are as follows: 3.0 g/L yeast extract, 3.0 g/L peptone, 3.0 g/L malt extract, and 20 g/L glycerol. To prepare the inocula, the spore suspension was inoculated into the seed medium at a level of 3% (v/v) and was incubated at 35°C and 100 rpm for 6 h.

Selective and fermentation medium

The selective medium containing: 50 g/L glucose, 1.24 g/L KNO_3, 0.16 g/L KH_2PO_4, 0.04 g/L $ZnSO_4{\cdot}7H_2O$, 0.25 g/L $MgSO_4{\cdot}7H_2O$, 5 g/L $CaCO_3$, 0.1 g/L bromocerol green and 50 mg/L nystatin. Fermentation medium containing: 85 g/L glucose, 1.24 g/L KNO_3, 0.16 g/L KH_2PO_4, 0.04 g/L $ZnSO_4{\cdot}7H_2O$, 0.25 g/L $MgSO_4{\cdot}7H_2O$, 0.01 mg/L biotin, and 15 mL/L methanol.

Mutagenesis procedure

UV irradiation was employed for mutagenizing the parent strain. It was performed as follows: the parent strain grew on YMP agar plates at 35°C for 3 days to form spores. Agar plates containing sporulated fungi were washed with sterile water to obtain spore suspension. The spore suspension was then filtered through sterile absorbent cotton to obtain single spore suspension and 8 mL single spore suspension was exposed to UV irradiation at 254 nm for 0–40 min at a distance of 20 cm.

Treated and untreated spores were diluted in sterile water and 0.2 mL spore suspension was spread onto the selective agar plate to calculate the percentage survival.

$$Percentage\ survival = \frac{A \times n_A}{B \times n_B} \times 100\%$$

A, B indicate the average number of the colonies on the selective agar plate of treated and untreated spores, respectively, and n_A, n_B indicate the dilution times of treated and untreated spores, respectively.

Strain selection procedure

A certain amount (0.2 mL) of UV treated spore suspension was spread onto the selective agar plates and cultivated at 35°C for 72 h. Then single colonies with larger diameter ratio of the color ring were selected for fumaric acid production. The selected mutants were first inoculated into a 250-mL flask containing 30 ml seed medium, the mycelium obtained were then transferred to 30 mL of fermentation medium in a 250-mL flask for the evaluation of fumaric acid production. The mutant with highest fumaric acid productivity was subjected to the next mutagenesis process. Strain mutagenesis and selection procedure is shown in Fig. 1.

Flask culture

For flask culture, 3 mL seed culture was transferred into a 250-mL flask containing 30 mL fermentation medium and cultivated at 35°C and 100 rpm for 48 h. Then, 0.6 g $CaCO_3$ autoclaved previously was then added to regulate

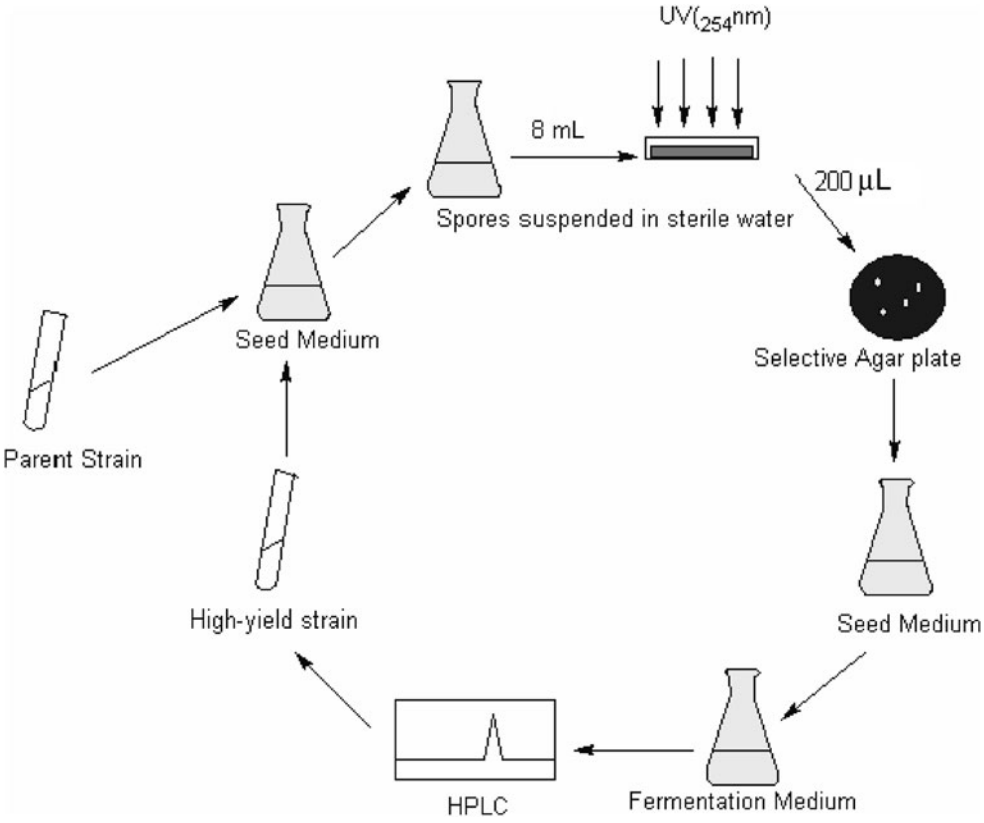

Fig. 1 Strain mutagenesis and selection procedure

pH, and allowed to grow at 35°C for another 72 h with shaking at 200 rpm.

Batch culture in stirred tank fermentor

A 5-L stirred tank fermentor (with a 3-L working volume) was used in this study. After 10% seed culture broth was inoculated, the fermentation was operated at an aeration rate of 3 vvm, 100 rpm and 35°C. After 29 h of incubation, the agitation speed was increased to 200 rpm and $CaCO_3$ (20 g/L) was added to regulate the pH. The batch fermentation was terminated after 120 h of incubation.

Analytical methods

A high performance liquid chromatograph (Agilent1100 Series, China) with a UV detector, a Hysperil BDS C18 column (Dalian Slite Scientific Instruments, Dalian, China) and an automatic injector was used to analyze fumaric acid. The mobile phase used was water and methanol at a ratio of

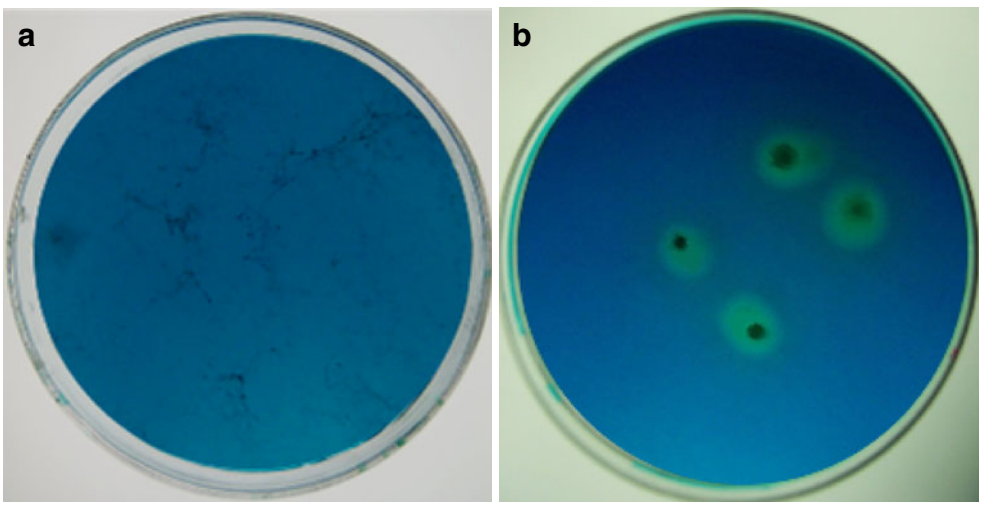

Fig. 2 Growth of *Rhizopus oryzae* on selective agar plate. **a** Selective agar plate containing 0.1 g/L bromocerol green, incubated at 35°C for 8 h; **b** selective agar plate containing 0.1 g/L bromocerol green and 0.05 g/L nystatin, incubated at 35°C for 72 h

9:1(v/v) and with pH value adjusted to 2.0 by $HClO_4$, and the flow rate was maintained at 0.8 mL/min.

Because of the low solubility of calcium fumarate, fumarate precipitated in the fermentation broth. The final culture broth was diluted by addition of hydrochloric acid and distilled water to neutralize excessive $CaCO_3$ and dissolve the fumarate before the samples were taken.

Glucose was analyzed by a glucose oxidase-peroxidase method using a glucose E-kit (Synermed, China).

Results and discussion

The appearance of single colonies in the medium supplemented with nystatin

We know that it is a useful approach to select a high-acid-producing strain by the area of colored rings, but this is not the case for *Rhizopus oryzae* due to the rapid spread of its growth mycelia on ordinary agar plates (Fig. 2a). In our work, an efficient procedure to screen for high level fumaric acid-producing strains was developed, based on the following. We found that the addition of nystatin to the medium can restrict the spread of *Rhizopus oryzae* hyphae on agar plates and make it possible to obtain a single colony for screening. The effect of nystatin on the growth of *Rhizopus oryzae* was investigated. It could not restrict the spread of the hyphae of *Rhizopus oryzae* efficiently when the concentration of the nystatin was below 40 mg/L. However, the hyphae of *Rhizopus oryzae* could be over-restricted, and the resulting colony was too small when the concentration of the nystatin was above 60 mg/L. As shown in Fig. 2b, the proper concentration of nystatin should be around 50 mg/L, which can restrict the spread of *Rhizopus oryzae* hyphae to obtain a isolated single colony but with no over-restriction.

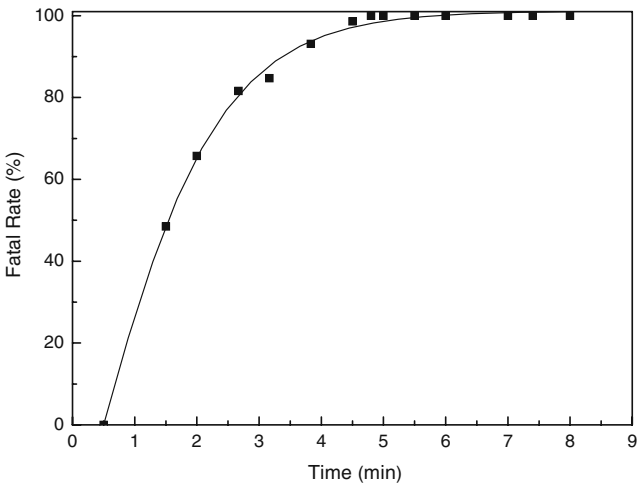

Fig. 3 Survival percentage after UV treatment

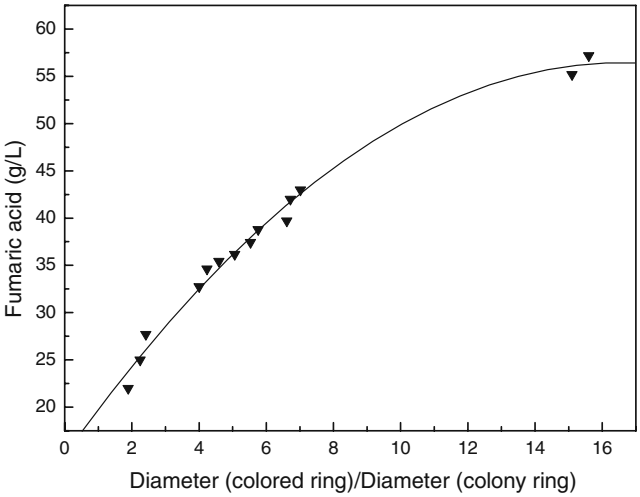

Fig. 4 Fumaric acid production by different strains of *Rhizopus oryzae*

Screening the mutants of *Rhizopus oryzae* for enhanced production of fumaric acid

The spore survival after UV treatment was shown in Fig. 3. In this work, 5% survival ratio was adopted, which corresponds to UV irradiation for 4 min. Nystatin (50 mg/L) was added into the sterilized selective medium and then the medium was poured into a 120-mm Petri dish. Figure 2b shows the growth of *Rhizopus oryzae* on the selective agar plate. It can be seen that nystatin effectively restricted the spread of *Rhizopus oryzae* hyphae and an apparent color ring appeared. Among the survivors, about 20 mutants with different color rings were isolated and further evaluated for fumaric acid production in flask. The relationship between fumaric acid yield and the size of the colored ring was then constructed. As shown in Fig. 4, the strain produced more fumaric acid with a higher ratio of the diameter of the color

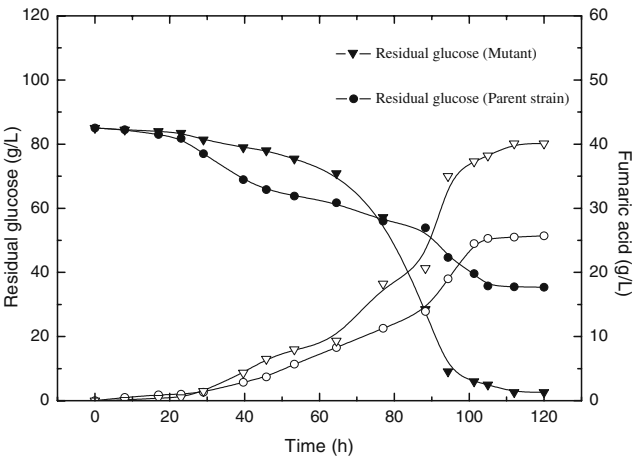

Fig. 5 Typical time course of fumaric acid production in fermentor by the mutant (*Rhizopus oryzae* ZJU11) and the parent strain (*Rhizopus oryzae* ZD-35)

ring to the diameter of colony. Therefore, it was feasible to screen the higher fumaric acid producing mutants by measuring the diameter ratio of the colored ring and the colony.

Fumaric acid production by *Rhizopus oryzae* ZJU11 in flask

After confirming that the highest fumaric acid productivity was achieved in flask culture, one mutant (*Rhizopus oryzae* ZJU11) was obtained after four rounds of UV mutagenesis, which was accordant with the observance of the largest colored ring on the selected medium. $CaCO_3$ was used to avoid acidification of the medium, which would affect the microbial growth and fumaric acid production. Starting with an optimized glucose concentration of 85 g/L, the *Rhizopus oryzae* ZD-35 produced 28.0 g/L fumaric acid after 120 h in flask, whereas the *Rhizopus oryzae* ZJU11 produced 57.4 g/L fumaric acid at the same time, which was 205% higher than that of the parent strain.

Furthermore, the stability of fumaric acid production of *Rhizopus oryzae* ZJU11 was studied by transfer generation. The production of fumaric acid remained almost at the same level (around 57 g/L) after three generations.

Fumaric acid production by *Rhizopus oryzae* ZJU11 in the bench-top fermentor

The fumaric acid production of parent strain and *Rhizopus oryzae* ZJU11 in a 5-L fermentor were investigated using optimized glucose concentration of 85 g/L. Figure 5 shows the time courses of fumaric acid production and residual glucose concentration. The *Rhizopus oryzae* ZJU11 consumed glucose more quickly than the *Rhizopus oryzae* ZD-35 and all the glucose was used up after 120 h in culture. During the 40–100 h time period, the production of fumaric acid gradually increased. Using $CaCO_3$ as the neutralizing agent in this fermentation has the advantage of avoiding base addition for pH control. But the precipitation of calcium fumarate in the broth results in higher viscosity of the medium. $NaHCO_3$ may be one alternative neutralizing agent due to the high solubility of the sodium fumarate, which offers the advantages of reuse of cells and simpler downstream separation (Zhou et al. 2002). Finally, the *Rhizopus oryzae* ZJU11 produced 41.1 g/L fumaric acid with an incubation period of 112 h, whereas 25.7 g/L fumaric acid was obtained by the *Rhizopus oryzae* ZD-35. The productivity of *Rhizopus oryzae* ZJU11 reached 0.37 g/L h, 60% higher than that of the parent strain. However, the fumaric acid production of the mutant in the stirred tank fermentor was 70% of that in flask culture. During the fermentation process, we found that the mycelia grew on the walls and on the stirrer of the fermentor, which should limit the substrate and oxygen diffusion and reduce the production level. An optimization of the fermentation conditions with *Rhizopus oryzae* ZJU11 is now under study, to further improve the fumaric acid production obtained.

Conclusion

In this study, an effective selection method for fumaric acid overproduction strain by measuring the diameter ration of the colored ring and the colony was established. To improve the production of fumaric acid from glucose by *Rhizopus oryzae*, we treated the strains by UV irradiation for 4 min. Through mutation and selection, a stable overproduction strain named *Rhizopus oryzae* ZJU11 was obtained, which produced 57.4 g/L fumaric acid in a flask and 41.1 g/L in 5-L fermentor, which was 105% and 60% higher than that of the parent strain, respectively. The heredity stability of the mutant was high because the fumaric acid production remained almost at the same level after three generations. This work will be helpful for large-scale production by fermentation of the increasingly demanded fumaric acid.

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