

Full Length Research Paper

Screening of allyl alcohol resistant mutant of *Rhizopus oryzae* and its fermentation characterization

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Ethanol is a main by-product in the fermentation broth of *Rhizopus oryzae* during the production of high-optical purity L-lactic acid. 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 L-lactic acid. Mutagenesis of *R. oryzae* As3.3461 was conducted in this study, and a high-producing lactic acid mutant HBF-12 was screened out with selective medium of Yeast-Peptone-Dextrose (YPD) containing allyl alcohol. This mutant showed higher conversion rate of 88.4 g/l to lactate in a 7 L bioreactor, which was an increase of 30.1% compared to original strain of 67.95 g/l, and the conversion rate to ethanol decreased 73.6%. The zymological analysis indicated HBF-12 had a lower ADH specific activity, but an appreciably higher lactate dehydrogenase (LDH) specific activity. The HBF-12 mutant also had a higher consumption rate of glucose and higher biomass collection.

Key words: Alcohol dehydrogenase, ethanol, L-lactic acid, *Rhizopus oryzae*.

INTRODUCTION

L-lactic acid is a versatile biological chemical, used as an acidulant, flavour and preservative in the food, pharmaceutical, leather and textile industries (Huang and Cui, 2005). Some *Lactic acid bacterium* strains that produce L-lactic acid were isolated from chicken faeces in sahelian region (Bayane et al., 2006), poultry farms in Senegal (Ibourahema et al., 2008). *Rhizopus oryzae* is an important filamentous fungus which synthesizes high optical-purity L-lactic acid under aerobic conditions (Yu et al., 2000), but the fungus suffers from some limitations such as longer period of fermentation, and lower conversion rate from glucose to lactate (Soccol et al., 1994). In recent years, some researchers (Bai and Zhao, 2004; Longacre et al., 1997) devoted themselves to the breeding by mutation of *R. oryzae* in order to find some strains with higher productivity of L-lactic acid, and some may succeed to some extent, but this success was acquired at the expense of tremendous procedures of mutagenesis-screening, and the target mutant obtained may encounter an instable heredity thus leading to strain

degeneration.

The glucose metabolism was analyzed in *R. oryzae* using a specific radioactivity curve-matching program. It proved that glucose was broken down into two molecules of pyruvate through Embden-Meyerhof-Parnas (EMP) pathway, and then most pyruvate fluxes were transformed into L-lactic acid with the catalysis of LDH, while other fluxes flowed to ethanol, oxaloacetate, malate, and fumarate (Wright et al., 1996). The investigation of carbon metabolic products of *R.oryzae* As3.3461 showed that ethanol was the main by-product of glucose metabolism during lactate production (Pan et al., 2006). So, screening the mutants with lower ADH activity may enhance the carbon flux conversion rate of pyruvate to L-lactic acid with a lower alcohol production. *R. oryzae* NRRL 395 has been mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and lower ADH activity was screened out with YPD agar medium containing 0.6% (v/v) allyl alcohol (Skory et al., 1998). The authors isolated a mutant that expressed only 5% of the wild type LDH activity under O₂ limiting conditions and produced nearly 40 g lactic acid/l in 70 h however, they did not investigate the lactate production of mutant and its related LDH and ADH activities under aeration conditions.

In the present research, a mutated *R. oryzae* strain with

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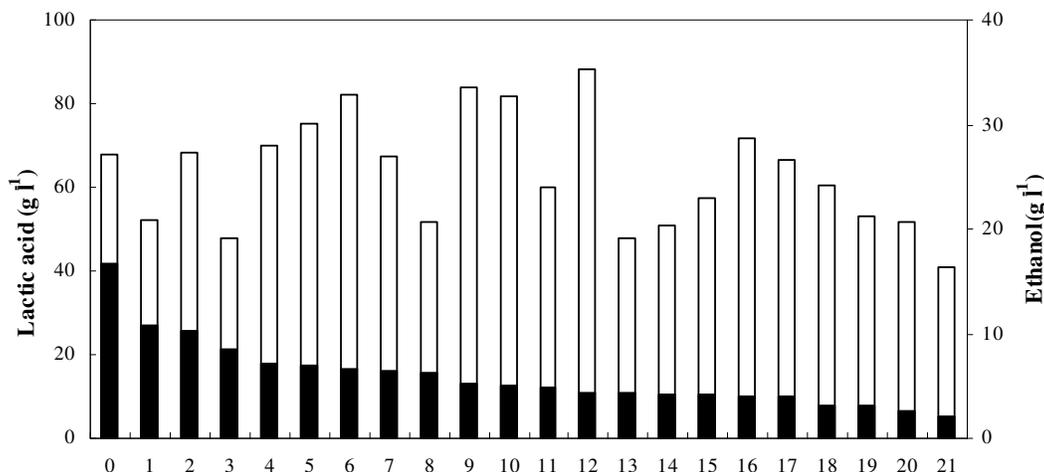


Figure 1. L-lactic acid (□) and ethanol concentrations (■) of 21 ADH mutants (1~21) and the wild type (0) of *Rhizopus oryzae* after cultured for 48 h in 250 ml shake flask which were run in the following conditions, 200 rpm and 32°C.

lower ADH activity was obtained in terms of the procedures employed by Skory et al. (1998), and the mutant's transformation characteristics of ethanol and lactate were investigated. These preliminary works are expected to provide theoretical basis for directed mutation breeding depending on metabolic network of microorganisms.

MATERIALS AND METHODS

Mutagenesis and selection of ADH mutants

Mutagenesis and selection of ADH mutants were performed in a microorganism lab of Hefei University of Technology, China, according to the procedures described by Skory et al. (1998). The strain employed was *R. oryzae* As3.3461, which was purchased from Type Culture Collection Center of Guangzhou Microorganism Institute in China.

Fermentation

Mutant isolates and the wild type control were inoculated in 50 ml preliminary medium consisting of (per liter of distilled water) 80 g glucose, 4 g (NH₄)₂SO₄, 0.4 g MgSO₄·7H₂O, 0.1 g ZnSO₄·7H₂O, 0.3 g KH₂PO₄, 60 g CaCO₃ in 250 ml shaking flask. The shaking flasks were run in the conditions of 200 rpm under 32°C for 48 h, then the fermentation was stopped and the broth was taken out for the analysis of lactic acid and ethanol concentrations. The ADH mutant which had the highest lactic acid conversion rate was selected and named, then was submitted for further fermentation research in a 7 L bioreactor. The selected ADH mutant and the wild type control were cultured respectively in 7 L stirred tank bioreactor. The compositions of seed medium were the same as the preliminary fermentation mentioned above. After being grown for 18 h, the seed medium was transferred to the 7 L stirred tank. The compositions of fermentation medium were the same as seed medium except glucose concentration which was 120 g/l. The stirred tank was run with 500 rpm stir speed and the aeration rate was 1.2-1.5 l/(l·min). Samples were withdrawn every 6 h during the fermentation period of 6 to 48 h. The samples were filtered and the filtrate was disposed for measurement of ethanol, lactic acid,

malate, fumarate and residual glucose concentrations. The mycelium was washed with deionised water; some were disrupted for determining the ADH and LDH specific activities, and the others were dried to constant weight for biomass measurement. The biomass was given as g dry cell wt per liter.

ADH and LDH specific activity assays

ADH and LDH activities were assayed as previously described (Skory et al., 1998; Skory, 2000). Protein concentration was determined by Coomassie Brilliant Blue G-250 method. The ADH and LDH specific activities were the values of total activities per mg protein.

Analytical methods

Reducing sugar was determined using the 3,5-dinitrosalicylate method. Ethanol was analyzed by gas chromatography with a column of GDX 102. Lactate, malate, and fumarate was tested using HPLC by a reversed-phase C₁₈ column as previously reported (Zheng et al., 2003). Cell growth was monitored by weighing the mass of dried mycelium. All determinations reported in this work were carried out in triplicate and experiments were executed at least in duplicate; the results are given as the mean values.

RESULTS AND DISCUSSION

The screening of ADH mutants and the influence on the production of lactic acid

After the mutagenesis of *R. oryzae* As 3.3461, 21 single clones were obtained on the YPD screening plate. The preliminary fermentation experiment was carried out with these 21 strains and the wild type was used as control. Figure 1 shows that lactic acid and ethanol concentrations of 21 ADH mutants were differed markedly. All mutants exhibited lower ethanol levels compared with the

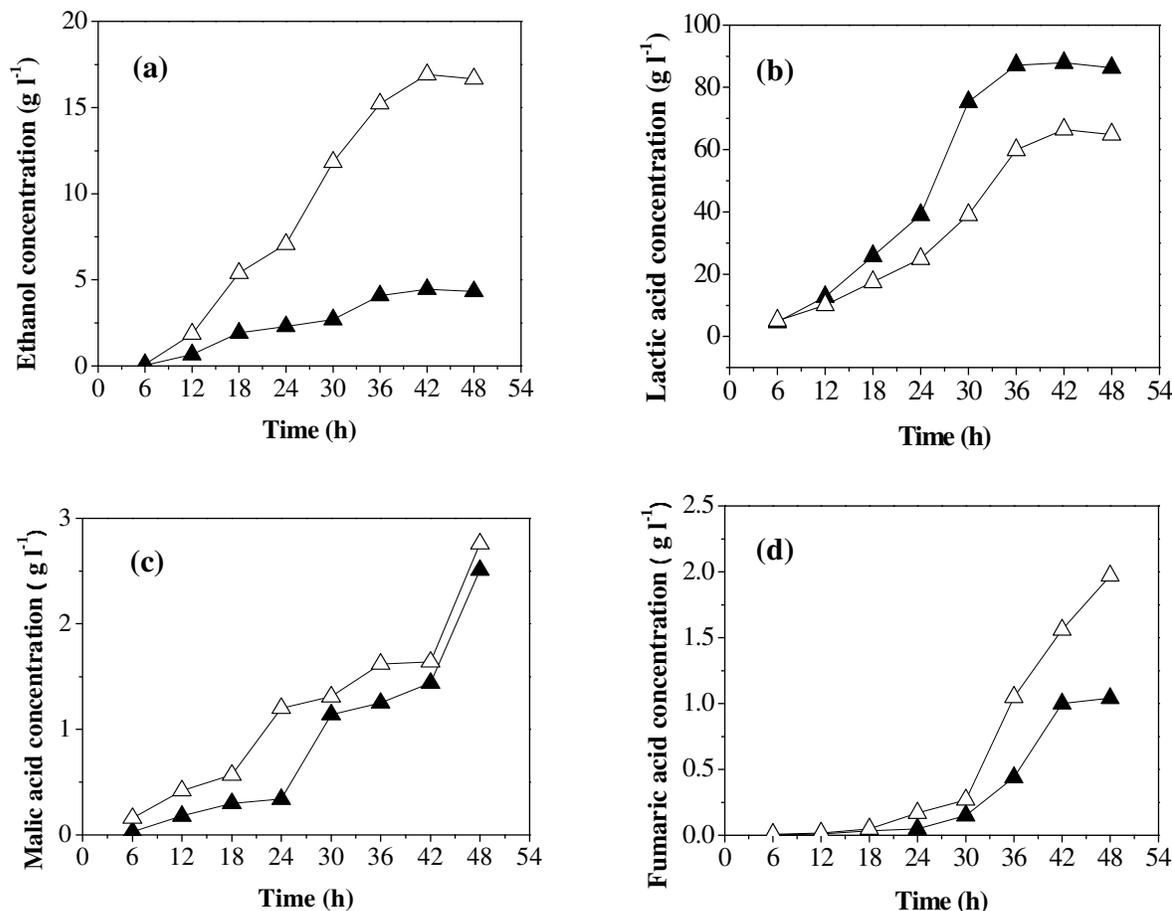


Figure 2. Ethanol, lactate, malate and fumarate concentrations of HBF-12 and the wild type *Rhizopus oryzae* AS3.3461 in a 7 L stirred tank fermenter at 500 rpm stir speed and aeration rate of 1.2 ~ 1.5 L/(L·min) (▲, HBF-12; and □, wild control).

wild type control of 16.75 g/l. Regarding the lactic acid conversion rate, there were 8 ADH mutants, namely No.2, 4, 5, 6, 9, 10, 12, 16, that had higher lactic acid levels than control. The No.12 mutant, which had the highest lactic acid concentration, designated as HBF-12, was selected for subsequent fermentation study.

The shifts characteristics of main metabolic products of ADH mutant of *Rhizopus oryzae*

Figure 2 (a) indicates that the accumulation of ethanol of HBF-12 was decreased significantly compared to the wild control type. The content of ethanol of wild type after fermentation for 42 h was 16.92 g/l, but the highest ethanol concentration of HBF-12 during the 48 h fermentation never exceeded 4.5 g/l. Figure 2 (b) showed that the yield of lactic acid of HBF-12 was always higher than that of the wild type during the fermentation process. Compared to the wild type, there was a 45.8% increased by the end of fermentation. It is speculated that the

specific activity of ADH of mutants decreased in comparison with the original strain, which resulted in impairing pyruvate fluxes to ethanol branch, and ultimately enhanced the carbon flow rate to the pathway of lactic acid through the catalysis of LDH. Figures 2 (a) and (b) also indicated that it was feasible to screen out the mutants of lower ADH activity to increase the yield of lactic acid.

Based on carbon metabolism network of *R. oryzae*, it is known that pyruvate can flow to malate and fumarate in addition to lactate and ethanol with the action of pyruvate carboxylase, malate dehydrogenase and fumarase. Thus, decreasing the pyruvate flux to ethanol may result in the increase of the flux to malate and fumarate with enhancing lactate production. So the levels of malate and fumarate of HBF-12 mutant and wild control in the bioreactor were detected. The ADH mutant had a slightly higher level of malate and fumarate than the original strain during fermentation. But the total content of malate and fumarate was very low compared with ethanol and lactate produced by HBF-12 mutant or wild type control

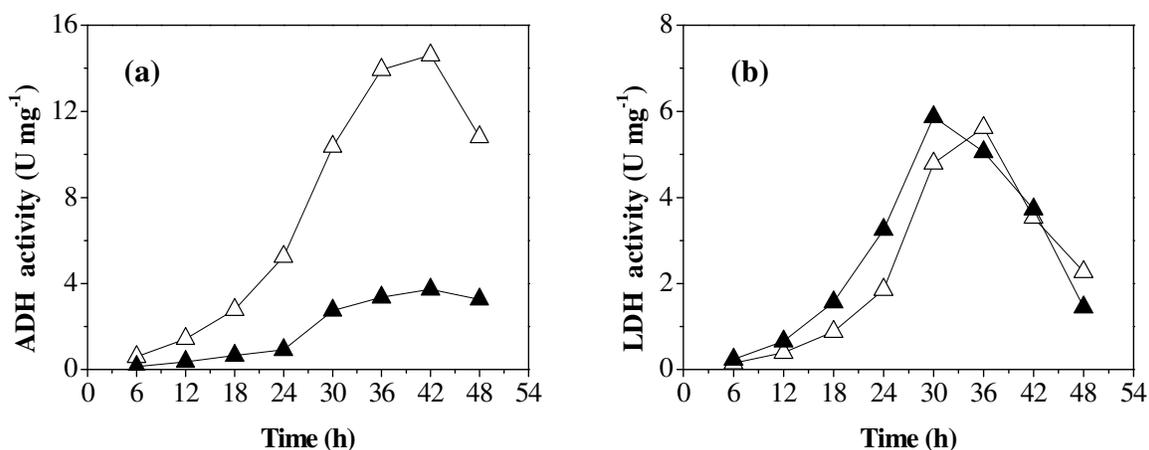


Figure 3. ADH and LDH specific activity of HBF-12 and the wild type *Rhizopus oryzae* AS3.3461 in a 7 L stirred tank fermenter at 500 rpm stir speed and aeration rate of 1.2~1.5 L/(L·min) (▲, HBF-12; and □, wild control).

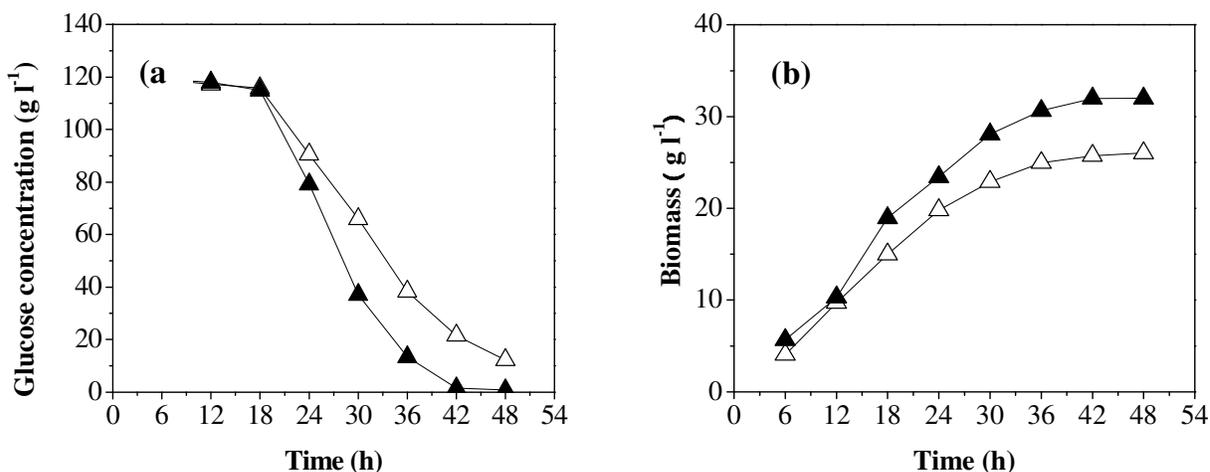


Figure 4. Glucose content and biomass concentrations of HBF-12 and the wild type *Rhizopus oryzae* AS3.3461 in a 7 L stirred tank fermenter at 500 rpm stir speed and aeration rate of 1.2~1.5 L/(L·min) (▲, HBF-12; and □, wild control).

(Figures 2 (c) and (d)).

The changes of ADH and LDH specific activities during the fermentation process

Figures 3 (a) and (b) demonstrate the ADH specific activity of HBF-12 which was always significantly lower than that of the wild type control during the fermentation process, while the specific activity of LDH was appreciably higher than the wild type. The maximum values of specific activities of ADH and LDH were apparent round 36 h of fermentation process. The trends shown by Figures 3 (a) and (b) correlated well with the results of Figures 2 (a) and (b). Other research on the relative

intensities of ADH zymogram from *R. oryzae* NRRL 395 correlated well with ADH activities (Skory et al., 1998).

The accumulation of biomass of mutants and the utilization of reducing sugar

The change of biomass and the utilization of reducing sugar with the wild type and mutant HBF-12 in fermentation were also studied. The results are shown in Figures 4 (a) and (b).

Figures 4 (a) and (b) indicate that the rate of glucose utilization of the wild type strains was 82.3% after fermentation of 48 h, but the utilization rate of the HBF-12 mutant was 94.5%, higher than that of the wild type. This

predicated that ADH mutant HBF-12 had a higher consumption rate of glucose than wild strain. Biomass curves of ADH mutant HBF-12 and wild strain showed that the former had higher biomass collections than wild type, so it was beneficial to increase the utilization rate of glucose.

Conclusion

In recent years, some researchers (Bai and Zhao, 2004; Longacre et al., 1997) devoted themselves to the breeding by mutation of *R. oryzae* in order to find some strains with higher productivity of L-lactic acid, and some may succeed to some extent, but this success was acquired at the expense of tremendous procedures of mutagenesis-screening, and the target mutant obtained may encounter a instable heredity thus leading to strain degeneration. In this study, a mutant of *R. oryzae* As 3.3461 with lower ADH activity was screened out; it had higher conversion rate of 88.4 g/l to lactate in a 7 L bioreactor, which was an increase of 30.1% compared to original strain of 67.95 g/l, and the conversion rate to ethanol decreased 73.6%. This indicates that we can achieve the goal of enhancing the lactate production by directed mutation breeding depending on metabolic network of carbon of *R. oryzae*.

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