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SHORT COMMUNICATION

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Reducing by-product formation in L-lactic acid fermentation by *Rhizopus oryzae*

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Abstract During L-lactic acid fermentation by *Rhizopus oryzae*, increasing the phosphate level in the fermentation medium from 0.1 g l^{-1} to 0.6 g l^{-1} KH_2PO_4 reduced the maximal concentration of L-lactic acid and fumaric acid from 85 g l^{-1} to 71 g l^{-1} and from 1.36 g l^{-1} to 0.18 g l^{-1}, respectively; and it decreased the fermentation time from 72 h to 52 h. Phosphate at 0.40 g l^{-1} KH_2PO_4 was suitable for both minimizing fumaric acid accumulation and benefiting L-lactic acid production.

Keywords Fermentation · Fumaric acid · L-Lactic acid · Phosphate · *Rhizopus oryzae*

Introduction

Rhizopus oryzae, an important microorganism in industrial fermentation, is widely used to produce L-lactic acid and other organic acids. Because it can produce only one stereospecific product (L-lactic acid) rather than a racemic mixture, it caters to the need for producing a food additive as both acidulant and preservative. The novel use of biodegradable plastic (polylactic acid) further promotes the application of L-lactic acid. It is estimated that the L-lactic acid market could exceed 10^6 t within a decade [1].

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Many programs on both fundamental studies and commercial developments have been carried out in order to increase the yield and productivity of L-lactic acid [2]. Besides the main product, L-lactic acid, the process of L-lactic acid fermentation can synchronously produce many other metabolites as byproducts, such as fumaric acid, ethanol, malic acid, etc. The amount of these metabolites can greatly influence the downstream process and the quality of the L(+)-lactic acid produced.

Fumaric acid is the main byproduct, due to the special metabolic pathway in L-lactic acid production by *R. oryzae* [3]. Its accumulation is affected by many factors, such as different neutralizing agents [4]. The main aim of this work was to investigate the variation in the production of fumaric acid and L-lactic acid under different initial phosphate concentrations. The optimal phosphate addition could decrease fumaric acid accumulation and consequently benefit L-lactic acid production.

Materials and methods

Microorganism

R. oryzae (CGMCC 3.1263) was employed in this research. The fungus grew and formed spores on potato/dextrose/agar slants at 32°C for 5 days. To prepare the inoculum, agar plates containing sporulated fungi were washed with sterile water to obtain a spore suspension.

Culture medium and growth conditions

The fermentation medium contained (per liter): 100 g glucose, 3.0 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.04 g $ZnSO_4{\cdot}7H_2O$ and different concentrations of KH_2PO_4 (0.10–0.60 g).

For each flask experiment, 1 ml spore suspension containing about 10^7 spores was transferred to a 250-ml flask containing 50 ml fermentation medium. $CaCO_3$ (2.5 g, sterilized separately) was added to the

fermentation medium in order to neutralize the organic acids produced in this process. The incubation was carried out for 72 h at 32°C, in a rotary shaker at 180 rev min^{-1}.

Analytical methods

Samples were withdrawn at intervals of 12 h, diluted by distilled water and filtered through preweighed filter paper to recover the mycelial biomass. The mycelia were washed twice with distilled water, dried at 80°C for 24 h and then weighed to determine the biomass. The supernatant was used to determine residual glucose by the dinitrosalicylic acid method and organic acids by HPLC with UV detector (Agilent). H_3PO_4 (0.01 mol l^{-1}, adjusted to pH 2.5 by adding 20% NaOH solution) was used as the mobile phase at a flow rate of 1.0 ml min^{-1} through a reverse column (Agilent) at 40°C. The organic acids were detected at 210 nm and the ethanol content was analyzed by GC (Agilent).

Results and discussion

To assess the effect of phosphate level upon the production of L-lactic acid, fumaric acid and mycelium biomass, flask fermentations with different initial phosphate levels (0.10–0.60 g l^{-1} KH_2PO_4) were carried out. The average results are summarized in Table 1. It was found that the highest L-lactic acid yield (0.81 g l^{-1}) and the lowest mycelial biomass (3.8 g l^{-1}) were obtained when using 0.10 g l^{-1} KH_2PO_4. As the initial phosphate concentration increased from 0.10 g l^{-1} to 0.60 g l^{-1} KH_2PO_4, the maximal L-lactic acid concentration in the broth decreased from 85 g l^{-1} to 71 g l^{-1}. Also, the fermentation time to maximal L-lactic acid concentration in the broth decreased from 72 h to 52 h, so that L-lactic acid productivity increased from 1.14 g l^{-1} h^{-1} to 1.37 g l^{-1} h^{-1}. But, the most interesting thing was that the fumaric acid concentration in the broth decreased greatly, from 1.36 g l^{-1} to 0.18 g l^{-1}. Undoubtedly, the metabolic flux in L-lactic acid fermentation was markedly affected by the phosphate concentration. However, the ethanol concentration under this condition fluctuated only slightly, between 3 g l^{-1} and 4 g l^{-1}. Comparing the results of yield and productivity of L-lactic acid and fumaric acid at different phosphate concentrations, the optimal phosphate concentration for obtaining a high L-lactic acid productivity and a low fumaric acid production was 0.40 g l^{-1} KH_2PO_4.

L-Lactic acid fermentation by *R. oryzae* using 0.40 g l^{-1} KH_2PO_4 in glucose-based medium is illustrated in Fig. 1. In the first 24 h fermentation (the mycelial growth phase), the mycelial biomass increased to 3.60 g l^{-1} and the phosphate concentration in the production medium fell sharply to about 0.06 g l^{-1}. After this period, the mycelial biomass remained almost constant throughout the fermentation and the fungus started to excrete L-lactic acid in large amounts. The highest L-lactic acid concentration in the broth (80 g l^{-1}) was obtained after 60 h fermentation, when the glucose was practically exhausted and the fumaric acid concentration was only 0.22 g l^{-1}. The L-lactic acid yield was 0.76 g l^{-1} glucose consumed and productivity was 1.34 g l^{-1} h^{-1}.

It was concluded that a high level of KH_2PO_4 in the medium can promote both mycelial growth and the

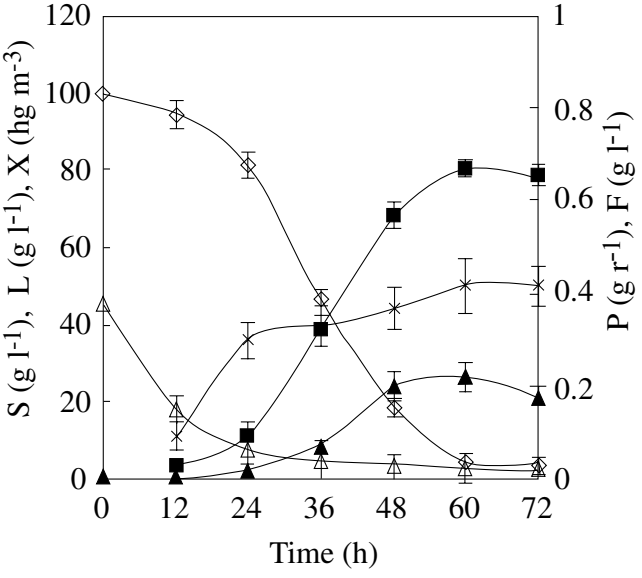

Fig. 1 Time-course of L-lactic acid production by *R. oryzae* at 0.40 g l^{-1} NaH_2PO_4 in glucose-based medium. *Filled squares* L-Lactic acid (*L*), *filled triangles* fumaric acid (*F*), *crosses* mycelial biomass (*X*), *open diamonds* glucose (*S*), *open triangles* phosphate (*P*)

Table 1 Average values at maximal L-lactic acid concentration in the broth, using data from three independent fermentation runs for each phosphate concentration. *T* Fermentation time, *L* L-lactic acid concentration, *F* fumaric acid concentration, R_L L-lactic acid production rate, *Y* yield of L-lactic acid vs glucose consumption, *Rx* mycelial growth rate, *E* ethanol concentration

KH_2PO_4 (g l^{-1})	*T* (h)	*L* (g l^{-1})	R_L (g l^{-1} h^{-1})	*Y* (g l^{-1})	*F* (g l^{-1})	Rx (g l^{-1})	*E* (g l^{-1})
0.10	75	85.0 ± 3.3	1.14	0.81	1.36 ± 0.15	3.8 ± 0.62	3.2 ± 0.2
0.25	70	82.0 ± 2.5	1.17	0.78	0.50 ± 0.09	4.5 ± 0.40	3.7 ± 0.4
0.40	60	80.0 ± 2.2	1.34	0.75	0.22 ± 0.03	5.0 ± 0.69	3.4 ± 0.4
0.60	52	71.0 ± 2.9	1.37	0.68	0.18 ± 0.04	6.2 ± 0.42	3.0 ± 0.3

consumption of substrate (glucose), thus facilitating the process of fermentation and shortening the duration of fermentation. Above all, the amount of fumaric acid in the broth can be greatly reduced, which benefits the downstream process and the quality of the L-lactic acid produced. However, the mechanism deserves further research.

Although the yield of L-lactic acid in a high phosphate concentration decreases slightly, there are advantages, such as higher volumetric productivity, shorter time of fermentation and lower quantity of byproduct. These advantages may offset the disadvantage of the lower yield. So, this is an economic method for the production of high-quality L-lactic acid.

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