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Enhancement of Fumaric Acid Production by *Rhizopus oryzae* Using a Two-stage Dissolved Oxygen Control Strategy

Yong-Qian Fu · Shuang Li · Yao Chen · Qing Xu · He Huang · Xiao-Yan Sheng

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Abstract Batch fermentative production of fumaric acid by *Rhizopus oryzae* ME-F12 was investigated in a 7-l stirred tank fermentor under different dissolved oxygen (DO) concentrations. High fumaric acid yield on glucose (0.56 g/g) was achieved under high DO concentration (80%), but the glucose consumption rate and fumaric acid productivity were rather low (0.91 and 0.51 g/l/h). Fumaric acid productivity was enhanced under low DO concentration (30%), but the fuamric acid yield on glucose decreased to 0.52 g/g. In order to achieve the high fumaric acid yield and productivity simultaneously, a two-stage dissolved oxygen control strategy was proposed, in which the DO concentration was controlled at 80% in the first 18 h and then switched to 30%. This was experimentally proven to be successful. Relatively high fumaric acid production (56.2 g/l), high fumaric acid yield on glucose (0.54 g/g), and high glucose consumption rate (1.3 g/l/h) were achieved by applying this strategy. The productivity (0.7 g/l/h) was improved by 37%, 21%, and 9%, respectively, compared with fermentations in which DO concentrations were kept constant at 80%, 60%, and 30%.

Keywords Batch fermentation · Fumaric acid · Kinetics · *Rhizopus oryzae* · Two-stage DO control strategy

Introduction

Fumaric acid is an important raw material of organic chemistry and has been applied in many fields such as materials, food, pharmaceutical, light industry, and so on. Current US consumption of fumaric acid was about 40 million pounds per year [1]. Most of the fumaric acid used nowadays is produced through the catalytic oxidation of benzene [2]. As benzene is a very well-known carcinogenic substance, there is a need for new routes to produce

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fumaric acid for the food and pharmaceutical industry and several trials have been made through fermentation with fungi using renewable resources.

The accumulation and excretion of fumaric acid, and to a lesser extent ethanol, lactic acid, and malic acid, by *Rhizopus* species occurs under aerobic conditions in a high-glucose medium containing a limiting amount of nitrogen [3–5]. In order to develop a cost-effective process for fumaric acid production by *Rhizopus* sp., researches were mainly focused on these aspects such as the improvement of applied microbial strain, the control of morphology, the exploitation of cheap feedstock and novel bioreactors [6–10]. However, in most studies of fumaric acid fermentation, the productivity of fumaric acid was only 0.32–0.63 g/l/h, with fumaric acid production of 17.5–43.5 g/l [7, 9, 11–13]. Although Cao et al. [3, 10] used a rotary biofilm contactor (RBC) as a fermentor with immobilized *Rhizopus oryzae* to produce fumaric acid and the fumaric acid productivity reached more than 3.78 g/l/h, the scalability potential of the RBC fermentor limits its commercial application. As fumaric acid is accumulated under aerobic conditions, it is therefore essential to study the effect of DO concentration on glucose consumption rate and fumaric acid formation rate. Ling and Ng [14] found that DO concentration was a critical factor for high-level production of fumaric acid. Higher DO concentration mainly led to the growth of cells at the expense of fumaric acid production [15, 16]. While when oxygen was limited, the main byproduct-ethanol would be then produced, and thus fumaric acid production was also decreased [17]. So they [14] patented an improved fermentation process to produce fumaric acid by controlling dissolved oxygen levels between 30% and 80%. When the dissolved oxygen concentration was lowered below 30% or heightened above 80% air saturation, a drastic decrease in the specific productivity of fumaric acid would be happened. All this information demonstrated the positive or negative effect of DO concentration on fumaric acid production, but no DO control strategy was set up for efficient fumaric acid production with high production, high yield, and high productivity.

Since *R.oryzae* is not commonly used in the fermentation industry, the physiological characteristics, including the demand for DO, are not yet well described. Therefore, in the present study, the processes of fumaric acid fermentation by *R. oryzae* were compared under different constant DO concentration. Subsequently, a two-stage DO control strategy, aimed at achieving high fumaric acid production, high yield, and high productivity, was designed based on the kinetic analysis of batch processes controlled by constant DO concentrations and was confirmed experimentally.

Materials and Methods

Microorganism

R. oryzae ME-F12, as the mutant of *R. oryzae* ATCC 20344, was used in this study. The fungus was grown on potato-dextrose agar slants at 35 °C for 6 days. For the experiments, the fungal spores in the slants were suspended in sterilized water maintained at 4 °C.

Culture Media

Preculture medium (grams per liter): glucose, 30; urea, 2.0; KH_2PO_4, 0.6; $MgSO_4{\cdot}7H_2O$, 0.5; $ZnSO_4$, 0.11; $FeSO_4{\cdot}7H_2O$, 0.0088. Fermentation medium (grams per liter): glucose, 100; urea, 0.2; $CaCO_3$, 50, with other ingredients similar as the preculture medium. The media were autoclaved at 115 °C for 30 min.

Cultivation

The preculture inoculated with spores (a final concentration of 10^7 spores/ml) was grown in a 250-ml shake flask containing 50-ml preculture medium and cultivated for 36 h on a reciprocal shaker with a rotation speed of 200 rpm at 35 °C. The preculture was then inoculated into 7.0-l stirred tank fermentor (New Brunswick Scientific, USA) containing 5.0-l fermentation medium with an inoculum size of 10% (*v*/*v*). Sterile excess $CaCO_3$ was added whenever needed to maintain the pH at 5.5. The dissolved oxygen concentration was monitored with an on-line DO sensor (InPro 6800, Mettler Toledo, Switzerland) and controlled at the set point by adjusting the agitation rate and the flow rate of filter-sterilized air.

Analytical Methods

Fumaric acid and ethanol 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; and column temperature, 60 °C. 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 mycelial mass after drying at 60 °C overnight.

Kinetic Parameters Calculation

The specific cell growth rate (μ, h^{-1}), specific glucose consumption rate (q_s, h^{-1}), and specific fumaric acid formation rate (q_p, h^{-1}) were estimated from experimental or fitted data of cell growth (*x*, grams per liter), residual glucose concentration (*s*, grams per liter), and fumaric acid production (*p*, grams per liter) by Eqs. 1, 2, and 3, respectively [18]. The fitted data were obtained by interposing between experimental data of cell growth, residual glucose concentration or fumaric acid production at definite time with the approximation method of cubic spline interpolation in Origin software (Version 7.5, OriginLab Corp., Northampton, Massachusetts, USA).

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta x}{\Delta t} \tag{1}$$

$$q_s = -\frac{1}{x}\frac{ds}{dt} = -\frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta s}{\Delta t} \tag{2}$$

$$q_p = \frac{1}{x}\frac{dp}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta p}{\Delta t} \tag{3}$$

Results and Discussion

All values presented in this study are averages of at least three independent trials.

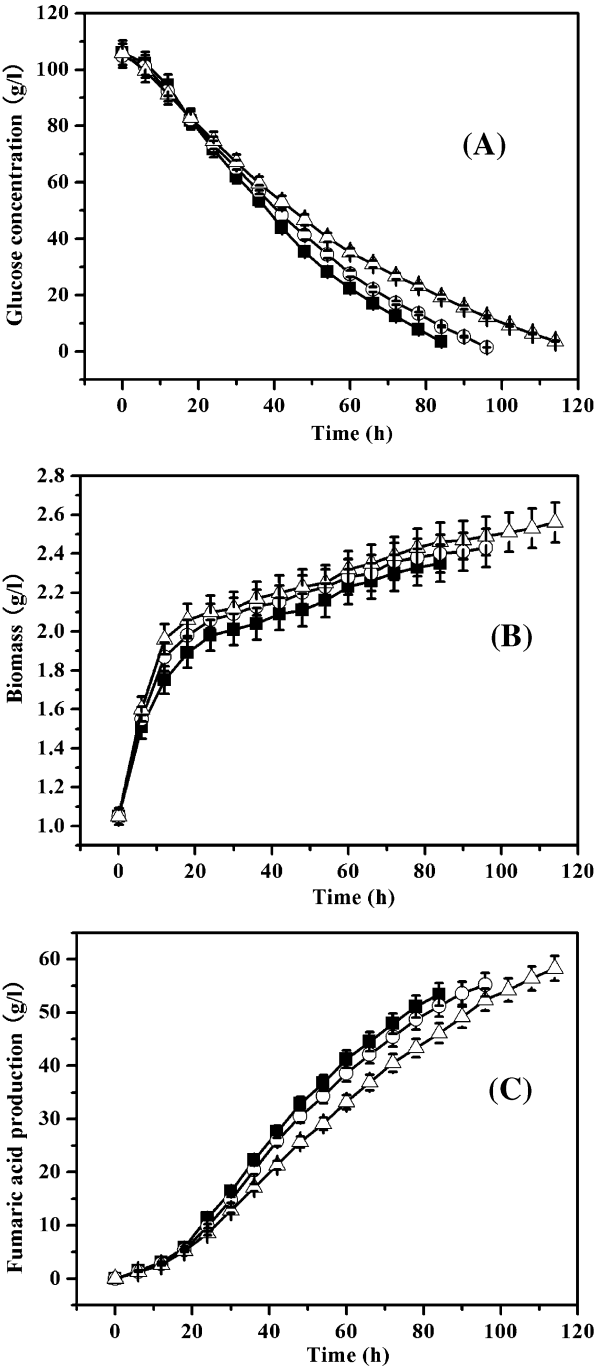

Fig. 1 Time courses of glucose consumption (**a**), cell growth (**b**), fumaric acid production (**c**), under various constant dissolved oxygen (DO) concentrations: 30% (*filled square*), 60% (*empty circle*), and 80% (*empty triangle*)

Effects of DO Concentrations on Fumaric Acid Fermentation

Effects of DO concentrations at the range of 30% to 80% on fumaric acid fermentation were investigated, respectively. The results indicated that DO concentration played a vital role in fumaric acid production. As shown in Fig. 1a–c, the relatively higher final fumaric acid production up to 58.3 g/l was obtained at the DO concentration of 80%. While when the DO concentration was lower, the final fumaric acid production decreased (55.2 g/l at 60% and 53.4 g/l at 30%). This indicated that lower DO concentration was not beneficial for fumaric acid production. The reason might be that the ethanol was relatively highly accumulated as the main byproduct respectively in these two cases (Table 1). Furthermore, although the final fumaric acid production at the DO concentration of 80% was higher compared with DO concentration of 60% and 30%, the former fumaric acid productivity (0.51 g/l/h) was lower than the latter (0.58 g/l/h at 60% and 0.64 g/l/h at 30%). The observations described above indicated that the optimum DO concentration for obtaining high fumaric acid production and high yield was not suitable for ensuring high fumaric acid productivity. It can therefore be concluded that high fumaric acid production, high yield, and high productivity could not be achieved simultaneously by controlling a constant DO concentration throughout the whole culture process.

Kinetic Analysis of Fumaric Acid Fermentation at Different DO Concentrations

To analyze the kinetic characteristics of the above two processes at the DO concentrations of 30% and 80%, three kinetic parameters, including specific glucose consumption rate (qs), specific cell growth rate (μ), and specific fumaric acid formation rate (qp), were calculated by an interpolation method based on the data of Fig. 1a–c and were shown in Fig. 2. Compared with the DO concentration of 30%, qs and μ were higher at the DO concentration of 80% at the beginning of fumaric acid fermentation (about 18 h). It showed that the DO concentration of 80% was better for glucose consumption and cell growth during the first 18 h. But after 18 h, the DO concentration of 30% was beneficial for fumaric acid formation with a high valve of qp. Based on the analysis of μ, qs, and qp, a two-stage DO control strategy was therefore proposed. In this strategy, the DO

Table 1 Comparison of process parameters under different DO concentration control modes.

Parameters	DO concentration (%)			
	30	60	80	80 (0–18 h), 30 (after 18 h)
Initial glucose concentration (g/l)	106	105	107	106
Residual glucose concentration (g/l)	3.5	1.5	3.7	2.4
Final fumaric acid production (g/l)	53.4	55.2	58.3	56.2
Final dry cell weight (g/l)	2.35	2.43	2.56	2.38
Final ethanol concentration (g/l)	15.2	10.5	5.1	7.4
Culture time (h)	84	96	114	80
Average fumaric acid yield on glucose (g/g)	0.52	0.53	0.56	0.54
Average glucose consumption rate (g/l/h)	1.22	1.08	0.91	1.3
Average fumaric acid productivity (g/l/h)	0.64	0.58	0.51	0.7

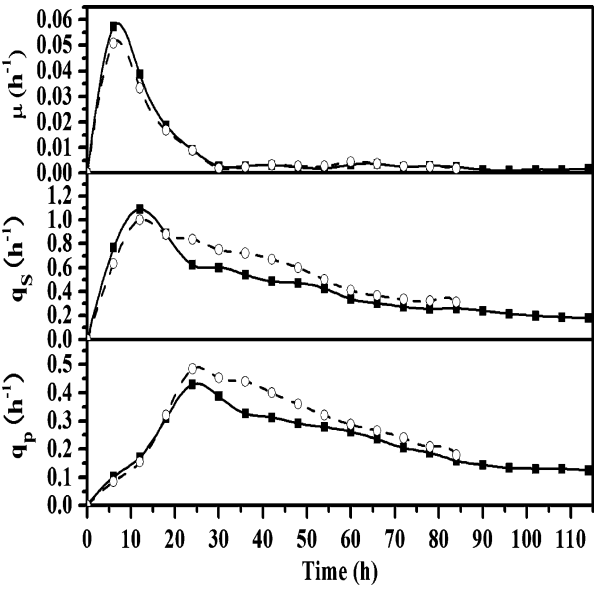

Fig. 2 Comparison of kinetic parameters in fumaric acid fermentation at the dissolved oxygen concentration of 80% (*filled square*) and 30% (*empty circle*): specific cell growth rate (μ); specific glucose consumption rate (q_s); specific fumaric acid formation rate (q_p)

concentration was controlled at 80% in the first 18 h to maintain high μ and q_s for fast cell growth and glucose consumption, and then switched to 30% after 18 h to maintain high q_p for high fumaric acid production.

Two-stage DO Control Strategy for Fumaric Acid Fermentation

Time course of two-stage DO control strategy for fumaric acid fermentation was shown in Fig. 3. To facilitate the comparison with other processes controlled by constant DO concentrations, the experimental results of using the two-stage DO control strategy were listed in the last column of Table 1. It could be seen from the last column of Table 1 that both higher fumaric acid yield on glucose (0.54 g/g) and higher glucose consumption rate (1.3 g/l/h) were achieved by applying the two-stage DO control strategy. Fumaric acid production reached 56.2 g/l at 80 h, achieving a high productivity of 0.7 g/l/h. This shows a 37%, 21%, and 9% improvement over cases controlled by constant DO concentrations of 80%, 60%, and 30%, respectively.

It can be concluded that the unification of relatively high fumaric acid production, high yield, and high productivity was achieved by applying the two-stage DO control strategy, combining the advantages of high fumaric acid production and yield under high DO concentration and high fumaric acid productivity under low DO concentration. The final fumaric acid production, yield and productivity reached 56.2 g/l, 0.54 g/g, and 0.7 g/l/h, respectively, which were at a relatively high level compared with values reported in the literature [11–13]. However, the reported high levels of fumaric acid fermentation were usually based on novel bioreactor such as rotary biofilm contactor, fluidized bed or bubble column, and high fumaric acid production, high yield, and high productivity were usually not able to be achieved simultaneously. Here, we achieved the goal just by applying a two-stage DO control strategy in the batch fermentation, which was easier to operate and control compared with batch fermentation in rotary biofilm contactor, fluidized bed or bubble

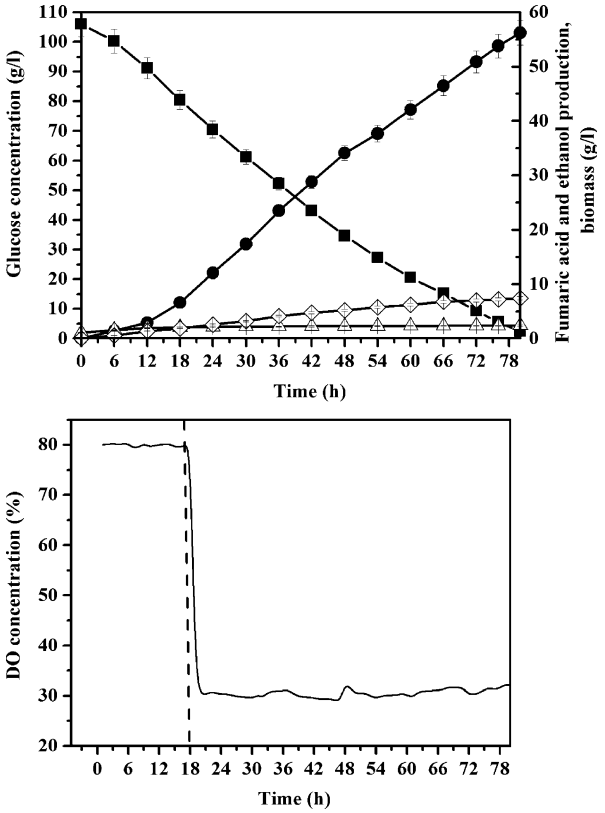

Fig. 3 Time course of glucose (*filled square*), fumaric acid (*filled circle*), ethanol (*empty diamond*), biomass (*empty triangle*) and dissolved oxygen (DO) concentration (curve 1) in batch production of fumaric acid applying the two-stage DO control strategy

column usually adopted in fumaric acid fermentation. The proposed two-stage DO control strategy was therefore proved to be successful to enhance fumaric acid production.

Conclusion

It was previously reported that DO concentration played an important role in fumaric acid fermentation [14–17]. In this study, we investigated the characteristics of fumaric acid fermentation under different constant DO concentrations by adjusting the agitation rate and the flow rate of filter-sterilized air, and it was interesting to find that significant differences occurred in μ, qs, and q_p in batch fermentation experiments using *R. oryzae* ME-F12 under different DO concentrations. And a proper DO concentration control method using a two-stage DO control strategy was therefore developed from a kinetic stand point. As shown in Figs. 1 and 3, the relatively high DO concentration in the first 18 h, resulted in an increase of cell growth and quick glucose consumption. While the decrease of DO concentration resulted in a quick fumaric acid formation rate after 18 h. Finally, relatively high fumaric acid production, high fumaric acid yield on glucose, and high glucose consumption rate were achieved by applying this strategy. The fumaric acid productivity (0.7 g/l/h) was also improved by 37%, 21%, and 9%, respectively, compared with fermentations in which DO concentrations were kept constant at 80%, 60%, and 30%.

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