Applications of One-Step Environmentally-Friendly Fermentation Method to Produce Fumaric Acid with Immobilized *Rhizopus arrhizus* in Stirred-Tank Reactor

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The traditional two-step cultivation mode for fungal cultivation is commonly divided into the seed stage (usually 24 to 48 h) and the production stage (usually 96 to 144 h). The use of two stages prolongs the total production cycle and generates excess wastewater. In this work, an efficient and environmentally friendly one-step fermentation method was applied for the production of fumaric acid by immobilized *Rhizopus arrhizus* in a stirred-tank reactor. The nitrogen source content and the agitation speed of the reactor were optimized as the two critical factors in the one-step fermentation process; the fumaric acid production of 51 g/L with a yield of 0.42 g/g glucose was comparable with the product from the traditional two-step fermentation process. Furthermore, the total production cycle was shortened to 96 h, and the amount of wastewater was reduced due to the avoidance of the seed culture process. Thus, utilization of the one-step fermentation method to produce fumaric acid was shown to be preferable feasibility and environmental-friendliness. This is a promising method for industrial production of fumaric acid and other high-value biochemicals by fungus.

Keywords: Fumaric acid; One-step fermentation; Two-stage regulation of agitation speed; Rhizopus arrhizus; Stirred-tank reactor

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INTRODUCTION

Given the aggravation of the environmental degradation and resource shortage, the development of environmentally friendly methods to produce clean energy is paramount (Fan *et al.* 2020). Fumaric acid, an important four-carbon dicarboxylic acid, is an intermediate in the TCA cycle, and it is a building block to synthesize more complex compounds in the chemical, food processing, agriculture, and pharmaceutical industries (Guo *et al.* 2020). Microbial fermentation using renewable biomass as raw material has the advantages of being environmentally friendly and sustainable (Abo *et al.* 2019; Liu *et al.* 2020). *Rhizopus, Saccharomyces cerevisiae*, *Torulopsis glabrata*, *Escherichia coli*, and their derivatives have been evaluated for their production capability of fumaric acid. *Rhizopus* is the most promising potential strain in industrial production due to its strong accumulation capacities of fumaric acid (Troiano *et al.* 2020).

To improve fumaric acid accumulation by *Rhizopus*, investigations have been carried out involving low-cost feedstock, isolation of high-performance strains, optimization of cultivation conditions, and mycelia morphology (Ilica *et al.* 2019). Few studies have paid attention to updating the cultural mode of *Rhizopus*. Currently, a two-step cultivation mode (divided into seed culture stage and fermentation stage) was utilized

in *Rhizopus* cultivation process by controlling different carbon/nitrogen ratios in seed and fermentation media. Sufficient cells are obtained in the seed culture stage with abundant nitrogen sources supplied, while nitrogen limitation in the production stage prevents the overgrowth of cells, leading to fumaric acid accumulation (Ding *et al.* 2011). Nevertheless, the residual seed culture broth (containing 4 g/L glucose, 3 g/L fumaric acid, and 5 g/L ethanol) is treated as waste after cultivation, which is a cost burden and a waste of water resources. A novel and superior culture is needed to alleviate these problems.

According to recent work, *Rhizopus* could be also used to produce lactic acid, and a convenient and economical one-step fermentation strategy has been applied to replace the conventional two-step fermentation of *Rhizopus*, which conducts the seed and fermentation culture stages together and leads to an increasing in lactic acid concentration and productivity (Fu *et al.* 2016, 2018). In this study, a similar one-step fermentation method was utilized to avoid generating excess waste water and to improve the efficiency of fumaric acid production by *Rhizopus arrhizus* in the stirred-tank reactor.

EXPERIMENTAL

Microorganism and Culture Medium

The strain used in this work was *R. arrhizus RH-7-13-9#*, which was cultured on an agar medium and stored at 4 ℃ (Liu *et al.* 2015). The medium used in one-step cultivation process contained 90 g/L glucose, peptone content optimized (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 g/L), 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1.76×10^{-3} g/L $ZnSO_4 \cdot 7H_2O$, and 4.98×10^{-4} g/L $FeSO_4 \cdot 7H_2O$. In addition, 50 g/L $CaCO_3$ and 0.2% (v/v) soybean oil were used as the neutralizer and antifoaming agent, respectively; these were added into the stirred tank reactor after cultivated for 24 h.

One-Step Fermentation Process

Mycelia immobilized fermentation was utilized for fumaric acid production in the one-step fermentation. Loofah fiber (prepared from dried *Luffa cylindrica* and purchased at Jinhua in the Zhejiang Province, China) was shown to be an available carrier for *R. arrhizus RH-7-13-9#* in the authors' previous work (Liu *et al.* 2017). Small loofah fiber cubes of 0.5 to 1 cm^3 were prepared, and 6.5 g/L loofah fiber cubes were added into a 5 L stirred-tank reactor as mycelia immobilized carriers. *R. arrhizus RH-7-13-9#* spores were inoculated as described previously (Liu *et al.* 2018b), as the ratio of 1% (v/v) into the 3 L medium. The one-step fermentation process was conducted at 30 ℃, 200 rpm, and 2.0 vvm for 108 h.

Analytical Methods

A high-performance liquid chromatograph (HPLC; Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector and UV detector (210 nm) was utilized to detect the titer of fumaric acid, by-product ethanol, and glucose consumption. The column used was a Bio-Rad Aminex HPX-87 H ion exclusion column (Richmond, CA, USA), and 0.005 M H_2SO_4 was used as eluent at 50 ℃ with a flow rate of 0.6 mL/min (Liu *et al.* 2018a). Due to the low solubility of fumaric acid and calcium fumarate, both accumulated as precipitated in the broth. To detect the total production of fumaric acid, the precipitates were dissolved in 1 M HCl (W_1) (Zhang *et al.* 2015). The concentration of fumaric acid in broth was recorded as C_1, and the volume of residual broth was recorded as V. The total

fumaric acid production (C_t) was calculated as the sum of the content in both precipitates and broth parts, as in Eq. 1.

$$C_t = \frac{C_1 V + W_1}{V} \quad (1)$$

The initial glucose addition content was recorded as (W_2), and the yield of fumaric acid (Y) was calculated as shown in Eq. 2.

$$Y = \frac{C_t V}{W_2} \quad (2)$$

After fermentation, the carriers with mycelium were washed with deionized water three times and dried at 50 °C to a constant weight. The biomass content was determined by subtracting the weight of carriers.

RESULTS AND DISCUSSION

Development of the One-Step Fermentation Method

In two-step cultivation, the seed culture broth is usually discarded as waste. It was suspected that the waste seed culture broth could be reused in the fermentation stage. In this work, one-step fermentation was investigated to complete the seed and production phases synchronously by immobilized *R. arrhizus* in the stirred-tank reactor. The one-step medium contained 90 g/L glucose with 4 g/L peptone as initial carbon and nitrogen sources to cultivate *R. arrhizus* RH 7-13-9#. The mycelia were clearly observed and immobilized on the carriers. The biomass reached 9.89 g/L after spore inoculum inoculated for 24 h, which was similar to the previous results (Xing *et al.* 2020). This result indicated that the growth of the strain was not impacted by the high-concentration glucose, which is necessary for fumaric acid production using one-step fermentation.

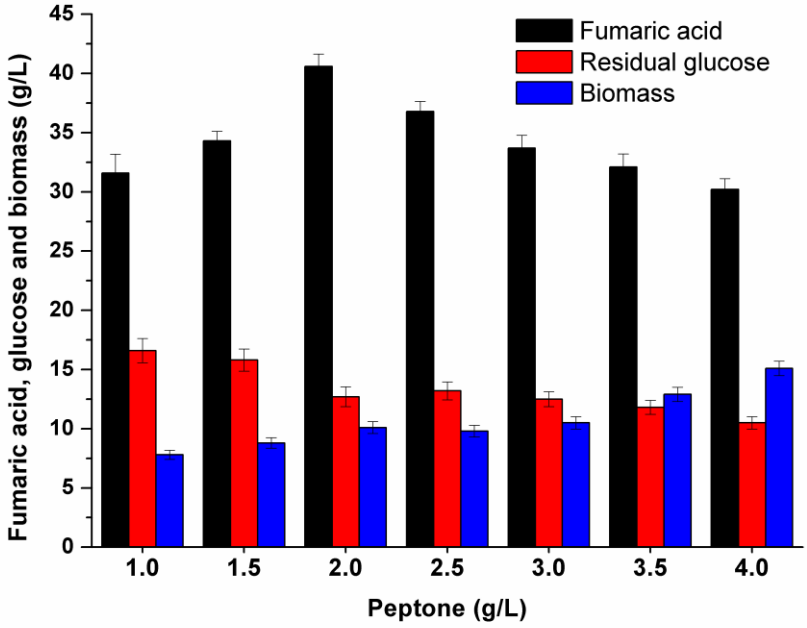

Fig. 1. One-step fermentation profiles under different peptone content

Optimization of Nitrogen Source Level in the One-Step Fermentation

In traditional two-step fermentation, the nitrogen source is the critical factor for fumaric acid production (Xing *et al.* 2020). Therefore, the level of nitrogen source peptone was optimized for one-step fermentation. The production of fumaric acid, consumption of glucose, and biomass were analyzed after cultivation for 84 h. As shown in Fig. 1, more than 30 g/L fumaric acid was produced in the nitrogen content optimization process, which illustrated that one-step fermentation had preferable implementation ability for fumaric acid production by immobilized *R. arrhizus* RH 7-13-9# in the stirred-tank reactor. The biomass displayed a gradually increasing trend with the increase of nitrogen source, which was similar to the previous results (Zhang *et al.* 2015). For the accumulation of fumaric acid, 40.6 g/L fumaric acid as the highest production was obtained when 2.0 g/L peptone was used as the nitrogen source. Either higher or lower peptone content was not favored for fumaric acid accumulation. This result was related to the balance between cell growth and fumaric acid synthesis. Limited nitrogen does not satisfy cell growth requirements and leads to low fumaric acid production; high nitrogen content causes the overgrowth of cells and has a negative effect on the transferring of oxygen and nutrients, resulting in the waste of carbon flux and decreased fumaric acid accumulation (Zhang *et al.* 2015). Therefore, 2 g/L peptone was the optimum level of nitrogen source in one-step fermentation.

Two-Stage Regulation of Agitation Speed in the Stirred-Tank Reactor

In addition to the nitrogen source, the agitation speed of the stirred-tank reactor is important in fumaric acid production. A higher agitation speed destroys the growth of the cells due to the shear force, while a lower agitation speed influences the oxygen transfer and inhibits the accumulation of fumaric acid (Fu *et al.* 2010; Liu *et al.* 2017). Therefore, different agitation speeds are applied in the seed culture phase (200 rpm) and fumaric acid production phase (400 rpm) during two-step fermentation (Singh *et al.* 2021; Wu *et al.* 2018). In this study, the agitation speed was maintained at 200 rpm throughout the one-step fermentation process, which possibly limited the accumulation of fumaric acid. To enhance fumaric acid production, the two-stage regulation of agitation speed strategy was developed for one-step fermentation, in which the agitation speed was controlled at 200 rpm in the first stage and then increased to 400 rpm in the second stage.

The maintenance time of the first stage was critical in this strategy, and it was optimized as shown in Table 1. When the maintenance time of the first stage was 12 h, the highest production of fumaric acid was 31.3 g/L at 108 h without any precipitates formed. It was suspected that the strain could not grow well due to the short maintenance time of the first stage, while the high agitation speed 400 rpm in the second stage was not conducive to cell growth, which resulted in the low biomass and poor production.

Table 1. Optimization of the Two-Stage Regulation of Agitation Speed

Maintenance Time of the First Stage (h)	Fumaric Acid in Solid Precipitate (g)	Total Production of Fumaric Acid (g/L)	Yield of Fumaric Acid from Glucose (g/g)	Highest Production of Ethanol (g/L)	Biomass (g/L)
12	0	31.3	0.26	4.76±0.35	8.32
24	31.32±1.21	51.0	0.42	7.81±0.43	10.21
36	24.31±0.97	43.5	0.36	10.23±0.61	13.34
48	21.19±0.93	41.2	0.34	16.74±0.65	15.15

The maintenance time of the first stage was extended so that the biomass gradually increased. However, even though the long maintenance time of the first stage was beneficial for cell growth, the high biomass resulted in more by-product ethanol, which affected the allocation of carbon flows and the production of fumaric acid.

The optimum maintenance time of the first stage was 24 h. As shown in Fig. 2, the first stage was within 24 h, and the agitation speed was controlled at 200 rpm, which was then regulated to 400 rpm to switch to the second stage for fumaric acid production. When the agitation speed was increased to 400 rpm after 24 h, both fumaric acid accumulation and glucose consumption rates were expedited. The highest titer of fumaric acid in broth reached 36.9 g/L at 96 h, and 31.32 g fumaric acid was obtained from the precipitates at the same time. After measuring the volume of residual broth, the total fumaric acid production was calculated, and it was 51 g/L with a yield of 0.42 g/g glucose, which was increased by 26% compared with the production without the two-stage regulation of agitation speed. Meanwhile, the accumulation of by-product ethanol was controlled, and its highest tier was 7.81 g/L. These data illustrated that fumaric acid production could be significantly promoted through two-stage regulation of agitation speed in the one-step fermentation process. The production of fumaric acid was higher than that obtained from two-step fermentation under the same conditions (Xing *et al*. 2020).

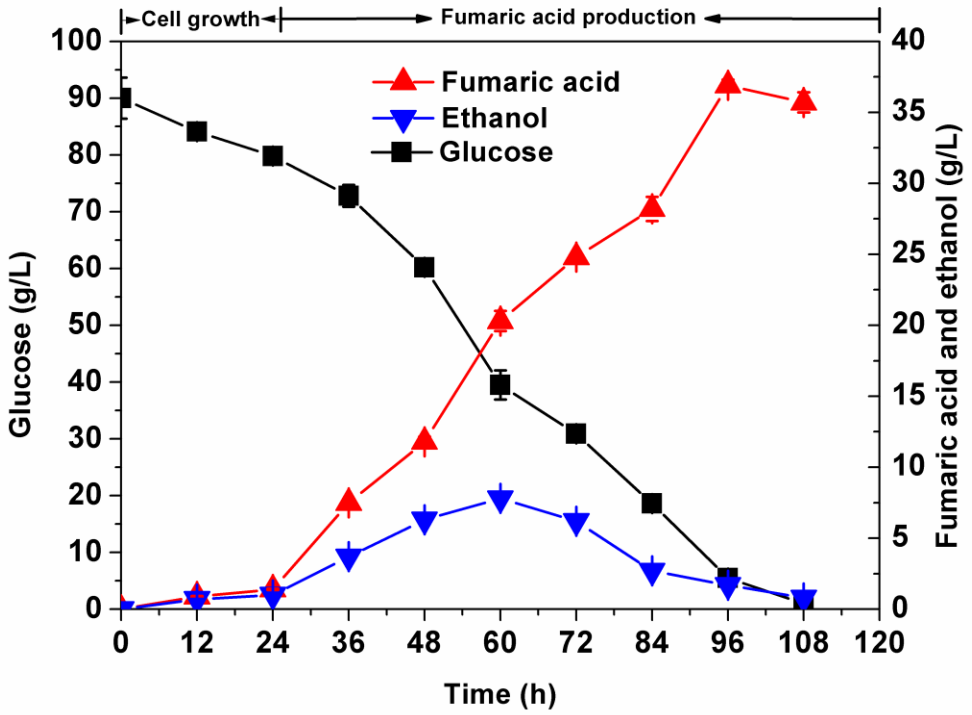

Fig. 2. Fumaric acid production profile using two-stage regulation of agitation speed in one-step fermentation process

In two-step fermentation, the seed culture stage is usually 24 to 48 h, and then *Rhizopus* cells are transferred into the fermentation medium for fumaric acid production for 96 to 144 h (Kowalczyk *et al.* 2018; Swart *et al.* 2020). In this process, not only the total production cycle is long (about 120-192 h), but also copious wastewater is produced after the seed culture stage. In this work, the one-step fermentation method was developed, so that the total production cycle was shortened to 96 h and the amount of waste water was

reduced due to the avoidance of the seed culture stage. The fumaric acid production was comparable between the one-step fermentation and the traditional two-step fermentation process (Fu *et al.* 2010; Singh *et al.* 2021). The results demonstrated that one-step fermentation had preferable feasibility, economical efficiency, and environmental-friendliness. The effective application of the method provided a promising and potential way for industrial production of fumaric acid as well as other high-value biochemical by fungus.

CONCLUSIONS

1. In this work, the one-step fermentation method was applied to fumaric acid production by immobilized *Rhizopus arrhizus* in the stirred-tank reactor.
2. Nitrogen source content and the agitation speed of the reactor were optimized as the two critical factors in one-step fermentation. The optimum level of nitrogen source was 2 g/L peptone, and the two-stage regulation of agitation speed strategy was developed to promote fumaric acid accumulation.
3. The highest fumaric acid production reached 51 g/L with a yield of 0.42 g/g glucose, which was comparable with the level of traditional two-step fermentation. The production cycle was shortened, and the amount of wastewater was efficiently reduced, which demonstrated the great advantages and application potential of the one-step fermentation method.

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