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Production enhancement of *Rhizopus arrhizus* lipase by feeding oleic acid

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Abstract

A strategy for *Rhizopus arrhizus* lipase production enhancement by feeding oleic acid was developed. The oleic acid was proved to have strong inducing effect on lipase production, but high concentration oleic acid could repress lipase production. The decrease rate of oleic acid concentration using peanut oil as initial carbon source was figured out according to the change of oleic acid concentration in the fermentation broth. Our feeding strategy designed based on the decrease rate of oleic acid could avoid the repression of lipase production that is caused by high concentration of oleic acid in the fermenting liquor, and this strategy worked as a new feeding method showing excellent performance. The maximum lipase activity was gained by feeding dilute oleic acid every 12 h starting at 60 h, which maintained the oleic acid concentration around 18 mg/L, and the lipase activity was 31% higher than that of no feeding.

Keywords: *Rhizopus arrhizus*; Feeding strategy; Lipase; Fermentation; Oleic acid

1. Introduction

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are hydrolytic enzymes, which can catalyze the hydrolysis of the ester bond of long-chain acylglycerols at the oil–water interface [1–3]. Several species of microorganisms, including bacteria, fungi, yeast, and actinomyces [4–9], are capable of producing lipase. Particularly, the lipase from *Rhizopus* has 1,3-regioselectivity, so it can selectively catalyze the hydrolysis of triacylglycerol to produce some specific industrial products [9]. As a result, this catalytic feature has drawn more attention in recent years.

However, the lipase fermentation with *Rhizopus* is relatively low-yield and high at cost. Various methods were employed to optimize the fermentation process to enhance production of lipase [4,10–12]. Xu et al. [10] optimized the medium components as well as fermenting conditions, including temperature, initial pH, rotating speed and aeration by *Rhizopus* Y-92, and 99.15 U/mL lipase activity (olive emulsion method) was gained under optimal medium constitution, which was obtained by orthogonal test. Elibol and Ozer [11] obtained 0.37 U/mL lipase activity (tributyrin as substrate) using response surface methodology with *Rhizopus arrhizus*. Repeated batch fermentation by immobilized mycelium was tested by Yang et al. [12]. In their work, the time to replace fermented broth, the volume and optimal composition of the medium for replacing were optimized. However, few attempts have been made to improve the fermentation process by investigating the variation of medium component during the fermenting process.

In contrast to suspension cultures, immobilized cell fermentation is capable of preventing mycelia to form pellets which results in poor substrate and oxygen transfer. Yang et al. [12] studied different carriers such as perlite, silk screen and polyurethane, and the results showed that polyurethane was the preferable support. Therefore, polyurethane was used as carrier throughout the work according to the previous study.

In view of the reports reviewed, the production of lipase is mostly inducer-dependent, and in many cases, oils and fatty acids act as good inducers of the enzyme [13], including vegetable oils such as corn oil and long-chain fatty acids such as oleic acid. This work, therefore, intended to explore the usage of oleic acid and to develop a new effective feeding method by means of monitoring the carbon source concentration, so as to enhance the lipase production.

2. Materials and methods

2.1. Microorganism and medium

R. arrhizus BUCT was reserved in the Key Laboratory of Bioprocess, Beijing University of Chemical Technology. The

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strain was inoculated on potato dextrose agar slants, grown at 27 °C for 3 days and stored at 4 °C. Lipase production was carried out in the production medium consisting of (per litre): 30.0 g soybean flour, 10.0 g peanut oil, 1 g $MgSO_4$, 5 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, pH natural 7.2.

The soybean flour and peanut oil was purchased from Beijing Secondary Soybean Products Factory and ShangHai Fortune Food Company, respectively. All other inorganic salts are of analytical grade and commercially available.

2.2. The support and immobilization

The polyurethane was cut into 5 mm cubic pieces and sterilized at 115 °C for 15 min three times, and then dried at ambient temperature. The production medium along with 0.4 g polyurethane were transferred into 250 mL flasks and sterilized at 121 °C for 15 min [12].

After inoculation, the germinated spores with hyphae were embedded into the mesh of polyurethane, so there were few mycelia in the fermented broth during the whole fermentation process.

2.3. Fermentation conditions in shaker

After 3 days of growth and sporulation, 20 mL sterilized water was added into the slant aseptically, the surface of which was then scraped to collect the spores. The spore suspension was used as inoculum.

One millilitre of the prepared inoculum was inoculated into 250 mL flasks containing 100 mL medium each. The flasks were placed on a rotary shaker at 26.5 °C and 130 rpm.

2.4. Lipase activity assay

Samples were drawn periodically during the fermentation from flasks, and the extracellular lipase activity was analyzed using an olive emulsion method [12]. The fatty acids released were determined by titration with 0.05 M NaOH. One lipase unit was defined as the enzyme required to release 1 μmol of fatty acid per minute under 43 °C, pH 7.0.

2.5. Oleic acid concentration assay

Three millilitre sample was centrifuged at 4000 × *g* for 10 min to discard the remaining mycelia. 2 mL hexane and 2 mL brine were added to the supernatant, centrifuged at 9000 × *g* for 15 min. The organic layer was separated and dried in the air, giving small amounts of white powder, which is a mixture of several species of fatty acids. The air-dry sample was added 1 mL mixture of boron trifluoride etherate and methanol (1:7, v/v), and the suspension was kept at 80 °C for 5 min for esterification. The resulting mixture was extracted with 1.5 mL hexane, and the upper layer containing fatty acid methyl esters was separated and analyzed by GC to determine the fatty acid assay.

A GC-2010 gas chromatograph (Shimadzu, Japan) equipped with a capillary column (DB-1ht from J&W Scientific, 30 m × 0.25 mm, 0.2 μm film thickness) and a flame ionizing detector (FID) was used. Injection was done in split mode (1/5) and the injector and detector temperatures were 350 and 360 °C, respectively. Samples (1 μL) were injected at an oven temperature of 100 °C, then the oven was heated at 15–180 °C/min, afterwards at 10–230 °C/min and then at 20–330 °C/min (holding for 5 min). Nitrogen was used as the carrier gas introduced at a rate of 6.21 mL/min.

3. Results and discussion

3.1. Effect of feeding different carbon sources on lipase production

Lipidic carbon sources (corn oil, olive oil, etc.) seem to be essential for obtaining high lipase yield due to their potential inducing ability [12,13]. As Elibol and Ozer [14] reported, in the presence of corn oil, lipase activity was approximate 2.5 times higher than in the absence of inducer. In order to study the inducing ability of different carbon sources, several kinds of

Table 1
Effect of feeding different carbon sources on lipase production (non-fat in initial medium)

Carbon source for feeing	Lipase activity before feeding (U/mL)	Lipase activity after feeding (U/mL)	%[a]
Glycerol	43.7 ± 1.5	62.5 ± 1.5	143
Butanol	46.0 ± 1.5	57.0 ± 1.5	124
Olein	52.5 ± 2.5	166.0 ± 4.0	316
Acetin	45.0 ± 1.5	54.0 ± 2.0	120
Behenic acid	51.2 ± 1.5	109.0 ± 2.7	213
Arachidic acid	46.0 ± 1.5	120.0 ± 2.5	261
Oleic acid	46.9 ± 0.5	170.0 ± 4.0	362
Palmitic acid	51.0 ± 1.5	110.5 ± 2.7	217
Octanoic acid	47.5 ± 1.7	40.0 ± 1.5	84
Butyric acid	49.0 ± 2.5	43.0 ± 1.5	88
Peanut oil	50.0 ± 1.5	163.0 ± 2.7	326
Olive oil	52.0 ± 1.5	157.0 ± 3.0	302

Initial medium: 0.5% glucose, 3% non-fat soybean flour, 0.5% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, feeding 0.5 mL different carbon source at 48 and 72 h. The lipase activity before feeding was obtained from the sample drawn at 48 h of cultivation before the feed operation, and the lipase activity after feeding was obtained from the sample drawn at 96 h of cultivation. 26.5 °C, 130 rpm, 100 mL liquid in 250 mL flask.

[a] The ratio of the lipase activity after feeding to that of before feeding.

carbon sources including triacylglycerols, alcohols, fatty acids and natural oils were fed to the fermentation process in the absence of any fat in the initial medium at 48 h of cultivation.

As shown in Table 1, alcohols like glycerol and butanol had no significant effect on lipase production. The lipase production was active on olein, which is a long-chain fatty acid ester, however, acetin, which is a short-chain fatty acid ester, had little effect on lipase production. We also found that the addition of C22∼16 fatty acids like behenic acid, arachidic acid, oleic acid and palmitic acid, boosted the lipase activity, which meant the long-chain fatty acids had inducing effect on lipase production; on the contrary, short-chain fatty acids such as octanoic acid and butyric acid, which are the fatty acids of C8 and C4, respectively, would repress lipase production. Additionally, natural oils such as olive oil and peanut oil could also advance the lipase production. Finally, we confirmed that oleic acid was the best inducer in our experiments, and the highest lipase activity gained was 3.62 times as high as before feeding.

Since oleic acid showed a satisfying inducing effect on lipase production, it was reasonable to use it as the main carbon source. In this case, different levels of oleic acid and 1% (v/v) peanut oil were added to respective batches of fermentation medium as carbon sources. As shown in Fig. 1(a), maximum activity was gained at the point of 0.4% among the five levels of oleic acid. Lower initial concentration oleic acid (<0.4%, observed in our experiments) is not sufficient to support the growth of cells thus resulting in a low lipase activity; on the other hand, oleic acid of higher initial concentration (>0.4%, observed in our experiments) is prone to repress the lipase production, which was also stated by previous literature [15]. It is noticeable that though oleic acid had strong inducing ability, it resulted in lower lipase activity compared with using peanut oil as carbon source, and the lipase activity declined sharply after the lipase activity maximized. A possible reason is that peanut oil could release oleic acid continuously by the lipase catalyzed hydrolysis, so the oleic acid in the fermentation broth could not be used up abruptly, which avoided a steep decrease of lipase activity.

The explanation above was verified as shown in Fig. 1(b), where the changes of oleic acid concentration in fermentation broth with 0.4% oleic acid and 1% peanut oil as initial carbon source were compared. When oleic acid served as initial carbon source, the cells could directly use it for growth, therefore, the oleic acid concentration declined as the fermentation proceeded. At 48 h, oleic acid was almost used up, so the increase of lipase activity after 48 h perhaps resulted from other nutrilites in the fermentation broth. The steep drop of lipase activity after 72 h was due to the depletion of all kinds of carbon sources. With the peanut oil as initial carbon source, however, the trend of oleic acid was changed related to the lipase production. Because the initial medium was free of oleic acid and the peanut oil was not hydrolyzed, the concentration of oleic acid was zero before cultivation. During the first 12 h of fermentation, the oleic acid concentration increased sharply, suggesting that an amount of lipase was synthesized to hydrolyze peanut oil to release oleic acid. After 12 h, the oleic acid concentration started to fall, implying that the consuming rate of oleic acid became greater than the hydrolysis rate of peanut oil, and the slope of the profile from 12 to 84 h was considered being proportion to the rate of cell growth, that is, the lower the growth rate, the smaller of the slope. The small rise in oleic acid concentration after 84 h implied that the consuming rate of oleic acid was lower than the hydrolysis rate of peanut oil at this stage, which may attribute to the cell aging. It is thus possible to remark that peanut oil generated oleic acid through a mode of controlled release, which could avoid the repression of lipase activity by high concentration oleic acid and the significant drop of lipase activity by the depletion of oleic acid.

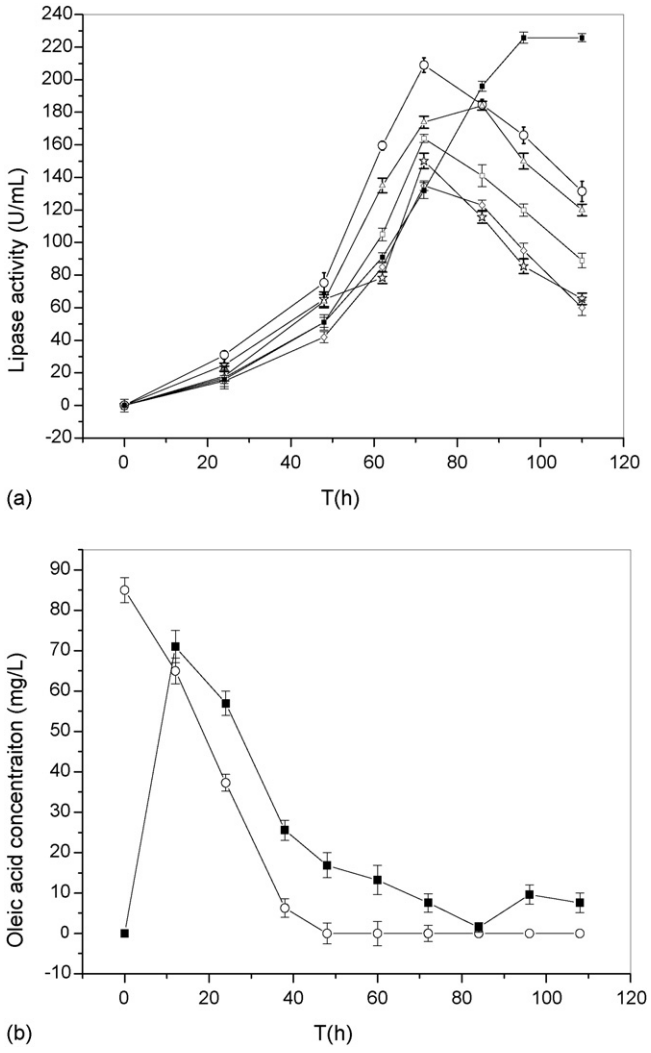

Fig. 1. Comparison of the effect on lipase production between oleic acid and peanut oil. (a) Lipase activity curves with different oleic acid concentrations and peanut oil as initial carbon source respectively. Concentration of oleic acid used: (☆) 0.2% (v/v); (◯) 0.4%; (△) 0.6%; (□) 0.8%; (◊) 1% oleic acid; (■) 1% peanut oil was used as control. (b) Oleic acid concentration curves. The initial carbon source was 1% peanut oil (■) and 0.4% oleic acid (◯), respectively. Other medium compositions and the culture conditions: 3% soybean flour, 0.5% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, 26.5 °C, 130 rpm, 100 mL liquid in 250 mL flask.

3.2. Feeding oleic acid based on the analysis of parameter

Owing to the excellence of peanut oil as initial carbon source (Fig. 1) and the strong inducing ability of oleic acid (Table 1),

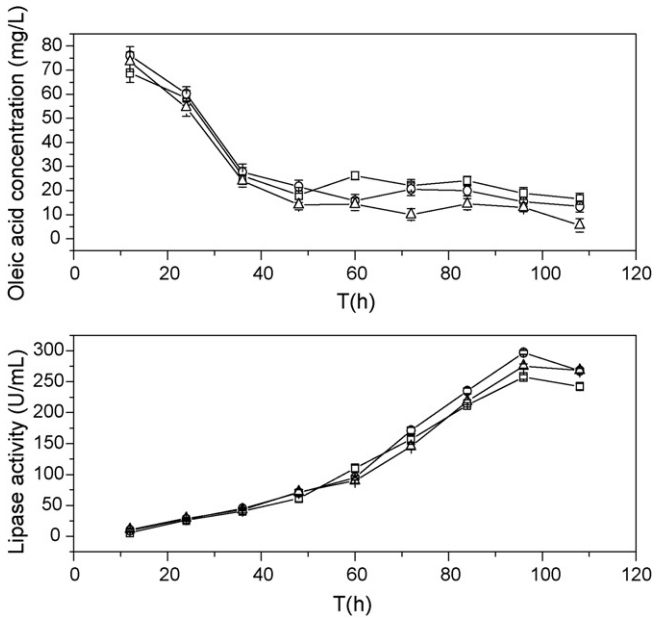

Fig. 2. Feeding oleic acid quantitatively starting from different time. Initial carbon and nitrogen source: 3% soybean flour, 1% peanut oil; 1.7 mL feeding solution which contained 350 mg/L oleic acid was fed starting from 48 h (□), 60 h (◯), 72 h (□), respectively, to 96 h every 12 h. Other medium compositions and culture condition: 0.5% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, 26.5 °C, 130 rpm, 100 mL liquid in 250 mL flask.

the fed-batch fermentation was carried out by feeding oleic acid using peanut oil as initial carbon source to enhance lipase production. From the lipase profile of peanut oil as carbon source (see Fig. 1(a)), we found that 48–84 h was the critical period during which lipase activity increased most quickly in the whole fermentation process. Therefore, the reasonable time to start feeding should be at this stage in order to keep the lipase activity at an increasing trend. According to Fig. 1(b), the decrease rate of oleic acid concentration during 38–84 h (approximately 0.0005 mg/mL h) was figured out, so oleic acid was reduced by 0.6 mg in 12 h/100 mL fermented broth. Therefore, equivalent amounts of oleic acid would be fed according to the decrease rate both to maintain the oleic acid concentration in the fermented broth at certain level and to avoid the repression of lipase production by high concentration of oleic acid.

The feeding solution was introduced at 48, 60 and 72 h, respectively, in order to maintain the oleic acid at different level. The result was shown in Fig. 2. As the oleic acid concentration curves suggested, when the feeding solution was introduced at 48, 60 and 72 h, the oleic acid level was maintained approximately at 23, 18 and 12 mg/L, respectively. As shown in lipase curves, the lipase activities were higher than no feeding involved, reflecting that a more stable environment of inducer level is favorable for lipase synthesis within the cells. The maximum lipase (297 U/mL) was obtained with feeding starting at 60 h, which was 31% higher than batches without feeding. So, 18 mg/L was the appropriate concentration of oleic acid which should be maintained during the process of lipase inducing.

4. Conclusion

Our feeding strategy designed on the basis of oleic acid concentration change during fermentation process could avoid the repression of lipase synthesis. The maximum lipase activity was gained by feeding dilute oleic acid every 12 h starting at 60 h, which maintained the oleic acid concentration around 18 mg/L, and the lipase activity was 31% higher than that of no feeding.

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