ORIGINAL

Effect of Lipid Addition on the Profile of Lipase Production by *Mucor javanicus*

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While increasing lipase production by *Mucor javanicus* IAM 6108, reduction in cultivation time and stabilization of lipase in the broth were investigated. The addition of 1 % soybean oil to the initial medium containing 3 % corn steep liquor, 1 % soluble starch, 0.2 % KH_2PO_4 , 0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 % KCl, and 1 % soybean-yuto reduced the cultivation time of lipase production from 50 h to 30 h.

Lipase activity in the broth decreased rapidly after reaching a maximum. To avoid this, the 1 % soybean oil was added to the broth at 25 h of cultivation before the maximum had been reached. As a result, maximum lipase activity was maintained for more than 10 h. This appeared due to the presence of fatty acids or triglycerides with double bonds on the broth.

Introduction

The relationship between lipase production and lipid has been reported on various kinds of microorganism. The addition of lipid to cultivation medium increased the lipase productivity of *Mucor javanicus*¹⁾, *Candida parailipolytica*²⁾, *Pseudomonas mephitica*³⁾, *Chromobacterium viscosum*⁴⁾ and *Pseudomonas* sp. No. 156⁵⁾. On the other hand, the lipase production of *Rhizopus stolonifer*⁶⁾ was inhibited by lipid. Furthermore, lipoprotein lipase of *Rhizopus japonicus*⁷⁾ was effectively excreted from its mycelia by lecithin added to the medium.

In the course of study on the scale-up of lipase production by *Mucor javanicus* IAM 6108, the authors found that the addition of soybean oil to the medium reduced the cultivation time with the change of fermentation profile. However, lipase activity in broth decreased rapidly after reaching the maximum. This report describes these facts and how to maintain the maximum value of lipase activity during the late phase of cultivation.

Materials and Methods

Microorganism and Cultivation : *Mucor javanicus* IAM 6108 in 100 mL/500mL-Sakaguchi flask grown by Saiki *et al.*'s method¹⁾ was inoculated into a 30 L-jar fermenter (Liquid volume : 15 L) and cultivated at 26 °C under 15 L/min of aeration and 300 rpm of agitation. *Mucor javanicus*

IAM 6108 and soybean-yuto were gifted by Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University.

Assay of Lipase⁸⁾ : The solution composed of 5.0 mL of olive oil-poly(vinyl alcohol) emulsion* and 4.0 mL of 0.1 M phosphate buffer (pH 7.0) was put into a reaction tube (30×120 mm) and preincubated for 5 min at 37 °C prior to the addition of enzyme solution.

The reaction mixture was incubated for 30 min at 37 °C and the reaction was stopped by the addition of 10.0 mL of acetone-ethanol mixture (1 : 1). To the mixture, 10.0 mL of 0.05 N NaOH solution and 10.0 mL of acetone-ethanol mixture (1 : 1) were added.

The solution was titrated with 0.05 N HCl solution under nitrogen gas bubbling. As the blank test, 1.0 mL of the enzyme solution was added after the termination of reaction with 10.0 mL of acetone-ethanol mixture (1 : 1).

* Olive oil poly (vinyl alcohol) emulsion : 75 mL of olive oil (JP-XI) and 225 mL of 2 % poly(vinyl alcohol) solution** was stirred vigorously (14 500 ± 300 rpm) with keeping at below 10 °C for 10 min in use of Homogenizer (Nippon Seiki Co., Ltd.).

** 2 % poly(vinyl alcohol) solution : 4.5 g of PVA 117 and 0.5 g of PVA 205 (Kurashiki POVAL) was suspended in 250 mL of the distilled water and heated at below 80 °C under stirring to become a clear solution and it was cooled, filtered and supplied.

to use.

One unit is defined as the amount of the enzyme which liberated 1 μmol of free fatty acid in 1 min under above conditions.

Stability of Lipase in Aqueous solution : *Mucor* lipase (Lipase M-AP, 20 000 u/g, Amano Pharmaceutical) was dissolved in McIlvain buffer (pH 7.0) to get the solution of 0.5 % concentration. The solution was kept at 37 °C and the residual activity after 30 min and 60 min was assayed.

Triglycerides or fatty acids were added to become $5/3 \times 10^{-3}$ M or 5×10^{-3} M respectively of final concentration.

Results

1 The change of fermentation profile

Mucor javanicus IAM 6108 was cultivated by the method described in Materials and Methods. Lipase production reached the maximum value at 50 h to get the same results as in Saiki *et al.*'s (Fig. -1). On the other hand, various kinds of

Medium : corn steep liquor 3 %, soluble starch 1 %, KH_2PO_4 0.2 %, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 %, KCl 0.05 % and soybean-yuto 1 % (0.2 % as phospholipid). Initial pH was 6.0.

Cultivation was carried out at 26 °C, under 300 rpm of agitation and 15 L/min (1 vvm) of aeration by use of 30 L-jar fermenter (Liquid volume : 15 L).

Fig. -1 Lipase production of *Mucor javanicus* IAM 6108 in the presence of limited lipids.

carbon source were studied by use of Czapek-Dox's medium to increase mycelia, or to decrease the cultivation time. Addition of soybean oil or oleic acid was most effective (Table-1), and the former was chosen in view of cost for the test at the fermentor scale.

Table. -4 Effect of carbon sources on the growth of Mycelia of *Mucor javanicus* IAM 6108

Carbon sources	Dry weight of Mycelia (mg/100 mL of Broth)
Glucose	100
Lactose	9
Maltose	101
Sucrose	13
Soluble starch	203
Glycerol	10
Stearic acid	15
Oleic acid	815
Soybean oil	722

Basal medium (Czapek-Dox's medium) : $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 %, NaNO_3 0.2 %, KCl 0.05 %, K_2HPO_4 0.1 % and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 % (initial pH : 6.0)

Cultivation was carried out for 72h at 26°C under shaking (110 strokes/min) by use of 500 mL-Sakaguchi flask (Liquid volume : 100 mL).

When cultivation was carried out in the medium containing 1 % of soybean oil, the lipase production was parallel to the growth of Mycelia and the fermentation profile changed from non-growth associated type (Fig. -1) to the growth associated one to reach the maximum value of lipase activity at 30 h. However, lipase activity in broth

Medium : corn steep liquor 3 %, soluble starch 1 %, KH_2PO_4 0.2 %, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 %, KCl 0.05 % soybean-yuto 1 % and soybean oil 1 %. Initial pH was 6.0.

Cultivation was carried out at 26 °C, under 300 rpm of agitation and 15 L/min (1 vvm) of aeration by use of 30 L-jar fermenter (Liquid volume : 15 L).

Fig. -2 Effect of initially added soybean oil on lipase production of *Mucor javanicus* IAM 6108.

decreased rapidly after reaching the maximum value (Fig. -2).

2 The stabilization of lipase by lipid

To solve the serious problem on the unstability of lipase in broth, the addition of lipid to the aqueous lipase solution was investigated. Triglycerides and fatty acids with double bonds in fatty acid were effective to stabilize lipase (Fig. -3 a, Fig. -3 b).

3 The Stabilization of Lipase during Cultivation

Among effective lipids shown above, soybean oil containing approximately 35 % of triolein was chosen because it was inexpensive and readily available. It was supplemented to become 1 % concentration at 25 h-cultivation prior to the time reaching the maximum value. Lipase activity maintained its maximum value over the range of 10 h (Fig. -4).

Discussion

When the enzyme is produced in aerobic submerged culture, 30-L jar fermenter is more effective than Sakaguchi flask with respect to the

Mucor lipase was dissolved in McIlvain buffer (pH 8.0) to get 0.5 % concentration, kept at 37 °C. The residual activities after 30 min and 60 min were assayed. Triglycerides or fatty acids were added to become $5/3 \times 10^{-3}$ M or 5×10^{-3} M respectively.

Fig. -3 The stabilization of *Mucor* lipase in the aqueous solution by addition of lipids.

Medium : corn steep liquor 3 %, soluble starch 1 %, KH_2PO_4 0.2 %, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 %, KCl 0.05 % soybean-yuto 1 % and soybean oil 1 %. Initial pH was 6.0.

Cultivation was carried out at 26 °C, under 300 rpm of agitation and 15 L/min (1 vvm) of aeration by use of 30 L-jar fermenter (Liquid volume : 15 L).

1 % of soybean oil was supplemented to the broth at 25 h-cultivation prior to the time reaching the maximum lipase activity.

Fig. -4 Effect of supplementation of soybean oil on the stability of lipase excreted in broth during cultivation.

supply of oxygen.

Lipase productivity by *Mucor javanicus* IAM 6108 increases in proportion to the amount of oxygen supplied⁹. Therefore, the experiments in this report were carried out under 300 rpm of agitation and 15 L/min (1 vvm) of aeration, which is practically the most aerobic condition. 1 % of soybean oil added to initial medium reduced the cultivation time of lipase production.

However, the rapid decrease of lipase activity in broth occurred as an unexpected phenomenon which led to the difficulty in deciding the best brothout time. To solve this problem, the improvement of stability in aqueous solution of *Mucor* lipase was investigated, and the addition of lipids with double bonds in fatty acids were most effective. On the basis of this result, the further addition of soybean oil (adopted from the reason as mentioned in Result-3) during cultivation was successfully carried out.

The mechanism of the stabilization of lipase by lipids is explained as follows. To obtain the residual activity above

50 % under the condition used, it is necessary that the lipids contain double bonds (trilinolein, triolein, linolic acid and oleic acid) or carbon number above approximately 12 in fatty acids (tripalmitin, trilaurin, palmitic acid and lauric acid). Lipids such as tripalmitin, trilaurin, palmitic acid and lauric acid, which are solid at 37 °C, were used after they were melted by heating at the temperature above their melting point prior to adding them to the aqueous lipase solution kept at 37 °C. Then these lipids dispersed into the solution with turbidity and without becoming hard. The difference of effects of those lipids on the stabilization of lipase is presumed not due to the difference of the dispersion rate of lipids, because single chain fatty acid esters which are liquid at room temperature showed the same result as responding free fatty acids or triglycerides. In conclusion, oil drop seems to attach to the surface of enzyme particle, to minimize the contact with water and to stabilize lipase. This phenomenon is similar to that of the improvement on stability by an addition of lecithin or triolein to the aqueous solution of *Pseudomonas* lipase¹⁰⁾.

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ムコール・ジャバニカスによるリパーゼの 生産様相への脂質の添加効果

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ムコールジャバニカス IAM 6108 のリパーゼをスケールアップ生産するにあたり、培養時間の短縮と培養液の安定化を試みた。コーンステープリカー 3 %, 可溶性デンプン 1 %, リン酸二水素カリウム 0.2 %, 硫酸マグネシウム 0.05 %, 塩化カリウム 0.05 % 及び大豆油糖 1 % の基本培地に大豆油 1 % を添加すると、培養時間は 50 h から 30 h に短縮された。一方、培養中のリパーゼ活性は最高値に達した後急速に低下した。これを防ぐため、最高値に達する時間に先だて、25 h で更に大豆油 1 % を添加すると、最高値のリパーゼ活性は 10 h 保持された。培養液のリパーゼ活性の安定化は、二重結合をもつ脂肪酸またはトリグリセリドによるものと考えられた。

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★大豆の供給、長期需要を上回るか

アメリカ大豆協会の 11 月 7 日のニュースレターで農業評論家が指摘したところによれば、大豆の長期的にみた供給量は需要を上回ろうという。農業データ源の B. Utterback 氏は“長期的にみて、供給が需要の伸びを上回って増加している。需要は横這いしないしはやや下向気味になっている”, と述べている。

★ブラジルの大豆収穫、2,000 万トンの最高値に

ブラジルの 1989 年大豆収穫量は、作付面積 1,170~1,210 万ヘクタールと単収 1.86 トン/ヘクタールから算出すると、2,050~2,200 万トンと予測される。アメリカ農務省の観測筋の報告によれば、多くのニュース源は 2,100 万トンの収穫を予測しているという。