

Characteristics of Cell-bound Lipase Production by a Newly Isolated Strain of *Aspergillus flavus*

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ABSTRAK

Pencilan *Aspergillus flavus* telah didapati sebagai penghasil lipase terikat sel yang berpotensi. Penghasilan maksimum sebanyak 32 U/g biojisim kering dicapai selepas 36 jam pengkulturan. Penghasilan enzim seterusnya menjadi malar. Aktiviti lipase daripada kulat ini tidak begitu dipengaruhi oleh kehadiran berbagai sumber karbon dan nitrogen dan bahan lipid di dalam medium basal. Dalam jangkamasa sintesis lipase terikat sel yang maksimum, peningkatan yang bererti dalam lipase ekstrasel telah juga dicerap di dalam medium kultur. Walau bagaimana pun lipase ekstrasel ini didapati amat tidak stabil. Aktiviti lipase terikat sel adalah stabil pada 55°C dan mengekalkan 100% daripada aktiviti nya selepas 24 j pada suhu ini. Aktiviti lipase dapat juga dirangsang dengan kehadiran natrium taurokolat dan ZnCl₂. Lipase ini juga menunjukkan kespesifikan substrat yang luas keatas hidrolisis bahan-bahan lipid.

ABSTRACT

An isolate of *Aspergillus flavus* was found to be a potential producer of cell-bound lipase. A maximum lipase production of about 32 U/g dried biomass occurred after 36 h of cultivation. The enzyme production remained constant thereafter. The lipase activity of the fungus was not greatly influenced by the presence of various carbon and nitrogen sources and lipid materials in the basal medium. During the period of maximum synthesis of cell-bound lipase, a significant increase in the extracellular lipase was also detected in the culture medium. However, extracellular lipase was found to be extremely unstable. The activity of cell-bound lipase was stable at 55°C and retained 100% of its activity after 24 h at this temperature. The lipase activity can also be stimulated by the presence of sodium taurocholate and ZnCl₂. The lipase exhibited a wide range of substrate specificity on the hydrolysis of lipid materials.

Keywords: cell-bound lipase, lipase production, *Aspergillus flavus*, lipase characteristics

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INTRODUCTION

Though there are numerous reports on the production of extracellular lipases (Macrae 1983), few reports are available on the fermentation of cell-bound lipases. A limited number of publications on the use of cell-bound lipases can be found (Patterson *et al.* 1979; Knox and Cliffe 1984; Leiger and Comeau 1992). Cell-bound lipases are known to demonstrate more advantages than extracellular lipases. The enzyme, which is basically confined within the periplasmic region of the cell, is subjected to protection against environmental perturbation during enzyme production. Therefore it is expected to show higher thermal stability and a higher resistance against ionic stress or pH change. At the same time, the lipase which is enclosed and bound within the cell wall may act as a convenient means of enzyme immobilization. The use of biomass as enzyme source not only allows ease of handling but eliminates numerous steps in downstream processing. Based on these advantages, an attempt was made to isolate a potential fungus for the production of cell-bound lipase for industrial applications. The obtaining of a new strain may be of interest because different properties of the lipase produced by different micro-organisms can be observed. For this reason we have isolated a strain of *Aspergillus flavus* for the production of cell-bound lipase.

MATERIALS AND METHODS

Source of Micro-organism

Soil samples were collected from the vicinity of Penang and Kedah, northern Malaysia. Initially, the fungi were isolated on potato dextrose agar. Plates were incubated at 30, 37 and 45°C for 24-96 h.

Cultivation Medium

In the screening experiments, purely isolated fungal cultures were grown in liquid cultures containing the basal medium comprising (% w/v): soluble starch 1.0, NaCl 0.5, CaCl₂.2H₂O 0.01 and olive oil 0.3, pH 7.0 (Ibrahim *et al.* 1987). A 100-ml Erlenmeyer flask containing 35 ml medium was incubated in a rotary shaker at 200 rpm (B. Braun, Certomat R, Rep. of Germany) for 24 h. The culture broth was centrifuged at 4000 rpm (4°C, 20 min) and the supernatant was used as the extracellular source of enzyme. Other substrates were added as indicated as carbon or nitrogen sources or as inducers in optimizing enzyme production.

Preparation of Biomass as a Source of Cell-bound Lipase

At the end of the cultivation, the biomass was filtered using a Whatman filter paper No. 3 and the mycelium was collected. For the determination of growth, the mycelium was washed twice with 10 ml n-hexane to remove excess lipid, dried at 60° for 24 h before weighing. As the enzyme source, the washed biomass was dried at 50°C to constant weight.

Assay of Cell-bound and Extracellular Lipase Activity

The reaction mixture consisted of 5 ml polyvinyl alcohol-olive oil emulsion (prepared using olive oil in 1% polyvinyl alcohol of ratio 1:3 which was emulsified for 10 min), 5 ml phosphate buffer (0.2 M) and 0.3 g of dried biomass was incubated with shaking of 200 rpm for 30 min at 30°C unless otherwise stated (see below). In the case of extracellular enzyme, the reaction mixture consisted of 5 ml emulsion, 4 ml buffer and 1 ml of the culture supernatant. The enzyme reaction was terminated by the addition of an equal volume of acetone-ethanol (1:1 v/v) to the reaction mixture. The free fatty acid liberated was determined by titration with 0.05 M NaOH solution using phenolphthalein as the indicator. One unit of lipase activity is defined as the amount of enzyme needed to liberate 1 μ mole of free fatty acid under the stated conditions. The cell-bound lipase activity was expressed as unit (U) per g dried biomass, while the extracellular lipase activity was expressed as U/ml culture supernatant. For comparison, activities are also expressed as total activity.

RESULTS AND DISCUSSION

Ninety-five strains of pure fungal isolates were screened at 3 temperatures. The results showed that the fungal strain *A. flavus* (Raper and Fennel 1965) is a potential producer of cell-bound lipase when grown at 30°C. Growth ceased gradually with temperature of more than 30°C.

The profile of biomass and lipase production using the optimized medium is given in Fig. 1. Cell-bound lipase was optimally produced after 36 h cultivation and remained constant thereafter although the biomass still

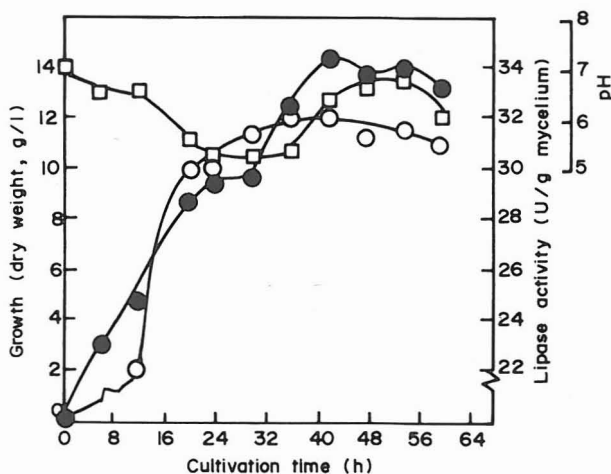


Fig. 1. Cell-bound lipase production by *Aspergillus flavus*. The fungus was grown in the basal medium containing sorbitol (1.0%, w/v) and PFAD (0.3%, w/v). Lipase activity (○), growth (●) and pH (□).

increased significantly. The figure also illustrated that there was a sudden increase in enzyme synthesis within the cell after 12 h cultivation. The results suggested that the synthesis of cell-bound lipase occurred simultaneously with the formation of cell mass. The pH profile varied from pH 5 to 6.5.

Studies on the effect of various carbon sources on the production of cell-bound lipase by *A. flavus* showed that the fungus was able to utilize a wide range of carbon sources besides soluble starch with an average activity of about 35.5 U/g biomass (Table 1). Based on the amount of biomass produced, sorbitol may be considered a significant carbon source. Of the nitrogen sources tested, yeast extract (1.0%, w/v) seemed to be the best. The growth was significantly inhibited in the presence of inorganic nitrogen sources such as NH_4Cl , NH_4NO_3 , NaNO_2 and NaNO_3 (data not shown). Based on the belief that most extracellular lipases are inducible (Ota *et al.* 1968; Ibrahim *et al.* 1987), the cell-bound lipase production by *A. flavus* was examined in the presence of lipids and related compounds in the basal medium (Table 2). As shown in Table 2, all lipid compounds examined showed an almost identical effect with activities ranging from 30-35 U/g biomass suggesting that induction is not a crucial criteria for production of cell-bound lipase.

All the results shown in *Fig. 1*, Table 1 and Table 2, conclusively illustrate that the enzyme activity within the cell is quantitatively similar (27-35 U/g biomass), regardless of the differences in carbon and nitrogen sources, inducers, cultivation duration or the amount of biomass produced

TABLE 1
Effect of carbon sources on the production of
cell-bound lipase by *A. flavus*

Carbon source (1.0%, w/v)	Growth* (g/l)	Lipase activity* (U/g)
Sorbitol	11.1	36.1
Glucose	8.4	34.4
Galactose	3.3	36.7
Maltose	7.6	33.3
Fructose	9.2	36.7
Mannitol	4.4	37.2
Mannose	8.7	34.4
Soluble starch	8.3	35.6
Xylose	8.7	36.7
Lactose	3.2	36.1
Sucrose	8.8	31.1

*determined after 48 h cultivation, data were presented as average value from three different cultivations

TABLE 2
Effect of lipid materials and related compounds on the growth and production of cell-bound lipase by *A. flavus*

Lipid material (0.3%, w/v)	Growth (g/l)	Lipase* (U/g)	Lipid material (0.3%, w/v)	Growth (g/l)	Lipase* (U/g)
Natural oil/fats			Triglycerides		
Corn oil	11.7	27.8	Triacetin	11.7	28.9
Coconut oil	8.8	31.1	Tributylin	3.9	31.7
Olive oil	13.5	33.3	Tricaproin	9.0	37.8
Beef tallow	5.4	32.2	Tricaprin	12.2	30.6
Castor oil	10.7	29.4	Trilaurin	13.0	30.6
Groundnut oil	10.2	30.0	Trimyristin	13.4	29.4
Soybean oil	13.2	33.3	Tripalmitin	5.9	34.4
Vegetable oil	9.9	32.7	Tristearin	11.2	29.4
Gingelly oil	10.2	31.7	Fatty acids		
Crude palm oil	12.0	31.7	Acetic acid	6.9	34.4
Palm RBD	13.1	27.2	Butyric acid	ND	ND
Palm FAD	15.0	32.7	Capric acid	5.4	30.6
Palm olein	7.1	30.0	Caproic acid	8.9	31.7
Palm stearin	4.4	33.9	Lauric acid	1.5	32.2
Monoesters			Myristic acid	13.5	28.9
Methyl laurate	6.8	31.1	Palmitic acid	8.5	33.3
Methyl myristate	9.3	35.6	Stearic acid	11.6	32.8
Methyl oleate	9.3	30.0	Oleic acid	10.9	33.4
Methyl stearate	6.6	35.0	Linoleic acid	4.1	31.1
			Control (without lipid addition)	8.9	27.2

* determined after 48 h cultivation, see Table 1.

RBD - Refined bleached and deodorised palm oil

FAD - Fatty acid ditillates

ND - no growth

in the culture system. These observations suggested that the synthesis of cell-bound lipase by *A. flavus* not only takes place simultaneously with cell mass formation, but is also limited within the cell containment at a limited capacity. Therefore, changing the substrate or increasing the cultivation time would only improve biomass formation and the enzyme concentration within the mycelium would remain constant. In the case of extracellular enzymes, the enzyme synthesized by the cell is continuously secreted into its environment, and changes in substrate concentration in the medium can influence the amount secreted and hence the enzyme concentration in the culture (Ibrahim *et al.* 1987). In order to obtain comprehensible information on the nature of the synthesis of cell-bound lipase, extracellular lipase activity of *A. flavus* was also determined.

Fig. 2 illustrates the distribution of the cell bound and extracellular lipase activities in the culture system of *A. flavus*. As shown in *Fig. 2*, the

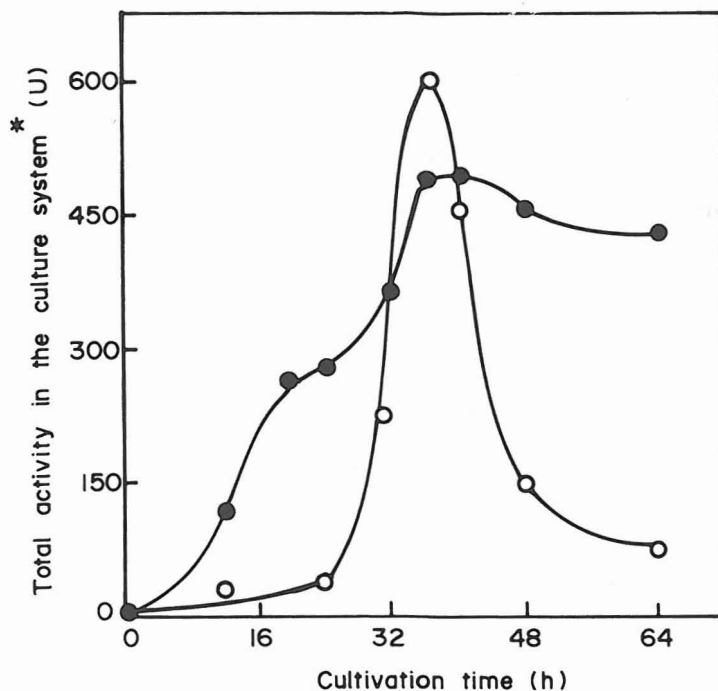


Fig. 2. Distribution of cell-bound lipase and extracellular lipase during growth of *A. flavus*. Total activity is expressed as the activity calculated from 1l of culture medium*, (●) cell-bound lipase, (○) extracellular lipase.

fungus initially synthesized the cell-bound lipase rapidly with increasing cell mass. The maximum activity of about 460 U was observed after about 40 h. At the same time, the production of extracellular lipase increased notably after 32 h. After the growth and enzyme synthesis of cell-bound lipase approached the maximum activity, the extracellular lipase was then secreted in the medium as indicated by the sudden increase in the enzyme activity of the broth supernatant. A maximum activity of about 600 U was observed after 36 h. The activity dropped tremendously thereafter and disappeared almost entirely 12 h later. The unstable phenomenon in the extracellular lipase activity may be due to several factors. The instability can be the result of pH dropping to about 5 in the culture system (see Fig. 1) due to excessive fatty acid liberation from active hydrolysis of lipid substrate at high enzyme concentration. Another possible reason is the degradation of lipase protein by proteolytic enzymes. Noor Izani and Ibrahim (1991) demonstrated that the instability of lipase production by *Aspergillus niger* after achieving maximum activity is due to the degradation of lipase by a thiol protease. A similar result was reported by Taylor (1989) in the production of extracellular lipase by *Thermomyces lanuginosus*. The extracellular

lipase of *A. flavus* may be similar to that of the cell-bound lipase. However, under unprotective conditions the extracellularly secreted lipase of *A. flavus* may be highly degradable. Such a phenomenon is definitely a disadvantage in downstream processing, especially for industrial applications of the enzymes. On the other hand, the cell-bound lipase, although with a lower total activity, remained stable for a relatively significant period of time. The lower activity in cell-bound lipase may be expected due to limitation in mass transfer into and out of the cell.

Table 3 shows some characteristics of the cell-bound lipase of *A. flavus*. The enzyme showed an optimum pH of 7 with a stability of pH 3-9. The optimum temperature was 45°C and the stability was 70°C for 30 min incubation time. The results clearly showed that although the fungus is mesophilic in nature, the lipase bound to the mycelium was thermostable, probably due to the protection effect by the cell. The thermal stability exhibited is identical to or higher than the lipases from most thermophilic lipase producers (Liu *et al.* 1972; Suzuki *et al.* 1986; Ibrahim *et al.* 1987; Samad *et al.* 1990). Fig. 3 shows the thermal stability of the cell-bound lipase during prolonged incubation period at 50, 55, 60 and 70°C. It was observed that after 24 h, complete activity (100%) was retained by the enzyme at 50 and 55°C. Nevertheless, the activity dropped to about 50% at 60°C. At 70°C, complete activity was retained for 2 h, dropping rapidly to about 20% after 10 h.

TABLE 3
Some physical properties of cell-bound lipase from *A. flavus*

Optimum pH	7.0
pH stability (45°C, 24 h)	3.0 - 9.0
Optimum temperature	45°C
Temperature stability	70°C (30 min incubation time)

The enzyme activity was stimulated^a by the presence of sodium taurocholate (1.0%, w/v) and ZnCl₂ (0.01%, w/v). At these concentrations, the enzyme activity was improved by 15 and 70%, respectively, compared to the reaction system without the presence of either sodium taurocholate or ZnCl₂. The enzyme also exhibited a wide range of substrate specificity on the hydrolysis of natural lipids, triglycerides and monoesters. Based on the carbon chain length, the cell-bound lipase demonstrated significant specificity between C10 and C14 (data not shown). The physical properties of microbial lipases with respect to requirements for optimum catalytic activity or its substrate and structural specificities varied greatly depending on the source of the enzyme (Macrae 1983).

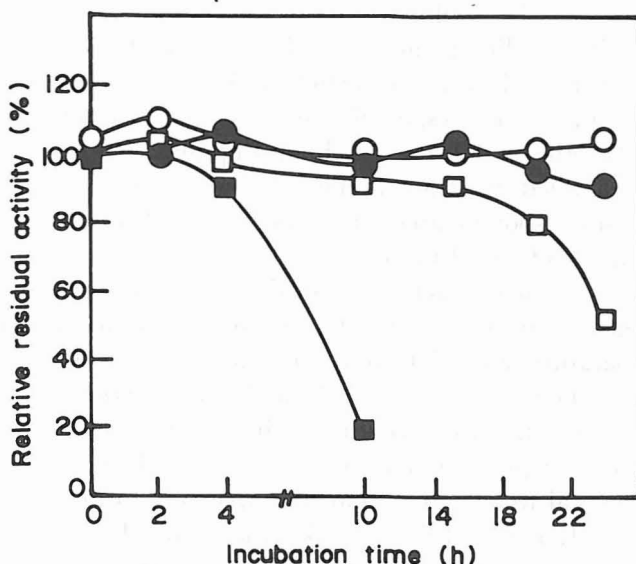


Fig. 3. Thermal stability of cell-bound lipase of *A. flavus*. The biomass was incubated at 50°C (○), 55°C (●), 60°C (□) and 70°C (■), at various times. Residual activity was assayed at 45°C (see Table 3) and calculated relative to the enzyme preparation without temperature treatment which was considered as 100% activity.

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REFERENCES

- IBRAHIM, C.O., N. NISHIO and S. NAGAI. 1987. Production of a thermostable lipase by *Humicola lanuginosa* grown on sorbitol-corn steep liquor medium. *Agricultural and Biological Chemistry* **51**: 2145-2151.
- KNOX, T. and K.R. CLIFFE. 1984. Synthesis of long chain esters in a loop reactor system using a fungal cell-bound lipase. *Process Biochemistry* **19**: 188-192.
- LEIGER, V. and L.C. COMEAU. 1992. Continuous synthesis of esters by cell-bound fungal lipases in an organic solvent. *Applied Microbiology and Biotechnology* **37**: 732-736.
- LIU, W.H., T. BEPPU and K. ARIMA. 1972. Cultural conditions and some properties of the lipase of *Humicola lanuginosa* S-38. *Agricultural and Biological Chemistry* **36**: 1919-1924.
- MACRAE, A.R. 1983. Extracellular microbial lipases. In *Microbial Enzymes and Biotechnology*, ed. W.H. Forgy, p. 225-250. New York: Appl. Sci. Publisher.
- NOOR IZANI, N.J. and C.O. IBRAHIM. 1991. Improvement of lipase production and enzyme stability of *Aspergillus niger* with p-chloro mercuribenzoic acid. *Malaysian Applied Biology* **20**: 125-127.

- OTA, Y., M. SUZUKI and K. YAMADA. 1968. Lipids and related substances inducing the lipase production by *Candida cylindracea*. *Agricultural and Biological Chemistry* **32**: 390-391.
- PATTERSON, J.D.E., J.A. BLAIN, C.E.L. SHAW, R. TODD and G. BELL. 1979. Synthesis of glycerides and esters by fungal cell-bound enzymes in continuous reactor systems. *Biotechnology Letters* **1**: 211-216.
- RAPER, K.B. and D.I. FENNEL. 1965. *The Genus Aspergillus*, p. 293-344. Baltimore: Williams and Wilkins.
- SAMAD, M.Y.A., A.B. SALLEH, C.N.A. RAZAK, K. AMPON, W.M.Z.W. YUNUS and M. BASRI. 1990. A lipase from a newly isolated thermophilic *Rhizopus rhizopodiformis*. *World Journal of Microbiology and Biotechnology* **6**: 390-394.
- SUZUKI, M., H. YAMAMOTO and M. MIZUGAKI. 1986. Purification and general properties of a metal insensitive lipase from *Rhizopus japonicus* NR 400. *Journal of Biochemistry* **10**: 207-213.
- TAYLOR, F. 1989. Stabilization of lipase from *Thermomyces lanuginosus* with p-chloro mercuribenzoic acid. *Journal of Fermentation and Bioengineering* **68**: 141-143.