J. Trop. Agric. and Fd. Sc. 28(2)(2000): 135–142

Physiochemical properties and specificity of mycelium-bound lipase from a locally isolated strain of *Aspergillus flavus* Link

(Ciri-ciri fisiokimia dan kespesifikan lipase terikat-miselium daripada strain tempatan *Aspergillus flavus* Link)

K. Long*, H. M. Ghazali**, A. Ariff** and C. Bucke***

Key words*: Aspergillus flavus,* mycelium-bound lipase, extracted lipase, positional specificity

Abstrak

Sifat-sifat lipase terikat-miselium daripada *Aspergillus flavus* Link yang dikulturkan selama 3 hari di dalam minyak olein dikaji. Lipase yang terikat-miselium mempunyai aktiviti optimum pada suhu antara 50–55 °C, dan 25–35 °C apabila diekstrak keluar. Lipase yang diekstrak keluar ini stabil pada pH alkali dan peka kepada haba. Pendedahan selama 4 jam pada suhu 30 °C akan menyebabkannya kehilangan aktiviti sebanyak 14%. Ion Ca^{2+} (1 mM) didapati meningkatkan aktiviti lipase, manakala EDTA (1 mM) tidak mempunyai kesan terhadap aktiviti. Enzim ini mempunyai aktiviti yang tinggi pada minyak kelapa berbanding dengan minyak sayuran yang lain dan lipase yang diekstrak keluar tidak berkeupayaan untuk melakukan hidrolisis terhadap tributirin. Nilai K_m bagi aktiviti lipase terikat-miselium (11.76 mg/mL) ialah tiga kali lebih tinggi daripada lipase yang diekstrak (3.92 mg/mL) dan ini menunjukkan adanya halangan untuk lipase terikat-miselium bertindak pada substrat. Lipase ini juga menunjukkan kespesifikan pada kedudukan 1,3.

Abstract

The properties of mycelium-bound lipase of *Aspergillus flavus* Link obtained after 3 days growth in palm olein were studied. The mycelium-bound lipase has an optimum activity at a temperature range of 50–55 °C and once extracted, the activity was optimum at a temperature range of 25–35 °C. The extracted lipase was stable at alkaline pH and heat labile, thus lost 14% of its activity after being exposed to 30 °C for 4 h. Ca^{2+} enhanced the lipase activity while EDTA (1 mM) had no effect. The enzyme hydrolysed coconut oil faster than other vegetable oils and tributyrin was not hydrolysed by the extracted lipase. The apparent K_m values obtained for mycelium-bound lipase (11.76 mg/mL) was three times higher than the extracted lipase (3.92 mg/mL) suggesting that there was a diffusion limitation of the substrate in reaching the bound lipase. The extracted lipase displayed 1,3 positional specificity.

*Food Technology Centre, MARDI Headquarters, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
**Dept. of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
***School of Biological and Health Sciences, University of Westminster, 115 New Cavendish Street WIM 8JS London, UK
Authors' full names: Kamariah Long, Hasanah M. Ghazali, Arbakariya Ariff and Christopher Bucke

Introduction

Interest in lipase from different sources has markedly increased in the last decade due to the potential application of lipases in industry and medicine. Lipases from microorganisms are widely diversified in their enzymatic properties and substrate specificity. The properties such as, positional specificity, fatty acid preferences, thermal stability and pH optima, determine their biotechnological importance in industry. The process of interesterification depended critically on the positional reactivity of lipase (Sonnet and Gazzillo 1991). The use of 1,3-regioselective lipase altered the fatty acids of 1-position and 3-position but left the fatty acid substituting in the 2-position unchanged.

Literature review showed that lipase of *Aspergillus flavus* received little attention although there were many papers reported on its ability to grow and utilise oil from storage seeds (Farag et al. 1981; Bose and Nandi 1985; Dart et al. 1985; Fernandez 1987). For industrial needs, this lipase should be investigated and characterized. Earlier studies by Long et al. (1996a) showed that *A. flavus* produced lipase that was largely bound to the mycelia during growth. However, the activity of lipase from the supernatant may not be easily detected due to the presence of a mixture of metallo-proteases (Long et al. 1996a) which actively degraded the lipase.

In most cases, the physiochemical properties of the free lipase showed some changes when in the immobilised state, such as the thermal-stability of the enzyme (Xavier et al. 1992), pH and temperature optima (Shaw et al. 1990), fatty acid specificity of the enzyme (Huge-Jensen et al. 1987) and their kinetics (Van der Padt et al. 1990). The immobilisation which is the main aim to reuse the enzyme does often confer greater operational stability. There are numerous papers reported on the physiochemical properties of the man-made immobilised lipase. In contra
st very little work had been done on the mycelium-bound lipase. Accordingly, a study was conducted to compare the characteristics of the mycelium-bound lipase and after the lipase was released from the cell wall.

Materials and methods

Fungus

The fungus used in the study was isolated from copra meal (Long et al. 1996b) and was identified as *A. flavus* Link (IMI 361648) by International Mycological Institute, United Kingdom.

Commercial lipase

Lipase from *Pseudomonas* sp. and *Rhizomucor miehei* was donated by Amano Pharm. Co. Japan and NOVO Nordisk Industry, Denmark respectively. *Candida rugosa* lipase was obtained from Sigma Chem Ltd. USA

Liquid culture conditions

The medium contained (w/v): 2% yeast extract, 2% palm olein, 0.3% $(NH_4)_2 SO_4$, 0.02% $CaCl_2$, 0.2% $KH_2 PO_4$, 0.1% glucose and 0.4% EDTA. The pH of the medium was adjusted to 6.0 using 1 M NaOH and sterilised at 121 °C for 15 min. Four mL of spore suspension containing an average of 10^7 spores per mL was used to inoculate 200 mL of the medium. Cultivation was carried out at 30 °C and agitated at 150 rev/min in an orbital shaker for 72 h.

Lipase preparation

Mycelium-bound lipase Mycelia were harvested and washed with 1 500 mL distilled water, then defatted with 100 mL n-hexane using a vacuum pump. The n-hexane defatted mycelia were used as the source of mycelium-bound lipase**.**

Extraction of mycelium-bound lipase freed from proteases Wet mycelia (4 g) were suspended in 80 mL of Tris-HCl buffer (pH 8.2, 0.05 M). The suspension was shaken in an orbital shaker (200 rev/min) for 90 min at 35 °C after which the mycelia were removed

by filtration through Whatman No. 4 filter paper. The extraction procedure was repeated the second time using the same mycelia. The extracted lipase from each extraction was combined together and later was fractionated with acetone at a ratio of 1:1.5 (lipase solution : acetone) (Long 1997). The mixture was centrifuged at 12000 x *g* for 10 min at 0 °C. The pellet containing active proteases was discarded and the supernatant was rotary evaporated for 30 min at room temperature to remove the solvent. The aqueous fraction was then concentrated to one-tenth of the original volume by ultrafiltration through a Diaflo PM 10 membrane filter (43 mm diameter) molecular cut-off 10,000. Retentate containing lipase was then used for characterization of soluble lipase.

Assay for lipase activity

Lipase activity was measured as described by Sugiura et al. (1975) with slight modifications (Long 1996a). The reaction mixture consisted of 2.5 mL of 10% refined, bleached and deodorised (RBD) coconut oil emulsified in 10% gum arabic, 2 mL of Tris-HCl buffer pH 8.2, 0.5 mL of 0.25% $CaCl_2$ (unless otherwise stated), 4 mL of deionised water and activity was assayed using 1 mL of enzyme. In the case of mycelium-bound lipase, activity was assayed using 0.15 g mycelium wet weight (approx. 36 mg dry weight). The reaction was conducted at 50 °C (unless otherwise stated) for 30 min in a shaker at 200 rev/min and 20 mm displacement. Free fatty acid liberated was determined by titration to pH 9.0 with 0.05 N aqueous NaOH using an automatic recording pH-stat (VIT 90, Video Titrator Radiometer, Copenhagen). One unit of activity is defined as 1 mmol fatty acids released/min (U). Control for the bound lipase activity was carried out as above but the mycelium-bound lipase was first inactivated by sonicating the mycelium for 5 min before boiling for 10 min.

Temperature and pH optimum

The optimum temperature was determined by incubating the mycelium-bound and extracted lipase at temperatures ranging from 22 °C to 75 °C for 30 min. The pH profiles of extracted and mycelium-bound lipase were determined over a pH range of 3.0 to 10.0. Three types of buffers, i.e. McIlvaine buffer (pH 3.0–7.0), Tris-HCl (pH 7.5–8.8) and Glycine-NaOH (pH 8.8–9.5) were used.

Thermal and pH stability

Extracted lipase obtained after ultrafiltration was diluted with 30 mL of 0.05 M Tris-HCl buffer (pH 8.2). The solution was then exposed to various temperatures ranging from 4 °C to 60 °C. Five mL of the solution was taken out each time and the activity of lipase was assayed for up to 240 min. For pH stability, three types of buffer mentioned above were used. One mL lipase solution was added into 9 mL of the appropriate buffer, mixed and then stored at 4 °C for 20 h before residual activity was assayed.

Effect of metal ions

Salts at various concentration were added at the beginning of incubation time to give a final concentration of 1 mM. The lipase activity was then measured and reported as the percentage of the enzymes without metal ion treatment.

K_m and V_{max} values

The Michaelis-Menten constant *(K_m)* and maximal velocities of the extracted and bound lipase would be determined, based on the double reciprocal plot initial velocities and substrate (coconut oil) concentration (Lineweaver and Burk 1934). The intercepts on the abscissa and ordinate of the plot were used to calculate the K_m and V_{max} values, respectively.

All the above experiments were carried out in triplicate.

Positional specificity

The positional specificity of extracted lipase was determined according to the method of Okumura et al. (1976) and Jensen et al. (1990). The reaction mixture containing 0.5 g triolein (99% pure), 8 mL 0.05 M Tris-HCl buffer, pH 8.2, 1 mL 0.1 M $CaCl_2$ and 1 mL extracted lipase. The reaction mixture was incubated at 30 °C for 2 h with constant agitation (200 rev/min). One mL of reaction mixture was withdrawn each time and 10 mL of acetone : ethanol (1:1) mixture was added immediately to stop the reaction. Hydrolysis products were separated and identified using thin layer chromatography on precoated silica gel 60 F254 plate (Merck) impregnated with 1.2% boric acid in absolute ethanol (1:1, v/v). Precoated silica gel 60 plate was dipped into the above solution followed by air-drying and activation at 100 °C for 15 min. The developing solvent system used was chloroform : acetone (6:4). Reaction products of triolein were visualised by spraying with 50% sulphuric acid and heated at 150 °C for 20 min. The hydrolysis reaction of triolein using other commercial lipases such as *Candida rugosa* (Sigma Chem. Co., USA), *Pseudomonas* sp. *LPSATO* 3516 (Amano Pharm. Co Ltd. Japan) and *Rhizomucor miehei* (Novo Nordisk Copenhagen, Denmark) was carried out following the above procedure as comparison.

Activity of mycelium-bound and extracted lipase towards triglycerides and vegetable oils The composition of the reaction mixture and assay conditions used are described above. Coconut oil was replaced with palm olein, sun flower oil, soybean oil, olive oil, grape seed oil, corn oil, tributyrin and triolein.

Results

Physiochemical properties

The extracted lipase was shown to have optimum temperature between 25–35 °C and its activity gradually decreased as temperature increased *(Figure 1a).* On the other hand, the mycelium-bound lipase had activity optimum at 50–55 °C and its activity decreased sharply at 60 °C *(Figure 1b).* The pH optima of both forms of lipase remained similar, pH 8.0 *(Figure 2)*. The thermal stability of extracted lipase at various temperatures ranging from 4 °C to 60 °C as shown in *Figure 3*. The enzyme quickly lost its activity (94%) after 30 min exposure to 60 °C. It was also noted that the extracted lipase was heat labile and thus lost 14% of its activity after being exposed to 30 °C for 4 h. *Aspergillus flavus* lipase was stable at alkaline pH ranging between pH 7 and 8.5 *(Figure 4)*. Below and above this range, there was a loss of activity e.g. 92% of activity was lost when the enzyme was exposed to pH 3 *(Figure 4).* The effect of metal ions and EDTA on the activity of

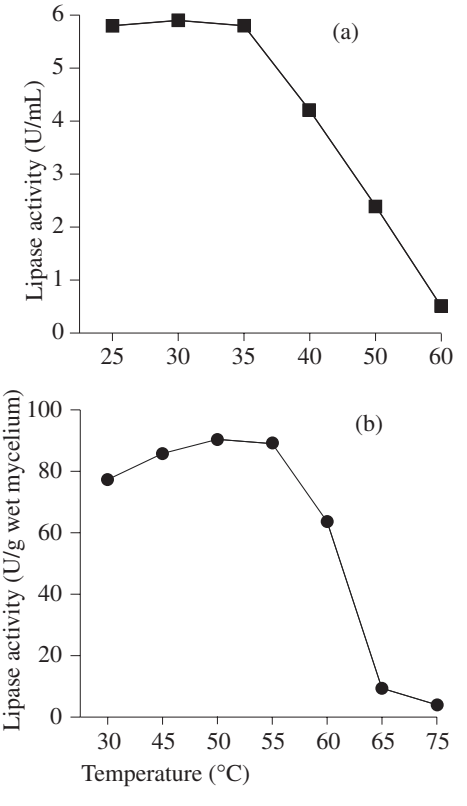


Figure 1. Temperature profile of (a) Extracted lipase (b) Mycelium-bound lipase of ***A. flavus*** *Link*

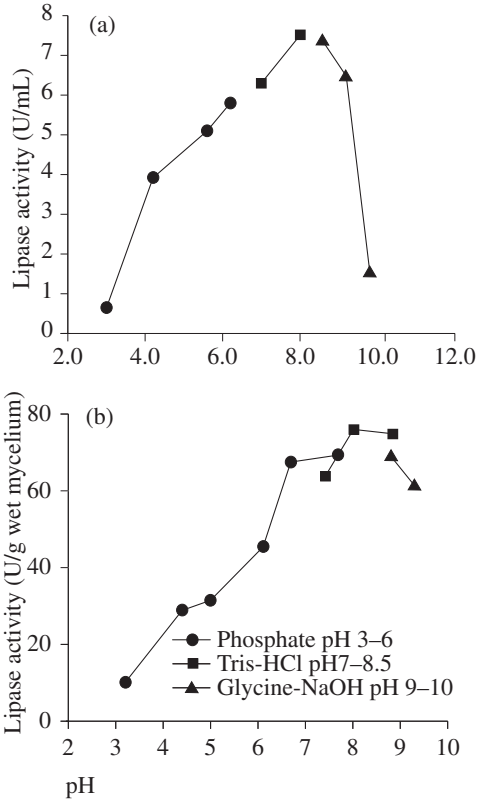


*Figure 2. pH profile of (a) Extracted lipase (b) Mycelium-bound lipase of **A. flavus** Link*

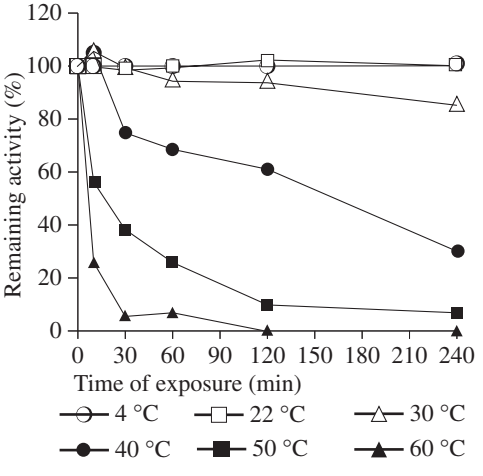


*Figure 3. Thermal stability of extracted lipase of **A. flavus** Link*

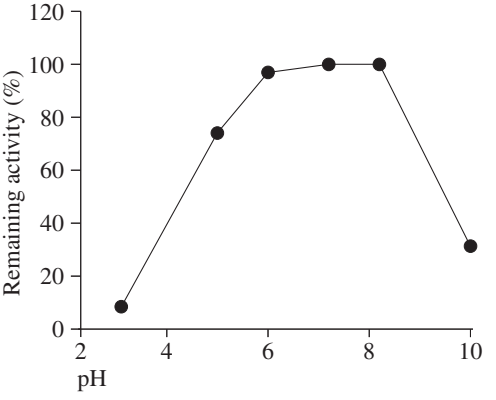


*Figure 4. pH stability of the extracted lipase of **A. flavus** Link*

Table 1. Effect of metal ions on the activity of extracted lipase

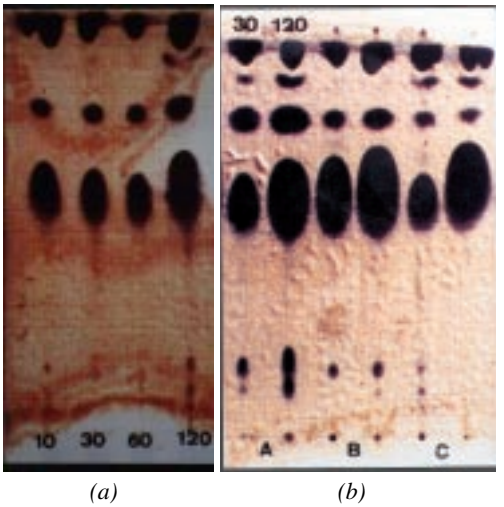
Metal ion (1 mM)	% of remaining activity
Control	100
Na^+	98.3
Ca^{2+}	113.9
Zn^{2+}	8.0
Fe^{3+}	68.7
Fe^{2+}	72.3
Cu^{2+}	23.8
K^+	100.4
EDTA	101.3
PMSF	97.8

extracted lipase is shown in *Table 1*. Among the metal ions tested, Zn^{2+} caused a severe inactivation to the activity, followed by Cu^{2+}, Fe^{3+} and Fe^{2+}. On the other hand, monovalent cations, such as K^+ and Na^+ do not have any significant effect on the activity of *A. flavus* lipase. Calcium ions increased the activity of lipase by 13.9%.

The apparent K_m and V_{max} values of the mycelium-bound lipase were 111.76 mg/mL and 68.03 U/g, respectively. On the other hand, the apparent K_m and V_{max} values of the extracted lipase were 3.92 mg/mL and 9.02 U/mL, respectively. Both the extracted and mycelium-bound lipase preferred to hydrolyse coconut oil followed by palm olein. It was also observed that once extracted, lipase losts its activity towards tributyrin but not to triolein and other vegetable oils *(Table 2).*

Table 2. Hydrolysis of triglycerides and vegetables oils using extracted and mycelium-bound lipase of *A. flavus* Link

Substrate	Tributyrin	Triolein	Coconut oil	Palm olein	Sun flower	Soyabean	Olive oil	Grapeseed	Corn oil
Activity of extracted lipase (U/mL)	0	7.15	8.34	7.78	5.78	5.61	4.90	6.13	5.80
Activity of mycelium-bound lipase (U/g wet weight)	44.13	52.60	71.58	66.56	39.66	31.31	35.60	41.64	41.18



(a) *(b)*

Plate 1. Thin layer chromatograms of the products of triolein hydrolysis of using (a) extracted lipases from ***A. flavus*** *Link, (b) Commercial non-specific lipase* ***Pseudomonas*** *sp. (A),* ***Candida rugosa*** *(C) and I,3 – specific lipase,* ***Rhizomucor miehei*** *(B)*

Positional specificities

Thin layer chromatogram (TLC) of the products of hydrolysis of triolein by *A. flavus* is shown in *Plate 1a*. Neither 1(3)-monolein nor 1,3-diolein was detected in the reaction mixture for the first 60 min of the reaction. However, 1,3-diolein and monolein were only detected following prolonged reaction time (120 min). The hydrolysis products of triolein by *A. flavus* was compared with the commercial lipase from *Pseudomonas* sp. (non-specific lipase), *C. rugosa* (non-specific lipase) and *R. miehei* (1,3-specific) *(Plate 1b*). It was noted that *A. flavus* lipase gave a similar pattern of hydrolysis products to that shown by *R. miehei* lipase which was known to be 1,3-positional specificity.

Discussion

As reported by many other researchers, immobilisation almost always leads to a positive shift in the optimum temperature (Omar et al. 1988; Shaw et al. 1990). This result implies that upon extraction, *A. flavus* lipase became more sensitive to thermal inactivation. Usually, immobilized lipases are more stable than their free counterparts because the enzyme molecule becomes more rigid after attachment to a solid carrier.

Hence unfolding is greatly hindered and disruption of the active centre of the protein becomes less likely to occur (Zaks and Klibanov 1985). Although immobilisation has also been shown to shift pH optimum of lipases (Omar et al. 1988; Shaw et al. 1990) in this study the pH optimum of *A. flavus* (pH 8.2) was not affected. A similar finding was also reported with *Candida cylindracea (C. rugosa)* lipase (Tahoun 1986). The variability of the results may be due to the different nature of the lipases, supports and immobilization procedures (Malcata et al. 1992).

Many researchers have reported activation of microbial lipase by Ca^{2+} (Susumu et al. 1969; Hoshino et al. 1992). The role of Ca^{2+} salts is to remove the free fatty acid or partial glycerides (Iwai and Tsujisaka 1984). Ca^{2+} might also play an important role in the structural stabilisation of *Pseudomonas* lipases (Barfoed et al. 1993). The addition of EDTA did not give any negative effect on the lipase activity of *A. flavus*, suggesting that the lipase is independent on a metal cofactor at its active site. Zn^{2+} and Fe^{2+} strongly inhibited many other lipases including *A. niger* whose activity was inhibited by 98% and 89%, respectively (Sugihara et al. 1988). Tsujisaka et al. (1972) showed that the inhibitory effect of Fe^{2+} ions on fungal lipases was more severe than those of other metal ions examined. They showed that *A. niger* lipases were instantly inactivated with even at very low concentration of Fe^{2+} ions, regardless of the pretreatment.

The apparent K_m value of the mycelium-bound lipase was three times higher than those of the extracted lipase, suggesting that the affinity of the mycelium-bound lipase towards the substrate was lower than the extracted lipase. This could be due to the diffusion limitations of the substrate in reaching the mycelium-bound lipase.

The observation that the extracted lipase loses its ability to hydrolyse tributyrin is of considerable interest. It is not certain why such a difference occurs, probably it could be due to conformational changes of the lipase either through denaturation or the enzyme that has activity towards tributyrin is tightly bonded and therefore was not released upon extraction. Long (1997) found that the lipase that is bound to the mycelia of *A. flavus* has various degrees of binding. About 28% of its activity was easily released after washing with n-hexane deffated mycelia with water. The rest was quite easily released by treating the mycelia with 0.05 M Tris-HCl buffer pH 8.2 at 35 °C for 90 min, 200 rev/ min. However, 7% is tightly bound and released only upon treatment with lytic enzyme preparation (Long 1997).

It was obvious that *A. flavus* lipase gave a similar pattern of hydrolysis products to that shown by *R. miehei* lipase. However, after prolonged reaction time (120 min) there is formation of 1,3 diolein. This formation is probably due to acyl-migration of the fatty acid in 2-position of the triolein towards the outer position. In the determination of positional specificity, it is advisable to perform the reaction in a short incubation time in order to minimize the acyl migration (Macrae 1983). Lipases that have no positional preferences attack fatty acid, regardless of their positions at any time of reaction and give hydrolysis products as shown by *Pseudomonas* sp. and *C. rugosa* lipases. Results from the above studies demonstrated that *A. flavus* displayed a strong positional specificity towards the outer chains of the triglyceride molecule and this was also shown by other *Aspergillus* sp. (Okumura et al. 1976).

Results from *Table 2* indicate that *A. flavus* lipase hydrolyses coconut oil and palm olein better than other vegetable oils suggesting that its lipase is less favourable in hydrolysing vegetable oils that contain more unsaturated fatty acid. The result supports the finding by Long et al. (1998) who shows that this enzyme has greater preference for saturated triglycerides than the unsaturated triglycerides.

References

Barfoed, M., Borch, K., Lund, H. and Patkar, S. A. (1993). Ca^{2+} induced enhanced of the molecular stability of *Pseudomonas* lipases. *Stud. Org. Chem*. ***47:*** 181–8

Bose, A. and Nandi, B. (1985). Role of enzymes of storage fungi in deterioration of stored safflower and sesame seed. *Seed Res*. ***13(2):*** 19–28

Dart, R. J., Dede, E. B. and Offem, J. O. (1985). Fungal damage to palm kernel oil. *Food Chem*. ***18:*** 113–9

Farag, R. S, Khalil, F. A., Taha, R. A. and Shihata, A. A. (1981). Comparison between the lipid classes and fatty acid composition of peanut and cottonseeds infected with *Aspergillus flavus*. *Grass Accites* ***32(1):*** 19–23

Fernandez, W. (1987). Oil loss in copra due to fungi: A review. *Philip. Agric*. ***70(1):*** 29–40

Hoshino, T., Sasaki, T., Watanabe, Y., Nagasawa, T. and Yamane, T. (1992). Purification and some characteristics of extracellular lipase from *Fusarium oxysporum. Biosci. Biotechnol. Biochem*. ***56(4):*** 660–4

Huge-Jensen, B., Galluzzo, D. R. and Jensen, R. G. (1987). Partial purification and characterization of free and immobilized lipases from *Mucor miehei. Lipids* ***22:*** 559–65

Iwai, M. and Tsujisaka, Y. (1984). Fungal lipase. In *Lipases* (Borgstrom, B. and Brockmad, H. L. eds.) p. 443–66. Amsterdam: Elsevier Applied Science publishers

Jensen, R. G., Gallyzzo, D. R. and Bush, V. J. (1990). Selectivity is an important characteristic of lipase (acylglycerol hydrolases). *Biocatalysis* ***3:*** 307–16

Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constant. *J. Am. Oil Chem. Soc.* ***56:*** 658–66

Long, K. (1997). Production, properties and application of mycelium-bound lipase from a locally isolated strain of *Asperigillus flavus* Link. Ph. D. Thesis, Universiti Putra Malaysia

Long, K., Ghazali, H. M., Ariff, A., Ampon, K. and Bucke, C. (1996a). Mycelium-bound lipase from a locally isolated strain of *Aspergillus flavus*: Pattern and factors involved in its production. *J. Chem. Tech. Biotechnol.* ***67:*** 157–63

Long, K., Ghazali, H. M., Bucke, C., Ampon, K. and Ariff, A. (1996b). Isolation of lipolytic fungi using coconut oil as a carbon source. *J. Bioscience* ***7(2):*** 143–52

Macrae, A. R. (1983). Lipase-catalysed interesterification of oil and fats. *J. Am. Oil Chem. Soc.* ***60:*** 291–4

Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill, C. G. and Amundson, C. H. (1992). Kinetic and mechanisms of reactions catalysed by immobilized lipases. *Enzyme Microb. Technol*. ***14:*** 426–46

Omar, I. C., Saeki, H., Nishio, N. and Nagai, S. (1988). Hydrolysis of triglycerides by immobilized lipase from *Humicola lanuginosa*. *Agric. Biol. Chem.* ***52:*** 99–105

Okumura, S., Iwai, M. and Tsujisaka, Y. (1976). Positional specificities of four kinds of microbial lipase. *Agric. Biol. Chem.* ***40(4):*** 655–60

Shaw, J. F., Chang, R. C., Wang, F. F. and Wang, Y. J. (1990). Lipolytic activities of a lipase immobilized on six selected supporting materials. *Biotechnol. Bioeng.* ***35:*** 132–7

Sonnet, P. E. and Gazzillo, J. A. (1991). Evaluation of lipase selectivity for hydrolysis. *J. Am. Oil Chem. Soc*. ***68(1):*** 11–5

Sugihara, A., Shimada, Y. and Tominaga, Y. (1988). Purification and characterization of *Aspergillus niger* lipase. *Agric. Biol. Chem*. ***52(6):*** 1591–2

Sugiura, T., Ota, T. and Minoda, Y. (1975). Effect of fatty acid lipase activator, phospholipids and related substances on lipase production by *Candida paralipolytica*. *Agric. Biol. Chem.* ***39(9):*** 1689–94

Susumu, O., Yamazaki, O., Sawada, A. and Satomura, Y. (1969). Purification and some properties of a fungal lipase preparation used for milk flavouring. *Agric. Biol. Chem.* ***33(5):*** 729–38

Tahoun, M. K. (1986) Large agarose- Lipase beads for the hydrolysis of triglycerides. *Food Chem.* ***22:*** 297–303

Tsujisaka, Y., Iwai, M. and Tuminaga, Y. (1972). A comparative study on some properties of fungal lipase. In *Fermentation Technology Today* (Terui, G., ed) p. 315–20. Society of Fermentation Technology Osaka

Van der Padt, A., Edema, M. J., Sewalt, J. J. W. and van't Riet, K. (1990). Enzymatic acyglycerol synthesis in a membrane bioreactor. *J. Am. Oil Chemist's Soc*. ***67:*** 347–52

Xavier, F., Reyes, H. R., Garcia, H. S., Hill, C. G. and Amundson, C. H. (1992). Kinetics and mechanisms of reactions catalysed by immobilised lipases. *Enzyme Microb. Technol*. ***14:*** 426–46

Zaks, A. and Klibanov, A. M. (1985). Enzymatic-catalysed process in organic solvents. *Proc. Natl. Acad. Sci.* USA ***82:*** 3192–6

Accepted for publication on 22 October 2000