ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 3 (1997) 153–159

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Some characteristics of lipases from thermophilic fungi isolated from palm oil mill effluent

C.N.A. Razak *, A.B. Salleh, R. Musani, M.Y. Samad, M. Basri

Enzyme and Microbial Technology Research, Fakulti Sains and Pengajian Alam Sekitar, Universiti Pertanian Malaysia, 43400 UPM Serdang, Malaysia

Received 20 April 1996; accepted 13 September 1996

Abstract

Rhizopus oryzae and *Rhizopus rhizopodiformis* are thermophilic fungi isolated from palm oil mill effluent (POME). They are able to grow up to 50°C and produce remarkable amounts of extracellular lipases. The extracellular lipases from both fungi displayed quite similar characteristics. The optimum pH and temperature for both lipases were 6.0 and 45°C, respectively. However, lipase from *R. rhizopodiformis* was slightly more thermostable than that of *R. oryzae* lipase but the latter was more stable over broader pH ranges compared to the former, specially at acidic pH. Both the enzyme showed rapid loss of activities at temperatures above 50°C and pH above 7.0. Lipase from *R. oryzae* showed broader specificities to substrates than *R. rhizopodiformis*, but both displayed little reactivity for triacetin and polyoxysorbitan (Tween 20, 40, and 60). Both the lipases are 1,3 specific.

Keywords: Lipases; *Rhizopus oryzae*; *R. rhizopodiformis*; Thermophilic fungi

1. Introduction

Lipases from various microorganisms have been reported and many of the lipases have been purified to homogeneity and their properties elucidated [1,2]. However, in view of their potential use in ester synthesis, interesterification reactions and as ester hydrolysers, work on microbial lipases still continues to receive much attention both academically and industrially.

Our group has carried out extensive screening programs for lipolytic microorganisms from our local environments. *Rhizopus oryzae* and *Rhizopus rhizopodiformis* are among the microbes isolated. Both fungi were isolated from POME and are able to grow up to 50°C [3,4]. The extracellular lipases from both the fungi have been purified and characterized. This paper describes the properties of the extracellular lipases from the two fungi.

2. Materials and methods

2.1. Microorganism

R. oryzae and *R. rhizopodiformis* were isolated from the effluent treatment ponds of two

* Corresponding author. Fax: (+60-3)9432508; E-mail: cnyonya@fsas.upm.edu.my

PII S1381-1177(96)00035-5

different palm oil mills in Malaysia. The former is now deposited at the American Type Culture Collection as *Rhizopus oryzae* S3, ATTC #96382 while the latter is kept at the International Mycological Institute, Surrey, England, who also carried out the fungal identifications.

2.2. Growth media and culture condition

The fungi were grown in 100 ml media in 250 ml conical flask shaken at 100 rpm for 72 h at 45°C. The media used comprised (g/l) peptone, 50; KH_2PO_4, 1.0; $NaNO_3$, 1.0 and $MgSO_4 \cdot 7H_2O$, 0.5. The initial pH of the media was adjusted to 6.0.

2.3. Enzyme activity

Lipase activity was determined according to Salleh et al. [3]. The reaction mixture comprised 2.5 ml olive oil emulsion in water (1:1; v/v), 1% (w/v) polyvinyl alcohol, 0.02 ml, 0.02 M $CaCl_2$ and 1.0 ml extracellular enzyme. The reaction was carried out for 30 min at 37°C with shaking at 200 rpm. The reaction was terminated by addition of 7.0 ml of ethanol:acetone (1:1; v/v). The remaining free fatty acid in the reaction mixture was determined by titration with 0.05 M to pH 10.0 using a pH autotitrator (Radiometer ABU 90). One unit of activity is equivalent to one μmole of free fatty acid released per minute.

2.4. Purification of extracellular lipase

After cultivation, the culture broth was filtered to remove the mycelium. The filtrate designated as crude lipase was further purified. Acetone precipitation was employed to precipitate the crude enzyme up to 80% saturation. The precipitate was harvested by centrifugation and washed twice with 0.02 M Tris HCl (pH 7.0) and resuspended in the same buffer. The concentrated sample was applied onto a Sephadex G-100 column (2.5 × 54 cm). The purified lipase was lyophilised and stored at −80°C.

2.5. Characterization of the enzyme

2.5.1. Effect of the temperature

The effect of temperature on the lipase activity was measured at various temperatures ranging from 28 to 70°C. The highest activity of the enzyme was noted as 100% activity. For thermostability, the enzyme was incubated at 40, 50, 60, 70, 80 and 90°C for 30 min. After each period of incubation, the enzyme was immediately cooled in an ice bath for 15 min and the residual activity determined.

2.5.2. Effect of pH

The effect of pH on the enzyme activity and stability was measured at pH ranges of 4.0 to 10.0. For pH optimum determination, the lipases activity was measured in various buffers (pH 4–10). For pH stability, the enzyme was incubated in 1 ml of various buffers at 37°C for 30 min. After incubation, the residual activity of the lipases was assayed. The activity was expressed as a percentage, with the highest activity as 100%. Buffer systems used were 0.05 M acetate buffer (pH 4–5), 0.05 M phosphate buffer (pH 6–7) and 0.1 M Clark and Lubs buffer (pH 8–10).

2.5.3. Substrate and positional specificity

The substrate specificity was determined following the method by Okumura et al. [5]. Various substrates (0.5 g) were incubated with 0.05 ml enzyme (2 mg/ml), 2.0 ml phosphate buffer pH 6.0 and 0.02 ml 0.02 M $CaCl_2$. The mixture was shaken at 200 rpm for 2 h at 37°C. The reaction was stopped by addition of 5.0 ml ethanol: acetone (1:1, v/v) and fatty acids liberated were titrated with 0.05 M NaOH to pH 10.0 using a pH autotitrator (Radiometer ABU 90).

For positional specificity, the hydrolysis of triolein was monitored with time. The reaction mixture containing 0.1 ml triolein, 4.0 ml 0.05 M phosphate buffer pH 5.0 and 0.05 ml extracellular lipase, was incubated with constant shaking at 37°C. Samples were taken at 2, 24

and 48 h and spotted onto silica gel TLC plates (60F-254, 0.25 mm, Merck). The solvent system used for the TLC comprised petroleum ether:diethyl ether:acetic acid (80:30:1, by volume). The dried plate was covered with concentrated sulfuric acid using a spray gun (CAMAG). The plates were dried at 80–110°C until spots became visible.

3. Results and discussion

Table 1 shows the summary of the purification procedures done on the extracellular lipase of *R. oryzae* and *R. rhizopodiformis*. For *R. oryzae* the specific activity of the purified enzyme was 189 U/mg, with an overall yield of 64% and a 158-fold purification. One activity peak obtained by separation through Sephadex G-100 chromatography (Fig. 1a). The crude enzyme showed 5 bands whereas the purified enzyme showed a single band on PAGE (Fig. 2a). The purified enzyme was used for further characterization. However, a much lower recovery and purification factor was obtained with the extracellular lipase of *R. rhizopodiformis*. The enzyme was purified 10-fold with 38% recovery and it had a specific activity of 11.1 U/mg. Similar to *R. oryzae*, the enzyme activity appeared in one peak after elution through Sephadex G-100 and the crude enzyme showed 5 protein bands. However, PAGE analysis of the purified lipase showed 2 protein bands (Fig. 2b). No attempt was made to check for the lipase activity in the 2 bands. One or both bands may contain the lipase activity. This enzyme preparation was used for further characterization. Most of the purified lipases reported by other workers showed homogeneity with one band appearing in PAGE [6,7]. However, the presence of multiple lipases was reported for *Pythium ultimatum* and *Penicillium cyclopium* [8,9].

3.1. Effect of pH on lipase activity

Fig. 3 shows the pH optima and pH stabilities of extracellular lipases of *R. oryzae* and *R. rhizopodiformis*. The activity of both lipases were optimal at pH 6.0. The extracellular lipase from *R. oryzae* was more active at acidic pH compared to that of *R. rhizopodiformis*. Both of the enzymes were not active at pH above pH 7.0. This result is in agreement with most fungal lipases which exhibit maximum activity in the acidic pH range, as shown by *Aspergillus niger* and *Rhizopus japonicus* NR 400 lipases which have pH optima of around pH 5.0 [6,7]. Two other lipases from *P. cyclopium* and *Syncephalastrum racemosum* had pH optima of 4.0 and 6.0, respectively [9,10].

The extracellular lipase of *R. oryzae* was stable at pH ranges from pH 4.0–7.0 whereby more than 80% of the activity was retained. At

Table 1
Purification of extracellular lipases from *R. oryzae* and *R. rhizopodiformis*

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity (fold)
Crude extract					
R. oryzae	7050.0	8433.5	1.2	100.0	1
R. rhizopodiformis	16200	18540	1.14	100.0	1
Acetone precipitation (80% v/v)					
R. oryzae	1680.0	6448.0	3.8	76.4	3.2
R. rhizopodiformis	3669	11592	3.2	62.5	2.8
Sephadex G-100					
R. oryzae	28.8	5451.0	189.0	64.0	158
R. rhizopodiformis	630	6990.4	11.1	37.7	9.7

pH 8.0, the lipase activity was reduced to 75%. In contrast, the extracellular lipase of *R. rhizopodiformis* was more pH labile, it was only stable at pH 7.0. The lipase activity dropped greatly at pH values above and below pH 7.0. At pH 8.0, the enzyme lost about 50% of its initial activity while the drop at acidic pH was less drastic, retaining about 65% of its initial activity at pH 5.0 and 6.0.

3.2. The effect of temperature on the lipase activity

The effect of temperature on the enzymes activities and stabilities were studied from 28 to 70°C (Fig. 4). The optimum temperature for both lipases was at 45°C. Earlier studies have shown that most fungal lipases exhibited optimum temperature in the range of 25 to 40°C.

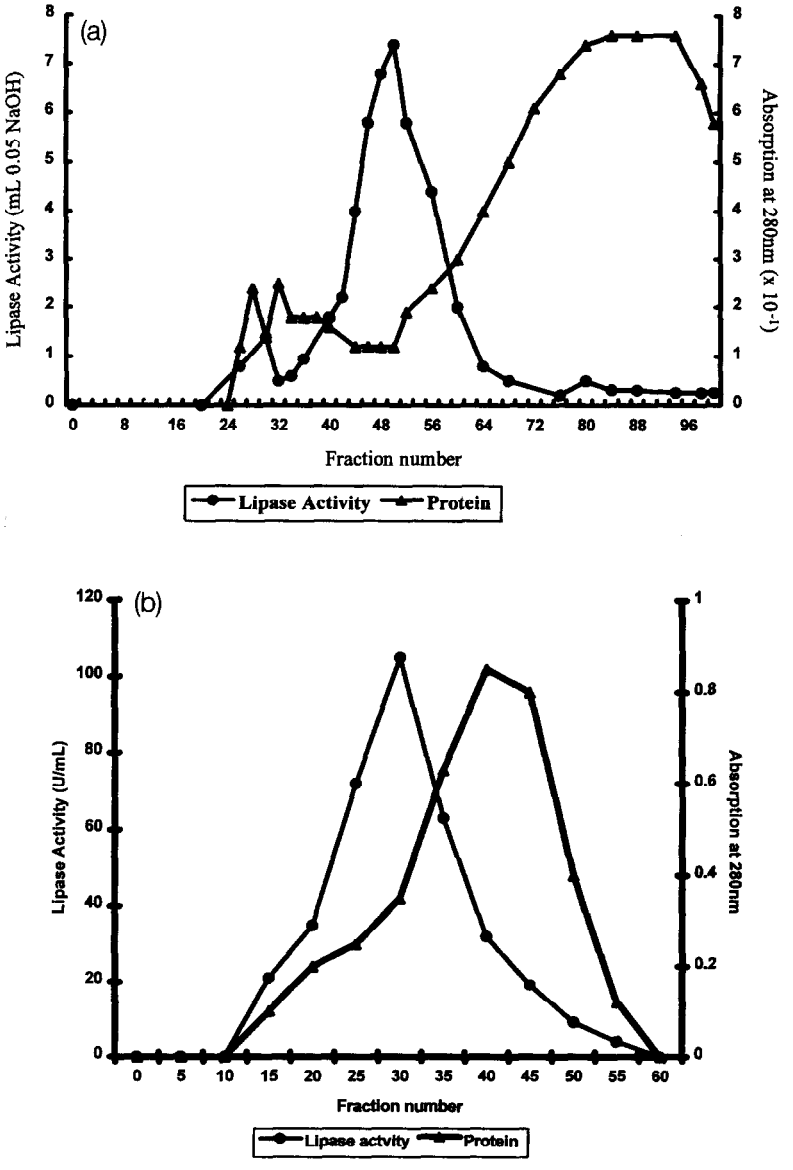

Fig. 1. (a) Gel filtration elution profiles of lipase activity of *R. oryzae* on Sephadex G-100 column. (b) Gel filtration elution profiles of lipase activity of *R. rhizopodiformis* on a Sephadex G-100 column.

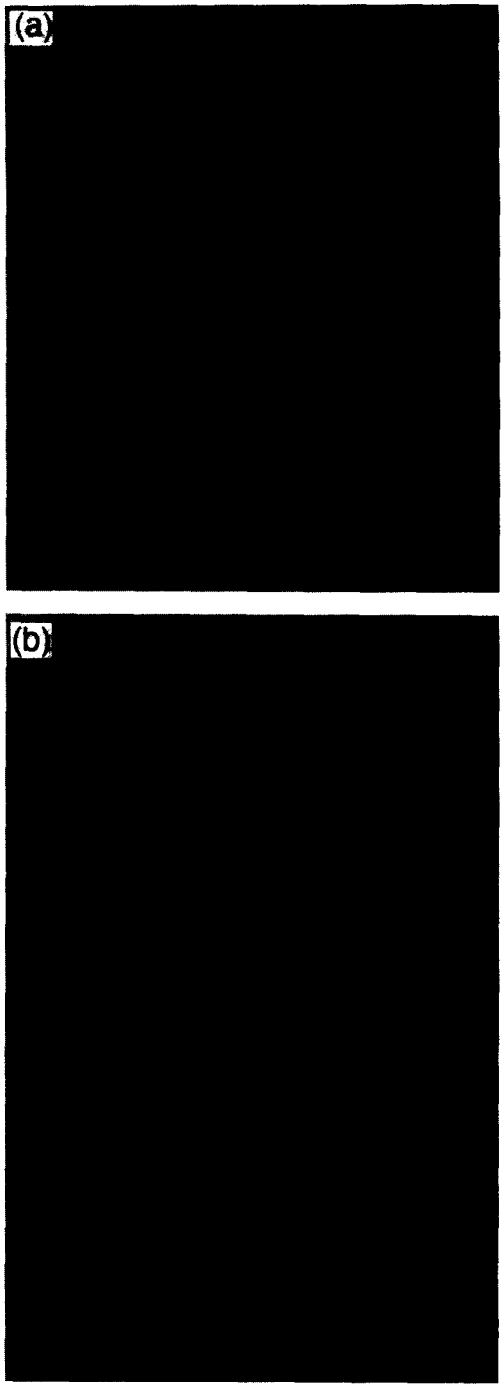

Fig. 2. (a) SDS–PAGE of crude (A) and purified extracellular lipase (B) of *R. oryzae*. (b) SDS–PAGE of crude (A) and purified extracellular lipase (B) of *R. rhizopodiformis*.

For example, lipases from *A. niger* [6] had an optimum temperature at 30°C, *S. racemosum* [10] at 37°C and *R. japonicus* NR 400 [7] at 40°C. Very few fungal lipases exhibited temperature optima above 40°C, thus the extracellular lipases from both the fungi may have potentials in high temperature reactions. Other lipases that exhibited temperature optima of above 40°C are from *Humicola lanuginosa* [11] and *Penicillium roqueforti* [15].

The temperature stabilities studies on the extracellular lipases showed that at 45°C, 100% of the activity of extracellular lipase from *R. rhizopodiformis* was still retained after 30 min, while the lipase activity of *R. oryzae* was reduced to 80%. Both enzymes were not stable at temperatures above 50°C. The stability of the lipases were better than that reported for *S. racemosum* [10] which was less stable above 30°C. However, they were less thermostable than lipases from *H. lanuginosa* No.3 [10] and *A. niger* [6] which were stable for 20 min at

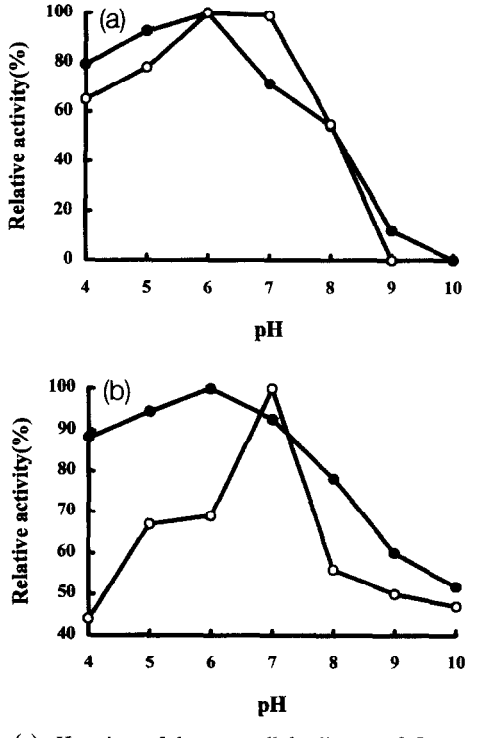

Fig. 3. (a) pH optima of the extracellular lipases of *R. oryzae* (t) (●) and *R. rhizopodiformis* (○). The optimum activity was taken as 100%. Each point represents a mean of triplicates. (b) pH stability of the extracellular lipases of *R. oryzae* (t) (●) and *R. rhizopodiformis* (○). The activity at pH 6.0 was taken as 100%. Each point represents a mean of triplicates.

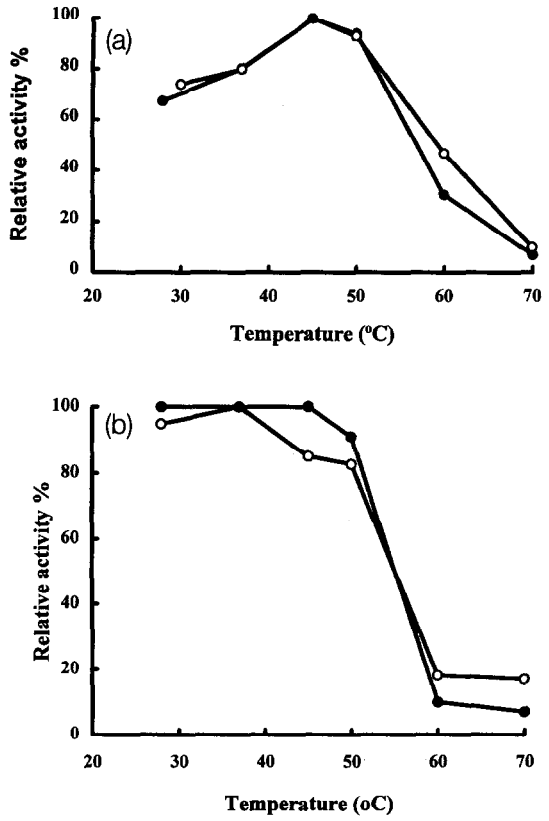

Fig. 4. (a) Temperature profiles of the extracellular lipases of *R. oryzae* (t) (●) and *R. rhizopodiformis* (○). Each point represents a mean of triplicates. (b) Temperature stability of the extracellular lipases of *R. oryzae*(t) (●) and *R. rhizopodiformis* (○). Each point represents a mean of triplicates.

60°C (pH 7.0) and 30 min at < 65°C (pH 5.6), respectively.

3.3. Regiospecificity and substrate specificity

The mode of reaction of the extracellular lipase from *R. oryzae* in triolein is shown in Fig. 5. The chromatogram showed that even after 48 h, the monoolein fraction could be observed, indicating that the ester at C2 was not cleaved suggesting the 1,3 preference of the enzymes. According to Liu et al. [13] and Okumura et al. [12], lipases with no regard for positional specificities would show no monoolein when the hydrolysates were chromatographed on silica gel TLC plates. Similar

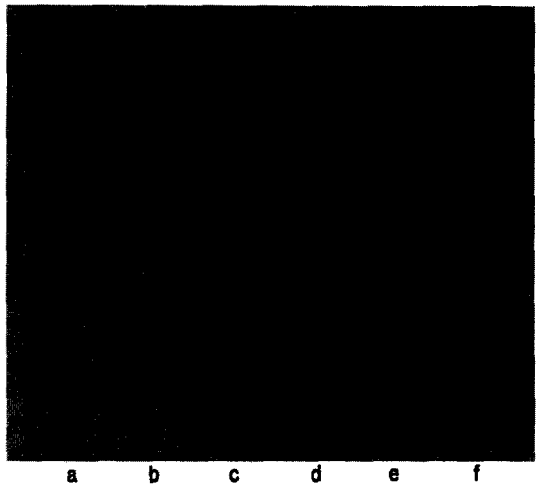

Fig. 5. Specificity of extracellular lipase of *R. oryzae* the lines are monoolein (a), 1,2-diolein (b), 1,3-diolein (c), oleic acid (d), triolein (e) and triolein hydrolysate after 48 h reaction (f).

Table 2
Substrate specificity of extracellular lipases from *R. oryzae* and *R. rhizopodiformis*

Substrates	Relative hydrolysis (%) [a]	
	R. rhizopodiformis	*R. oryzae*
Monoacid triglycerides		
triolein (16C)	100	100
triacetin (2C)	0	3.6
tributyrin (4C)	227	71.8
tricaproin (6C)	40	54.5
tricaprylin (8C)	129	160.0
Polyoxysorbitan		
Tween 20	0	8.2
Tween 40	0	27.3
Tween 60	0	10.0
Tween 80	43	7.3
Tween 85	74	1.8
Sorbitan		
Span 20	n.d.	36.4
Methyl ester		
methyl oleate	0	50.5
methyl palmitate	71	44.1
methyl myristate	n.d.	78.5
methyl stearate	n.d.	43.0
methyl caprylate	49	257.0
methyl laurate	46	103.0

[a] Reaction rate with triolein under condition of assay was taken as 100%.
n.d. – not determined.

results were obtained with lipases from *Rhizopus delemar* and *A. niger* [12] and *H. lanuginosa* [11]. The extracellular lipase of *R. rhizopodiformis* also showed a similar chromatogram pattern to that of *R. oryzae* indicating similar positional specificities

The reactivity of the lipases on different substrates is presented in Table 2. Using triolein as the standard substrate, the extracellular lipases from *R. oryzae* did not show chain length specificity. It showed very little reactivity for triacetin but the rate increased substantially for the other monoacid triglycerides with tricaprylin being the most preferred followed by triolein and tributyrin. Similarly, the extracellular lipase of *R. rhizopodiformis* showed no activity with triacetin but showed the most preference for tributyrin followed by tricaprylin and triolein. Both lipases showed moderate preference for tricaproin. Other lipases that did not show any chain length specificity were *P. cyclopium* [14], *R. japonicus NR 400* [7], *Aspergillus niger* and *R. delemar* [1]. Both lipases showed little reactivity for polyoxsorbitan (Tween) and sorbitan (Span) compared to triolein standard. This is in contrast to the lipases from *Humicola lanuginosa* [13,11] which were able to hydrolyze Tween, Span, monoester as well as triglycerides. Although both the extracellular lipases showed similar trends, the lipase from *R. oryzae* showed a broader substrate specificity than that of *R. rhizopodiformis*.

Acknowledgements

This project was financed by the Ministry of Science, Technology and Environment Malaysia (IRPA No: 1-07-05-086)

References

[1] M. Iwai and Y. Tsujisaka, in B. Borgstrom and H.L. Brockman (Eds.), Lipases, Elsevier, Amsterdam, 1984, p. 443.
[2] A. R. Macrae, in W.M. Forgarty (Ed.), Microbial Enzymes and Biotechnology, Applied Science Publishers, London, 1983, p. 225.
[3] A. B. Salleh, R., Musani, M. Basri, K. Ampon, W.M.Z. Yunus, and C.N.A. Razak. Can. J. Microbiol., 39 (1993) 978.
[4] M.Y.A. Samad, A. B. Salleh, C.N.A. Razak, W.M.Z. Yunus, K. Ampon and M. Basri, World J. Microbiol. Biotechnol., 6 (1990) 390.
[5] S. Okumura, M. Iwai and Y. Tsujisaka, J. Biochem., 87 (1980) 205.
[6] A. Sugihara,, Y. Shimada and Y. Tominaga, Agric. Biol. Chem., 52 (1988) 1591.
[7] M. Suzuki, H. Yamamoto and M. Mizugaki, J. Biochem., 100 (1986) 1207.
[8] Z. Mozaffar and J. D. Weete, Lipid, 28 (1993) 377
[9] D. Druet, N. El Abadi and L.-C. Comeau, Appl. Microbiol. Biotechnol., 37 (1992) 745.
[10] A. K. Chopra, H. Chander and J. Singh, J. Dairy Sci., 65 (1982) 1890.
[11] C.O. Ibrahim, N. Nishio and S. Nagai, Agric. Biol. Chem., 51 (1987) 2153.
[12] S. Okumura, M. Iwai and Y. Tsujisaka, Agric. Biol. Chem., 40 (1976) 655.
[13] W. H. Liu, T. Beppu and T. Arima, Agric. Biol. Chem., 37 (1973) 1349.
[14] K. Isobe, T. Akiba and S. Yamaguchi, Agric. Biol. Chem., 52 (1988) 41
[15] L. B. Loyreva and A.I. Marchenkova. Microbiologiya, 50 (1980) 90.