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Substrate specificity of lipases from four species of *Aspergillus* towards hydrolysis of homoacid triacylglycerols and vegetable oils in non-aqueous system

(Kespesifikan substrat lipase daripada empat spesies *Aspergillus* terhadap hidrolisis triasilgliserol homoasid dan minyak sayuran dalam sistem tanpa air)

L. Kamariah*, H. Nurul Huda*, M.D. Azlina*, O. Rosmawati*, M.G. Hasanah**, O.M. Lai** and H. Ainon***

Key words: *Aspergillus* species, discriminate, homoacid triacylglycerols, lipases, substrate specificity, saturated short, medium and long chain fatty acids

Abstract

Among the four species of *Aspergillus*, *Aspergillus oryzea* lipase demonstrates high preference towards medium chain triacylglycerols (C_{10}) and discriminates against triunsaturated triacylglycerol (TAG) e.g. triolein. The great discriminating power of its lipase against triolein was shown in comparison with its ability to catalyse the hydrolysis of medium chain (C_{10}) TAG e.g. tricaprin and less shown when hydrolysing saturated long chain TAG i.e. tripalmitin. The discriminating power of these lipases are in this order: triolein > tripalmitin > trilaurin > tricaprin.

Similar phenomenon was noted when mycelium-bound lipases of *Aspergillus* sp. were used to catalyse the hydrolysis of coconut oil, palm olein, olive oil and flaxseed oil. In most cases, relative percentage of monounsaturated fatty acid ($C_{18:1}$) in non-hydrolysed fractions of oils increased after 12 days of hydrolysis. Hydrolysis of flaxseed and olive oil showed that *Aspergillus murarum, Aspergillus oryzea* and *Aspergillus flavus* lipases have high preference towards polyunsaturated fatty acids i.e. linoleic acid ($C_{18:2}$) in olive oil and linolenic acid ($C_{18:3}$) in flaxseed oil and no preference for monounsaturated and saturated long chain fatty acids.

Introduction

Lipases are widely diversified in their enzymatic properties and substrate specificities. The need for novel lipases is obvious, and industry continues to look for lipases with high activity, from less expensive sources, high selectivity towards fatty acids and high regioselectivity towards *sn*-2 position of acylglycerol. The lipase selectivity (fatty acid selectivity, positional specificity, selectivity towards different classes of lipid, stereo-selectivity) is often crucial to their applications for analytical and industrial purposes.

Many papers regarding fatty acid selectivity of lipases from microbes had been published. For example, lipase generated from *Geotrichum candidum* has

*Biotechnology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
**Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
***Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
Authors' full names: Kamariah Long, Nurul Huda Hashim, Azlina Mohd. Danial, Rosmawati Osman, Hasanah Mohd. Ghazali, Lai Oi Ming and Ainon Hamzah
E-mail: amai@mardi.my

strong specificity for unsaturated fatty acids $C_{18:1}$, n-9 regardless of their positions in the triacylglycerol (Jensen 1974). Its lipases were used to obtain rapeseed oil enriched with polyunsaturated fatty acid especially docosahexaenoic acid (DHA) (Shimada et al. 1995), and *Candida parapsilosis* lipase is highly specific for long chain fatty acids and particularly for polyunsaturated fatty acids (Riaublanc et al. 1993). A few microbial lipases discriminate against n-3 polyunsaturated fatty acids (PUFA) e.g. *G. candidum*, *Candida rugusa* and *Rhizomucor miehei* (Shimada et al. 1995). The lipase of *Candida cylindracea* has less preference in hydrolysing the DHA of tuna oil and therefore, the amount of DHA in the glyceride mixture after 70% hydrolysis was twice that of the original oil (Tanaka et al. 1993). On the other hand, Sonnet et al. (1993) found that *Pseudomonas cepacia* lipase exhibit neither positional nor fatty acid selectivity towards rapeseed oil.

This paper reports on the fatty acid preference of mycelium-bound lipase of four types of *Aspergillus* species during hydrolysis of homoacid triacylglycerols and vegetable oils in water saturated n-hexane system. Such studies are important prerequisites to the design of a system employing lipase-catalyzed fat splitting.

Materials and methods

Unless otherwise stated, all chemicals were obtained from BDH, Chemical Ltd. Poole Dorset, England and were of analytical grade. Homoacid triacylglycerols were purchased from Sigma Chem. CO., USA and 99% pure. Coconut oil, palm olein, olive oil and flaxseed oil were purchased from local supermarket.

Microorganisms

Fungi were isolated from palm oil mills in Malaysia and were identified as *Aspergillus niger, Aspergillus flavus, Aspergillus oryzea* and *Aspergillus murarum.* Fungi were identified by lacto phenol cotton blue staining and observation of slide under a light microscope.

Lipase preparation

Mycelium-bound lipase was prepared according to the methods of Long et al. (1996). Mycelia defatted by n-hexane were freeze-dried for 3 h followed by homogenization in a blender for 90 s and stored over silica gel before use.

Hydrolysis of homoacid TAG

The reaction mixture comprised 10 ml n-hexane containing mixtures of 10 mM homoacylglycerol, 0.25 g mycelium dry weight and 0.25 ml water. The reaction was carried out in duplicate at 40 °C, shaken at 200 rev/min. At various times, 2.5 ml of mixture was withdrawn and filtered. The filtrates containing triacylglycerol were analysed by high performance liquid chromatography (HPLC). The percentage of homoacid TAG remained after reaction indicates the degree of discrimination. High amount of TAG remained reflect strong degree of discrimination by lipases.

Hydrolysis of vegetable oils

The reaction mixture contained 90 ml of 10% vegetable oil in water saturated n-hexane, 0.5 ml deionised water and 0.5 g mycelium dry weight. The mixture was incubated at 40 °C 200 rev/min, 2 ml of reaction mixture was withdrawn at intervals and 20 ml acetone:ethanol mixture added to stop the reaction. The amount of fatty acid released was determined by titration with 0.1 M NaOH to a phenolphthalein end-point.

The amount of FFA present was determined according to the method of Cocks and van Rede (1966). At the end of reaction period, 5 ml of acetone:ethanol mixture (1:1) was added to 2 ml of the sample and the mixture was titrated with 0.05 N NaOH to a phenolphthalein end-point. Duplicate runs were carried out for each sample.

Removal of free fatty acids from reaction mixtures

Free fatty acids were removed by the method described by Foglia et al. *(*1993). Reacted samples (15 ml) were placed in 250 ml conical flasks and 70 ml acetone:ethanol (1:1, v/v) was added. The mixture was neutralized with 0.1 M NaOH to a phenolphthalein end-point. The neutralized samples were transferred into a 100 ml separating funnel. After shaking and standing for several minutes, the bottom aqueous layer was discarded and the top layer, which was the organic phase, was collected. The organic phase which contained glycerides mixtures was transferred into a bottle and dried overnight in an oven at 60 °C prior to fatty acids analysis by gas liquid chromatography.

***Fatty acids analysis by gas liquid chromatography (GLC*)**

The fatty acid compositions of non-hydrolysed samples were analysed with GLC after removal of free fatty acids of the oil samples. Fatty acid methyl ester (FAME) was prepared by dissolving 0.05 g of the sample in 0.95 ml hexane to which 0.05 ml of 1 M sodium methoxide was added and the samples were analysed on a Hewlett Packard Gas Chromatography Series II. A polar capillary column BPX 70 (0.25 mm internal diameter, 30 m length and 0.25 μm film thickness, SGE Australia Pty. Ltd.) was used to separate the esters.

TAG profile by high performance liquid chromatography (HPLC)

TAG of non-hydrolysed fractions was determined by HPLC (Shimadzu Co. Kyoto, Japan) with a commercially packed RP-18 column (250 x 4 mm) with 5 μm particle size (E. Merck, Darmstadt, Germany). TAG was eluted with acetone/acetonitrile (60:40) at 1 ml/min flow rate. The identification of TAG of the oil was according to Long et al. (1998).

Results and discussion

Hydrolysis of homoacid triacylglycerols

The relative percentages of TAG after hydrolysis between two homoacylglycerols in different combination against times are shown in *Figure 1A–E*. Generally, as the faster reacting fatty acid residues were hydrolysed, the enzyme became exposed to an increasing amount of slower reacting fatty acid residues. Consequently, the content of slower reacting fatty acid residues increased with time *(Figure 1A–B)*. In some cases where the lipase does not have any preference towards both homoacid TAG, it will hydrolyse the substrates equally and at the end of reaction time the ratio between the homoacid TAG remained as it is *(Figure 1D)*.

Apparently, *A. oryzea* lipase shows strong discriminating power towards triolein followed by *A. murarum* and *A. niger (Figure 1A–B)*. At the end of reaction period the amount of triolein in the mixture hydrolysed by *A. oryzea* lipase increased from 57.2−91.9% *(Figure 1A)*. Unlike *Aspergillus* lipases, *G. candidum* lipase hydrolysed triolein more rapidly than tripalmitin and discriminates against tristearin at a greater degree (Plazza et al. 1992). On the other hand, it was noted that *A. flavus* lipase did not have any preference towards either the medium molecular chain homoacid TAG or the long monounsaturated molecular chain homoacid TAG (triolein) *(Figure 1A–B)*. This was indicated by the percentage of homoacid TAG that remained after 4 h of hydrolysis.

However, earlier work by Long et al. (1998) have shown that *A. flavus* Link, isolated from copra meal, had some degree of preference towards medium chain fatty acid especially in the presence of triunsaturated triolein and trisaturated long chain fatty acids. Most likely, the degree of preference towards short and medium chain fatty acids depends upon the strain of *A. flavus*. However, when medium chain homoacid TAG was replaced with saturated long chain homoacid TAG, i.e. tripalmitin,

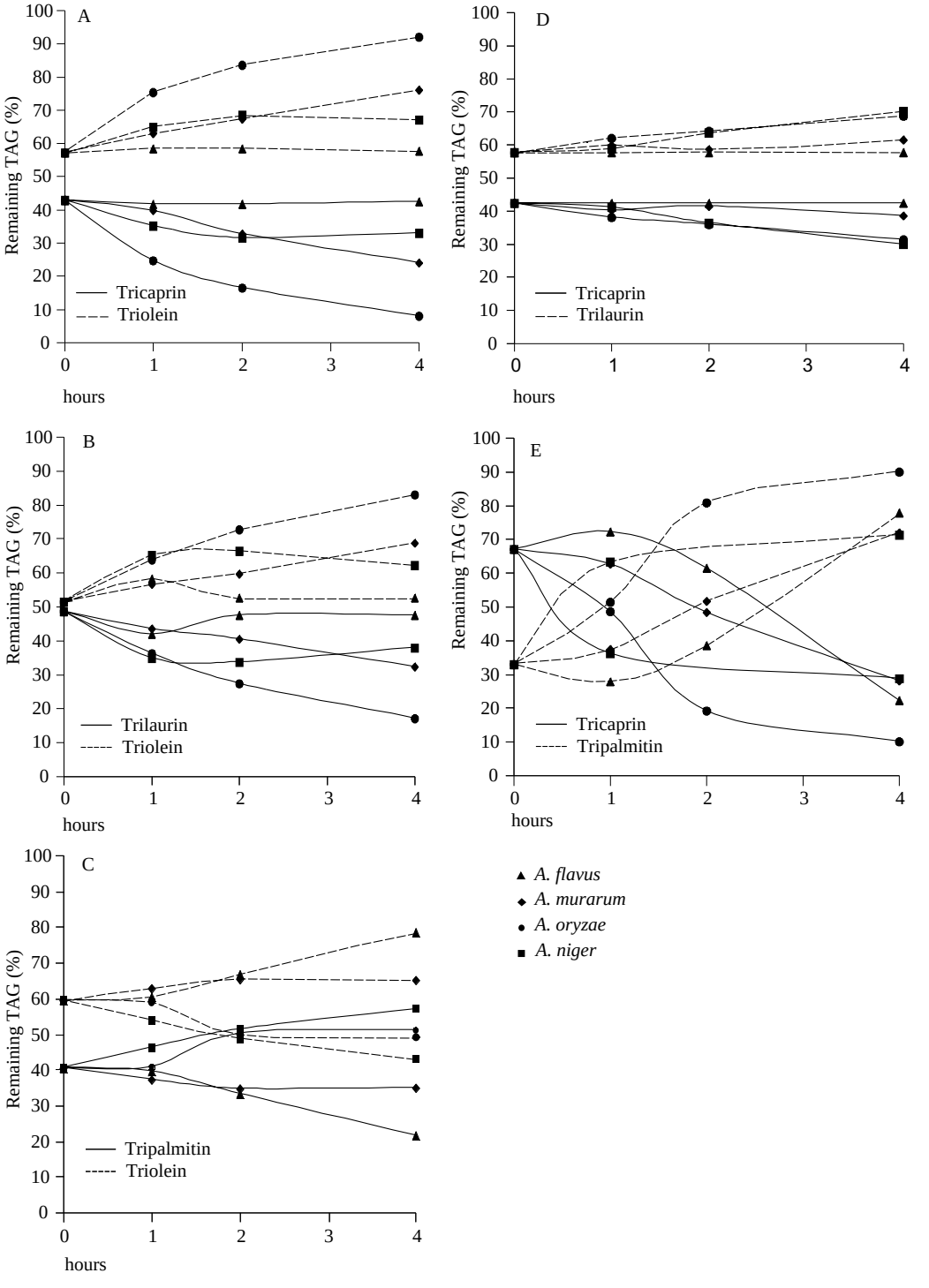

Figure 1. Percentages of homoacid triacylglycerol of (A) tricaprin and triolein, (B) trilaurin and triolein, (C) tripalmitin and triolein, (D) tricaprin and trilaurin, and (E) tricaprin and tripalmitin remained after hydrolysis against time

A. flavus lipase showed its preference towards hydrolyzing this saturated long chain TAG *(Figure 1C)* and this consequently left the non-reacted fraction enriched in triolein and depleted in tripalmitin. Conversely, *A. oryzea* and *A. niger* lipase showed low degree of preference for triolein. On the other hand, *A. murarum* lipase did not show preference for either tripalmitin or triolein *(Figure 1C)*. The preferences towards tricaprin by lipases from all four species of *Aspergillus* were less seen in the presence of trilaurin *(Figure 1D)*. However, its degree of preference towards tricaprin was increased when trilaurin (medium molecular chain) was replaced with tripalmitin (long saturated molecular chain). In each reaction, tripalmitin was less hydrolyzed than that of tricaprin *(Figure 1E)*. Again in this reaction, it was noted that *A. oryzea* lipase hydrolyzed tricaprin better than any of the other lipases *(Figure 1E)*.

Unlike lipase from *Aspergillus* species, oat seed lipase acted most rapidly upon those TAG containing oleate, long–chain Δ9 unsaturated fatty acyl groups (Plazza et al. 1992). Oleic acid was enriched in the non-hydrolyzed fraction and palmitic and stearic acids were depleted (Plazza et al. 1992). Preference for monounsaturated fatty acid, i.e. oleic acid and other polyunsaturated fatty acids were also shown with lipase from *Candida parapsilosis* (Riaublanc et al. 1993) and *G. candidum* (Sonnet et al. 1993).

Based on these findings, it may be possible to conclude that lipase from *Aspergillus* species displays different substrate specificity from that of *C. parapsilosis* (Riaublanc et al. 1993) and *G. candidum* (Sonnet et al. 1993).

Hydrolysis of vegetable oils

The above finding was further investigated with another experiment using vegetable oils as a substrate for hydrolysis. Different types of vegetable oil containing different types of fatty acid were subjected to hydrolysis. The substrate selectivity of all four *Aspergillus* on hydrolysis of coconut oil, palm olein, olive oil and flaxseed oil were studied in non-aqueous solution. The reaction was carried out for 12 days. The amounts of free fatty acids released were analysed against time. In addition, the amounts of fatty acids left in non-hydrolysed fractions were analysed at the time where lipase activity reached equilibrium. In general, microbial lipases showed little fatty acid specificity when incubated with most natural oils and fats. However, in non-aqueous system, some degrees of selectivity towards certain fatty acid were shown *(Table 1)*.

Preference towards short and medium chain fatty acids was shown when coconut oil was used as substrate. These phenomena were demonstrated with mycelium-bound lipase from *A. flavus* and *A. niger* and less shown with *A. murarum* and *A. oryzea*. On the other hand long saturated (C_{14}-C_{18}) and unsaturated chain fatty acids ($C_{18:1}$ and $C_{18:2}$) were not preferred. Consequently, the amount of these fatty acids in the non-hydrolysed fractions increased. Earlier work by Long et al. (1998) also indicated that *A. flavus* lipase has preference for oils containing saturated medium chain fatty acids rather than unsaturated fatty acids.

In most cases, strong degrees of preference towards polyunsaturated fatty acids were shown when flaxseed, olive oil and palm olein were used as substrate. The relative percentages of polyunsaturated fatty acids (PUFA) such as linoleic and linolenic acid in the non-hydrolysed fractions were depleted. The relative percentage of PUFA in palm olein and olive oil decreased from about 10% to 3.5% and 2.4%, respectively when lipase from *A. murarum* was used. As a result, the relative percentage of saturated long chain fatty acids and monounsaturated chain fatty acids in non-hydrolyzed fractions increased.

Other than that, lipase from *A. oryzea* also showed some degree of preference towards PUFA. At the end of 12 days of hydrolysis, the relative percentages of PUFA decreased from about 10% to 7% in palm

Table 1. Fatty acids composition of non-hydrolyzed coconut oil, palm olein, olive oil and flaxseed oil

Fatty acid (%)	Control	*A. murarum*	*A. flavus*	*A. oryzae*	*A. niger*
Coconut oil					
SCFA (C_6-C_8)	11.20 ± 0.49	10.20 ± 0.16	9.58 ± 1.18	10.27 ± 0.58	9.82 ± 0.46
MCFA (C_{10}-C_{12})	56.78 ± 1.21	55.58 ± 0.66	54.05 ± 1.79	55.91 ± 0.38	56.06 ± 1.40
LCFA (C_{14}-C_{18})	27.30 ± 1.04	28.86 ± 0.21	29.77 ± 2.34	28.23 ± 0.43	27.44 ± 0.85
MUFA ($C_{18:1}$)	4.14 ± 0.56	4.91 ± 0.37	5.81 ± 0.55	4.76 ± 0.27	4.96 ± 0.26
PUFA ($C_{18:2}$)	0.57 ± 0.08	0.46 ± 0.06	0.79 ± 0.16	0.83 ± 0.05	0.85 ± 0.04
Palm olein					
MCFA (C_{12})	0.31 ± 0.02	0.35 ± 0.05	0.30 ± 0.08	0.52 ± 0.12	0.45 ± 0.02
LCFA (C_{14}-C_{18})	45.34 ± 0.40	50.79 ± 0.18	45.73 ± 0.72	49.28 ± 3.47	45.96 ± 0.73
MUFA ($C_{16:1}$-$C_{18:1}$)	43.56 ± 0.36	45.40 ± 0.23	43.32 ± 0.57	43.05 ± 2.72	43.10 ± 0.61
PUFA ($C_{18:2}$)	10.80 ± 0.06	3.47 ± 0.08	10.66 ± 0.31	7.14 ± 1.08	10.50 ± 0.15
Olive oil					
LCFA (C_{16}-C_{18})	16.70 ± 0.98	17.12 ± 0.52	18.05 ± 0.96	19.96 ± 2.26	16.90 ± 0.86
MUFA ($C_{16:1}$-$C_{18:1}$)	73.23 ± 0.87	80.49 ± 0.74	72.42 ± 0.91	75.33 ± 1.73	72.81 ± 1.46
PUFA ($C_{18:2}$-$C_{18:3}$)	10.07 ± 0.12	2.39 ± 1.27	9.53 ± 0.12	4.73 ± 0.52	10.30 ± 0.61
Flaxseed oil					
LCFA (C_{16}-C_{18})	10.44 ± 0.31	11.26 ± 0.42	13.17 ± 0.88	14.93 ± 0.04	11.97 ± 0.16
MUFA ($C_{16:1}$-$C_{18:1}$)	18.18 ± 0.03	19.96 ± 0.41	21.49 ± 1.17	23.18 ± 2.56	19.72 ± 0.13
PUFA ($C_{18:2}$)	16.06 ± 0.35	16.66 ± 0.08	16.42 ± 0.13	16.78 ± 0.25	16.58 ± 0.09
($C_{18:3}$)	55.33 ± 0.08	52.12 ± 0.08	48.92 ± 1.75	45.11 ± 2.86	51.74 ± 0.37

SCFA = Short chain fatty acids MCFA = Medium chain fatty acids
LCFA = Long chain fatty acids MUFA = Monounsaturated fatty acids
PUFA = Polyunsaturated fatty acids

olein and 4.7% in olive oil. All lipases from *Aspergillus* sp. showed some degree of preference towards PUFA especially the linolenic acid ($C_{18:3}$) in flaxseed oil. The relative percentages of linolenic acid ($C_{18:3}$) decreased from 55.3% to 45.1%, 48.9%, 51.7% and 52.1% with lipase from *A. oryzea*, *A. flavus*, *A. niger* and *A. murarum*, respectively.

Conclusion

Lipase obtained from all four species of *Aspergillus* demonstrated less preference towards hydrolysing cis-9 monounsaturated fatty acid. Hydrolysis of homoacid triacylglycerol and vegetable oils in non-aqueous medium showed that lipase from all four species of *Aspergillus* have no preference towards oleic acid and triolein. Some degrees of discrimination towards oleic acid were noted in the presence of saturated short chain fatty acids, medium chain fatty acids and polyunsaturated fatty acids. These results may indicate a competitive inhibitory action of short chain fatty acids and monounsaturated fatty acids (i.e. oleic acid). This property has determined their biotechnological importance in industry as they can be used for the production of oil containing high concentrations of monounsaturated fatty acids especially the oleic acids. It is possible to utilize such fatty acid selectivities for enrichment of certain fatty acid from naturally occurring oils by selective hydrolysis.

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Abstrak

Antara empat spesies *Aspergillus* yang dikaji, *Aspergillus oryzea* lipase mempunyai kecenderungan yang tinggi terhadap triasilgliserol tepu berantai sederhana (C_{10}) dan didapati mempunyai tahap keupayaan hidrolisis yang rendah terhadap triasilgliserol tri-tak-tepu sebagai contoh triolein ($C_{18:1}$). Tahap ketidakupayaan untuk menghidrolisis triasilgliserol tri-tak-tepu dapat dilihat dengan lebih jelas dengan kehadiran TAG tepu berantai sederhana dan akan kurang ditunjukkan sekiranya TAG tepu berantai panjang, contoh tripalmitin, hadir semasa tindak balas. Tahap ketidakupayaan lipase melakukan hidrolisis berkurangan mengikut susunan berikut: triolein > tripalmitin > trilaurin > tricaprin.

Fenomena yang sama dapat dilihat apabila lipase terikat miselium daripada spesies *Aspergillus* digunakan untuk memangkin tindak balas hidrolisis minyak kelapa, minyak olein, minyak zaitun dan minyak biji flax. Peratusan bandingan asid lemak oleik dalam fraksi yang tidak dihidrolisis bertambah selepas 12 hari. Hidrolisis minyak biji flax dan minyak zaitun menunjukkan lipase daripada *Aspergillus murarum, Aspergillus oryzea* dan *Aspergillus flavus* mempunyai kecenderungan yang lebih tinggi terhadap asid lemak poli-tak-tepu seperti asid linoleik ($C_{18:2}$) daripada minyak zaitun dan asid linolenik ($C_{18:3}$) daripada minyak biji flax dan tidak mempunyai keupayaan menghidrolisis asid mono-tak-tepu dan asid tepu berantai panjang.

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