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Screening, isolation and identification of microbial lipase producers from palm oil mills

(Penyaringan, pemencilan dan pengenalpastian mikrob penghasil lipase dari kilang sawit)

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Key words: screening, isolation, identification, microbial lipase producer, palm oil mill

Abstract

Screening, isolation and identification of microbial lipase producers from 16 locations of palm oil mills in Malaysia were successfully conducted. A total of 52 different strains were found to be lipase producers. These isolates were identified and divided into three different groups i.e. bacteria, yeast and mould. Twenty-five isolates were bacteria, 10 were yeast and the remainder were mould. Out of the 52 isolates, four were found to be new lipase producers and identified as *Klebsiella pneumoniae rhinoschlero, CDC gr. IV C-2, Geotrichum penicillatum* and *Rahnella aquaticus*. Lipases from *Bacillus lentus, Bacillus sphaericus, Tatumella ptyseos, Geotrichum capitatum* and *Thielaviopsis* sp. have been less reported and studied as lipase producers. Both *Bacillus lentus* and *Bacillus sphaericus* were found to be thermophilic bacteria and produce alkaline tolerant lipase.

Introduction

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are enzymes that catalyse the hydrolysis of triacylglycerol at the oil-water interface. They are produced by many organisms and higher eukaryotes. Most commercially useful lipases are of microbial origin (Sztajer and Maliszewska 1988; Sharma et al. 2001). Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oil seeds, and decaying food (Sztajer and Maliszewska 1988).

Interest on lipase from different sources (microorganisms, plants) has markedly increased in the last decade due to its potential application in industries such as pharmaceutical, cosmetic and food. Plant and microbial enzymes have the added advantage over animal enzymes owing to their availability, their apparent ease of purification and their particular selectivity. The lipase selectivity is often crucial to its application for analytical and industrial purposes, e.g. lipase generated from *Geotrichum candidum* was used to obtain rapeseed oil enriched with polyunsaturated fatty acids, *Rhizomucor miehei* lipase was selected by Novo industry for interesterification based on its high regioselectivity and good thermostability. When immobilized on an anion-exchange

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resin, the enzyme was able to meet the extreme requirement without loss of productivity or selectivity.

Lipases are widely diversified in their enzymatic properties and substrate specificities. The need for novel lipases is obvious, and industry continues to look for new lipases with high activity, from less expensive sources, high selectivity towards fatty acids and high regioselectivity towards the sn-2 position of the acylglycerol. To date there are no authenticated reports of lipases which catalyse the release of fatty acids selectively from the central sn-2 positions of the acylglycerol.

Screening and selection of microorganisms based on their ability to hydrolyse different types of vegetable oil and fat has never been reported before. In this work, effort has been made to screen and isolate microbial lipase producers from various locations of palm oil mills in Malaysia. Six different agars containing various types of vegetable fat and oil were used to isolate positive strains. Positive strains were identified through the formation of insoluble blue droplets entrapped within the agar underneath a positive colony.

Materials and methods

Source of microorganism

Palm oil wastes were taken from 16 different locations of palm oil mills in Malaysia, namely, Felcra Bukit Kepong Palm Oil Mill, Labis; Felda Kahang Palm Oil Mill, Kluang; Felda Lok Heng Palm Oil Mill, Kota Tinggi, Johor; Tanah Merah Palm Oil Mill, Port Dickson, Negeri Sembilan; Malpom Industries Berhad Palm Oil Mill, Nibong Tebal, Penang; Talico Company Sdn. Bhd. Palm Oil Mill, Padang Serai, Kedah; Malmaju Bina Sdn. Bhd. Palm oil Mill, Selama, Perak; Felda Selendang Palm Oil Mill, Kuala Rompin, and FPISB Neram Palm Oil Mill, Kuantan, Pahang; Felda Kertih Palm Oil Mill, Ketengah Jaya, Terengganu; Lubuk Antu Palm Oil Mill, Sri Aman; Mukah Palm Oil Mill, Sibu; and Saratok Palm Oil Mill, Sarikei, Sarawak; and Beaufort Palm Oil Mill, Beaufort; Felda Kalabakan Palm Oil Mill, Tawau; and Apas Balung Palm Oil Mill, Tawau, Sabah.

Isolation of lipase producing microorganisms

One gramme of homogenized sample was added into 100 mL yeast extract-malt extract-nutrient broth (YME-NB) medium (contains 0.3 g yeast extract, 0.3 g malt extract, 0.5 g nutrient broth, 1 g glucose). The mixture was incubated for 48 h at 30 °C and 200 rpm. Serial dilution was performed before plating 0.1 mL of an appropriately diluted culture broth (10^{-5} to 10^{-6}) on Nile blue sulphate (NBS) agar containing fat and oil. The plates were incubated at 30 °C for 5–7 days and positive colonies were identified through the formation of insoluble blue droplets underneath the colonies. Well-isolated producers were selected and purified using Potato Dextrose Agar (PDA) and Nutrient Agar (NA) medium. Selected pure colonies were tested again on NBS agar medium containing fat and oil for confirmation. Pure colonies were inoculated on PDA or NA slants as stock cultures and kept at 4 °C.

Preparation of agar medium containing vegetable oils and fats

A medium similar to that described by El-Azzabi et al. (1981) modified by Long et al. (1996) was used for the detection of lipolytic activity except that rapeseed oil was replaced by corn oil, palm olein, palm stearin, soybean oil, coconut oil and ghee respectively. The composition of the medium was oil (8 mL), 0.1% aqueous NBS (4 mL), Difco 'Bacto' tryptone (0.5 g), Difto 'Bacto' agar (2 g), Tween 80 (0.08 g) and deionised water to make the volume up to 100 mL and adjusted to pH 6. The medium was autoclaved at 121 °C for 15 min.

Identification of isolates

Identification of bacteria and yeast was done using the Gram staining and API kit strips (API 50CHB, API 20 NE, API 20 E, API 20

C AUX) (bio Mérieux, France). Fungi were identified by lacto phenol cotton blue staining and observation of slides under a light microscope.

Lipolytic activity at different pHs and temperatures

Selected isolates were inoculated onto oil medium and incubated at five different temperatures (30, 35, 40, 45 and 50 °C) with pH adjusted to 6. The effect of pH on lipolytic activity of selected microbes were tested at pH 6, 7, 8, 9 and 10 by adjusting the pH of the medium with 1.0 M HCl or 1.0 M NaOH.

Results and discussion

A total of 250 cultures were found to be positive isolates. The isolates were put into groups and categorized as bacteria, yeast and mould. Further identification tests showed that there were 52 different strains of which 25 isolates were found to be bacteria, 10 isolates were yeast and the remainder were mould. The lipolytic activity could easily be detected through the formation of insoluble blue droplets entrapped within the agar underneath a positive colony (*Plates 1* and *2*). Six types of vegetable oil and fat were used as a medium for screening and isolation of lipolytic producers. It was noted that the insoluble blue droplets formed on each medium tested were different in size and form *(Plate 1)*.

Some isolates could grow and show lipolytic activity on all media tested, whereas other isolates could grow on all media but only showed lipolytic activity on specific medium. Nearly half of the isolated strains were able to grow on all media tested *(Table 1)*. *Bacillus lentus* is an example of bacteria that could grow and show lipolytic activity on all media *(Plate 1)*. On the other hand, about seven isolated strains showed lipolytic activity on at least one medium. *Rahnella aquaticus* is an example of bacteria that could show lipolytic activity only on coconut oil whereas *Enterococcus faeciem* showed lipolytic activity only on media containing ghee.

Several types of substrate have been used for screening lipase producers such as tributryin (Fryer et al. 1966; Arima et al. 1972; Lawrence et al. 1972), triolein (Kouker and Jaeger 1981), rapeseed oil (El-Azzabi et al. 1981), palm olein (Ibrahim et al. 1991), chromogenic lipid substrate (Yeoh et al. 1986) and surfactants like Tween 80 (Karnetova et al. 1984; Mohd. Yusof et al. 1989). Substrates other than triolein and fats and oils are sometimes not suitable because they are also hydrolysed by esterases. There have been many cases where organisms that were reported to produce clear zones on tributryin agar but apparently failed to hydrolyse milk fat (El Sadek and Richards 1957; Franklin and Sharpe 1963; Alford and Steinle 1967; Brockerholf and Jensen 1974).

It was interesting to observe that lipolytic activity of specific isolates i.e. *Geotrichum penicillatum* known previously as *Trichosporon penicillatum*, was clearly shown on medium containing soybean oil but not on coconut oil and ghee, although growth was noted on both media *(Plate 2)*. These results were very useful findings as these reactions could be related to the selectivity of the lipase of each isolate. For example *G. penicillatum* showed its lipolytic activity only on medium rich in unsaturated fatty acid like corn oil, soybean oil and RBD olein but not on medium containing palm stearin, coconut oil and ghee *(Table 1)*. The latter media are found to be rich in saturated fatty acids.

Most of the bacteria were identified as Enterobacteriaceae. There were nine bacteria which were isolated and found only from single locations such as *Serratia marcescens, Aeromonas salmonicida, Rahnella aquaticus, Tatumella ptyseos, Serratia odorifera, Acinetobacter junii/ johnsonii, Brevundimonas vesicularis, Bacillus coagulans* and *Flavobacterium breve*. Most yeast were identified as *Candida* and were successfully isolated from

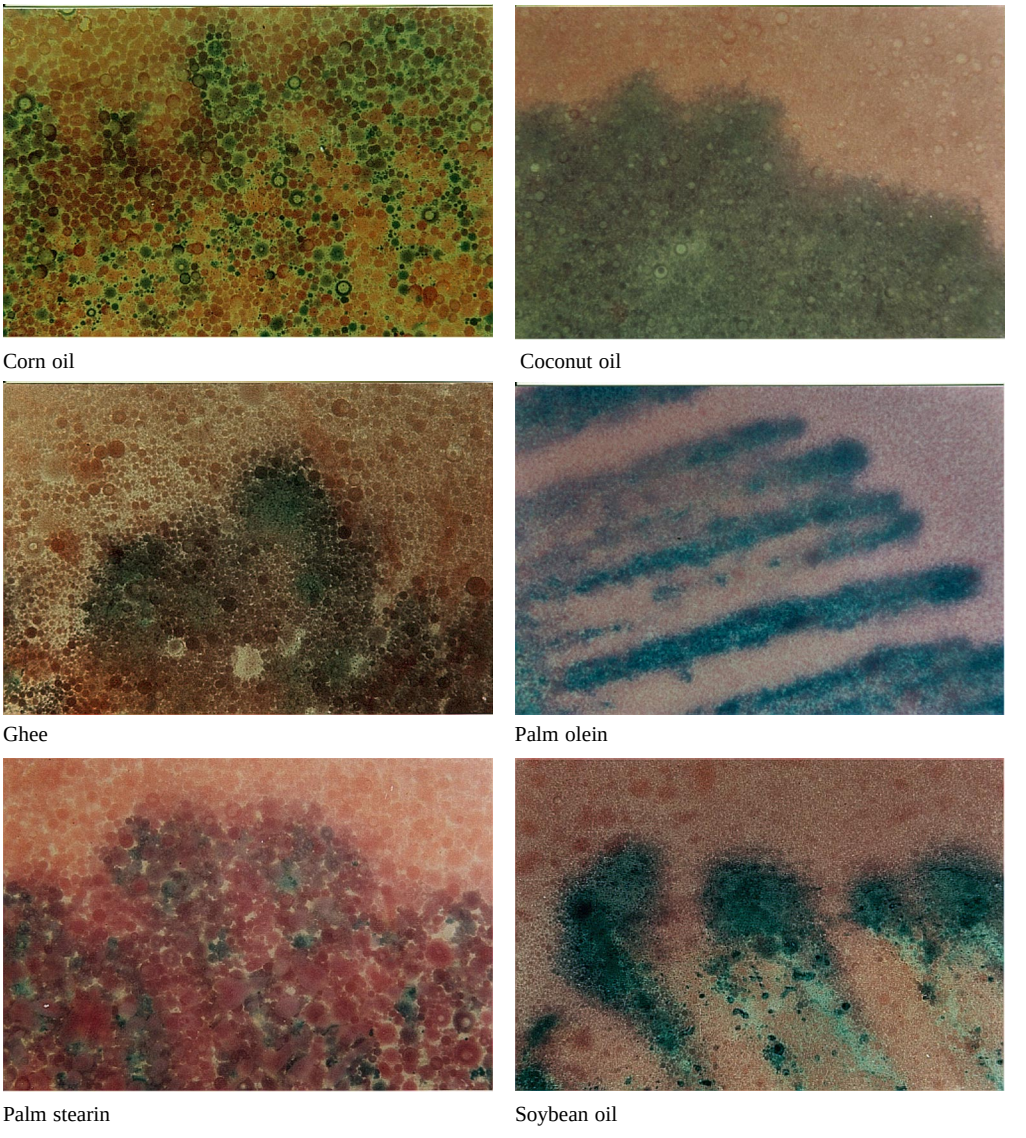
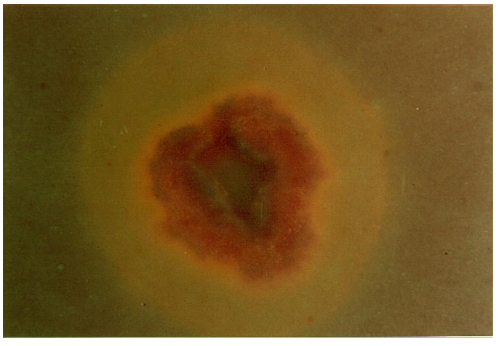


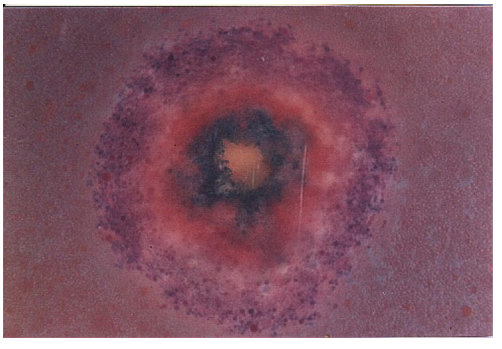
Plate 1. Lipolytic activity of ***Bacillus lentus*** *on different types of vegetable fat and oil*

each identified location. Out of 17 types of mould, 7 could not be identified up to species level and isolates will be sent to International Mycological Institute (IMI), United Kingdom for further identification. The most common mould found in almost all palm oil mill locations was *Neurospora crassa*. Studies on this mould lipase were done in the eighties (Chakravarti et al. 1980; Chakravarti et al. 1981; Kundu et al. 1987) but no recent research related to it was available.

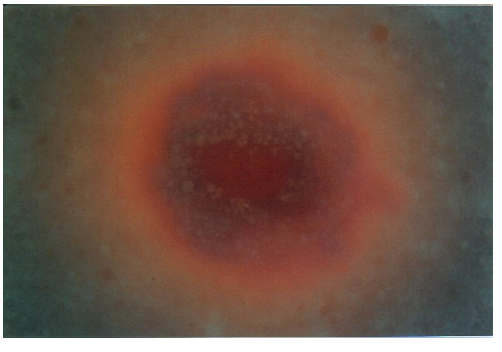
There were four new lipase producers that were isolated and identified, namely, *Klebsiella pneum. rhinoschlero, CDC gr. IV C-2, Geotrichum penicillatum* and *Rahnella aquaticus*. Two bacillus species i.e. *B. lentus* and *B. sphaericus* have been less reported and studied. *Bacillus sphaericus*, which was isolated from soil samples, has recently been identified as a lipase producer by Hun et al. (2003). Literature survey also showed that *Tatumella ptyseos, Geotrichum capitatum* and *Thielaviopsis* sp. lipases have been less



Coconut oil



Soybean



Ghee

*Plate 2. Lipolytic activity of **Geotrichum penicillatum** on soybean medium as compared with coconut oil and ghee which showed growth but no lipolytic activity*

studied and reported. The rest of the isolates were commonly known as lipase producers and work on their lipases had been frequently published.

The effects of pH and incubation temperature on the lipolytic activity of seven isolates are presented in *Table 2*. *Geotrichum penicillatum* was able to grow on palm stearin at 30 °C and 35 °C, and pH 6 to 7; however, no lipolytic activity was detected on this medium. *Bacillus lentus, B. sphaericus, N. crassa* and *CDC gr. IV C-2* were able to grow and showed lipolytic activity at extreme alkaline conditions up to pH 10. On the other hand, *Klebsiella pneum. rhinoschlero* and *R. aquaticus* were found to show lipolytic activity only up to pH 7 although growth was detected up to pH 10. *CDC gr. IV C-2, B. lentus* and *B. sphaericus* were found to be thermophilic bacteria and able to grow up to 50 °C, however only *B. lentus* was able to show lipolytic activity at 45 °C.

Conclusion

A total of 52 different strains of lipolytic microorganism were isolated and identified. Out of that, 4 strains have never been reported as lipase producers. They were identified as *Klebsiella pneum. rhinoschlero, CDC gr. IV C-2, Geotrichum penicillatum* and *Rahnella aquaticus*. Five strains i.e. *Bacillus lentus, Bacillus sphaericus, Tatumella ptyseos, Geotrichum capitatum* and *Thielaviopsis* sp. were less reported and studied as lipase producers. The results indicate that screening of lipase producers, using different types of vegetable fat and oil, is useful as an indicator for lipase selectivity towards different types of vegetable fat and oil.

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Table 1. Lipolytic activity of identified bacteria, yeast and mould on different types of vegetable oil and fat at pH 6, 30 °C

Microorganism	Vegetable fat and oil					
	PO	PS	CN	CO	SB	GE
Bacteria						
Acinetobacter baumannii	+	+	+	+	+	+
Acinetobacter baumannii/calco	+	+	+	+	+	+
Acinetobacter junii/johnsonii	+	+	+	+	+	+
Aeromonas hydra/caviae	+	–	+	–	+	+
Aeromonas salm. salmonicida	+	+	+	+	+	+
Bacillus coagulans	+	–	+	–	+	–
Bacillus lentus	+	+	+	+	+	+
Bacillus sphaericus	+	+	–	–	–	–
Brevibacillus brevis	+	–	–	–	–	–
Brevundimonas vesicularis	+	+	+	+	+	+
Burkholderia cepacia	+	+	+	+	+	+
CDC gr. IV C-2	+	+	+	+	+	+
Enterobacter cloacae	+	–	+	–	–	+
Enterobacter sakazakii	+	+	+	+	+	+
Enterococcus faeciem	–	–	–	–	–	+
Flavobacterium breve	+	+	+	+	+	+
Klebsiella pneum. rhinoschlero	–	+	–	+	+	–
Klebsiella pneum. pneumoniae	+	+	+	+	–	–
Providencia alcalifaciens/rustig	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+
Rahnella aquaticus	–	–	–	+	–	–
Serratia marcescens	+	+	+	+	+	+
Serratia odorifera	+	–	+	–	+	+
Stenotrophomonas maltophilia	+	+	+	+	+	+
Tatumella ptyseos	+	+	+	–	+	+
Yeast						
Candida ciferrii	+	+	+	+	+	+
Candida famata	–	–	–	+	–	–
Candida guilliermondii	+	+	+	+	–	+
Candida utilis	–	+	+	–	+	–
Candida zeylanoides	+	+	+	–	+	+
Cryptococcus humicolus	+	+	+	+	+	+
Cryptococcus terreus	–	+	+	–	+	–
Geotrichum capitatum	+	+	+	+	+	+
Geotrichum penicillatum	+	–	+	–	+	–
Trichosporon asahii	–	–	–	+	+	+
Mould						
Aspergillus flavus	+	+	+	–	+	+
Aspergillus murarum	+	+	+	–	+	+
Aspergillus niger	+	+	+	+	+	+
Aspergillus oryzae	+	+	+	+	+	+
Aspergillus sp.	+	+	+	+	+	+
Fusarium longipus	+	+	+	+	+	+
Fusarium oxysporum	+	+	+	–	+	+
Fusarium solani	–	+	–	–	–	–
Fusarium sp.1	+	+	+	+	+	+

(cont.)

Table 1. *(cont.)*

Microorganism	Vegetable fat and oil					
	PO	PS	CN	CO	SB	GE
Fusarium sp. 2	+	+	+	–	+	–
Fusarium sp. 3	+	+	+	+	+	+
Mucor sp.	+	+	+	+	+	+
Neurospora crassa	+	+	–	+	+	+
Paecilomyces sp.	–	+	–	–	–	–
Rhizopus oligosporus	+	+	+	–	+	+
Thielaviopsis sp.	–	+	–	–	–	–
Trichoderma viride	+	+	+	+	+	+

PO = Palm olein, PS = Palm stearin, CN = Corn oil, CO = Coconut oil, SB = Soybean oil, GE = Ghee
+ = Positive reaction, formation of blue droplets
– = Negative reaction, no formation of blue droplets

Table 2. Lipolytic activity of selected microbes on palm stearin at different temperatures and pH

Microorganism	Incubation temperature (°C) at pH 6					Incubation using different pH at 30 °C				
	30	35	40	45	50	6	7	8	9	10
Bacillus lentus	+	+	+	+	–	+	+	+	+	+
Bacillus sphaericus	+	+	–	–	–	+	+	+	+	+
CDC gr. IV C-2	+	+	+	–	–	+	+	+	+	+
Geotrichum penicillatum	–	–	NG	NG	NG	–	–	–	–	–
Klebsiella pneum. rhinoschlero	+	+	NG	NG	NG	+	+	–	–	–
Neurospora crassa	+	+	NG	NG	NG	+	+	+	+	+
Rahnella aquaticus	+	+	NG	NG	NG	+	+	–	–	–

NG = No growth
+ = Positive reaction, formation of blue droplets
– = Negative reaction, no formation of blue droplets

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Abstrak

Penyaringan, pemencilan dan pengenalpastian mikrob penghasil lipase daripada 16 lokasi kilang kelapa sawit di Malaysia telah dilakukan. Sejumlah 52 strain berbeza didapati menunjukkan aktiviti lipase. Pencilan-pencilan tersebut telah dikenal pasti dan dibahagikan kepada tiga kumpulan berbeza, iaitu bakteria, yis dan kulat. Dua puluh lima pencilan ialah bacteria, 10 yis dan selebihnya kulat. Empat daripada 52 organisma yang dipencilkan, merupakan penghasil lipase baru dan telah dikenal pasti sebagai *Klebsiella pneumoniae rhinoschlero*, *CDC gr. IV C-2, Geotrichum penicillatum* dan *Rahnella aquaticus.* Survei terdahulu menunjukkan lipase yang dihasilkan daripada *Bacillus lentus, Bacillus sphaericus, Tatumella ptyseos, Geotrichum capitatum* dan *Thielaviopsis* sp. kurang dilaporkan. Kedua-dua *Bacillus lentus* dan *Bacillus sphaerius* ialah bakteria termofilik yang berupaya menghasilkan lipase yang toleran dalam keadaan alkali.

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