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<https://doi.org/10.5109/9315>

出版情報：九州大学大学院農学研究院紀要. 52 (2), pp.281-286, 2007-10-29. Faculty of
Agriculture, Kyushu University

バージョン：

権利関係：

Culture Conditions of Psychrotrophic Fungus, *Penicillium chrysogenum* and Its Lipase Characteristics

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(Received May 15, 2007 and accepted July 17, 2007)

Among 97 fungal strains from the soil collected from the high mountain areas in the Jeju Island, Korea, *Penicillium chrysogenum* 9 was found to be the best lipase producer. Its lipase productivity reached 42 U/ml in the culture medium. Factors affecting lipase production by *Penicillium chrysogenum* 9 were studied using fermentation media of different chemical compositions. Under optimal conditions we noted a 1.6-fold increase of lipase activity. The maximum lipase activity was 68 U/ml of culture medium on the 5th day of incubation at pH 6.0 and 20 °C; therefore, *Penicillium chrysogenum* 9 was classified as a psychrotrophic microorganism. The enzyme from *Penicillium chrysogenum* 9 showed a maximum activity at 30 °C and pH 5.0 for synthetic substrates (triacylglycerols) or at pH 7.0 for natural substrates (oils).

INTRODUCTION

Lipases (triacylglycerol acylhydrolase EC 3.1.1.3.) are enzymes that catalyze the hydrolysis of fats and mono- and di-glycerides to free fatty acids and glycerol but are also unique serine esterases which catalytic activity is significantly enhanced at the liquid water interface. Although lipases have been found in many species of animals and plants, the enzymes from microbial sources (such as bacteria, yeast and fungi) are currently receiving particular attention because of their actual and potential applications in industry mainly in the detergents, oils and fats, dairy and pharmaceutical industries (Sarkar *et al.*, 1998; Costa and Peralta, 1999; Cardenas *et al.*, 2001).

Enzymes from extremophiles are valuable objects of research of biotechnologists from the point of view of their widest capabilities of technological utilization. Among enzymes from cold-adapted microorganisms, lipases and proteases have a considerable potential, mainly in the food industry and as additives to detergents. Specificity of lipases provides numerous applications such as flavour-modifying enzymes, detergent additives or stereospecific catalysts (Feller *et al.*, 1991). Microorganisms living in extreme environments have adapted to their habitats in such a way that metabolic process permits them to survive and function (Prins *et al.*, 1990).

All microorganisms have a characteristic optimal growth temperature at which they exhibit their highest growth and a range of temperatures they can tolerate.

Psychrophilic microorganisms have been defined as organisms, which have optimal growth temperature below 16 °C and an upper temperature limit of 20 °C, and psychrotrophic microorganisms, which are able to divide at 0 °C and grow optimally at temperatures around 20–25 °C. High microbial growth rates as well as high enzymatic activities and catalytic efficiencies in the temperature range of 0–20 °C of cold-adapted microorganisms (at which homologous mesophilic enzymes are not active) prevent the risk of microbial contamination (Margesin and Schinner, 1994). Enzymes from psychrophils and psychrotrophs may require even shorter reaction times as compared to mesophilic enzymes used near their low temperature limit (Ludlow and Clark, 1991).

The present paper deals with the screening of lipase producing psychrotrophic strains isolated from the cold high mountain area, culture conditions for the enzyme production by the selected strain and some properties of the enzyme that are important for its industrial application.

MATERIALS AND METHODS

Microorganisms

Fungi were isolated from soils of the high Mt. Halla areas in the Jeju Island situated in southern part of Korea. Compositions of Martin medium used for screening of microorganisms were as follows: glucose, 1%; peptone, 0.5%; K₂HPO₄, 0.1%; MgSO₄·7H₂O, 0.05%; rose bengal (sterilized separately) 0.003%. After sterilization 0.003% of streptomycin was added.

Isolation of microorganisms from soil

Isolation of microorganisms was completed by means of a dilution plate method. Portions of 1.0 g of representative soil were placed in a dilution tube containing 9 ml of sterile distilled water, and shaken for 60 min. Serial 10-fold dilutions were made and surface

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inoculated onto agar plates of Martin medium plates were incubated for 8–10 days at 15 and 30 °C. Fungi were isolated from plates that contained no more than 50 well-separated colonies.

Taxonomic characterization of fungi

Identification of isolated moulds strains was achieved on the basis of their micro- and macro-morphological features, expressed in microcultures, and on agar plates containing known diagnostic media for moulds, by the method of Domsch *et al.* (1980) and Onions and Brady (1987).

Preliminary selection of strains for lipase activity

The first selection of lipase active strains was done on the basis of the magnitude diffusion zone of the enzyme (mm, in diameter) secreted into agar medium by growing colonies. As similar diffusion technique alternately used for lipase formed by culture, supernatants were added to agar plates (2%, 20 ml) containing in 0.1 M phosphate buffer (pH 7.0) and 1% tributyrin. In this approach, the liquids (0.3 ml) were dropped into a well (10 mm diameter) previously cut out in agar medium. During the enzymatic reaction (96 h at 28 °C) the clear zones were formed.

Growth media and culture conditions

Seven different media were used for optimization:

- GPN – glucose 1%; peptone 5%; NaNO₃ 0.1%; KH₂PO₄ 0.1%; MgSO₄·7H₂O 0.05%; olive oil 1%.
- PEK – peptone 5%; yeast extract 1%; KH₂PO₄ 1.4%; Na₂PO₄ 0.24%; MgSO₄·7 H₂O 0.04%; olive oil 1%.
- GKM – glucose 1%; KH₂PO₄ 0.6%; MgSO₄·7H₂O 0.1%; urea 0.4%. Micronutrients: FeCl₃·6H₂O 0.001%; biotin; thiamin HCl; inositol; olive oil 1%.
- EKM – yeast extract 0.5%; KH₂PO₄ 1%; MgSO₄·7H₂O 0.1%; olive oil 1%.
- NKM – NaH₂PO₄ 1.2%; KH₂PO₄ 0.2%; MgSO₄·7H₂O 0.03%; CaCl₂ 0.03%; olive oil 1%.
- PKK – peptone 1%; KH₂PO₄ 1.4%; K₂HPO₄ 0.24%; MgSO₄·7H₂O 0.04%; olive oil 1%.
- SKN – starch 0.1%; KH₂PO₄ 0.2%; NH₄NO₃ 0.1%; MgSO₄·7H₂O 0.1%; olive oil 1%.

Initial pH was adjusted to 6.0 or 7.0. The media were autoclaved at 121 °C (15 psi) for 25 min. The flasks containing 25 ml of medium were inoculated with 2 ml spore suspensions (about 2 × 10⁶ spores) and cultured for 5 days at 20 °C on a rotary shaker operating at 150 rpm.

Analytical procedure

The mycelium was separated by filtration through Miracloth quick filtration material (Chicopee Mills, Inc., New York, USA) and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was used as the crude enzyme for the estimation of lipase activity. Lipase activity was determined by the method of Sokolovska *et al.* (1998). One unit (U) of lipase activity is defined as the amount of enzyme releasing 1 μmol of free fatty acids per minute under assay conditions. Protein con-

centration was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Biomass was determined by estimation of dry weight of the mycelium by washing and drying the sample at 105 °C for 24 h. Cultivations were performed in 3 replicate culture flasks, and analysis carried out in duplicate. The data given here are the means of the measurements. The mean standard error of the enzyme estimate was ± 0.23 and ranged from ± 0.003 to ± 0.32 U/ml.

Effect of different parameters on lipase production

Various parameters were studied in order to achieve maximum lipase production from *Penicillium chrysogenum* 9.

- a. Effect of inducers at a concentration of 1% – fatty acids (oleic and caprylic acid), triglycerides (tributyrin, tricaprillin, triolein) and different oils, such as olive, corn, soybean and rape.
- b. Effect of carbon sources (sugar) at a concentration of 1%: monosaccharides (fructose, galactose, xylose and glucose), disaccharides (sucrose, maltose and lactose) and polysaccharide (starch).
- c. Effect of various nitrogen sources: organic nitrogen sources (yeast extract, peptone, casein, asparagine and urea), inorganic nitrogen sources (ammonium nitrate, sodium nitrate and diammonium hydrogen orthophosphate).
- d. Effect of initial culture pH: pH of the medium varied from 3.0 to 11.0.

RESULTS AND DISCUSSION

All 97 strains isolated from soil samples in preliminary experiments were tested to determine their lipolytic activity by employing screening techniques on agar plates, containing 1% tributyrin as substrate.

For forty-seven fungal strains smaller or bigger halozones (14–27 mm) were observed around circular wells with culture supernatant. Nine strains showed a big zone above 18 mm. The results are summarized in Table 1. Out of 9 microorganisms, two strains, *Penicillium chrysogenum* 9 and *Cladosporium*

Table 1. Preliminary evaluation of lipase activity of moulds grown in on basal medium. Relative activity was expressed in mm of enzymatic zone size formed on agar plates

Organisms	Number of strains tested	Lipase activity (mm)
<i>Cladosporium cladosporoides</i>	16	23
<i>Penicillium chrysogenum</i>	9	21
<i>Cylindrocarpum magnusianum</i>	40	20
<i>Aspergillus versicolor</i> (Vull.)	16	20
<i>Cladosporium cladosporoides</i>	43	19
<i>Chrysosporium pannorum</i>	24	18
<i>Penicillium lanosum</i>	29	18
<i>Chrysosporium pannorum</i>	53	18
<i>Chrysosporium pannorum</i>	57	18

cladosporoides 16, were found to produce high activity of extracellular lipase, as they showed the biggest clear zone (diameter over 21 mm). Since the isolates were obtained from representative samples in the environment, we can presume that lipase-producing microorganisms are well distributed in the cold mountain area soil.

Seven media with different constituents were tested to maximize lipase production by the selected strains *P. chrysogenum* and *C. cladosporoides*. The initial pH of the media was adjusted to pH 6.0 or 7.0. Fungal biomasses and lipolytic activities of both strains were estimated and compared in 5-day cultures. Table 2 shows the medium GKM of pH 6.0 to be the best for lipase production by microorganisms used in this study. Consequently, only *Penicillium chrysogenum* was selected for further studies because it produced the highest level of lipase activity (42 U/ml).

Lipolytic enzyme production by microorganisms depends mainly on the incubation conditions and the composition of the media. *Penicillium chrysogenum* growth on GKM medium was performed for seven days under the conditions given in Materials and Methods, and at a temperature varying from 10 °C to 30 °C. The aim of this experiment was to establish the influence of temperature and time of cultivation on fungal biomass increase and lipolytic activity level in *P. chrysogenum*. Hence, it was possible to determine what kind of extremophile this fungal strain was. The results summarized in Table 3 show that *P. chrysogenum* grows well at temperatures of 10–25 °C, which was testified by

the level of dry fungal mass. The maximum amounts of fungal mycelia were observed on the 7th day of culture at 10 °C, and the 5th day at 20 °C. When performing the culture at 30 °C, the increase in mycelium dry weight was minimal. Simultaneously the lipolytic activity in culture filtrates was estimated. The maximum lipase activity was obtained on the 5th day of incubation at 20 °C (40 U/ml), which was accompanied by an increase in mycelium dry weight. A low lipase activity was observed when *P. chrysogenum* was incubated at 10 °C (about 10 U/ml). A similar result was reported for *Penicillium citrinum* (Maliszewska and Mastalerz, 1992) incubated at 22 °C. Psychrotrophic fungi of the genus *Penicillium* were also isolated from the softwood in Sweden by Land *et al.* (1987).

In further experiments, the lipase activity under different growth conditions was tested on the 5th day of incubation. Different lipidic substrates (fatty acids, triacylglycerols and oils) were tested as activators for lipase production. As shown in Table 4, among few lipids used in the experiment, corn oil appeared to be the best substrate for lipase production. The activity of lipase synthesized under these conditions was of 55 U/ml. Still, *Penicillium chrysogenum* presented a high lipolytic activity when incubated with triolein, olive oil and oleic acid. Studies on varying concentrations of corn oil in the culture medium showed lipase production to improve to 55 U/ml, when using 1% corn oil (data not shown). Lipolytic activity decreased dramatically when higher concentrations of corn oil were added to GKM medium. This is similar to a report on lipase production

Table 2. Comparison of biomass and lipase activity from *P. chrysogenum* 9 and *C. cladosporoides* 16 measured in different culture media

Culture media	<i>Penicillium chrysogenum</i>				<i>Cladosporium cladosporoides</i>			
	pH 6.0		pH 7.0		pH 6.0		pH 7.0	
	Activity U/ml	Biomass g/l	Activity U/ml	Biomass g/l	Activity U/ml	Biomass g/l	Activity U/ml	Biomass g/l
SKN	0	6.4	0	4.4	10	5.2	5	4.8
GKM	42	11.6	0	12.8	22	32.0	5	11.6
PEK	0	35.6	16	26.0	0	22.8	0	21.6
GPN	31	32.8	0	11.6	0	24.0	2	24.0
EKM	0	10.4	0	10.8	0	11.6	0	15.6
NKM	0	0.8	2	0.48	0	4.0	0	1.12
PKK	12	14.0	0	13.2	1	17.2	3	11.2

Table 3. Effect of growth temperature and incubation time on lipase activity

Day of incubation	Growth Temperature							
	10 °C		20 °C		25 °C		30 °C	
	Activity U/ml	Biomass g/l	Activity U/ml	Biomass g/l	Activity U/ml	Biomass g/l	Activity U/ml	Biomass g/l
3 rd	4	10.4	2	9.6	1	10.8	17	2
4 th	5	11.6	5	11.6	16.5	10.4	28	1.04
5 th	6	10.6	40	10	10	8.8	25	0.8
6 th	4	11.6	6	8	8	8.6	8	0.68
7 th	0	14.0	4	0	0	8.0	0	0.56

Table 4. Selection of best lipidic substrates for lipase production by *P. chrysogenum* 9

Inducers	Activity, U/ml	Biomass, g/l	Protein, mg/ml
Control	0	6	0.19
Tributylin	0	2	0.12
Tricaprylin	0	1.2	0.34
Triolein	41.0	4	2.46
Soybean oil	21.5	11.2	1.25
Rape oil	0	9.2	1.03
Corn oil	55.0	6.8	2.80
Olive oil	41.5	8	1.28
Oleic acid	38.0	10.4	1.64
Caprylic acid	0	0	0.30

* Control: basic medium without inducer

by *Aspergillus terreus* when corn oil was used as an inducer, but at a higher concentration of 2% (Gulati *et al.*, 1999). In contrast, *Penicillium restrictum* (Gombert *et al.*, 1998), *Penicillium citrinum* (Maliszewska and Mastalerz, 1992) and *Penicillium wortmanii* (Costa and Peralta, 1999) presented the highest lipolytic productivity when incubated with olive oil.

Lipase production depends on several environmental factors, such as nitrogen, carbon and lipid sources and initial pH of the medium. Various carbohydrates at concentration of 1% were tested. The results summarized in Table 5 show that among various carbon sources, sucrose proves to be the best carbohydrate for high lipase production, but a low biomass (72 U/ml). However, the biomass was significantly increased (16 g/l) when glucose was added to the medium instead of sucrose. Different sucrose concentrations were tested for lipase production. A significant increase in lipase activity (72 U/ml) was observed in the presence of 1% of sucrose (data not shown). In contrast, glucose and starch were the best carbon source for the production of lipase by other *Penicillium* strains *P. citrinum* (Maliszewska and Mastalerz, 1992) and *P. restrictum* (Gombert *et al.*, 1998), relatively.

Table 5. Effect of carbon source on growth and lipase production by *P. chrysogenum* 9

Carbon source (1%)	Activity, U/ml	Biomass, g/l	Protein, mg/ml
Control	14	0.4	1.64
Lactose	16	14.4	0.6
Maltose	14	14.4	0.32
Starch	20	1.2	1.32
Sucrose	72	7.6	3.13
Xylose	25	15.2	0.5
Glucose	40	16	0.27
Fructose	6.5	13.6	0.43
Galactose	15	15.2	0.24

* Control: basic medium without carbohydrate

Different types of organic and inorganic nitrogen sources were tested in order to determine their effect on fungal growth and lipase production. As shown in Table 6, out of various organic nitrogen sources, urea (concentration of 0.4%) was the best nitrogen source for increasing the lipolytic activity in the fungal culture. In this case, the highest enzyme activity (68 U/ml), but a low biomass (7.2 g/l) was observed. This is similar to the report on lipase production by *Yarrowia lipolytica* strain, where the maximum lipase activity was reached after adding urea as nitrogen source (Corzo and Revah, 1999). Moreover, asparagine was also found to be good nitrogen for lipase activity (25.5 U/ml), while the lipase production was inhibited with other organic nitrogen sources. Among inorganic nitrogen sources sodium nitrate was found to be the most effective for the lipase production (63.5 U/ml).

Table 6. Effect of nitrogen source on growth and production of lipase by *P. chrysogenum* 9 (urea at a concentration of 0.4%, others at a concentration of 1%)

Nitrogen source (1%)	Activity, U/ml	Biomass, g/l	Protein, mg/ml
Control	0	0.04	0.15
Yeast extract	4	11.2	2.02
Peptone	5	14.8	2.43
Urea	68	7.2	2.39
Casein	6	10	1.64
Asparagine	25.5	10	2.85
NaNO ₃	63.5	8.4	1.91
(NH ₄) ₂ HPO ₄	0	16.8	1.23
NH ₄ NO ₃	0	14.8	0.18

* Control: basic medium without nitrogen source

The production of lipase was not detected in the culture medium containing ammonium dihydrogen orthophosphate or ammonium nitrate. Lipase production by *Cryptococcus* sp. S-2 was neither observed on the culture medium containing ammonium nitrate (Kamini *et al.*, 2000). Corzo and Revah (1999) reported ammonium sulfate not to enhance the production of extracellular lipase by *Yarrowia lipolytica*. Medium without nitrogen source did not induce lipase production or growth of *P. chrysogenum*. According to the results obtained in the following experiments 0.4% urea was used.

The enzyme secretion is greatly influenced by a change in the initial pH of medium. To observe the effect of the initial pH of the GKM medium on lipase production, pH of the medium was varied from 3.0–7.5. As shown in Fig. 1, the maximum extracellular lipase production (68 U/ml) was achieved at pH 6.0 by *P. chrysogenum*. This result was in accordance with studies on *Penicillium citrinum* (Maliszewska and Mastalerz, 1992) and *Candida lipolytica* (Pereira and Rocha, 1997). Lipase activity from *P. chrysogenum* was not observed when the initial pHs of the medium were 3.0–5.0. The lowest lipase activity was obtained when the initial pH was adjusted to 7.5 (5 U/ml). The

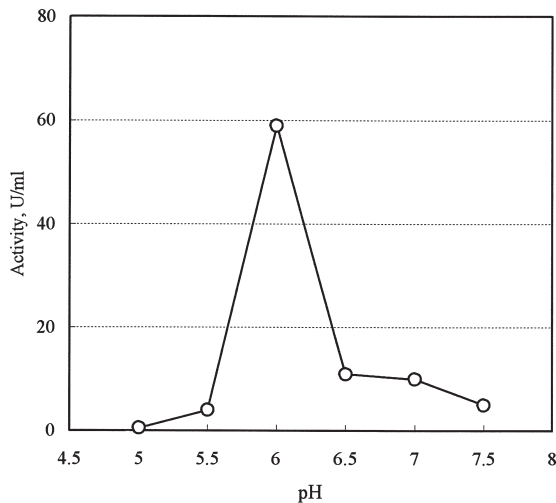


Fig. 1. Effect of initial pH on *P. chrysogenum* lipase production.

lipase from other *Penicillium* strains – *P. cyclopium* (Vanot *et al.*, 2002) and *P. restrictum* (Gombert *et al.*, 1998) showed the highest activity when the initial pH of the media were adjusted to 5.0. Excretion of lipase by *Rhodotorula glutinis* (Papaparaskevas *et al.*, 1992) was the highest at initial pH 8.0.

The enzyme activity is determined by the reaction conditions, such as temperature, pH and presence of inhibitory compounds. The extracellular lipase of *P. chrysogenum* was studied regarding the optimum of both pH and temperature according to different substrates. As it can be seen in Fig. 2, the optimal activity for lipase was found in the range of temperature 30–37°C. Optimum temperature depends on the substrate used in the reaction. The highest activity of lipase with tributyrin as a substrate was observed at 30°C. Therefore, this substrate was selected to be the best for lipase activity of extracellular lipase from *Penicillium chrysogenum*.

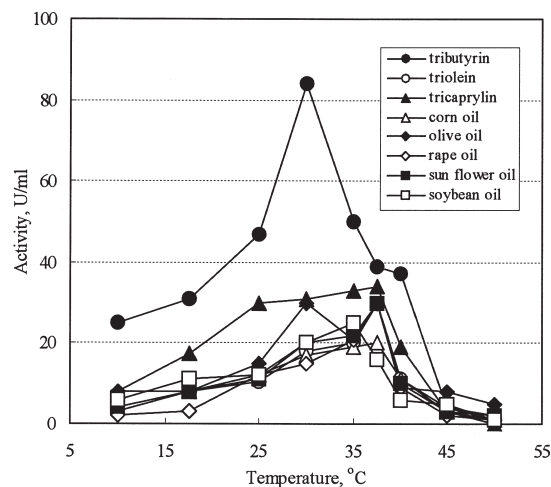


Fig. 2. Effect of reaction temperature on lipase activity according to different substrates. Lipase activity was estimated at temperature in the range of 20–80°C.

The optimal pH for lipase activity was found in the range of 5.0–7.0 and depends mainly on the kind of substrates tested in this experiment. In the presence of synthetic substrates (tributyrin, tricaprylin and triolein) the maximum activity of lipase was found at pH 7.0 (Fig. 3a). However, in the presence of natural substrates (oils), the maximum lipolytic activity was achieved at pH 5.0 (Fig. 3b). Similar results have not been found by now in available literature concerning this protein. Therefore, the mechanism of such reactions is still unknown. The enzyme from *Penicillium cyclopium* (Vanot *et al.*, 2002) exhibited a maximum activity at pH 7.0 at 50°C. Lipase from *P. chrysogenum* (Ferrer *et al.*, 2000) showed the greatest activity at pH 7.9–8.1 and 45°C. These results were in accordance with studies on *Penicillium citrinum* (Maliszewska and Mastalerz, 1992). The importance of the data presented here point to the possibility of obtaining an active lipase producer from psychrotrophic microorganisms. *Penicillium chrysogenum* as the fungus having the highest lipolytic activity will be great interest in biotechnological research.

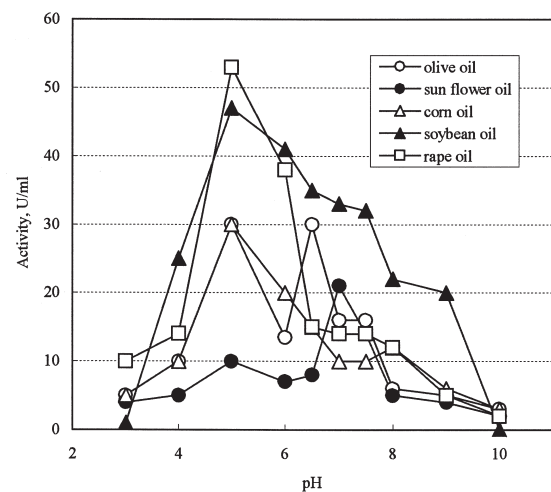
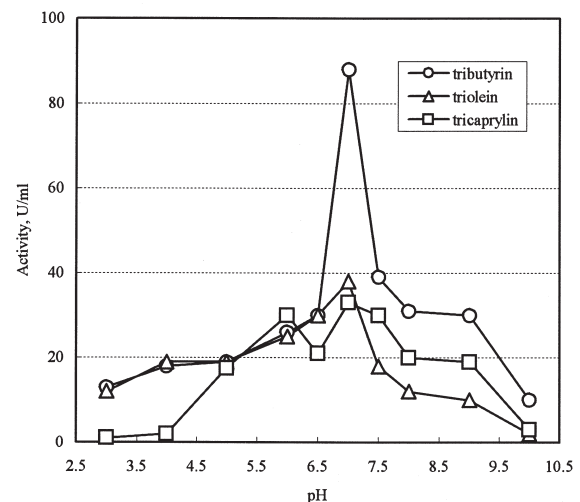


Fig. 3. Activity of lipase from *P. chrysogenum* against synthetic substrates (a, above) or natural substrates (b, under) depending on pH. Lipase activity was estimated at pH values in the range of pH 3.0–10.0.

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