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RESEARCH ARTICLE

SCREENING FOR NOVEL COLD-ACTIVE LIPASES FROM WILD TYPE BACTERIA ISOLATES

Svetlana Laura LEONOV*

Faculty of Food Science and Engineering (Department of Applied Microbiology), Dunărea de Jos University of Galați, 111 Domneasca, 800201 Galati, Romania

Abstract:
Soil from different cold regions in Romania was sampled and used to isolate lipase producing bacterial strains. The screening process was a three step one using straightforward methods in order to ensure the isolation of highly active lipase producing bacteria. The isolates were shown to have significant yield comparable to their growth rate at moderate to low temperatures. The results indicate the novel lipase as a good candidate for further testing as it may turn to be of use in oil waste treatment.

Keywords: Psychrotrophs, soil isolates, *Pseudomonas* strains, lipase

Introduction

Triacylglycerol hydrolases (E.C. 3.1.1.3), also known by their trivial name as lipases, are enzymes that catalyse the hydrolysis of long-chain acylglycerols in aqueous emulsions (*Lee et al., 2003*). They are also capable of catalyzing other reactions namely trans-esterification, inter-esterification and esterification between a fatty acid and an alcohol which is the reverse reaction of hydrolysis (*Macrae, 1983*). The broad range of substrate and reaction specificity, the ability to catalyse reaction in the absence of a cofactor and the versatility of these enzymes for industrial applications have emerged in time with multiple approaches towards their utilization. Their largest industrial use nowadays still remains the hydrolysis of fats and oils. Lipases are used as additives in detergent formulations, in cleaning solutions and in waste treatment cocktails for downstream industrial processes and for domestic use also (*Lai et al., 2007*). Fats and oils are known to clog tubes and pipes systems and filters by forming deposits on them.

Fats and oils discarded in the environment cause the contamination of soil and surface waters having a negative impact on the environment. If there is no intention for recycling natural oils and fats a simple pre-treatment for their digestion would be recommended. Although the use of lipases in waste treatment was proved to be highly effective, the costs this step would imply is a setback. This paper offers an alternative to waste emissions for local and regional companies whose business implies the disposal of used natural oils and fats and for sewage treatment plants. This measure involves a hydrolysis of the mentioned potential pollutants, using a crude lipase preparation active at low temperatures. The low-moderate temperature activity profile would be of special use for the waste treatment process because it involves low economic costs and versatility of the preparation through seasonal temperature changes. This paper presents the isolation and identification of cold-active lipase producers from soil, having as result the obtaining of a crude lipase extract with considerable lipolytic activity.

Materials and Methods

Isolation and screening of microbial strains. Soil samples were collected from Bucegi Mountains (Omu Peak, ~2500m) in Romania where the annual maximum temperature does not raise above 20°C. This safety measure was adopted in order to enhance the isolation of psychrotrophic

*Corresponding author: svetlana.leonov@ugal.ro

microorganisms capable of growing at low temperature and producing cold-active enzymes. An amount of 1g of soil was used for enrichment in a medium that consisted of 0.5% glucose, 0.4% NH_4NO_3, 0.1% yeast extract, 0.025 KH_2PO_4, 0.025 Na_2HPO_4, 0.1% $MgSO_4$, 0.5% Tween 80 (*Choo et al,. 1998*). The culture medium was supplemented with 3% extra virgin olive oil as lipase inducer (a cheaper alternative to triolein). A volume of 75 mL of this medium was placed in 300 mL Erlenmeyer flasks and after 15 days of enrichment cultivation at 15 °C at 100 rpm, samples were taken and spread on agar plates after a suitable dilution with cold Milli-Q water.

The agar plates contained nutrient broth (1% tryptone, 0.3% yeast extract, 0.5% beef extract, 0.5% NaCl, 0.7% $MgSO_4$, 0.7% KH_2PO_4, 0.5% Tween 80) and 2% agar. Plates were incubated at 4 ºC and the colonies spotted after 3 days were selected. The bacterial isolates were used for subsequent study.

Qualitative hydrolysis assay was performed by using agar plates in which olive oil (2% v/v) and Rhodamine B (0.1% w/v) have been added. After 2-3 days of incubation at room temperature the plates were examined under UV light illumination. The fluorescent orange halo around the lipase producing colony served as a preliminary screening step (data not showed).

Organism and culture. The most active bacterial isolates were cultured in 300 mL flask containing 75 mL of nutrient broth at 15 ºC for several days and samples were taken from time to time to establish a growth curve and lipase activity (*Chakraborty and Paulraj, 2009; Kasana et al., 2008*). The growing rate of the selected strains was determined by measuring the optical density of culture broth samples at 600 nm wavelength and plotted as a function of time.

Selected isolates were identified on the basis of colony and cell morphology, gram reaction, motility and several differentiation tests (*Thomas & McMeekin, 1980*).

The biomass was separated from the culture broth by centrifugation at 12000 rpm for 10 minutes at 4 °C followed by a filtration intended to separate the solidified fatty phase. The cell-free supernatant was used as crude lipase extract. The samples after 72h of cultivation could not be used because the production of exo-polysaccharides made impossible the separation of cells from supernatant and by that the obtaining of the lipase extracts.

Lipase activity assay. The hydrolytic activity was tested by titrimetric method as described previously *(ACS Specifications, 1993*) with slight modifications. Ultrapure water, Arabic gum (1% w/v) and the substrate (olive oil) 1:1 (v/v) were brought up to a 6 mL total volume. The reaction cocktail was thoroughly mixed and equilibrated at 20°C and 1 mL of crude enzyme was added after being previously incubated at the same temperature. The reaction was left at room temperature for exactly 1 hour and stopped by adding 3 mL of 95% ethanol. The released fatty acids from oil substrate during enzymatic hydrolysis were titrated to neutralization with 50 mM NaOH in presence of thymolphtalein as indicator. A blank was prepared for each sample where the enzyme was inactivated by heating at 95 °C for 15 minutes. One unit of lipase activity was expressed as micro equivalents of fatty acid released from a triglyceride in one hour at pH 7.2 at 20 °C.

Results and discussion

The enrichment and preliminary agar plate tests resulted with the isolation of 36 bacterial strains with lipolytic activity. After testing them on agar plates with olive oil and Rhodamine B only three were chosen for further study. These were given identification codes MP11, MP3 and M61. The three isolates were strictly aerobic, Gram negative, high motile, rod shaped bacteria (size of app. 1.5-2.5µ) (Figure 1). The temperature profile was different for the three but all were well capable of growing at temperatures even below 2°C.

Some characteristics belonging to strains MP11, MP3, M61 are listed in Table1.

Table 1. *Description of the strains selected through preliminary screening steps*

Strains / Tests	MP11	MP3	M61
Configuration of colony	Circular	Circular with scalloped margins	Circular
Margins	Smooth	Wavy	Smooth
Elevation	Convex	Hilly	Convex
Pigments	Blue greenish when grown in medium with no iron; Yellow, fluorescent under UV light (see Figure 2)	Blue greenish when grown in medium with no iron Yellow, fluorescent under UV light (see Figure 2)	Blue greenish when grown in medium with no iron Yellow, fluorescent under UV light (see Figure 2)
Gram reaction	Negative	Negative	Negative
Shape	Rods	Rods	Rods
Size	~ 1.8-2µm	~ 1.5-2µm	~1.5-2.5µm
Arrangement	Single	Single	Single
Motility	+	+	+
Growth at ≤ 5 °C	+	+	+
Growth at 10 °C	+	+	+
Growth at 20 °C	+	+	+
Growth at 30 °C	-	-	+
Hydrolysis (H) of Tween 80	+	+	+
H of gelatin	-	-	+
H of casein	+	+	+
H of lipids	+	+	+
Fermentation of glucose	-	-	-
Assimilation (A) of glucose	+	+	+
A of maltose	-	-	-
A of arabinose	+	+	+
A of mannose	+	+	+
A of manitol	+	+	+
A of N-acetyl-glucosamine	+	+	+
A of gluconate	+	+	+
A of citrate	+	+	+
Oxidase	+	+	+

Lipase-producing, gram negative, rod-shaped, psychrotrophic bacteria isolated from soil commonly belong to the genus *Pseudomonas* (*Jaeger & Reetz, 1998, Dong-Won et al, 1998; Männistö & Häggblom, 2006; Srinivas et al., 2009*). As a consequence to this and the results of biochemical tests employed, also with reference to Bergey's Manual of Determinative Bacteriology (*Holt et al., 1994*) the selected psychrotrophs were considered to be *Pseudomonas fluorescens*.

Growing curves and the activity patterns are presented in Figure 3.

The three isolates showed lipase activity after 24 hours of cultivation, except strain M61 which exhibited lipase activity after only 12 hours of submerged fermentation, a characteristic in lipase

synthesis usually encountered in *Pseudomonas* sp. (*Rajmohan et al., 2002*).
The highest peak for lipolysis was in the late exponential phase for all the selected strains, *Pseudomonas fluorescens* MP11 being the most active.

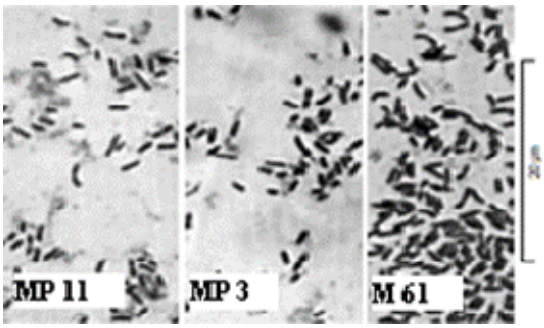

Figure 1. *Gram stain of the three isolates cultured as described above. The image was obtained with an Olympus phase contract microscope (100x)*

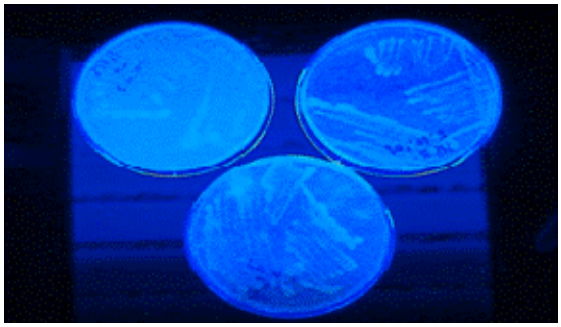

Figure 2.*Streak plates of MP11, MP3 and M61 strains after 48 hours of growth, observed under UV light (notice the fluorescence of the colonies*

In order to minimize the inhibition effect on the lipase activity it is indicated that the cultivation time should be stopped when the activity profile reaches its maximum rate. *Pseudomonas fluorescens* produces lipases at chill temperatures, lower than the optimum for growth (*Guillou et al., 1995; Matselis & Roussis, 1998*) and because of this, the recommended temperature used for cultivation should be 10-15 °C.
The screening steps revealed a highly active psychrotrophic bacterium, comparable to previous reported data (*Kandasamy et al., 2009*).
The crude preparations exhibit considerable hydrolytic activity on oily substrates at moderate temperature.
The difference between the activity pattern for the three isolates could be explained by the synthesis of proteases, *Pseudomonas* sp. being well known as a protease producer (*Matselis & Roussis, 1998; Rajmohan et al., 2002; Stevenson et al., 2003*).
The presence of newly secreted lipase would induce the synthesis of the proteases. Decrease in lipase activity after 40-48 hours of cultivation would indicate that the submerged cultivation time should be of maximum 48h at 15 °C , before the protease activity would affect the yield in lipase. Another solution to this problem would be the addition into the culture broth of protease inhibitors.

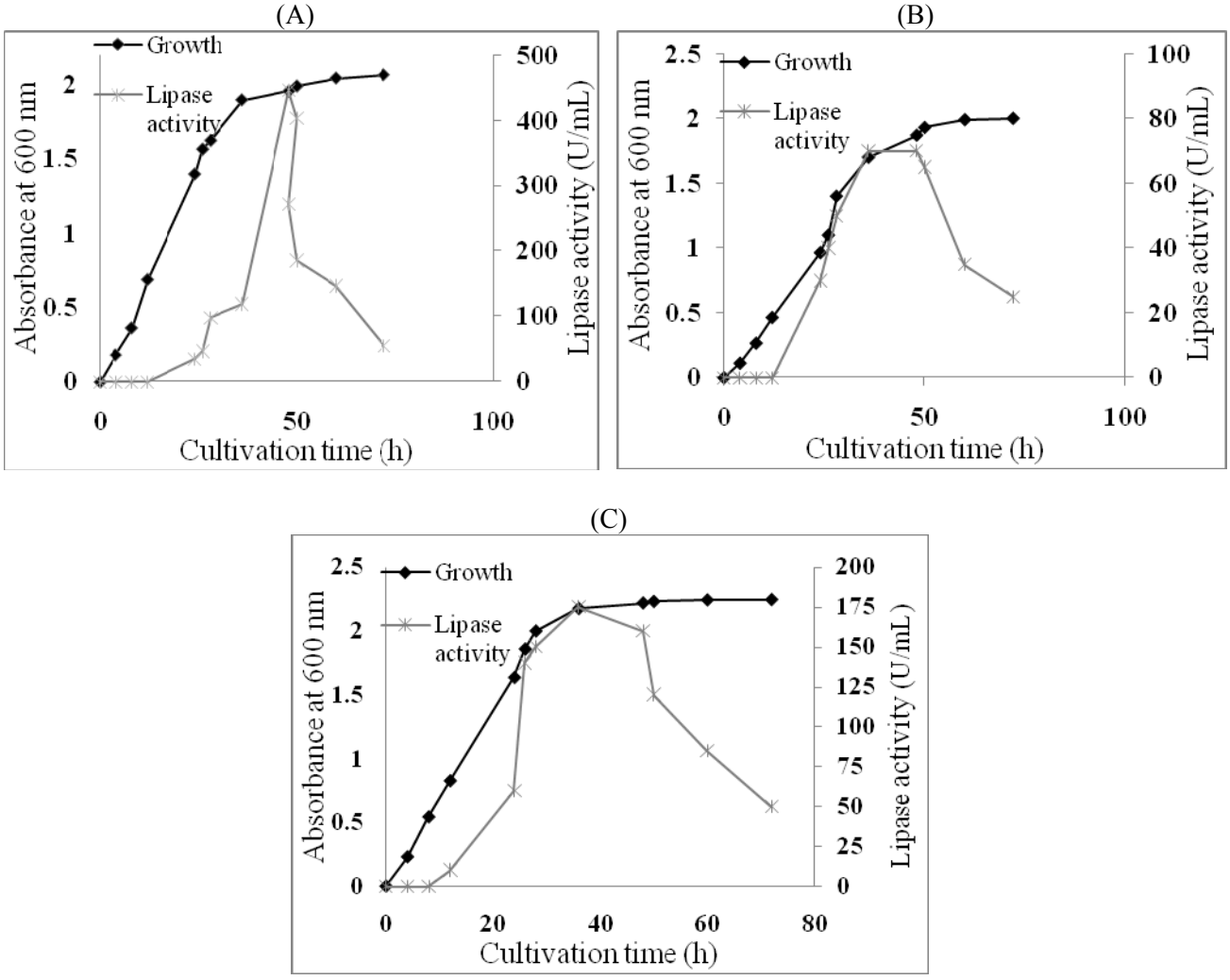

***Figure 3**. Growth curve (on nutrient broth suplimented with 2% olive oil at 100 rpm at 15 °C) and lipase activity of P.fluorescens strains MP11 (A), MP3 (B), M61 (C)*

The pattern of activity for strain MP11 suggests that the protease activity has a considerable inhibiting effect on the lipase produced by the same microorganism. Strains MP3 and M61 are also affected by the proteolytic enzymes, but the drop of activity is slower. Even so, the high activity exhibited by strain MP11 makes it the best candidate out of the three isolates as a lipase producer.

In conclusion, the isolation technique and screening methods revealed to be effective to isolate and identify a new microorganism capable to grow and produce lipase at low and moderate temperatures. The characteristic of this presumably new enzyme of acting hydrolytically on fatty substrates without having to go through complicated, expensive and time consuming purification steps indicate that this enzyme would be suitable for low-scale production and utilization. It would be recommended that the research involved in this enzyme's characterization should be continued in order to get a full image of its potential and its use in application.

Further study must be performed in order to fully characterize the new lipase and design its production to serve environmental friendly purposes.

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