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ORIGINAL PAPER

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A thermostable lipase produced by a newly isolated *Geotrichum*-like strain, R59

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Abstract Growth and production of lipase by a new *Geotrichum*-like strain, R59, were studied. Production of extracellular lipase was substantially enhanced when the initial pH of the culture medium, types of carbon and nitrogen sources, substances probably stimulating the lipase biosynthesis, the temperature, and time of growth were optimized. Sucrose and triolein were the most effective carbon sources for lipase production. Maximum lipase activity (146 U/ml^{-1}) was obtained with urea as the nitrogen source. Growth at 30°C, an initial pH of 6.0 and incubation time of 48 h were found as optimum conditions for cell growth and production of lipase by *Geotrichum*-like strain R59. The enzyme was thermostable and exhibited very high activity after 1 h incubation at 60°C.

Keywords *Geotrichum*-like R59 · Medium optimization · Thermostable lipase

Introduction

Lipases are defined as glycerol ester hydrolases (EC 3.1.1.3) disrupting the bonds in tri-, di- and monoacylglycerides present at oil-water interfaces. Lipases are produced by many microorganisms and higher eukaryotes. Enzyme-producing microorganisms include bacteria [12], fungi [8, 22], yeast [4] and actinomycetes [24]. Lipases from microorganisms have drawn much attention for their potential use in biotechnology, mainly due to their availability and stability [10, 26]. Microbial lipases have been used in a wide range of practical applications in industry, e.g., for the hydrolysis of fats in food processing, for the production of food additives and pharmaceuticals, in diagnostics, or as detergent additives [2, 21].

The aim of the present work was to determine the culture conditions for maximum lipase production by a newly isolated *Geotrichum*-like fungus and to characterize the enzyme. Medium composition, initial pH, temperature, and time of incubation were examined for the optimization of lipase production. Lipase characteristics with respect to the optimal temperature and pH for both activity and stability are described.

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Materials and methods

Materials

All reagents were of analytical grade and were purchased from POCh (Gliwice, Poland), Fluka (Buchs, Switzerland), Sigma (St. Louis, Mo.) and Difco (Detroit, Mich.). Various oil substrates were from the local market.

Pulp (*Streptomyces peucetius* mycelium) after industrial daunomycin extraction was kindly provided by the Biotechnology and Antibiotics Institute in Warsaw (Poland).

Samples of soil for fungus isolation (Phaeozems acc. FAO) of medium loam texture came from a field near Lublin (South-Eastern Poland). The samples contained (in percentage): humus 3.95, N_{Tot} 0.210; pH_{KCl} 7.45.

Fungal isolation

The sample of soil was passed through a 0.2 cm-mesh sieve, then humidified to 50–60% total water capacity and placed in 1,000 cm^{-3} glass vessels (1 kg/vessel). Fungi were isolated using a trapping method. Portions (15 g) of pulp after industrial production of daunomycin (*Streptomyces peucetius* mycelium), used as trapping material for fungal isolation, were placed into bags

(5×5×6 cm; pores of 0.5 mm in diameter) made of polyamide-6 (15 g/bag), placed into the vessels and covered with soil. Vessels were incubated for 3 months of 20 ± 2°C in conditions of controlled humidity.

The next isolation step was performed on Petri dishes with medium containing homogenized (in Potter homogenizer; 1,000 rpm; 10 min) daunomycin pulp [10%, KH_2PO_4 (0.15%), agar (2%) and streptomycin (300 ppm)]. The pulp from polyamide-6 bags, with fungal mycelia entrapped from the soil, was spread on the Petri dishes. After 3 weeks of growth at 20 ± 2°C, mycelial colonies capable of decolorizing daunomycin were found on approximately 1% of the plates.

The fungal mycelia were then transferred to, and maintained on, potato-glucose agar medium (PGM). PGM contained (per liter): potato, 300 g; glucose, 20 g; agar, 20 g. Potatoes were cooked in 1 l distilled water for 1 h. After cooling, the solution was filtered through Whatman no. 1 filter paper. Glucose and agar were then added to the filtrate.

Growth media preparation

Basal GKM medium for lipase production contained (per liter): glucose, 10 g; KH_2PO_4, 6 g; $MgSO_4 \cdot 7\ H_2O$, 1 g; urea, 4 g; $FeCl_3 \cdot 4\ H_2O$, 10 mg; D-biotin, 8 μg; myo-inositol, 4 μg; thiamine, 200 μg, and olive oil, 10 g [18]. The culture medium, except vitamins, urea and olive oil, was autoclaved at 121°C (15 psi) for 25 min. Olive oil and urea were autoclaved separately at 110°C for 15 min, vitamins was sterilized by UV light for 24 h. After autoclaving, the urea, olive oil and the vitamins were added to the medium, and the pH was adjusted to 6.0 by addition of sterile KOH solution.

Inoculum preparation and culture conditions

For inoculation, fragments of mycelium previously cultured on 2% agar-stabilized PGM medium, were suspended in 5 ml sterile distilled water. A 0.5 ml aliquot of this suspension was used to inoculate a 100 ml Erlenmeyer flask containing 25 ml sterile liquid GKM medium. The flasks were incubated on a rotary shaker (WL-972, JWElectronics, Poland) at 150 rpm at 30°C for 48 h.

When studying the effects of lipid substrates, olive oil was replaced by similar concentrations of other lipid substrates (fatty acids, triacylglycerols and oils). To test the effect of carbon source on lipase production, glucose was replaced by other sugars (maltose, galactose, xylose, fructose, lactose, sucrose and starch at concentrations of 1%). To test the effect of different nitrogen sources on lipase biosynthesis, urea was replaced by other nitrogen-containing compounds (peptone, yeast extract, casein, asparagine, ammonium nitrate, sodium nitrate and diammonium hydrogen orthophosphate) at concentrations of 1%.

Experiments were performed in duplicate and samples were analyzed in triplicate. The results reported are average values; standard deviation was less than 10% in all cases.

Assay method

The *Geotrichum*-like fungus was grown in the optimized medium for 48 h at 30°C, and the mycelia removed by centrifugation at 10,000 *g* for 15 min at 4°C. The supernatant, used as the source of lipolytic activity, was called crude lipase.

Lipase activity was determined by the method of Sokolovska et al. [23] with the following modifications: 0.5 ml crude lipase solution was mixed with 1 ml 50 mM phosphate buffer, pH 7.0, and 1.5 ml tributyrin in 25 ml Erlenmeyer flasks; after 60 min incubation at 37°C the emulsion was mixed with 1 ml acetone:ethanol mixture (1:1) to extract free fatty acids; free fatty acid concentration was determined by titration (0.05 *N* KOH) using phenolphthalein as an indicator. Controls were carried out as above, but the enzyme solution was inactivated by boiling for 20 min. Enzyme activity was calculated from the slope of the straight lines obtained. Measurements were performed in triplicate; they differed by less than 8%. One unit (U) lipase activity was defined as the amount of the enzyme releasing 1 μmol free fatty acids per minute by 1.0 ml crude lipase solution under assay conditions. Activities were expressed in units per milliliter.

Protein concentration was estimated by the method of Lowry with bovine serum albumin as a standard [13].

Biomass was determined by estimation of dry weight. Samples of mycelium were washed twice with 50 ml distilled water and dried at 105°C for 24 h. The biomass was weighed and expressed as dry weight (g) per 1 l medium. The results were duplicate determinations.

Effect of the initial pH, temperature, and time of incubation

To observe the effect of the initial pH of GKM medium on lipase production, the pH of the medium was varied from 3.0 to 11.0. The pH was adjusted after autoclaving. The effect of temperature (10–40°C) and time (1–6 days) of incubation on growth and lipase production was studied in the optimized medium.

Characterization of the lipase

Lipase thermostability was tested by preincubating the reaction mixture (0.5 ml crude lipase solution and 1 ml 50 mM phosphate buffer, pH 7.0) at temperatures ranging from 30°C to 90°C for up to 60 min. The pH optimum was tested used 50 mM Britton and Robinson buffer [6] in the lipase assay at a wide range of pH values (3.0–10.0).

The pH stability of the crude enzyme preparation was determined by preincubating the enzyme in 50 mM acetate-borate-phosphate buffer of different pH in the range of 3.0–11.0 for 1 h at 30°C, followed by activity estimation at pH 7.0.

Results and discussion

Identification and characterization of *Geotrichum*-like fungus R59

The investigated strain R59 ranks among quick-growing fungi: after a 5-day growth on GKM medium, it forms colonies reaching 9 cm in diameter. These hyaline fungi are characterized by good development of surface mycelium, and a fibrous structure. Microscopic micromorphological studies of the colony showed strain R-59 to produce forked ramifications of vegetative hyphae and arthroconidia formed by hyphal fragmentation. Considering that these characteristics suggest that this strain resembles the genus *Geotrichum* [7], belonging to imperfect fungi (subgroup *Deuteromycotina*, class *Hyphomycetes*), it was initially defined as *Geotrichum*-like R59. An essential morphological difference in the character and structure of their colonies was found between a typical representative of *Geotrichum*, i.e. *Geotrichum candidum* and strain R59. The strain we isolated from soil forms typical mold colonies consisting of a high, relatively loose and well-developed mycelium, in contrast with *G. candidum*, which forms flat, yeast-like, colonies with a highly reduced vegetative surface mycelium, covered with a thick, hoarfrost-like, layer of conidia.

Effect of the initial pH of the culture medium

Geotrichum-like R59 was grown at pH 3.0–11.0 on GKM medium. Maximum lipase activity and biomass were obtained when the initial pH of the GKM medium was 6.0 (40 U^{-1} and 2.3 g^{-1}, respectively), although a pH of 3.0–9.5 was preferred for lipase production. No growth was observed at pH 9.5–11.0. The lipase from *G. candidum* showed the highest activity when the initial pH of the medium was adjusted to pH 7.0 (3.7 U ml^{-1}) [1]. Lipase production from the newly isolated strain of *Geotrichum* sp. was maximum at pH 5.0 [14].

Effect of lipid substrates

Among the various oils, triglycerides and fatty acids tested, triolein allowed for maximum lipase production (Table 1). A similar result was reported for production of lipase from *Cryptococcus* sp. S-2 [11]. Rape oil, soybean oil and olive oil were also shown to be effective lipid substrates for lipase production by *Geotrichum*-like R59. Soybean oil was also used by *G. candidum*, ATCC 34614 (lipase activity was 5.2 U ml^{-1}) [1]. In the presence of triacylglycerides with saturated short-chain fatty acids (tributyrin and tricaprylin) *Geotrichum*-like R59 showed little production of lipase (6.5^{-1} and 3.0 U ml^{-1}, respectively). Obradors et al. [17] reported that caprylic acid was the best lipase inducer for *Candida rugosa*, but at low concentrations (0.05 and 0.1%). *C. rugosa* could not metabolize this substrate at high concentrations (0.2%, 1%) probably due to its toxicity. Similar results were observed in our experiment. Saturated short-chain caprylic acid at a concentration of 1% did not induce lipase production. These results were also in accordance with studies on *G. candidum* ATCC 34614 [22].

Table 1 Effect of lipid substrates on lipase production. *Geotrichum*-like R59 was grown on basal GKM medium to which different fatty substrates were added. Activity of lipase and protein concentration were assayed in the culture supernatant

Lipid substrates	Activity (U ml^{-1})	Protein (mg ml^{-1})	Biomass (g l^{-1})
Control[a]	10.0 ± 0.4	0.30	1.0
Tributyrin	6.5 ± 0.2	0.28	0.9
Tricaprylin	3.0	0.40	0.35
Triolein	91.0 ± 3.5	0.31	1.2
Soybean oil	71.0 ± 4.3	1.20	1.3
Rape oil	82.4 ± 5.7	1.30	1.6
Corn oil	40.0 ± 2.2	0.76	1.4
Olive oil	69.0 ± 3.0	0.24	1.2
Oleic acid	13.5	0.40	1.3
Caprylic acid	0	0.26	0.1

[a]Without fatty substrate

Influence of carbon source on lipase production

In GKM medium, glucose was replaced by other carbon sources. *Geotrichum*-like R59 strain grew and produced lipase on all media tested. The best result was observed for sucrose (Table 2). However, Baillargeon et al. [1] showed that lipase production was not detected in medium containing glucose or sucrose. The lowest lipase activity was obtained when fructose was added to the

Table 2 Effect of carbon source on lipase activity. Cells were grown on basal GKM medium with the addition of the carbon sources indicated at a concentration of 1%. Lipase activity and protein concentration were assayed in the culture supernatant

Carbon source (%)	Activity (U ml^{-1})	Protein (mg ml^{-1})	Biomass (g l^{-1})
Control[a]	28.0	0.23	1.2
Lactose	29.5 ± 0.8	0.19	1.2
Maltose	24.5 ± 0.4	0.19	1.0
Starch	30.0	0.12	1.6
Sucrose	118.0 ± 4.2	0.47	1.4
Xylose	26.0 ± 1.1	0.57	1.4
Glucose	92.4 ± 3.7	0.40	2.2
Fructose	10.0 ± 0.6	0.16	1.6
Galactose	82.0 ± 4.1	0.34	1.8

[a]Without carbohydrates

basal medium. Chander et al. [3] also found that lipase production by *Penicillium chrysogenum* was inhibited by fructose. According to Mates et al. [15], inhibition of lipase production by some sugars may be ascribed either to the ability of microorganisms to metabolize carbohydrates, or to the production of certain lipids during metabolism of these carbohydrates. It is possible that some fungi do not possess effective transporters for fructose uptake. In contrast, fructose was reported to be the best carbohydrate for production of extracellular lipase by *Rhodotorula glutinis* [19].

The highest level of lipase activity was obtained using sucrose at a concentration of 0.5% (146 U ml^{-1}). When higher concentrations of sucrose were added to GKM medium, lipolytic activity decreased (data not shown).

Effect of nitrogen source on lipase production

The effect of various nitrogen sources on lipase activity is shown in Fig. 1. Best results were obtained with urea. In general, microorganisms provide high yields of lipase when an organic form of nitrogen is used. *Yarrowia lipolytica* produced an extracellular lipase in a medium containing urea at a concentration of 0.2% [4]. Salleh et al. [20] obtained maximal production of extracellular lipase by the fungus *Rhizopus oryzae*, when the medium contained peptone as nitrogen source.

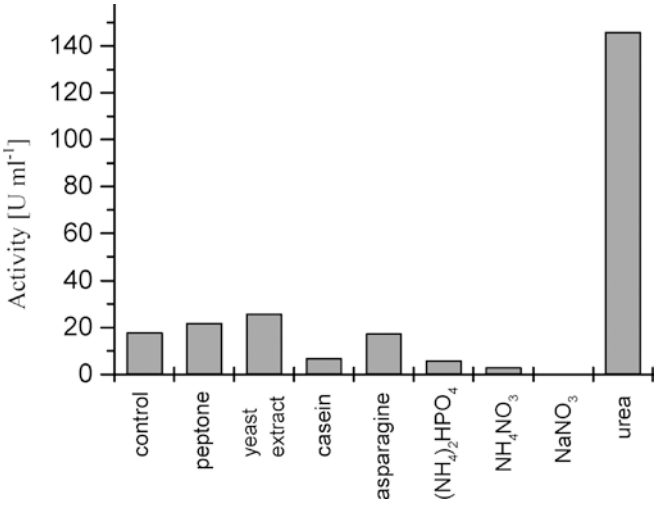

Fig. 1 Effect of nitrogen source on lipase activity. Activity of the crude enzyme preparation was assayed as described in Materials and methods. *Control* Basal GKM medium without nitrogen source. Nitrogen source concentrations: *urea* 0.4%, *others* 1%

The inorganic nitrogen sources (see Fig. 1) showed an inhibitory effect on lipase production. Similar results have been reported by Sarkar et al. [21].

Effect of temperature and time of incubation on lipase production

Table 3 shows the effect of temperature, and time of culturing, on lipase production. The best conditions for lipase production and cell growth were 30°C and 48 h of incubation. Similar results for lipase activity were reported for *Geotrichum* sp., which was incubated at 30°C for 48 h [14]. Baillargeon et al. [1] also reported that maximal lipase activity from *G. candidum* ATCC 34614 was found at 30°C, but after 24 h (5.2 U ml^{-1}). *Geotrichum*-like species also presented high lipolytic activities when incubated at 25°C for 4 days, but in that case the biomass was low (0.65 g l^{-1}). *Geotrichum*-like R59 was also grown at a temperature of 5°C, but maximum lipase production was observed after a 10-day incubation (30 U ml^{-1}) (data not shown).

Optimal pH and stability

Temperature and pH optima were detected in the presence of tributyrin as substrate. The lipase activity estimation at different pHs showed the optimum pH of lipase from *Geotrichum*-like R59 to be 7.0. As shown in Fig. 2, lipase activity decreased significantly above pH 8.0. The results were in accordance with studies on lipase from *Y. lipolytica* 681 [4]. Conversely, a crude lipase from *Geotrichum* sp. [14] was most active between pH 7.5 and 9.0.

Table 3 Effect of the temperature and time of growth on lipase activity. *Geotrichum*-like R59 was grown at temperatures from 5°C to 40°C on optimized GKM medium for 6 days. Lipase activity and biomass were measured at 24 h intervals. Biomass at time zero after inoculation was 0.23 g l^{-1}

Time of incubation (h)	Growth temperature (°C)									
	18		25		30		35		40	
	Activity (U ml^{-1})	Biomass (g l^{-1})	Activity (U ml^{-1})	Biomass (g l^{-1})	Activity (U ml^{-1})	Biomass (g l^{-1})	Activity (U ml^{-1})	Biomass (g l^{-1})	Activity (U ml^{-1})	Biomass (g l^{-1})
24	5.0 ± 0.2	0.63	10.0 ± 0.6	0.80	32.0 ± 2.8	0.89	59.0 ± 1.6	0.89	25.0 ± 1.3	0.83
48	8.0 ± 0.5	0.71	17.0 ± 1.2	0.82	150.0	1.04	75.0 ± 5.7	0.95	53.0 ± 2.1	0.97
72	24.5 ± 1.3	0.74	34.0	0.82	82.0 ± 1.5	0.91	49. 0 ± 3.4	0.92	31.0 ± 1.6	0.95
96	41.0 ± 2.2	0.77	85.0 ± 2.7	0.65	61.0 ± 0.4	0.79	29.0 ± 1.2	0.69	25.0 ± 0.9	0.89
120	30.0 ± 2.1	0.79	76.0 ± 6.3	0.63	28.0 ± 1.3	0.79	19.0 ± 0.8	0.89	13.0 ± 0.8	0.72
144	24.0 ± 0.9	0.97	43.0 ± 2.9	0.55	10.0 ± 0.4	0.75	8.0 ± 0.3	0.70	6.0 ± 0.2	0.71

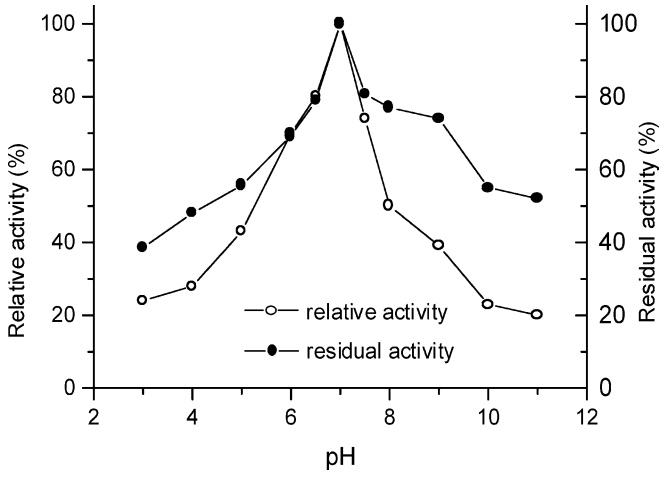

Fig. 2 Effect of pH on lipase activity and stability. The buffer used for activity and stability determination was general purpose Britton and Robinson buffer pH 3.0–11.0. The optimal pH was determined by assaying as described in Materials and methods but with Britton and Robinson buffer (pH 3.0–11.0). The high activity at pH 7.0 (150 U ml^{-1}) was taken as 100%. For pH stability experiments, the crude enzyme preparation was pre-incubated in Britton and Robinson buffer (pH 3.0–11.0) for 1 h at 23°C before assaying

The enzyme was the most stable when held at pH 6.5–8.0, retaining 75% of its initial activity at pH 9.0 (Fig. 2).

Effect of temperature on lipase activity and stability

The crude lipase from *Geotrichum*-like R59 exhibited two optimal activities: at 37°C and 50°C, suggesting that this strain produces multiple forms of lipase with different optimum temperatures. This is similar to the report on lipase production by *G. candidum* strain, where the optimum temperatures of lipase I and II were 30°C and 40°C, respectively [25].

Lipase from *Geotrichum*-like R59 species was shown to have good thermostability, with full activity after 1 h incubation at 60°C. It retained 50% of its initial activity when heated at 70°C for 45 min. The enzyme from *Bacillus alcalophilus* was thermostable at 60°C and retained 70% of its activity after incubation for 60 min at 75°C [9]. The thermostability was similar to other reported lipases from bacteria—from *Bacillus* J 33 [16] and from *Pseudomonas fluorescens* NS2 W [12]. On the contrary, mainly lipases with only moderate thermostability have been found in different strains of fungi. Lipase from *Penicillium wortmanii* was stable at 40°C and retained 20% of the initial activity after 60 min preincubation at 60°C [5]. The enzyme produced by *R. glutinis* was stable at 35°C, and at 60°C it retained 15% of its initial activity [19].

Geotrichum-like strain R59 isolated from soil is a very good producer of extracellular lipase, exhibiting very high lipolytic activity. In optimized growth conditions, this strain produced a 3-fold increase in lipase activity, when compared with non-optimized conditions. Although these observations will require confirmation in large-scale production, with its short fermentation time (48 h) and high thermostability, R59 could be a very useful industrial strain.

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