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Ginalska, Gryzyna
Marie Curie-Sklodowska University

Cho, Hee-Yeon
Dental Research Institute, School of Dentistry, UCLA

Cho, Nam-Seok
School of Eorest and Rural Engineering, Chungbuk National University

Bancerz, Renata
Marie Curie-Sklodowska University

他

<https://doi.org/10.5109/9276>

出版情報：九州大学大学院農学研究院紀要. 52 (1), pp.29-34, 2007-02-28. 九州大学大学院農学研究院
バージョン：
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Effect of Culture Conditions on Growth and Lipase Production by A Newly Isolated Strain, *Geotrichum*-like R59 (Basidiomycetes)

Gryzyna GINALSKA¹, Hee-Yeon CHO², Nam-Seok CHO³, Renata BANCERZ¹,
Teresa KORNŁOWICZ¹, Andrzej LEONOWICZ¹, Soo-Jeong SHIN³
and Shoji OHGA^{4*}

Laboratory of Forest Resources Management, Division of Forest Ecosystem Management,
Department of Forest and Forest Products Sciences, Kyushu University,
Sasaguri, Fukuoka 811–2415, Japan

(Received November 9, 2006 and accepted December 1, 2006)

Growth and production of lipase by a new isolant *Geotrichum*-like R59 were studied. Production of extracellular lipase was substantially enhanced when the initial pH of the culture medium, types of carbon and nitrogen sources, those of inducers of lipase biosynthesis, temperature and time of growth were optimized. Sucrose and triolein were the most effective compounds for lipase production. Maximum lipase production was obtained when urea was used as nitrogen source (146 U/ml). Temperature of 30 °C, the 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 R59 strain. The enzyme presented a good thermostability and still exhibited very high activity after 1 h incubation at 60 °C.

INTRODUCTION

Lipases are defined as glycerol ester *hydrolases* (EC3.1.1.3) hydrolyzing tri-, di- and mono-glycerides present at oil–water interface (Saxena *et al.*, 1999). Some lipases are also able to catalyze esterification, trans-esterification and enantioselective hydrolysis reactions (Nini *et al.*, 2001; Shintre *et al.*, 2002; Nagayama *et al.*, 2002; Piao *et al.*, 2003; Kui *et al.*, 2003; Raku *et al.*, 2003). The interest in microbial lipase production has increased in the last decades, because of its large potential in a wide range of industrial applications as additives in food processing (flavor modification), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), waste water treatment (decomposition and removal of oil substances), diagnostics, cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skins) and medical (blood triglyceride assay) (Sarkar *et al.*, 1998; Cardenas *et al.*, 2001; Elibol and Ozer, 2000; Kamini *et al.*, 2000).

Lipases are produced by many microorganisms (Kamimura *et al.*, 2001; Elibol and Ozer, 2000) and higher eukaryotes. Enzyme-producing microorganisms include bacteria (Kulkarni and Gadre, 2002), fungi (Fodiloglou and Erkmén, 1999; Shimada *et al.*, 1992), yeast (Corzo and Revah, 1999) and actinomycetes (Sommer *et al.*, 1997). Especially lipases from microor-

ganisms have drawn much attention for their potential use in biotechnology, mainly due to their availability and stability (Ghosh *et al.*, 1996; Wang *et al.*, 1995). The lipase from *Geotrichum candidum* is currently the subject of intense investigation and industrial interest because it shows a strong preference for fatty acids that contain a cis-9 double bond (Bertolini *et al.*, 1994; Jacobsen and Poulsen, 1995; Macedo *et al.*, 1997). Recently optimization of extracellular lipase production by *Geotrichum* sp. using factorial design was conducted (Burkert *et al.*, 2004). The response surface methodology was applied for the optimization of the nutrient concentrations in the culture medium for the enzyme production, at 30 °C. The optimum medium composition for lipase production by *Geotrichum* sp. was ammonium nitrate 2.1–2.5%, corn steep liquor 13–15% and soy oil 0.6% as carbon source, which lead to a lipase activity of about 20 U/ml. Using olive oil as carbon source, the optimum composition was ammonium nitrate 0.8–1%, corn steep liquor 13–15% and olive oil 0.6%, leading to an activity of 17 U/ml.

The aim of this study was to determine the culture conditions for maximum lipase production by a newly isolated *Geotrichum*-like R59 (Basidiomycete) and to characterize the lipase enzyme. Medium composition, initial pH, temperature and time of incubation were examined for optimization of the production of lipase. Lipase was characterized with respect to the optimal temperature and pH, as well as to its stability.

MATERIALS AND METHODS

Isolation of fungus

The new strain of *Geotrichum*-like R59 (Basidiomycetes) was isolated from black soil (pH 7.6, 50–60% of humidity), by a method of trapping soil microorganisms on daunomycine-rich pulp, buried in caprone bags for the period of 3 months. The trapped

¹ Department of Biochemistry, Marie Curie-Skłodowska University, Lublin 20–031, Poland

² Dental Research Institute, School of Dentistry, UCLA, LA, California 90095, USA

³ School of Forest Resources and Rural Engineering, Chungbuk National University, Cheongju 361–763, Korea

⁴ Dept. of Forest and Forest Products Science, Kyushu University, Sasaguri, Fukuoka 811–2415, Japan

* Corresponding author (E-mail: ohgasfor@mbx.nc.kyushu-u.ac.jp)

organisms were sieved on agarized medium consisting of 1.5 g/l of KH_2PO_4 , 300 ppm of streptomycin and 10% of daunomycin-rich solid material in a dispersed form as main source of carbon and nitrogen. After three-week growth, only one place on a number of Petri dishes was decolorized and the fungal mycelium was isolated from this place. After isolation, the fungus was cultivated on agarized GZ medium (spud 300 g/l; glucose 20 g/l; agar 20 g/l).

Growth media and culture conditions

Basal GKM medium for lipase production contained: 10 g/l glucose; 6 g/l KH_2PO_4 ; 1 g/l $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$; 4 g/l urea; 10 mg/l $\text{FeCl}_3 \times 4 \text{ H}_2\text{O}$; 8 $\mu\text{g/l}$ d-biotin; 4 $\mu\text{g/l}$ myo-inositol; 200 $\mu\text{g/l}$ thiamin and 10 g/l olive oil (Ota *et al.*, 1968). The pH of the medium was adjusted to 6.0 by addition of KOH. The culture medium without urea and vitamins was sterilized at 121 °C for 25 min. After cooling, the urea and the vitamins were added to the medium.

To observe the effect of the initial pH of GKM medium on lipase production, pH of the medium varied from 3.0–11.0. While studying the effects of inducers, olive oil was replaced by similar concentrations of other inducers (fatty acids, triacylglycerols and oils). Next glucose was replaced by other sugars (maltose, galactose, xylose, fructose, lactose, sucrose and starch at concentrations of 1%) for the effect of carbon source. Then to test the effect of different nitrogen source on lipase production, 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%).

The flasks containing 25 ml of medium were inoculated with 1 ml suspension of *Geotrichum*-like mycelium, grown earlier in the basal medium and cultured for 2 days at 30 °C on rotary shaker operating at 150 rpm.

Lipase and protein assays

Lipase activity was determined by the method of Sokolovska *et al.* with some modifications. 0.5 ml of lipase solution were mixed with 1 ml 50 mM phosphate buffer pH 7.0 and 1.5 ml of tributyrin in 25 ml Erlenmeyer flasks. After 60 min. incubation at 37 °C the emulsion was mixed with a 1 ml acetone: ethanol mixture (1:1) to extract free fatty acids. Finally, their concentration was determined by titration (0.05 N KOH) using phenolphthalein as indicator. One unit (U) of lipase activity is defined as amount of the enzyme releasing 1 μmol of free fatty acids per minute under assay conditions.

Protein concentration was estimated by the method of Lowry with bovine serum albumin as a standard (Lowry *et al.*, 1951).

Effect of the initial pH, temperature and time of incubation

The effect of temperature and time of incubation on growth and lipase production was studied in the opti-

mized medium. For selection of the optimum temperature for the production of lipase, the temperature of the culture varied from 10–40 °C. The effect of incubation time on lipase production was studied in shake flasks incubated for 6 days.

Characterization of lipase

Geotrichum-like fungus was grown in the optimized medium for 48 h at 30 °C and the mycelium was removed by centrifugation at 12,000 rpm for 15 min at 4 °C. The temperature optimum for the activity of the crude enzyme was evaluated using lipase activity assay with tributyrin at different temperatures. The activity assay was performed at pH 7.0. Enzyme stability at various temperatures was studied by incubating lipase within the range of 30–90 °C for 30 and 60 min each, followed by activity estimation at 37 °C.

Lipase activity was estimated following pH values within the range pH 3.0–10.0 to determine the optimal pH. The substrate specificity towards different triacylglycerols and oils was analyzed by titration method as described above.

RESULTS AND DISCUSSION

Identification and characterization of *Geotrichum*-like fungus

The investigated strain R59 is ranked among quick growing fungi: after a 5-day growth on GZ medium, it forms colonies reaching a 9-cm diameter. These hyaline fungi are characteristic of a good development of the surface mycelium, and of a fibrous structure. Microscopic micromorphological studies of the colony showed strain R59 to produce forked ramifications of vegetative hyphae and arthroconidia formed by hypha fragmentation. Having considered that the aforesaid characteristics bring this strain closer to genus *Geotrichum* (Domsch *et al.*, 1980), belonging to imperfect fungi (subgroup *Deuteromycotina*, class *Hyphomycetes*), it was initially defined as *Geotrichum*-like. There was found an essential morphological difference between typical representative of *Geotrichum*, i.e. *Geotrichum candidum*, and strain R59 in distinct character and structure of their colony. The strain we isolated from the soil forms typical mold colonies consisting of a high, relatively loose and well-developed mycelium, in contrast with *Geotrichum candidum* forming flat yeast-like colonies with a highly reduced vegetative surface mycelium, hoarfrost covered mass conidia.

Effect of initial pH of the culture medium

Fig. 1 shows the effect of the pH of medium on lipase production. The maximum lipase activity was obtained when the initial pH of the GKM medium was 6.0, although pH 3.0–9.5 favored lipase production. However, lipase production dropped significantly at pH 9.5. No production of lipase was observed at pH 9.5–11.0. The lipase from *G. candidum* (Baillargeon *et al.*, 1989) showed the highest activity when the initial pH of the medium was adjusted to pH 7.0. On the con-

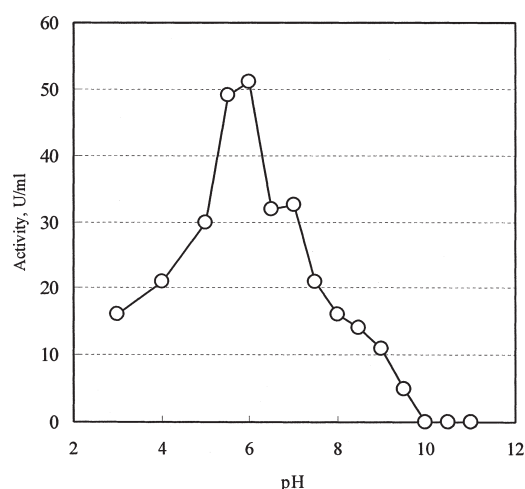


Fig. 1. Effect of pH medium on lipase production by *Geotrichum*-like grown on GKM medium.

trary, lipase production from the newly isolated strain of *Geotrichum* sp. was maximum at pH 5.0 (Macedo *et al.*, 1997).

Effect of inducers

Among various oils, triglycerides and fatty acids tested at concentrations of a 1%, triolein allowed for maximum lipase production ~91 U/ml (Table 1.). A similar result was reported for synthesis of lipase from *Cryptococcus* sp. S-2 (Kamini *et al.*, 2000). Rape oil, soybean oil and olive oil were also proved to be effective inducers for lipase production by *Geotrichum*-like R59 (lipase activity were 82.4 U/ml, 71 U/ml and 69 U/ml, respectively). For lipase production, soybean oil was also used from strain *Geotrichum candidum* ATCC 34614 (Baillargeon *et al.*, 1989). In the presence of triglycerides with saturated short-chain fatty acids (tributylin and tricapylin) *Geotrichum*-like species showed little production of lipase (6.5 U/ml and 3.0 U/ml, respectively). Saturated short-chain caprylic acid did not induce lipase production. These results were in accordance with studies on *Geotrichum candidum* ATCC 34614 (Shimada *et al.*, 1992).

Table 1. Effect of inducers on lipase production

| Inducers | Activity [U/ml] | Protein [mg/ml] |
|---------------|-----------------|-----------------|
| Control * | 10.0 | 0.30 |
| Tributylin | 6.5 | 0.28 |
| Tricaprylin | 3.0 | 0.40 |
| Triolein | 91.0 | 0.31 |
| Soybean oil | 71.0 | 1.20 |
| Rape oil | 82.4 | 1.30 |
| Corn oil | 40.0 | 0.76 |
| Olive oil | 69.0 | 0.24 |
| Oleic acid | 13.5 | 0.40 |
| Caprylic acid | 0 | 0.26 |

*control-without inducers

Influence of carbon source on lipase production

In GKM medium, glucose was replaced by other carbon sources. These carbohydrates were optimized at concentrations of 1%. *Geotrichum*-like strain grew and produced lipase on all tested medium. The best result was observed for sucrose present in the medium (Table 2).

Table 2. Effect of carbon source on lipase activity

| Carbon source [1%] | Activity [U/ml] | Protein [mg/ml] |
|--------------------|-----------------|-----------------|
| Control * | 28.0 | 0.23 |
| Lactose | 29.5 | 0.19 |
| Maltose | 24.5 | 0.19 |
| Starch | 30.0 | 0.12 |
| Sucrose | 118.0 | 0.47 |
| Xylose | 26.0 | 0.57 |
| Glucose | 92.4 | 0.40 |
| Fructose | 1.0 | 0.16 |
| Galactose | 82.0 | 0.34 |

*control-without carbohydratess

The activity of lipase synthesized under these conditions was of 118 U/ml. Glucose and starch were also good carbon source for growth and production of lipase. However, Baillargeon *et al.* (1989) showed that lipase production was not detected in the medium containing glucose or sucrose. The lowest lipase activity was obtained, when adding fructose to the basal medium (only 1 U/ml). In contrast, fructose was reported to be the best carbohydrate for production of extracellular lipase by *Rhodotorula glutinis* (Papaparaskevas *et al.*, 1992). Fig. 2 showed data for lipase production for the range of sucrose concentration tested in the experiment. The highest level of lipase activity was obtained after using sucrose at a concentration of 0.5% (146 U/ml). When higher concentrations of sucrose were added to GKM medium, lipolytic activity decreased.

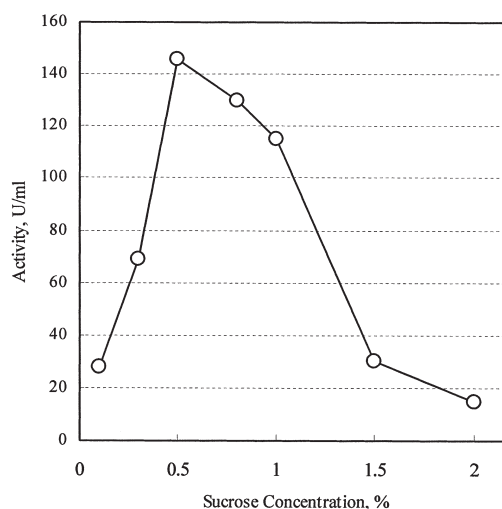


Fig. 2. Lipase production by *Geotrichum*-like R59 at various concentration of sucrose.

Effect of nitrogen source on lipase production

The effect of nitrogen source was tested in the GKM medium by removing urea and replacing it with selected nitrogen sources (both organic and inorganic). As shown in Fig. 3, the best result was observed for urea at a concentration of 0.4% (lipase production improved to 146 U/ml). Generally, microorganisms provide high yields of lipase when organic nitrogen is used. *Yarrowia lipolytica* produced extracellular lipase in a medium containing urea at a concentration of 0.2% (Corzo and Revah, 1999). Salleh *et al.* (1993) obtained maximal production of extracellular lipase by fungi *Rhizopus oryzae*, when the medium contained peptone as nitrogen source. Among other organic nitrogen sources tested (peptone, yeast extract, casein and asparagine), none increased lipase production significantly (in each case lipase activity was below 30 U/ml).

The inorganic nitrogen sources (see Fig. 3) showed an inhibitory effect on lipase production. Similar results have been reported by Sarkar *et al.* (1998).

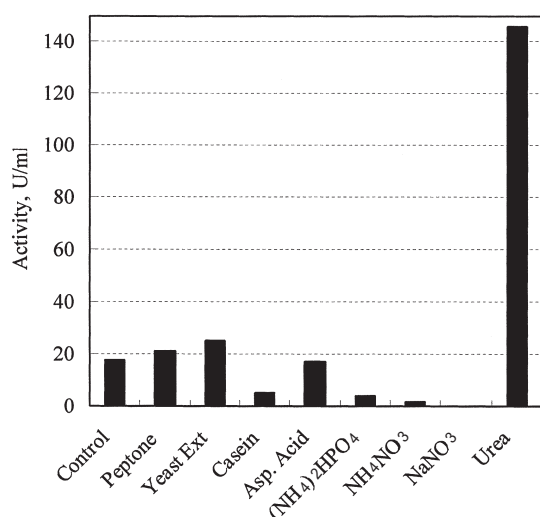


Fig. 3. Effect of nitrogen source on lipase activity.

Effect of the temperature and time of incubation on lipase production

Table 3 shows the effect of temperature and incubation time on lipase production. *Geotrichum*-like strain was grown at temperatures from 5 to 40 °C on optimized

GKM medium. The best conditions for lipase production and cell growth were 30 °C and 48 h of incubation (the total lipase activity and the biomass were maximum—150 U/ml and 1.04 g/l, respectively). The results of biomass were affected principally by the temperature and time of incubation. Similar results were reported for *Geotrichum* sp., which was incubated at 30 °C for 48 hours (Macedo *et al.*, 1997). Baillargeon *et al.* (1989) also reported that maximal lipase activity from *Geotrichum candidum* ATCC 34614 was found at 30 °C, but after 24 hours. *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). *Geotrichum*-like fungus was also grown at a temperature of 5 °C, but maximum lipase production was observed after 10-day incubation (30 U/ml) – data not shown.

Enzyme characterization

A comparison of the results of hydrolysis of several triglycerides and oils showed tributyrin to be the best substrate for *Geotrichum*-like extracellular lipase (Fig. 4). Lipase activity against tributyrin was taken for 100%. Lipase was more active on synthetic triacylglycerides (tributyrin, tricaprylin and triolein) than on natural oils. A high relative activity of lipase was observed for tricaprylin (70%). Lipase from *Yarrowia lipolytica* 681 (Corzo and Revah, 1999) and *Bacillus thermoleovorans* ID-1 (Lee *et al.*, 1999) also showed the greatest activity for tricaprylin as substrate.

Temperature and pH optimum were detected in the presence of tributyrin as substrate. The optimum pH for lipase activity was examined in the range of pH 3.0–11.0. The lipase activity estimation at different pHs showed the optimum pH of lipase from *Geotrichum*-like R59 to be pH 7.0. As seen on Fig. 5, lipase activity decreased significantly above pH 8.0. The results were in accordance with studies on *Yarrowia lipolytica* 681 (Corzo and Revah, 1999). Conversely, crude lipase from *Geotrichum* sp. (Macedo *et al.*, 1997) was most active between pH 7.5–9.0.

Fig. 6 showed that the crude lipase from *Geotrichum*-like R59 exhibited two optimal activities: at 37 °C and 50 °C. The results suggested that this strain produced multiple forms of lipase with different optimum temperatures. This is similar to the report on

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

| Day of incubation | Growth temperature | | | | | | | | | |
|-------------------|--------------------|---------------|-----------------|---------------|-----------------|---------------|-----------------|---------------|-----------------|---------------|
| | 18 °C | | 25 °C | | 30 °C | | 35 °C | | 40 °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] | Activity [U/ml] | Biomass [g/l] |
| 1 st | 5.0 | 0.63 | 10.0 | 0.80 | 32.0 | 0.89 | 59.0 | 0.89 | 25.0 | 0.83 |
| 2 nd | 8.0 | 0.71 | 17.0 | 0.82 | 150.0 | 1.04 | 75.0 | 0.95 | 53.0 | 0.97 |
| 3 rd | 24.5 | 0.74 | 34.0 | 0.82 | 82.0 | 0.91 | 49.0 | 0.92 | 31.0 | 0.95 |
| 4 th | 41.0 | 0.77 | 85.0 | 0.65 | 61.0 | 0.79 | 29.0 | 0.69 | 25.0 | 0.89 |
| 5 th | 30.0 | 0.79 | 76.0 | 0.63 | 28.0 | 0.79 | 19.0 | 0.89 | 13.0 | 0.72 |
| 6 th | 24.0 | 0.97 | 43.0 | 0.55 | 10.0 | 0.75 | 8.0 | 0.70 | 6.0 | 0.71 |

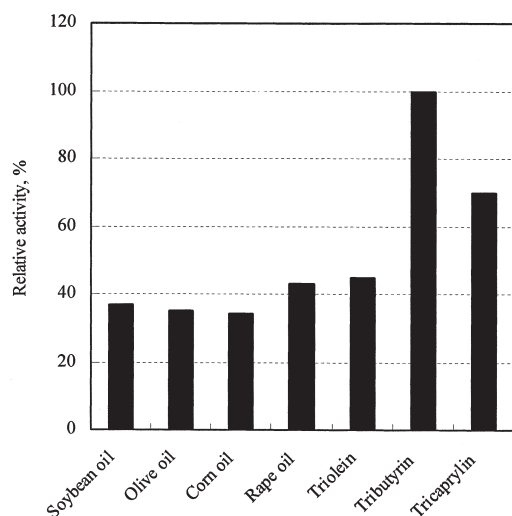


Fig. 4. Substrate specificity of lipase from *Geotrichum*-like R59.

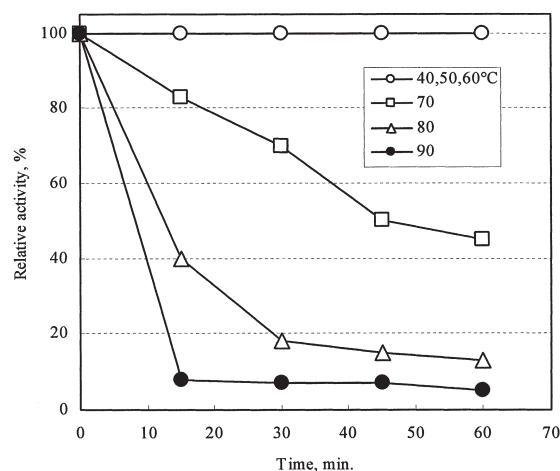


Fig. 7. Thermostability of lipase.

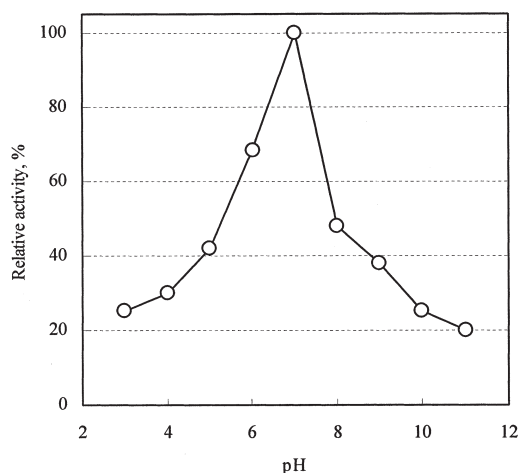


Fig. 5. Effect of pH on lipase activity.

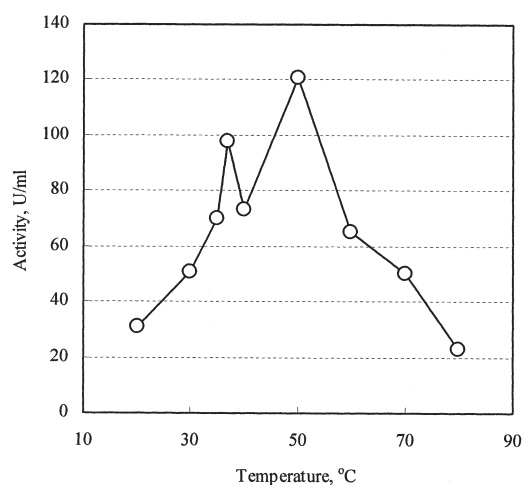


Fig. 6. Effect of temperature on lipase activity.

lipase production by *Geotrichum candidum* strain, where the optimum temperatures of lipase I and II were 30°C and 40°C, respectively (Sugihara *et al.*, 1990).

Fig. 7 shows that lipase from *Geotrichum*-like species presented a good thermostability. The enzyme appeared to be stable and fully active after incubation at 60°C. It retained 50% of its initial activity when heated at 70°C for 45 min.

It is noteworthy that *Geotrichum*-like strain R59 isolated from soil is a very good producer of extracellular enzyme exhibiting a very high lipolytic activity. Especially owing to its short period of growth (48 h) and high thermostability, it seems to be of a high biotechnological significance. With respect to the enzymatic processing of lipids, thermostable lipase from different strains seems very useful in industry and lipase-producing microorganisms can also be used to develop the process of lipid hydrolysis.

CONCLUSIONS

This study was carried out to determine the culture conditions for maximum lipase production by a newly isolated *Geotrichum*-like R59 (Basidiomycete) and to characterize the enzyme. Medium composition, initial pH, temperature and time of incubation were also examined for optimization of the production of lipase. Lipase was characterized with respect to the optimal temperature and pH, as well as to its stability.

Geotrichum-like strain R59 isolated from soil was confirmed is a very good lipase producer of extracellular enzyme. Sucrose and triolein were the most effective compounds for lipase production. The maximum lipase activity was obtained when the initial pH of the GKM medium was 6.0, although pH 3.0–9.5 favored lipase production. Among various oils, triglycerides and fatty acids tested at concentrations of a 1%, triolein allowed for maximum lipase production. Maximum lipase production was obtained when urea was used as nitrogen source (146 U/ml). Temperature of 30°C, the initial pH

of 6.0 and incubation time of 48 h were found as optimal conditions for cell growth and production of lipase by *Geotrichum*-like R59 strain.

The crude lipase from *Geotrichum*-like R59 exhibited two optimal activities: at 37 °C and 50 °C. The results suggested that this strain produced multiple forms of lipase with different optimum temperatures. The enzyme appeared to be a very good thermostability and fully active even after 1 h incubation at 60 °C.

ACKNOWLEDGEMENT

This work was supported by the Polish Science Committee BS/BiNoZ/4 and European Community (Contract ICA2-CT-2000-10050) and the Polish Committee for Scientific Investigations 139/E-339/SPUB-M-5PR-UE/DZ 280/200.

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