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Isolation and Purification of Lipases from Geotrichum Candidum Grown on a Sunflower Oil Waste as a Carbon Source

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An isolation of extracellular lipases from the endemic fungal strain Geotrichum candidum-M2 was performed by vacuum evaporation, precipitation with cold acetone and vacuum drying. The lipases were produced by submerged fermentation on a medium containing sunflower oil waste in a concentration of 10 mL/L. The isolation of lipases was performed at the 48th h of fermentation when the process of cell autolysis has already been started. Almost 17 fold increase of the total enzyme activity and 2 fold increase of the specific activity was achieved when concentrating the enzyme solution from 10.74 mg proteins per liter (in the supernatant) to 106.01 mg proteins per liter (in buffered solution of the previously precipitated enzyme). The crude enzyme preparation was further purified by an ion exchange chromatography using the Q-Sepharose FF resin and the hydrophobic interactive resin Phenyl-Sepharose Cl-4B. When purifying the enzyme preparation with the anion exchange resin Q-Sepharose FF, a lipolytic activity was detected only in the fractions representing the last two peaks with values of 0.27 and 0.20 U/mL. When those two collected fractions were further purified on the hydrophobic interactive resin Phenyl-Sepharose Cl-4B, an enzyme activity was detected only in the fractions representing the second peak, with a value of 0.22 U/mL. The factor of purification that characterized the whole purification procedure was 11.27. The purified enzyme preparation was stable in a broad temperature interval from 30-80 °C showing highest activity at 50 °C. At 80 °C, the enzyme preparation derived from solution with pH=9, showed activity of 0.15 U/mL. Those properties indicate that isolated lipases are especially suitable for usage in bioremediation processes and in detergent formulations.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) comprise a group of enzymes able to catalyze both the hydrolytic and the synthetic esterification reactions and are applicable in water, water organic two-phase and organic mono-phase media, equally Those enzymes have many applications, starting from synthesis of specially created fine chemicals via the bioorganic synthesis (Persson et al., 2002), purification of oil polluted waste waters and soil (Sharma et al., 2001) and especially in biofuel production via esterification and interesterification reactions (Caetano et al., 2012). There are many reports on lipase production with some endemic microbial strains and subsequent isolation, purification and characterization of lipases. Bacteria (Sirisha et al., 2010), yeasts (Kebabci et al., 2012) and fungi from both Trichoderma (Ülker et al., 2012) and Aspergillus genera (Brooks et al., 2011) are all used equally as producers of lipases on some synthetic lipid containing media. However, there are scarce reports about utilization of some kind of biodegradable oil waste as a medium for production of lipases by the isolated endemic strain and on further isolation, purification and characterization of the produced lipases. In this work the isolation, purification and partial characterisation of lipases produced by the endemic yeast-like fungus Geotrichum candidum-M2 is described. The possibility for application of these novel lipases in bioremediation processes is also discussed.

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2. Materials and Methods

2.1 Sunflower oil waste
The sunflower oil waste was obtained from the edible oil factory „Blagoj Gorev" in Veles, R. Macedonia. It contained: 63 % fat, 3.90 % moisture, 2.59 % nitrogen and 30.51 % suspended solids.

2.2 Microorganism
The microbial strain encoded as M2 was identified in the Institute for Health Protection in Stip. The identification was performed on Mini API apparatus (BioMérieux, France). Strips and the biochemical tests were also supplied from BioMérieux, France. The microorganism was deposited at a Culture collection of the Faculty of Technology and Metallurgy in Skopje and was maintained at 4 °C on malt extract agar slants.

2.3 Preparation of culture media
The mineral medium had the following composition (g/L): KH_2PO_4 2, $MgSO_4x7H_2O$ 1, $CaCl_2xH_2O$ 1 and NaCl 1. It was supplemented with carbon and nitrogen sources: yeast extract (10 g/L) and sunflower oil refining waste used in a concentration of 10 mL/L. The medium was sterilized at 121 °C for 35 min.

2.4 Production of lipases
A l0 mL portion of seed culture (3.5 x 10^6 spores/mL) was inoculated into 500 mL Erlenmeyer flasks containing 100 mL of the culture medium and stirred on a rotary shaker at 170 rpm. Initial pH was adjusted with HCI and NaOH solutions. At the end of the cultivation, the culture broth was filtered and centrifuged at 4,000 rpm for 50 min. The growth of the fungus was determined gravimetrically by drying the biomass at 105 °C. The supernatant was used as a source of crude enzymes for estimation of lipolytic activity.

2.5 Determination of the lipolytic activity
The lipase activity was measured by the method of Kwon and Rhee (1986). One unit (1U) of lipolytic activity is the amount of enzyme, which liberates 1 μmol free fatty acids per minute under the assay conditions.

2.6 Isolation of lipases
The supernatant obtained with the centrifugation of the cultural liquid was filtrated and vacuum evaporated at the temperature of 35-40 °C with an aim to achieve a concentrated enzyme solution. The precipitation of the proteins with a cold acetone was performed with a volume ratio acetone: enzyme solution 4:1. The precipitation was performed at the temperatures below 0 °C, in order to prevent the enzyme denaturation, since the reaction was an exothermic one. After the precipitation, the acetone layer together with the precipitated enzyme, were filtrated through a Buchner funnel and the drying continued in a desiccator. Than the enzyme preparation was pulverised and diluted in 8 mL pyridine buffer with pH=5.

2.7 Purification on an ion-exchange chromatography
For the enzyme purification in a first phase, an anion exchange chromatographic resign Q-Sepharose was used, with following characteristics: height H=15.5 cm, diameter D = 1.5 cm, gel volume 27.4 cm^3, buffer 10 mM pyridine buffer pH 5, flow rate of 100 mL/h, fraction volume of 3 mL. The gel was equilibrated with the basic buffer at 20 °C in a duration of 30 min. Elution was performed with a linear gradient of NaCl in the basic buffer (0-0.3 M).

2.8 Purification on a hydrophobic-interactive chromatography
For the enzyme purification in a second phase, a hydrophobic-interactive chromatographic resign Phenyl-Sepharose Cl-4B was used, with following characteristics: height H=14 cm, diameter D=1.5 cm, buffer 50 mM phosphate buffer pH = 7, flow rate of 120 mL/h, fraction volume of 8 mL. The gel was equilibrated with the basic buffer at 20 °C in duration of 30 min. The elution was performed with a linear gradient of a phosphate buffer without NH_4SO_4 and a gradient obtained with combinations of the buffer and a 30 % isopropanol solution.

2.9 Partial characterisation
The pH and thermostability of the purified enzyme preparation was determined by measuring the residual enzyme activity in the reaction media with certain pH and at certain temperatures for different durations.
All experimental data are obtained from, at least, 3 replicates of a run, and the values were estimated as an average value of all replicates of a run.

3. Results and Discussion

3.1 Isolation and precipitation of the enzymes
The isolation of the extracellular lipases produced via submerged fermentation of the endemic strain identified as Geotrichum candidum penicillatum, was performed by using a several step procedure. At the beginning of the isolation process a proper duration of the fermentation process was selected. This step

was very important, not only for achieving highest product yield, but also to establish an uncomplicated isolation process. When the time course of the biomass and lipase production was studied, it has been seen that the maximal lipolytic activity of the fungus, grown on the medium with 10 mL/L sunflower oil waste, was highest at the 24th h of cultivation (Mladenoska and Dimitrovski, 2013). However, in this work, the duration of the fermentation process of 48 h has been chosen because by that time the sunflower oil waste was almost completely spent and the cultural liquid obtained was relatively clear. The pH value of the medium measured at the 48 h of cultivation was 6.0, which indicated that the cell autolysis of the fungus started and that the cell-bound enzymes were also released in the cultural liquid. The flow chart of the isolation process is presented in Figure 1.

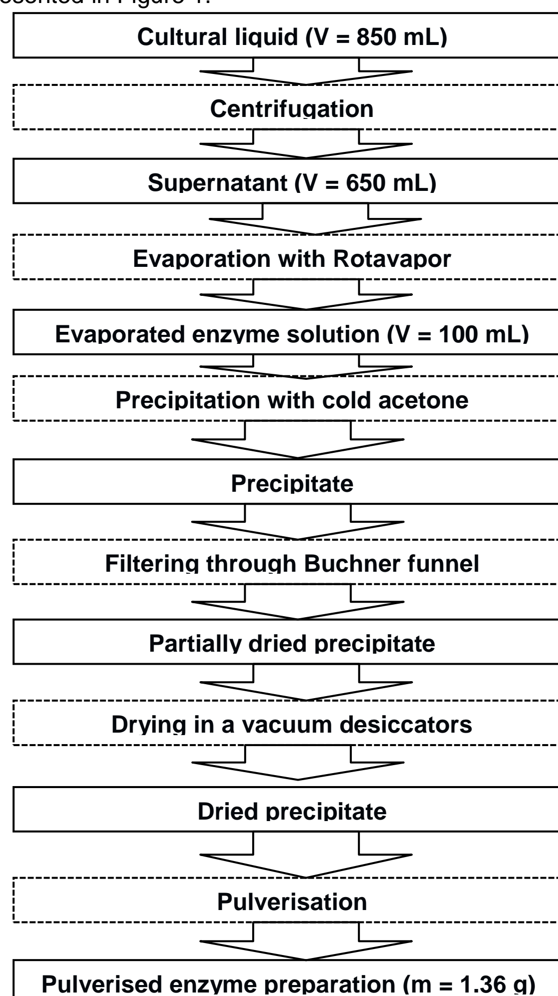

Figure 1: Flow chart of the isolation of lipases produced by G. candidum-M2

This several step isolation process resulted in achievement of three different enzyme preparations: the centrifuged enzyme preparation, the evaporated enzyme solution and the precipitated enzyme dissolved in a buffer. The total and the specific lipolytic activity of the different enzyme preparations are presented in the Table 1. When the specific lipolityc activity of the enzyme preparation obtained after centrifugation of the cultural liquid was compared to that of the preparation achieved by the subsequent evaporation, an increase of the enzyme activity of the second one of more than 2 times, has been noticed. It can be speculated that this increase of the activity was a result of the enzyme activation due to the temperature increase during the evaporation process. Namely, when the determination of the optimal temperature has been determined (data not shown) it was shown that the enzyme was very active at relatively high temperatures in the interval between 40 and 50 °C. However, the specific activity of the precipitated enzyme dissolved in a buffer was 1.2 times lower than the specific activity of the evaporated enzyme preparation. It can be assumed that the reason behind this phenomenon is the denaturation of the enzyme that appeared during the precipitation process by the very hydrophilic solvent acetone. Since the

concentration of proteins in the evaporated enzyme solution was 49.97 mg/mL, theoretically, the achieved protein yield during the precipitation and the drying processes was calculated to be 4.4 g proteins.

Table 1: Lipolytic activity of the different types of enzyme preparations achieved via the isolation process (maximal values for the experimental errors were: total activity U/mL±0.01, prot. conc. mg/mL±0.02)

Enzyme preparation	Lipolytic activity (U/mL)	Protein concentration (mg/mL)	Specific lipolyticactivity (U/mg)
Centrifuged	0.60	10.74	0.0056
Evaporated	5.00	43.97	0.113
Precipit. & dissolved	10.00	106.00	0.094

But, at the end of the precipitation and the drying processes, the amount of the powdered enzyme preparation of only 1.36 g was achieved, which means that the loss of the precipitation and the drying processes were even 69.10 %. As a possible explanation of this loss a very sticky nature of the enzyme preparation, that is not easily removable from the walls of the precipitation vial, might be pointed out. Besides the mass loss, during the precipitation process, loss due to the enzyme denaturation, was also present. The regeneration of the enzyme activity, after the precipitation with acetone, was 83.18 %.

3.2 Purification with ion-exchange chromatography

For further purification of the produced fungal lipases, the anion exchange resign Q-Sepharose FF was used. This exchanger was very stable in broad pH interval from pH=2 till pH=12 and compact enough to sustain high flow rates above 100 mL/h. The enzyme solution that was used for purification procedure was obtained by filtration and centrifugation of the cultural liquid. The concentration of the proteins of this solution was 10.90 mg/mL and the lipolytic activity was 0.50 U/mL. The flow rate of 100 mL/h was used. Elution was performed by utilisation of a linear gradient of NaCl in the basic buffer with a concentration between 0 and 0.3 M. The graph of the enzyme purification on the anion exchange resign Q-Sepharose FF is presented at the Figure 2.

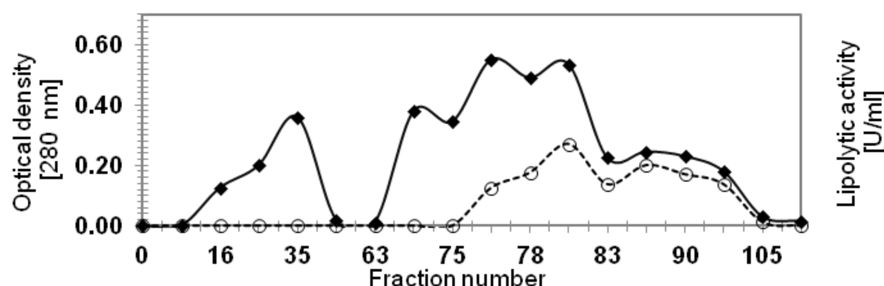

Figure 2: The chromatogram of the enzyme purification on the anion exchange resign Q-Sepharose FF. The optically active protein fractions (-■-) were evaluated regarding their lipolytic activity (-o-)

With this purification process 118 fractions were eluted, each in quantity of 3 mL. Fractions with optical density detected with the UV detector were examined for their lipolytic activity. The results from this experiment are presented in the Table 2.

Table 2: Selection of the lipolyticaly active fractions eluted on the Q-Sepharose FF resign (maximal values for the experimental errors were: total activity U/mL±0.01, prot. conc. mg/mL ± 0.02)

Peak number	Nu. of fractions	Total volume (mL)	Protein concentration (mg/mL)	Lipolytic activity (U/mL)
First (1/2)	12-26	42	/	0
First (2/2)	27-34	27	/	0
Second	63-75	36	/	0
Third	76-78	9	/	0
Forth	79-83	15	7.17	0.27
Fifth	84-110	81	4.30	0.20

From the results shown at the Figure 3 and in the Table 2, it can be noticed that the first wide peak was representing the non-lipolytically active fractions (12-34) that were, probably, some high molecular weight proteins. A lipolytic activity was detected only in the two last peaks and had values of 0.27 U/mL for the first peak and 0.20 U/mL for the second peak, respectively. The appearance of two peaks with lipolytic

activity might indicate an existence of two different lipases, which might be confirmed by an electrophoresis technique. Those lipolitycally active fractions were collected and were subject of further purification with hydrophobic-interactive resign. The total quantity of proteins in these lipolytically active fractions was 455.81 mg, which means that almost the half of the total proteins belonged to the lipases.

3.3 Purification with hydrophobic-interactive chromatography

The total volume of the lipolytically active fractions that were further purified with the hydrophobic interactive chromatography with the Phenyl Sepharose Cl-4B resign was 75 mL (fractions 79-110). The phosphate buffer with pH=7 containing 1M NH_4SO_4 was used as a basic buffer. The elution was performed with a linear gradient of a phosphate buffer without NH_4SO_4 and a gradient obtained with combinations of the buffer and a 30 % isopropanol solution. The chromatogram is shown on the Figure 4.

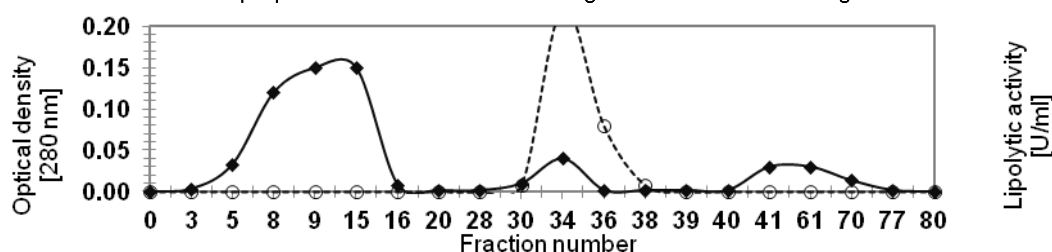

Figure 3: A chromatogram of the purification on the Phenyl Sepharose Cl-4B resign. The optically active protein fractions (-■-) were evaluated regarding their lipolytic activity (-o-)

With this purification phase 83 fractions were obtained, each with a volume of 8 mL. The results from the analysis of the lipolytic activity and the protein concentration of the eluted fractions are presented in the Table 3. Lipolytic activity was detected only in the fractions representing the second peak.

Table 3: Selections of the lipolyticaly active fractions eluted on Phenyl Sepharose Cl-4B resign (maximal values for the experimental errors were: total activity U/mL±0.01, prot. conc. mg/mL±0.02)

Peak number	Nu. of fractions	Total volume of the fractions (mL)	Protein concentration (mg/mL)	Lipolytic activity (U/mL)
First (1/2)	8-11	32	/	0
First (2/2)	12-15	32	/	0
Second	29-34	48	0.43	0.22
Third	39-68	224	/	0
Forth	69-83	128	/	/

3.4 Balance of the whole purification procedure

In the Table 4 the lipolytic activity and the protein concentration of the purification products obtained from the cultural liquid of Geotrichum candidum M2, via the whole purification processes are presented. The results obtained for the enzyme yield and the purification factor of each purification step are also shown.

Table 4: Lipolytic activity (total and specific), yield and purification factor of the separate purification phase (maximal values for the experimental errors were: total activity U/mL±0.01, prot. conc. mg/mL±0.02)

Purification step	Total activity (U)	Total proteins (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Centrifuged	50.00	1,090	0.046	100	1
Q-Sepharose	24.25	455.80	0.052	96	1.15
Phenyl Sepharose	20.91	10.80	0.510	48	11.27

From the results presented in the Table 4 it can be concluded that via the whole purification process 11 fold purification was achieved with 48 % yield and more than 11 times increase in the lipolytic activity. For comparison, when Lee and his co-workers (2003), have purified the cold-active lipase from psychrotrophic Aeromonas sp. LPB 4 strain, with acetone precipitation and chromatographic purification on QAE-Sephadex resign, 53.5 fold purification was achieved, but with a yield of only 7.5 %.

3.5 Preliminary partial characterisation

The purified enzyme preparation was stable in a broad temperature interval from 30-80 °C showing highest activity at 50 °C.

The enzyme derived from the solution with initial pH 9 showed highest activity of 0.36 U/mL for a duration of 60 min, which is the highest activity expressed among the all three enzyme preparations examined. This enzyme was also very stable at 80 °C, showing activity of 0.145 U/mL for duration of 90 min. The residual activity of this enzyme was even 40.29 %, when compared to the activity that this enzyme had at 50 °C for duration of 60 min.

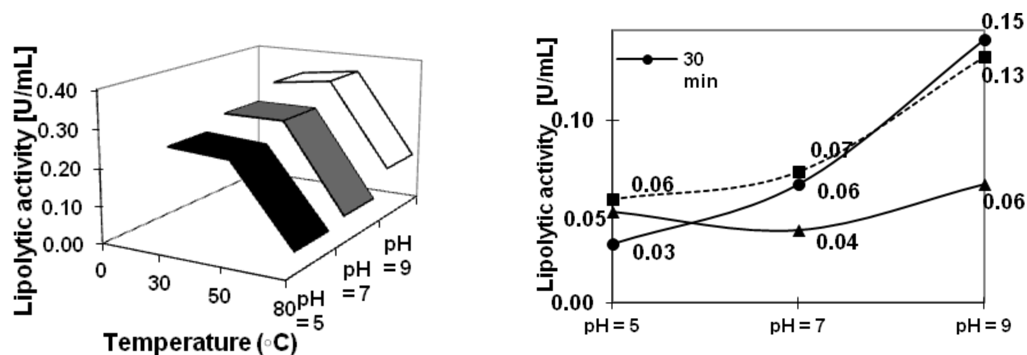

Figure 4: Activity of the lipases derived from solutions with different initial pH in duration of 60 min (left) and for different durations at a temperature of 80 °C (right)

4. Conclusion

A successful method for isolation and purification of extracellular lipases, produced by the newly isolated Geotrichum candidum penicillatum strain, grown on a sunflower oil waste, was developed. The enzyme preparation after the whole purification process was more than 11 times purified and with more than 11 times increased specific lipolytic activity compared to the purity and activity of the enzyme preparation obtained with only centrifugation. The preliminary experiments for the pH and thermostability of the obtained enzyme preparations showed that the isolated lipases were very active at relatively high temperatures, having highest activity at 50 °C. The enzyme isolated from the solution with initial pH=9 was the most active at this temperature and showed activity as high as 0.36 for a duration of 60 min. These characteristics make those enzymes particularly suitable for application in bioremediation processes and as components of detergent formulations. Further characterisation of isolated lipases is yet to be performed.

References

Brooks A. A., Asamudo N. U., 2011, Lipase production by strains of Aspergillus species isolated from contaminated body creams, J. Toxicol. Environ. Health Sci. 3, 311-316.

Caetano N., Teixeira J.I.M., Mata T.M., 2012, Enzymatic catalysis of vegetable oil with ethanol in the presence of co-solvents, Chemical Engineering Transactions, 26, 81-86.

Kebabci Ö., Cihangir N., 2012, Comparison of three Yarrowia lipolytica strains for lipase production: NBRC 1658, IFO 1195, and a local strain. Turk. J. Biol., 36, 15-24.

Kwon D. Y., Ree J. S., 1986, A simple and rapid colorimetric method for determination of free fatty acids for lipase assay, JAOCS, 63, 89-91.

Lee H. K., Ahn M. J., Kwak S. H., Song W. H., Jeong B. C.,2003, Purification and Characterization of Cold Active Lipase from Psychrotrophic Aeromonas sp. LPB 4, The J. Microbiol., 41, 22-27.

Mladenoska I., Dimitrovski A., 2013, Microbial production of lipases on media containing vegetable oil waste: process development, Chemical Engineering Transactions, 34, 103-108.

Persson M., Mladenoska I., Wehtje E., Adlercreutz P., 2002, Preparation of lipases for use in organic solvents. Enzym. Microb. Techn., 31, 833–841.

Sharma R., Chisti Y., Banerjee U. C., 2001, Production, purification, characterization, and applications of lipases, Biotechnol. Adv., 19, 627–662.

Sirisha E., Rajasekar N., Narasu M. L., 2010, Isolation and Optimization of Lipase Producing Bacteria from Oil Contaminated Soils, Advan. Biol. Res., 4, 249-252.

Ülker S., Özel A., Çolak A., Karaoğlu Ş. A., 2011, Isolation, production, and characterization of an extracellular lipase from Trichoderma harzianum isolated from soil, Turk, J. Biol. 35, 543-550.