ABSTRACT

Lipases are serine hydrolases, defined as triacyl-glycerol acylhydrolases. They catalyze both hydrolytic and synthetic reactions showing substrate, regio- and stereo selectivity. The psychrophilic strain Pseudozyma antarctica, isolated in the cold conditions of the Antarctica, can produce a thermostable extracellular lipase. In this work we report the synthesis of an extracellular lipase from Pseudozyma antarctica. The strain was cultured in flasks and various parameters were monitored during the proliferation process. Several cultivation procedures were done in order to find optimal conditions. Further, activity of the crude lipase, its temperature and pH optimum were determined using a potentiometric method. We found temperature optimum at 60°C and pH optimum at pH 8.0. The determined specific activity of the crude lipase was 97.2 U/mg. Activity and protein content of the “crude” post cultivation liquid were also determined.

Keywords: Pseudozyma antarctica, lipase, Pseudozyma antarctica cultivation, lipase optimums, lipase activity.

INTRODUCTION

Lipases are serine hydrolases, defined as triacyl glycerol acylhydrolases (EC 3.1.1.3). Lipolytic enzymes currently attract a considerable amount of attention because of their unique characteristics: substrate specificity, regio-specificity and stereo-selectivity. Novel biotechnological applications have been successfully established using lipases in the synthesis of biopolymers and the production of biodiesel, agrochemicals, biosensors, enantiopure pharmaceuticals and flavour compounds. Unlike esterases, lipases can hydrolyze/synthesize esters of long chain fatty acids in organic medium [1]. The production of lipolytic enzymes has been performed by bacteria, fungi and yeasts. It was revealed that yeasts (Candida rugosa, Candida antarctica, Candida cylindracea, Yarrowia lipolytica) can produce such enzymes under different growing conditions and using different approaches to the fermentation process [2, 3]. Production of lipases by yeasts can be done starting from different raw materials (feedstock), and inducers for increase of lipase activity [3 - 6]. The strain Pseudozyma antarctica, isolated in the cold conditions of the Antarctica, can produce thermostable extracellular lipases [7]. Many methods and techniques for optimization and obtaining of higher yields of lipase are reported [1, 3, 5, 6].

In this work we report the synthesis of extracellular lipase from Pseudozyma antarctica NBIMCC 8340 strain.

EXPERIMENTAL

Microorganism

Pseudozyma antarctica NBIMCC 8340 (Candida antarctica) was obtained from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC). The strain is included in the Bulgarian National Collection. The strain was isolated in 1997 from the Livingston
Island, Bulgarian Base, Antarctica. Stock cultures were cultivated for 2 days at 25°C on the standard for yeast YM agar medium containing 3 g dm⁻³ yeast extract, 3 g dm⁻³ malt extract, 5 g dm⁻³ peptone from soybeans and 10 g dm⁻³ glucose. The culture was stored at 4°C and renewed every 4 weeks [4].

Media preparation, culture conditions and cultivation
Initially the strain was developed for 24 hours in tubes with sloped YM agar medium at 25°C. Pre-cultures were prepared by inoculating cells grown on slants into a liquid growth medium 1 % glucose, 0.2 % NaNO₃, 0.02 % MgSO₄·7H₂O, 0.02 % KH₂PO₄ and 0.1 % yeast extract at 25°C on a rotary shaker (300 rpm) for 24 hours. Pre-cultures (1 cm³) were transferred into six 250 cm³ Erlenmeyer flasks containing 100 cm³ liquid nutrient media with the same medium, but with 4 % glucose. The strain was incubated at 25°C on a rotary shaker (300 rpm) for 7 days. Aliquots were withdrawn each day and the following parameters were determined: absorbance at 610 nm; dry biomass, protein concentration, consumption of glucose. The mean value of each parameter was calculated from the data obtained in the triplicate trials. After seven days of cultivation, the cells were removed by centrifuging (3000 rpm, 30 min). The lipase activity, protein concentration, pH and temperature optimum in the cultivation media were determined [4].

Determination of growth parameters
The absorbance at 610 nm of 1 cm³ suspension was measured. In addition, for dry biomass determination, 1 cm³ of fermentation broth was centrifuged at 14,000 rpm for 15 min, the supernatant was removed and the remaining biomass was dried in the moisture analyzer Kern at 105°C. The consumption of glucose was measured by HPLC – Dionex Ultimate 3000 with lead column Varion (PL Hi- Plex Pb 300x7.7mm), isocratic elution with bi-distilled water at a rate of elution 0.500 cm³ min⁻¹ and temperature 70°C. In order to make quantitative analysis, a standard curve of glucose was constructed [4]. Dissolved protein concentration was determined according to the Lowry method, using bovine serum albumin (BSA) as a standard [8].

Determination of enzyme activity
The activity of the obtained lipase was analyzed at every stage using the potentiometric method. Potentiometric method is based on monitoring the rate of hydrolysis of olive oil emulsion by potentiometric titration. Initially, the oil must be purified in order to remove free fatty acids. For this purpose 2 g Al₂O₃ is added in 20 cm³ extra virgin olive oil. After homogenization, the solution was centrifuged at 3000 rpm/min for 30 min. The precipitate is removed and the procedure was repeated two more times. 1 g of gum arabic was added to 10 ml of the resulting purified oil. The emulsion was stirred at 4°C for 24 hours. The sample was prepared in the following way: 2 cm³ (15 mg cm⁻³) taurocholate, 5 cm³ emulsion, 5 cm³ d.H₂O, 2 cm³ (3M) NaCl and 1 cm³ (0.075M) CaCl₂ were mixed. The resulting mixture was stirred continuously on a magnetic stirrer and an autotitrator at 25°C in order to homogenize it. The pH was also adjusted to 8.0. 0.5 ml of enzyme was added at zero time and the during 5 to 6 minutes at every 30 sec. The quantity of the expended sodium hydroxide was recorded. The specific activity of the lipase was calculated, using an equation described by Yotova et al. [9].

RESULTS AND DISCUSSION

Pseudozyma antarctica culturing procedure
After the initial development in a solid nutrient medium, the strain was grown in liquid medium, as described above. Increasing of biomass and glucose consumption is shown to Fig. 1. The main stages of growth of the cells are in a lag phase up to 8 hours, and the exponential phase is from 9 to 22 hours. The exponential phase is characterized by rapid development of biomass. Then the growth of culture slows down, entering in the stationary phase after 22 hours. Glucose consumption is measured by HPLC as described above. From the calibration curve the amounts of glucose in the sample were determined.

Determination of temperature, pH optimum and lipase activity in the supernatant
The supernatant was tested for lipase activity after incubation and centrifugation. pH and temperature optimum of the crude lipase were determined potentiometrically as already described. At the beginning all measurements for determination of pH optimum were performed in the range 7.7 to 8.3 at room temperature (Fig. 2). The specific lipase activity of the post ferme-
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The supernatant was determined to be 56 U mg⁻¹ at pH 8 and room temperature.

The temperature optimum of the crude lipase was also determined at different temperatures (25 - 70°C) at pH 8 (Fig. 3). The temperature optimum of the crude lipase is 60°C (Fig. 3), where the specific activity is 97.2 U mg⁻¹ (Table 1). The enzyme is thermostable and it has a high activity even at 70°C (Fig. 3). The obtained results are in accordance with those of Dimitrijevic et al. [4, 7].

### CONCLUSIONS

The *Pseudozyma antarctica* NBIMCC 8340 strain is suitable for the production of the enzyme lipase. The resulting lipase is extracellular and it can be used even in a „raw“ form. The strain has a relatively long cultivation time, which varies depending on the medium and cultivation conditions. Our experimental results revealed that the crude lipase produced by the strain had a temperature...
optimum at 60°C, where it had activity of 97.2 U mg⁻¹. The pH optimum of the enzyme is at pH = 8. It was also established that the *Pseudozyma antarctica* strain could be used successfully for large-scale production of the lipase.

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