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Isolation and purification of lipase produced from *Rhizopus arrhizus* in solid state fermentation by fractional precipitation

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A study on the selection of precipitating agent and determination of the optimal conditions for precipitation of lipase, produced from *Rhizopus arrhizus* in solid-state fermentation, was conducted with the aim of enzyme isolation and purification. The highest purification degree of 3.8-fold was achieved, when the enzyme was precipitated with 65% isopropanol and the activity yield was above 80%. The precipitation of lipase with acetone also allowed enzyme isolation and purification. With 50% acetone, 3.6-fold purification and 70% activity yield were achieved. Isolation and purification of the enzyme by salting-out with $(NH_4)_2SO_4$ was an inappropriate technique due to the low yield and the absence of purification. With the use of polyethylene glycol 4000 (PEG 4000) as a precipitating agent, low yields of lipase activity of less than 20% were obtained, but about 2-fold degree of purification was achieved, indicating a high selectivity of PEG 4000 to lipase precipitation. As a result of the various techniques used for lipase isolation and purification by fractional precipitation and the performed zymographic analysis of the resulting enzyme preparations, the presence of two multiple forms of lipase with a molecular weight of 28 000 Da and 55 000 Da was found.

Keywords: Lipase, Solid state fermentation, *Rhizopus arrhizus*, Purification

INTRODUCTION

Lipases (EC 3.1.1.3) catalyze the hydrolysis reaction of emulsified triglycerides to glycerol and composition of free fatty acids. The hydrolase activity of lipases determines their wide industrial application in the detergents. Lipases are also used in the dairy industry to develop a specific flavor in the products due to the oxidation of the free fatty acids obtained. Under certain conditions, lipases also catalyze the esterification, transesterification and aminolysis reactions in organic solvent media, which allows them to be used as a tool for developing "green" technologies in organic synthesis.

The possibilities for lipases applications make them one of the most important classes of industrially produced enzymes, accounting for about 5% of the world's enzyme market [1].

The study of new microbial strains producing lipases and the study of the specific characteristics of purified microbial lipase enzymes is an important scientific task with the possibility of practical application.

Lipases obtained from different sources are usually subjected to certain pre-purification steps before they are further purified. Typically, this is a one-step procedure involving precipitation by saturation with an $(NH_4)_2SO_4$ solution. The yield and the degree of purification depend on the concentration of $(NH_4)_2SO_4$ used [2].

Borkar *et al.* [3] obtained a relatively low yield of lipase (13.18%) but a significant degree of purification (20.84-fold) at 30% $(NH_4)_2SO_4$. Ji *et al.* [4] precipitated lipase from *Pseudomonas* with 20% $(NH_4)_2SO_4$ and achieved 1.8-fold purification and 58.7% enzyme yield. Pabai *et al.* [5] reported that the maximum increase in lipase activity in the precipitate occurred at 20-40% saturation with $(NH_4)_2SO_4$, with a 18.9-fold increase in purification and a yield of 52.93%.

Another method for selective precipitation of proteins is the use of water-soluble organic solvents with a low dielectric constant such as ethanol, acetone and isopropanol. As the principles causing precipitation are different, it is not necessarily an alternative to $(NH_4)_2SO_4$, but can be used as an additional step. Addition of a solvent such as ethanol or acetone to an aqueous extract containing proteins has a variety of effects which, combined, lead to protein precipitation. The principal effect is the reduction in water activity. The solvating power of water for a charged, hydrophilic enzyme molecule is decreased as the concentration of organic solvent increases [6].

Ameri *et al.* [7] achieved a 7.21-fold purification degree and an activity yield of 40.12% in the

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precipitation of lipase from *Bacillus atrophaeus* FSHM2 by 80% saturation of the culture liquid with chilled ethanol. Isolation of lipase by ethanol precipitation was also applied by Rua *et al.* [8], achieving a 1.8-fold purification degree and lipase activity yield of 84%.

Cao *et al.* [9] isolated lipase by specific precipitation from the culture medium at 60% acetone concentration. The precipitation was carried out at 0°C for 4 h. A 2.9-fold purification of the enzyme and a 68.7% activity yield were achieved. Razak *et al.* [10] used saturation of the culture liquid of two *Rhizopus* strains with 80% acetone to isolate lipase. As a result of the procedure, the authors obtained 3-fold purification and 62-76% yield of the precipitated lipases.

In the investigated literature no results for the use of isopropanol for specific lipase precipitation have been found.

Salts and organic solvents are not the only precipitation agents that can cause aggregation of proteins without denaturation. Polson *et al.* [11] investigated the ability of a variety of high-molecular-weight, neutral, water-soluble polymers to precipitate plasma proteins. The authors obtained best results with polyethylene glycol (PEG) with a molecular weight of 4000-20000. Although several types of PEG were effective in precipitation, the high viscosity of most solutions made their use as protein precipitants impractical [6]. PEG is not as easy to remove from a protein fraction as either salt or organic solvent. Nevertheless, a residual low level of PEG is not detrimental to many procedures – salting-out, ion exchange, affinity chromatography, or gel filtration can be carried out without having to remove the PEG first [6].

Görgün and Akpinar [12] applied 30% PEG 6000 precipitation to isolate and purify lipase from carp liver, yielding an enzyme activity of 60% and a minimum purification degree of 1.01-fold.

The aim of the present work is selection of precipitation agent and optimization of the conditions for isolation and purification of lipase by selective precipitation by salting-out with $(NH_4)_2SO_4$, ethanol, acetone, isopropanol and PEG.

EXPERIMENTAL

Microorganism

The studied *Rhizopus arrhizus* strain used in this study was provided by Biovet® Peshtera. It was grown in the following medium, g/l: malt extract 10.00; yeast extract 4.00; glucose 4.00; agar-agar 20.00. pH was adjusted to 7.0. The strain was cultivated at 28°C for 14 days and stored at 4°C.

Lipase biosynthesis

Solid state fermentation (SSF) was carried out in 500-ml Erlenmeyer flasks. The flasks contained nutrient medium of 10 g wheat bran and salt solution (g/l): $NH_4H_2PO_4$ 6.5, $(NH_4)_2C_2O_4$ 0.90, $MgSO_4$ 0.95, KCl 0.95. pH of the salt solution was adjusted to 7.0, and the final moisture content was adjusted to 66% with the salt solution before autoclaving. The solid substrate was supplemented with 1% (w/w) of glucose and 5% (w/w) of tryptone. After sterilization at 121°C for 30 min, the flasks were inoculated with 5 ml inoculum with 10^7 spores/ml and incubated at 30°C for 168 h.

Following SSF, extraction of lipase from the fermented solids was performed with 50 ml of eluent containing 1% commercial surfactant Disponil NP3070 for 30 min with constant agitation. The solids were removed by filtration, the filtrates were centrifuged and the resulting extract solution, containing lipase was tested for enzyme precipitation.

Fractional precipitation with $(NH_4)_2SO_4$

To the extract solution containing lipase, known volume of $(NH_4)_2SO_4$ was added, in such quantity that 20-70% degree of saturation to be reached. The samples were incubated at 4°C for 1 h for precipitate stabilization, and were centrifuged at 4000 rpm for 30 min. The precipitates were diluted with distilled water to a defined volume and lipase activity and protein content were determined.

Fractional precipitation with organic solvents

Organic solvents (acetone, ethanol or isopropanol) were added to the extract solution containing lipase, in such quantity that defined concentration (20-80%) to be achieved. The precipitates were stabilized at 4°C for 1 h, centrifuged at 4000 rpm for 30 min, and tested for lipase activity and protein content.

Fractional precipitation with PEG 4000

PEG 4000 was added to the lipase extract solution in a final concentration of 5-60%, the samples were kept at 4°C for 1 h, centrifuged at 4000 rpm for 30 min, and the precipitates were analyzed for lipase activity and protein concentration.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 15% polyacrylamide gel in the presence of SDS on Cleaver Scientific Ltd. OmniPAGE electrophoresis system CVS10DSYS by the method of Laemmli [13].

Lipase zymogram

The enzymes with lipase/esterase activity were detected by zymographic analysis. After non-denaturing PAGE, the gel was treated with 0.02% (w/v) α-naphthyl acetate and 0.05% (w/v) Fast Blue RR salt in 0.05 M Tris-HCl buffer with pH 7.2, revealing bands with lipase activity [14].

Determination of lipase activity

For lipase activity determination the method proposed by Babu *et al.* [15] and Saifuddin *et al.* [16] was adapted. Substrate solution was prepared by dissolving 30 mg of ρ-nitrophenyl palmitate in 10 ml of isopropanol, mixed with 90 ml of 0.05 M Tris-HCl buffer with pH 7.2, 0.4 g of Triton X-100 and 0.1 g of gum arabic. 2.4 ml of the substrate solution were incubated at 35°C for 10 min and 0.1 ml of suitably dissolved enzyme was added. The enzyme reaction was performed at 35°C for 30 min and the enzyme was inactivated by addition of 1.0 ml of 0.5 M solution of EDTA with pH 8.0. The absorbance at 405 nm was measured against a reference sample with an inactivated enzyme. One unit (U) of lipase activity was defined as the amount of enzyme releasing 1.0 μmol of ρ-nitrophenol for 1 min at 35°C and pH 7.2.

Protein assay

Protein content was determined by the method of Bradford [17].

RESULTS AND DISCUSSION

Fractional precipitation with various concentrations of $(NH_4)_2SO_4$ is the most commonly used technique for pre-purification and isolation of enzymes and proteins. The results for salting-out with $(NH_4)_2SO_4$ for isolation of lipase produced in SSF by *Rhizopus arrhizus* are presented in Fig. 1.

It should be noted that at all tested concentrations of $(NH_4)_2SO_4$, the yields of lipase activity were relatively low - below 40%, which determined the low degree of enzyme purification - below 1.0. At 40% concentration of the salt, about 80% of the protein in the extract solution was precipitated, but the yield of lipase activity was about 30%, which determined a low degree of purification. Low yields of lipase activity were probably due to enzyme inactivation at higher salt concentrations or the enzyme had not been precipitated. It was clear from this study that this classical isolation and purification step was not applicable to the lipase studied.

The fractional precipitation of lipase by ethanol is shown in Fig. 2. At ethanol concentrations above 70%, significantly higher yields of lipase activity were obtained. At 80% ethanol concentration, the yield of lipase activity was about 65%, and the yield of protein was about 46.5%, which defined a purification degree of 1.44-fold.

Isolation and purification of the examined lipase with different concentrations of acetone are shown in Fig. 3.

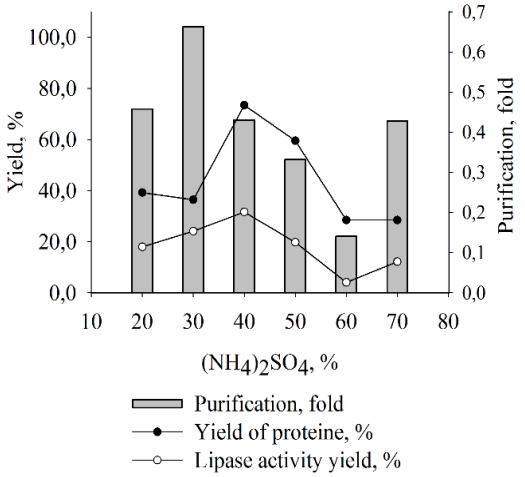

Fig. 1. Precipitation of lipase with $(NH_4)_2SO_4$

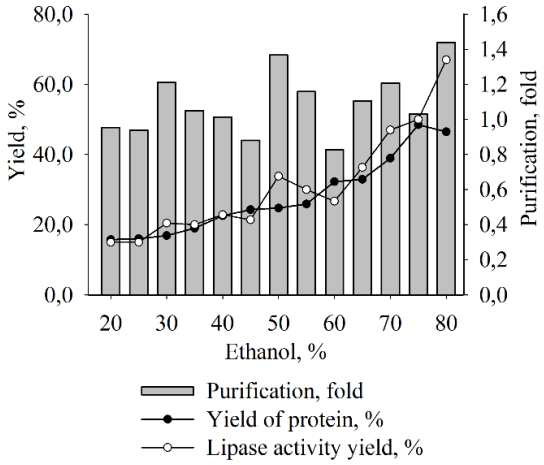

Fig. 2. Precipitation of lipase with ethanol

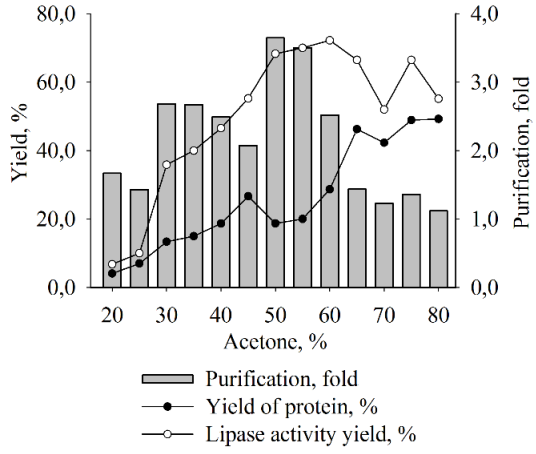

Fig. 3. Precipitation of lipase with acetone

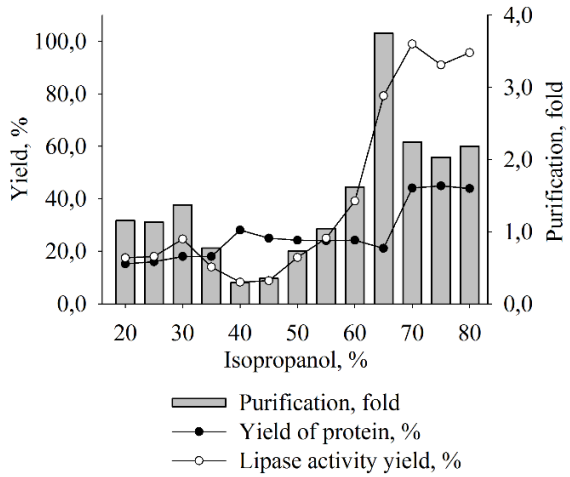

Fig. 4. Precipitation of lipase with isopropanol

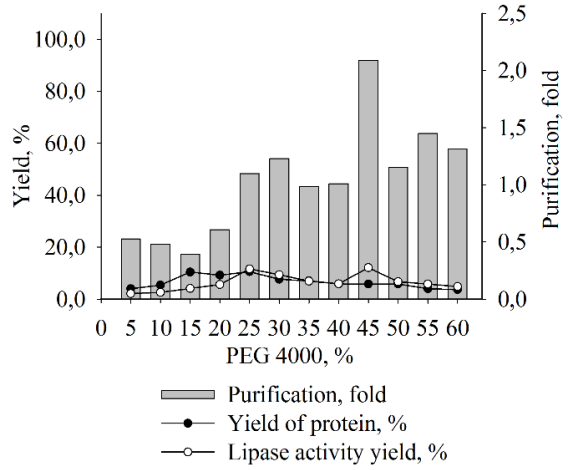

Fig. 5. Precipitation of lipase with PEG 4000

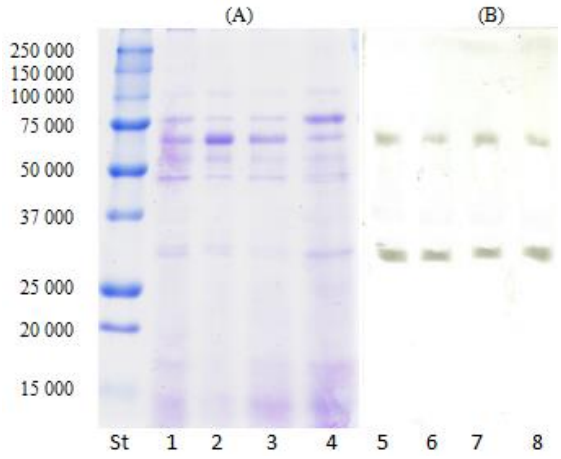

Fig. 6. (A) SDS-PAGE of lipase in denaturing conditions: 1-precipitation with 65% isopropanol, 2-precipitation with 50% acetone, 3-precipitation with 80% ethanol, 4-crude enzyme; (B) Zymogram of lipase in non-denaturing conditions: 5-crude enzyme, 6-precipitation with 65% isopropanol, 7-precipitation with 50% acetone, 8-precipitation with 80% ethanol.

The highest purification degree of about 3.5-fold was achieved at 50-55% acetone and the yield of lipase activity was about 70%. Similar results were obtained by Cao *et al.* [9] and Razak *et al.* [10], they achieved a degree of enzyme purification of about 3-fold and a yield of lipase activity of about 70%, at acetone concentration of 60% and 80%, respectively. Singh and Banerjee [18] obtained yield of 42% and 6.85-fold purification after precipitation with acetone in a ratio supernatant:acetone = 1:2.

Very good results on the isolation and purification of lipase produced in SSF of *Rhizopus arrhizus* were obtained by using isopropanol to selectively precipitate the enzyme (Fig. 4). At concentrations of isopropanol 65-80%, yields of lipase activity were above 80%. The maximum purification degree was 3.8-fold, achieved at 65% isopropanol. The high degree of purification was determined by the lack of concomitant proteins – the maximum yield of protein was only 40%.

An alternative method for fractional protein precipitation is the use of high-molecular-weight, neutral, water-soluble polymers [11]. The influence of PEG 4000 concentration on lipase precipitation is presented in Fig. 5.

After precipitation of lipase with PEG 4000, low yields of enzyme activity, below 20% were found. The maximum degree of purification was about 2.1- fold.

The process of lipase purification was monitored by SDS-PAGE and zymographic analysis (Fig. 6).

The analysis with SDS-PAGE (Fig. 6A) showed that as a result of the precipitation (line 1-3) a portion of the proteins with low molecular weight of about 15 000 Da and some proteins with high molecular weight of about 100 000 Da were removed in comparison to the crude enzyme (line 4). Zymographic analysis indicated that *Rhizopus arrhizus* produced in SSF 2 multiple forms of lipase with a molecular weight of 28 000 Da and 55 000 Da (Fig. 6B).

CONCLUSIONS

As a result of the research on the selection of a suitable precipitation agent and optimization of the conditions for isolation and purification of lipase, it was found that the highest degree of lipase purification (3.8-fold) was achieved by precipitation of the enzyme with 65% isopropanol with the yield of enzyme activity being above 80%. No data were found so far on the use of isopropanol as a precipitation agent in the isolation and purification of lipase. The precipitation of lipase tested with acetone also allowed isolation and purification of the enzyme. At 50% acetone, 3.6-

fold purification and about 70% activity yield were achieved. Salting-out with $(NH_4)_2SO_4$ was an inappropriate technique tested for isolation and purification of lipase. Despite the high yield of precipitated protein, about 80%, the lipase activity of the precipitates was low, below 40%. The use of PEG 4000 as a precipitation agent was characterized by very low yields of lipase activity of less than 20%, but about 2-fold purification was achieved, indicating a high selectivity of PEG 4000 to lipase precipitation. As a result of the procedures of isolation and purification of lipase, produced by *Rhizopus arrhizus* in SSF, the presence of two multiple forms of lipase with molecular weights of 28 000 Da and 55 000 Da was found.

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