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Isolation and purification of lipase from *Rhizopus arrhizus* by ultrafiltration and fractional precipitation

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Isolation and purification of lipase with ultrafiltration, fractional precipitation with $(NH_4)_2SO_4$, organic solvents and PEG 400 of culture broth containing lipase from *Rhizopus arrhizus* was investigated. By using polyacrylonitrile membranes with different size of pores (10-100 kDa) low yields of lipase activity were achieved. A possible reason was adsorption of the protein molecules on the membranes. During fractional precipitation with 60% $(NH_4)_2SO_4$ purification fold 1.3 and lipase yield about 80% were reached. Fractional precipitation with ethanol, acetone and isopropanol led to inactivation of the enzyme which was the reason for the low purification factor. Probably the low yields were occurred due to the polarity of the medium because in these conditions dielectric constant remained constant – 1.83. The highest lipase yield (95%) and purification factor (3.5-fold) were accomplished by fractional precipitation with 60% PEG 400. In this case dielectric constant of the medium was 1.97.

Keywords*: lipase, purification, ultrafiltration, fractional precipitation, dielectric constant*

INTRODUCTION

Lipases (E.C. 3.1.1.3) are a group of enzymes that in presence of water catalyze hydrolysis of triacylglycerol to mono-, diacylglycerol, free fatty acids and glycerol and in anhydrous medium – reactions of esterification, transesterification and interesterification. Also lipases catalyze reactions of alcocholysis, acidolysis and aminolysis [1].

Ultrafiltration, fractional precipitation with $(NH_4)_2SO_4$ and organic solvents are classical approaches and common first step in many schemes for purification of different enzymes.

Ultrafiltration is a wide used method for concentration of enzymes and takes part in many schemes for different enzyme purification, including lipase. Ultrafiltration is an effective technique which may lead to 5-fold concentration with high yield of the target protein because it is held at room temperature and thermal inactivation is not possible. This method also leads to partial purification as proteins with lower molecular masses pass through the membrane into permeate [2-5].

The first step in many purification schemes is fractional purification, when $(NH_4)_2SO_4$ is the most common substance. Fractional precipitation with acetone, ethanol and organic acid is also used in practice [6].

By using $(NH_4)_2SO_4$ for fractional precipitation high yields and purification folds of lipases are achieved. Gaikaiwari *et al.* [7] accomplished 90.67% lipase yield and more than 5 purification fold and Bose *et al.* [8] – 82.9% lipase yield and purification fold 4.48.

Another option for isolation and purification of enzymes is fractional precipitation with organic solvents. Dandavate *et al.* [9] used acetone for fractional precipitation and achieved high purification fold – 13.77 but low lipase yield - 37.0%.

Polyethylene glycol (PEG) is another option for fractional precipitation of enzymes. Degerli *et al.* [10] used 10% PEG 8000 for fractional precipitation and achieved 97.92% lipase yield and 13.42 purification fold. Romero *et al.* [11] used PEG 20000 for concentration of the enzyme solution and native electrophoresis was then applied. After these two steps purification fold 8.4 and lipase yield 47% were achieved.

The aim of this study is isolation and partial purification of lipase from *Rhizopus arrhizus* by ultrafiltration, fractional precipitation with $(NH_4)_2SO_4$, organic solvents and PEG 400.

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EXPERIMENTAL

Microorganism and lipase production

The studied *Rhizopus arrhizus* strain used in this study was provided by Biovet® Peshtera. Growth medium, inoculum preparation and submerged cultivation conditions were described in previous article [12]. Fermentation medium was containing: corn starch 10.5; tryptone 6.6; $NH_4H_2PO_4$ 7.1; $(NH_4)_2C_2O_4$ 1.0; $MgSO_4$ 1.5; KCl 1.9.

Ultrafiltration

Ultrafiltration of 50 cm^3 cultural broth with ultrafiltration cell Amikon® was applied and 5-fold concentration was performed. Polyacrylonitrile ultrafiltration membranes 10, 20, 25, 50 and 100 kDa were used. The process was carried out at room temperature and work pressure 0.5 MPa. Lipase activity and content of protein in concentrate, permeate and ultrafiltration membrane were analyzed.

Fractional precipitation with $(NH_4)_2SO_4$

To a cultural broth with known volume $(NH_4)_2SO_4$ was added in such quantity that defined degree of saturation (10-80%) to be achieved. Solutions were let for stabilization of the precipitate at 4°C for 1 h and then were centrifuged at 4000 rpm for 40 min. The precipitates were diluted with distilled water to a defined volume and lipase activity and content of protein were analyzed.

Fractional precipitation with organic solvents

To a cultural broth with known volume acetone, ethanol and isopropanol were added in such quantity that defined concentration (10-80%) to be achieved. Solutions were let for stabilization of the precipitate at 4°C for 1 h and then were centrifuged at 4000 rpm for 40 min. The precipitates were diluted with distilled water to a defined volume and lipase activity and content of protein were analyzed.

Fractional precipitation with PEG 400

To a cultural broth with known volume PEG 400 was added in such quantity that concentration (20-70%) to be achieved. Solutions were let for stabilization of the precipitate at 4°C for 1 h and then were centrifuged at 4000 rpm for 40 min. The precipitates were diluted with distilled water to a defined volume and lipase activity and content of protein were analyzed.

Lipase assay

Lipase activity was measured by spectrophotometric method using p-nitrophenyl palmitate as substrate buffered with Tris-HCl pH 9.0 [13]. The reaction mixture, containing 2.4 cm^3 of 0.8 mM substrate and 0.1 cm^3 of enzyme solution, was incubated for 15 min at 35°C. The enzyme reaction was stopped by adding 1.0 cm^3 saturated solution of lead (II) acetate. After centrifugation absorbance was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that released one μmol of p-nitrophenol per minute under the assay conditions described.

Lipase assay of ultrafiltration membrane

A part of the ultrafiltration membrane with size 1.0 cm^2 was cut and placed in a test tube containing 2.4 cm^3 of 0.8 mM substrate and 1.0 cm^3 water and the reaction mixture was incubated for 15 min at 35°C. The enzyme reaction was stopped by adding 1.0 cm^3 saturated solution of lead (II) acetate after the membrane was subtracted. After centrifugation absorbance was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that released one μmol of p-nitrophenol per minute under the assay conditions described.

Protein assay

Protein was quantified by Lowry assay [14].

SDS-PAGE electrophoresis

The purified lipase was analyzed electrophoretically on Cleaver Scientific Ltd; OmniPAGE Electrophoresis System CVS10DSYS, at 20 mA. 15% polyacrylamide gels in the presence of SDS as described by Laemmli [15].

Dielectric constant measurement

Dielectric constant was measured refractometric using a laser microrefractometer. Laser pointer generating at wavelength of 532 nm is used as a light source. The sample was placed between a glass prism and metal diffraction grating. At angles smaller than the critical angle of total internal reflection, the laser beam passes through a glass prism, sample and diffract from the metal grating. The critical angle (φ_{cr}) was measured in the air and the refractive index of the sample (n) in calculated by the formula [16, 17]:

$$n = N \sin\left[A - \arcsin\left(\frac{\sin \varphi_{cr}}{N} \right) \right] \qquad (1)$$

where $A = 64.7°$ is the refraction angle of the prism,

N is the refractive index of the prism for the used wavelength (1.7480).

Dielectric permittivity of the samples can be obtained using the Maxwell relation [18]:

$$\varepsilon = n^2 \qquad (2)$$

RESULTS AND DISCUSSION

Isolation and concentration of lipase from cultural broth obtained by submerged fermentation of *Rhizopus arrhizus* was performed.

Table 1 represents the results from ultrafiltration of the studied enzyme. As seen from the table, when membranes 10, 20 and 25 kDa were used very low lipase yield in the concentrates were reached while there were no lipase activity in the permeates. When ultrafiltration membranes 50 and 100 kDa were used there were enzyme activity in concentrates and in permeates as well. However, the lipase yields in concentrates and permeates were between 55 – 60%. This is low yield for this technique because during the procedure there is no increasing in the temperature which could result in a thermal inactivation of the enzyme. These results are comparable with other reports. For example Gaur *et al.* [3] achieved 60.8% lipase yield and 0.9 purification fold by ultrafiltration of lipase from *Pseudomonas aeruginosa* with ultrafiltration membrane with pore size 30 kDa.

As lipases are water soluble enzymes and the substrate – fat soluble, catalysis proceeds in the interfacial surface between water and oil phases. This is possible because of the hydrophobic domain in the lipase molecules, which allows the enzyme to adsorb on the substrate. It is possible adsorption of the lipase on the ultrafiltration membrane to be a reason for low activity yields [1, 5].

Another possible reason for inactivation of lipase is protein-protein interaction in the concentrate. During concentration protein molecules interact each other with their hydrophobic domains [19] which may inactivate the enzyme.

In order to confirm the hypothesis for adsorption of the molecules of the enzyme on the ultrafiltration membrane, lipase activity of the membranes was examined after the ultrafiltration was performed (Table 2). As seen from the results lipase activity was recorded at all of the membranes. However, these results could not be used the adsorbed lipase to be quantified because of changes in kinetics of the reactions during immobilization of the biocatalyst. As a result of this experiment the hypothesis that some of the lipase was adsorbed on the ultrafiltration membranes was confirmed.

From the obtained results we can conclude that ultrafiltration with polyacrylonitrile membranes 10-100 kDa is not suitable technique for concentration of lipase from *Rhizopus arrhizus.*

Fractional precipitation with $(NH_4)_2SO_4$ is a traditional method for isolation of enzymes.

$(NH_4)_2SO_4$ is more commonly used for fractional precipitation of lipase because it leads to high lipase yield. At 80% degree of saturation a lipase yield over 50% was accomplished. The highest purification fold – 1.17 and yield 30% was achieved (Fig. 1).

Table 1. Ultrafiltration

	V, cm^{-3}	Total lipase activity, U	Specific activity, U/mg	Yield, %	Purification factor
Crude enzyme	50	86.23	26.75	100	1.00
			Membrane 10 kDa		
Concentrate	10	16.62	11.09	19.27	0.41
Permeate	40	0.01	0.00	0.01	0.00
			Membrane 20 kDa		
Concentrate	10	23.72	13.07	27.50	0.49
Permeate	40	0.09	0.16	0.10	0.01
			Membrane 25 kDa		
Concentrate	10	40.67	15.18	47.16	0.57
Permeate	40	0.07	0.11	0.08	0.00
			Membrane 50 kDa		
Concentrate	10	45.35	23.77	52.59	0.89
Permeate	40	13.07	19.28	15.15	0.75
			Membrane 100 kDa		
Concentrate	10	45.08	20.11	52.27	0.75
Permeate	40	11.01	4.71	12.77	0.18

Table 2. Lipase activity on ultrafiltration membranes

Membrane, kDa	Area, cm^2	Total activity, U
10	16	1.42
20	16	2.23
25	16	1.02
50	16	1.57
100	16	1.04

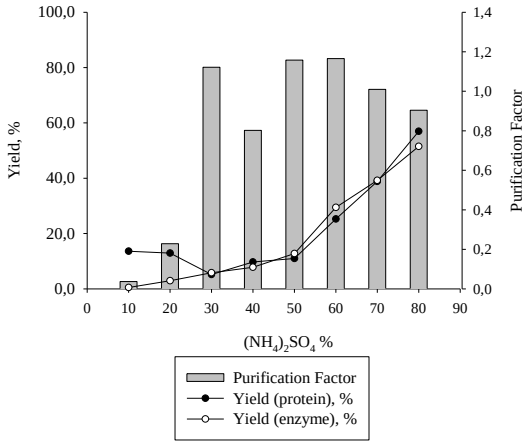

Fig. 1. Fractional precipitation with $(NH_4)_2SO_4$

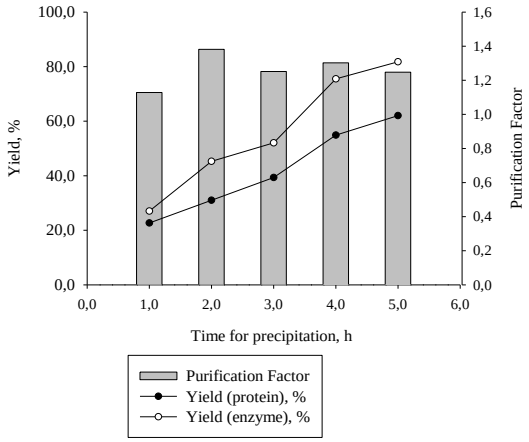

Fig. 2. Time for fractional precipitation with 60% $(NH_4)_2SO_4$

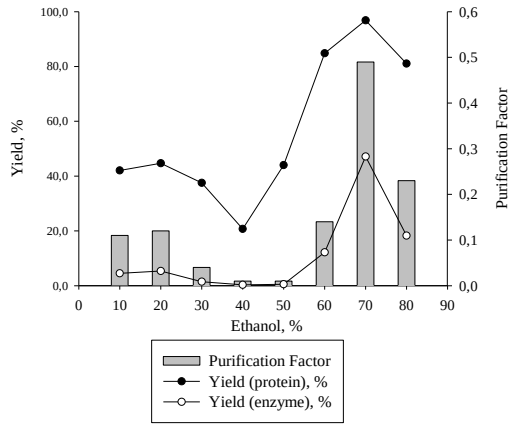

Fig. 3. Fractional precipitation with ethanol

Additional experiment was obtained in order to enhance the time for precipitate formation with 60% $(NH_4)_2SO_4$. Fig 2 reveals that with the enhancement of time (1-5 h) for precipitation, lipase yield also increased – from 30% to 80%. As seen from the chart lipase yield at 4^{th} h was 75.50% and at 5^{th} h – 81.75%. Protein yield also enhanced and the purification factor remained relatively constant – between 1.15 and 1.30 fold.

As a result of this experiment we could conclude that when fractional precipitation with $(NH_4)_2SO_4$ for 4 h was used lipase yield 75.50% and purification factor 1.30 was accomplished.

Traditional method for isolation of enzymes is their selective fractional precipitation with organic solvents such as ethanol, acetone and isopropanol.

Fig. 3 reveals the results for fractional precipitation with ethanol. The highest yield (almost 50%) was achieved when 70% ethanol was used. It can be noticed that at 40% and 50% ethanol the enzyme is almost completely inactivated. Because of the activity loss, the purification fold decreased.

Similar results were obtained for fractional precipitation of lipase with isopropanol and acetone (Fig. 4 and Fig. 5).

When isopropanol and acetone were used the highest lipase yield was accomplished at 80% of the organic solvent – respectively 52.98% and 74.83%. In both cases a decrease in the lipase activity was noticed between 30 and 40% of each of the solvents.

Yu *et al.* [20] explained the inactivation effect with changes of polarity of the medium and the influence of the polarity on the hydrophobic effects, which have a crucial role in formation of the tertiary structure of the proteins. Hydrophobic effects in the protein molecules are a result from the pressure of the water medium. Addition of organic solvent removed a part of the hydration shell, which results in changes in the native conformation which may lead to inactivation [20]. Respectively polarity of the medium, which impact on the hydrophobic effects of formation of the three dimensional structure of the proteins may be the factor which leads to inactivation of lipase at these conditions.

In order to examine the hypothesis, dielectric constant, which is a measure for the polarity of the medium, was measured for the samples where the strongest inhibition was noticed (Table 3). As seen from the table dielectric constants at the stated conditions were with the same values – 1.83.

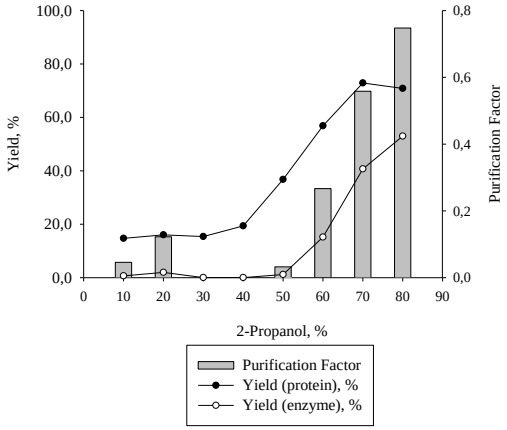

Fig. 4. Fractional precipitation with isopropanol

Table 3. Dielectric constant of water and some concentrations of organic solvents

Solution	Dielectric constant ε at 532 nm	Lipase activity, U.dm^{-3}
Lipase aqueous solution	1.79	1009.71
Lipase in 50 % Ethanol	1.83	4.30
Lipase in 40 % Ethanol	1.83	2.55
Lipase in 40 % 2- Propanol	1.83	0.02
Lipase in 30 % 2- Propanol	1.83	0.02
Lipase in 40 % Acetone	1.83	90.15
Lipase in 30% Acetone	1.83	96.64
Lipase in 40 % PEG 400	1.89	1351.53
Lipase in 50 % PEG 400	1.97	2165.43

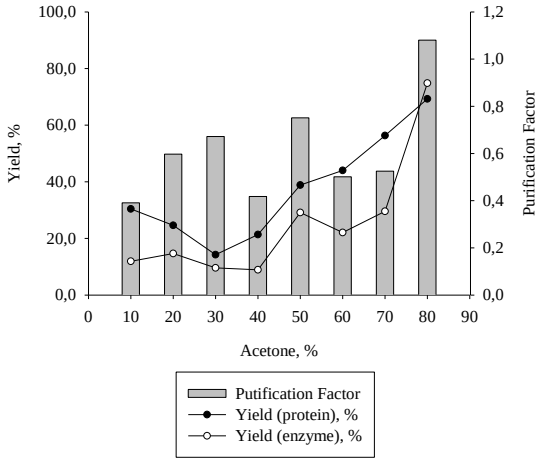

Fig. 5. Fractional precipitation with acetone

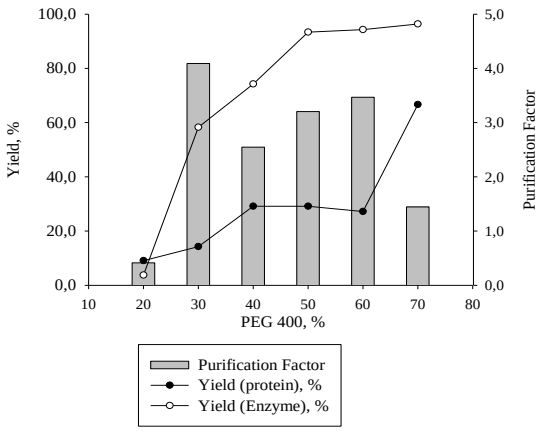

Fig. 6. Fractional precipitation with PEG 400

The obtained results are a partial proof of the hypothesis that the reached polarity at usage of these concentrations of organic solvents was a possible reason for lipase inactivation.

Some authors describe usage of PEG for fractional precipitation of lipase and relatively high yields are reported [10]. High yield and purification fold were achieved by fractional precipitation of lipase by PEG 400 (Fig. 6).

The highest purification factor, more than 4.0 fold, was accomplished at 30% PEG 400. Lipase yield in this case was almost 60%. Yield over 95% was obtained by using 60% and 70% PEG 400 but in these cases purification factor decreased – respectively 3.5 and 1.4 fold.

Degerli *et al.* [10] also used polymer and by using 10% PEG 8000 97.92% yield and purification factor 13.42 fold was achieved.

Dielectric constants of the solutions of PEG (Table 3) differed from those of the organic solvents. Dielectric constant 1.97 was measured at lipase solution containing 50% PEG 400. In this case lipase yield 93.36% was reached and purification factor 3.20 was accomplished.

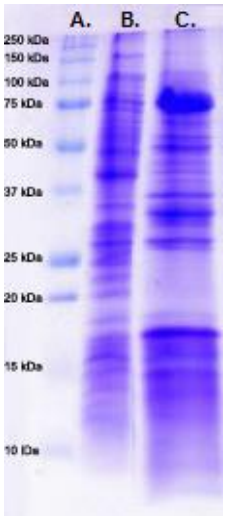

Fig. 7. SDS-PAGE electrophoresis – fractional precipitation with 60% PEG 400: A- protein markers; B -cultural broth; C - after fractional precipitation with PEG 400

In order to confirm the purification of the enzyme after fractional precipitation with 60% PEG 400 SDS-PAGE was performed (Fig. 7). As seen from the figure, many protein bands with molecule masses between 20 and 30 kDa and higher than 100 kDa were absent. That indicates that fractional precipitation with PEG 400 leads to partial purification of lipase from *Rhizopus arrhizus*.

CONCLUSION

In this study was established that ultrafiltration was not suitable for concentration of lipase from *Rhizopus arrhizus*. Lipase activity on the ultrafiltration membrane proved that a reason for low lipase yield was adsorption of the enzyme on the membranes. When fractional precipitation with ethanol, isopropanol and acetone was applied very low lipase yields were reached because of inactivation of the enzyme. The highest lipase yield – 94.29% and purification factor 3.47 were accomplished when 60% PEG 400 was used for fractional precipitation.

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REFERENCES

1. P.Y.Stergiou, A.Foukis, M.Filippou, M.Koukouritaki, M.Parapouli, L.G.Theodorou, E.Hatziloukas, A.Afendra, A.Pandey, E.M.Papamichael, *Biotechnol. Adv*., **31**, 1846 (2013).
2. M.F.Jesus, R.N.Branco, G.L.Sant'anna, D.M.G.Freire, J.G.Silva, *Braz. J. Chem. Eng*., **16**, 113 (1999).
3. R.Gaur, A.Gupta, S.K.Khare, *Process Biochem.*, **43**, 1040 (2008).
4. W.Stuer, K.E.Jaeger, U.K.Winkler, *J. Bacteriol*., **168**, 1070 (1986).
5. Y.Fang, X.J.Huang, P.C.Chen Z.K.Xu, *BMB Rep*., **44**, 87 (2011).
6. R.K.Saxena, A.Sheoran, B.Giri, W.S.Davidson, *J. Microbiol. Methods*, **52**, 1 (2008).
7. R.P.Gaikaiwari, S.A.Wagh, B.D.Kulkarni, *Bioresour, Technol.*, **108**, 224 (2012).
8. A.Bose, H. Kehara, *Biocatal. Agric. Biotechnol.*, **2**, 255 (2013).
9. V.Dandavate, J.Jinjala, H.Kehria, D.Madamwar, *Bioresour. Technol*., **100**, 3374 (2002).
10. N.Dagerli, M.A.Akpinar, *Turk. J. Biol.*, **26**, 133 (2002).
11. C.Romero, L.Pera, F.Loto, C.Vallejos, G.Castro, *Biocatal. Agric. Biotechnol.*, **1**, 25 (2012).
12. H.Strinska, V.Dobreva, B.Zhekova, G.Dobrev, *Int. J. Biochem. Res. Rev.*, **5**, 178 (2015).
13. R.Kaushik, S.Saran, J.Isar, R.K.Saxena, *J. Mol. Catal. B: Enzym*., **40**, 121 (2006).
14. O.H.Lowry, N.J.Rosebrough, A.L.Farr, R.J.Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15. U.K. Laemmli, *Nature*, **227**, 680 (1970).
16. S.Sainov, N.Dushkina, *Appl. Opt.*, **29**, 1406 (1990).
17. I.Vlaeva, T.Yovcheva, K.Zdravkov, G.Minchev, E.Stoykova, in: Proc. SPIE 7027 (2008).
18. W.C.Tan, K.Koughia, J.Singh, S.O.Kasap, in: Optical Properties of Condensed Matter and Applications (ed J. Singh), John Wiley & Sons, Ltd, Chichester, UK, 2006.
19. S.Jones, J.M.Thornton, Proc. Natl. Acad. Sci USA 93, 1996.
20. G.Barth, *Yarrowia lipolytica*: Biotechnological Applications. Springer Science & Business Media, 2013.

ИЗОЛИРАНЕ И ПРЕЧИСТВАНЕ НА ЛИПАЗА ОТ *Rhizopus arrhizus* С УЛТРАФИЛТРАЦИЯ И ФРАКЦИОННО УТАЯВАНЕ

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(Резюме)

Проведено е изолиране и пречистване на липаза чрез ултрафилтрация, фракционно утаяване с $(NH_4)_2SO_4$, органични разтворители и ПЕГ 400 от културална течност на *Rhizopus arrhizus.* При ултрафилтрацията с полиакрилонитрилни мембрани с различен размер на порите (10-100 kDa) е постигнат нисък добив, което вероятно се дължи на адсорбция на белтъчните молекули върху мембраните. При фракционно утаяване с 60% $(NH_4)_2SO_4$ е получена степен на пречистване 1.3 пъти и добив на липаза около 80%. Фракционното утаяване с етанол, изопропанол и ацетон води до инактивиране на ензима, в резултат на което е отчетена и ниска степен на пречистване. Вероятно това се дължи на промяна в полярността на средата, тъй като диелектричната константа при тези условия има една и съща стойност – 1.83. Най-висок добив (95%) и степен на пречистване (3.5 пъти) са получени чрез фракционно утаяване с 60 % ПЕГ 400. В този случай диелектричната константа на средата е 1.97.

Ключови думи: *липаза, пречистване, ултрафилтрация, фракционно утаяване, диелектрична константа*