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Purification of lipase from *Aspergillus carbonarius* NRRL369 by ATPS PEG/potassium phosphate

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In this research purification of lipase from *Aspergillus carbonarius* NRRL369 by aqueous two-phase system (ATPS) was studied. Binodal curves of PEG 400/potassium phosphate and PEG 4000/potassium phosphate were developed and appropriate concentrations of the two components were chosen for examination of the purification process. For all ATPS some thermodynamic parameters were calculated to describe the process. Purification of lipase by different ATPSs was tested. It was noticed that increasing of molecular weight of PEG leads to increasing of purification factor of lipase. The results revealed that for lipase purification an ATPS 24% PEG4000/15% potassium phosphate was the most suitable. By using this ATPS high purification factor (10.18) and lipase yield (90.68%) were achieved. Addition of NaCl affects lipase purification. Including 6% NaCl in ATPS 24% PEG4000/15% potassium phosphate increasesthe purification factor from 10.18 to 14.08 and final lipase yield 88.81% was achieved.

Keywords*: lipase, purification, aqueous two-phase system, Asp. carbonarius*

INTRODUCTION

Lipases (triglycerol acyl-hydrolases E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of water insoluble triglycerides to di and monoacylglycerides, free fatty acids and glycerol. Lipases catalyze also the reverse reactions of esterification and transesterification in organic solvent medium [1-3]. Because of the high number of reactions catalyzed by these enzymes, lipases have widespread application in many industries including organic synthesis, paper manufacturing, oleochemistry, dairy, cosmetics, biosensors, and detergents [4].

The main procedure for lipase production is fermentative process of microbial producers. Fungus are preferred sources for lipase production because the enzyme is extracellular which facilitate the technological scheme [4, 5].

Development of specific methods for lipase purification is important because highly purified enzymes are able to be applied in medicine and analytical chemistry also that gives opportunity the mechanism of action to be studied [6].The aqueous two-phase system (ATPS) technique is an ideal method for protein separation and purification, because it is fast and economical. The processes are easy to implement because the clarification, concentration and partial purification of the target product can be carried out in one step [7]. This system, consisting of two liquid phases that are immiscible beyond a critical concentration, has high selectivity and recovery yield of biomolecules. That makes the ATPS possible strategy for purification of a desired protein (including enzymes as lipases) in large-scale. The ATPSs that are currently in use are usually based on, polymer/polymer system (polyethylene glycol (PEG)/dextran), or polymer/salt system (PEG/potassium phosphate). The partition of proteins in the ATPS is influenced by many factors, such as protein properties (e.g. hydrophobicity, molecular size, weight and conformation, net electrical charge), type and concentration of salts, polymer molecular mass and environmental conditions [8].

In the present study, the partitioning behavior of lipase in various PEG/potassium phosphate ATPSs and the feasibility of utilizing these systems for purification of lipase produced from *Aspergilus carbonarius* NRRL369 was investigated.

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EXPERIMENTAL

Microorganism and lipase production

A strain of *Aspergillus carbonarius* NRRL369 from ARS Culture Collection was used for lipase biosynthesis. Storage of the strain and inoculum preparation were accomplished as described by Dobrev *et al.* [9]. Submerged cultivation of the strain was performed in 500-mL Erlenmeyer flasks, containing 50 mL nutrient medium containing (g/L): rapeseed oil 20.0, meat extract 5.6, $MgSO_4$ 1.0, KH_2PO_4 4.0 and Tween 80 20.0. After sterilization the medium was inoculated with 5.0 mL inoculum and the strain was grown at 27°C for 64 h with a 180 rpm rotary shaking.

Lipase activity assay

Lipase activity was determined by titrimetry using olive oil emulsion (25 mL of olive oil and 75 mL of 7% Arabic gum in a homogenizer for 2 min). The reaction mixture containing 2 mL of olive oil emulsion, 2 mL of phosphate buffer with pH 6.0, and 1 mL of enzyme was incubated at 35ºC for 20 min. The reaction was immediately stopped after the incubation period by the addition of 7 mL acetone : ethanol mixture (1:1 v/v), and the released free fatty acids were titrated with 50 mM NaOH. One unit (U) of lipase activity was defined as the amount that released 1 μmol of fatty acid per min [10].

Protein assay

Protein concentration was measured by the method of Bradford [11].

Phase diagrams and APTS preparation

The binodal curves were estimated using turbidometric titration [12].

All ATPS were prepared in 15 mL graduated centrifuge tubes. To study the effects of PEG molecular weight and salt concentration on the partitioning of lipase from the cell-free fermentation broth, different concentrations of PEG (400, 4000) and 40% phosphate salts (K_2HPO_4/KH_2PO_4) were mixed. The tubes were shaken on vortex for 2 min, followed by centrifugation for 20 min at 4000 rpm to assist phase separation.

After phase separation and visual estimation of the top and bottom phases, the volumes of the phases were used to estimate the volume ratio. The samples of the top and bottom phases were carefully withdrawn. Aliquots from each phase were analyzed to determine the enzyme activity and protein concentration [13].

Determination of selectivity, purification factor and yield

The protein partition coefficient (K_p) in the ATPS was defined as:

$$K_{p} = C_{p,t}/C_{p,b} \quad (1)$$

where $C_{p,t}$ and $C_{p,b}$ are the concentrations of protein in the top and bottom phases.

The partition coefficient for lipase activity (K_e) in the ATPS was calculated:

$$K_{e=} A_t/A_b \quad (2)$$

where A_t and A_b are the enzyme activity in the top and bottom phases.

Yield (Y, %) of lipase in the top phase was also calculated:

$$Y\% = \frac{100}{((VrK_e)^{-1} + 1)} \quad (3)$$

where Vr is the volume ratio of the top phase to the bottom phase (V_t/V_b).

Selectivity (S) was calculated as:

$$S = \frac{K_e}{K_p} = \frac{A_\tau}{A_b}\frac{C_{p,b}}{C_{p,\tau}} \quad (4)$$

The purification factor in the top phase (PF_{top}) was defined as:

$$PF_{top} = SA_t/SA_i \quad (5)$$

where SA_t and SA_i are the specific activities in the top phase and the crude enzyme, respectively. Also, the specific activity (SA) represents the ratio:

$$SA = \frac{A}{C_p} \quad (6)$$

where A and C_p are the enzyme activity and the total protein [13].

Thermodynamic parameters of ATPS

The enthalpy change (ΔH) was calculated by van Hoff's equation (7), free energy of Gibbs change (ΔG) and the entropy change (ΔS) were calculated using classical thermodynamic equations (8, 9) [13]:

$$lnK_e = -\frac{\Delta H}{r} \times \frac{1}{T} + \frac{\Delta S}{R} \quad (7)$$

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

$$\Delta G = -RTlnK_e \quad (9)$$

SDS-PAGE electrophoresis

SDS-PAGE was performed in a Cleaver Scientific Ltd; OmniPAGE Electrophoresis System CVS10DSYS, at 20 mA using a method described by Laemmli [14]. The acrylamide gel was prepared as a 15% resolving gel. Protein samples recovered from the top phase were concentrated and precipitated using 10% trichloroacetic acid (TCA) solution, which removed the salts that affect the electrophoresis process. The gel was stained with a

buffer solution consisting of 0.2% (v/v) Coomassie® Brilliant Blue G-250.

RESULTS AND DISCUSSION

Phase diagrams for PEG/potassium phosphate systems

PEG/potassium phosphate APTSs were formed by PEG 400 and PEG 4000 and potassium phosphate. Single phase and two-phase systems were separated by the binodal curves. Two phases were formed above the binodal curve (Fig.1). As the compositions of ATPS below the binodal curve, the system becomes homogenous. The concentration of PEG and salt solution required for formation of ATPSs decrease, when PEG with high molecular weight (PEG 4000) was used. The concept of stability ratio was applied to select appropriate APTS.

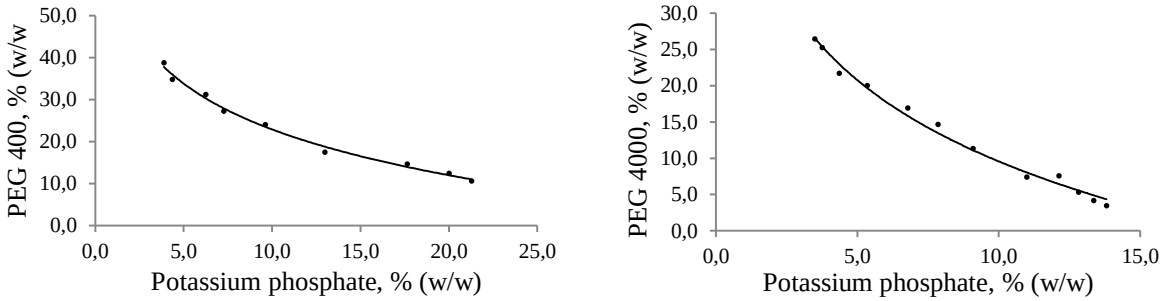

Fig. 1. Phase diagrams for PEG/potassium phosphate ATPSs: a. PEG 400, b. PEG 4000

Table 1. Characteristics of ATPSs PEG 400/potassium phosphate

PEG,%	Potassium phosphate,%	R_v	K_e	K_p	S	ΔG, kJ/mol	ΔH, kJ/mol	TΔS, kJ/mol
22	15	8.10	133.30	0.14	952.14	-12.12	-22.60	-10.48
24	15	3.58	50.00	0.07	714.29	-9.69	-21.60	-11.86
26	15	3.65	5.00	0.09	55.56	-3.98	-19.10	-15.08
28	15	4.29	4.00	0.06	66.67	-3.43	-18.80	-15.40
22	17	2.80	1.40	0.29	4.83	-0.83	-17.70	-16.87
24	17	2.33	0.37	0.18	2.06	2.33	-16.32	-18.66
26	17	2.20	0.39	0.22	1.77	2.46	-16.27	-18.73
28	17	2.82	2.40	0.19	12.63	-2.16	-18.28	-16.11
22	19	1.83	1.40	0.38	3.68	-0.83	-17.70	-16.87
24	19	1.89	0.62	0.16	3.88	1.18	-16.82	-18.01
26	19	1.93	9.00	0.18	50.00	-5.44	-19.70	-14.26

Table 2. Characteristics of ATPSs PEG 4000/potassium phosphate

PEG%	Potassium phosphate,%	R_v	K_e	K_p	S	ΔG, kJ/mol	ΔH, kJ/mol	TΔS, kJ/mol
20	15	1.94	10.00	0.10	100.00	-5.70	-19.82	-14.11
22	15	2.44	6.00	0.10	60.00	-3.99	-19.07	-15.08
24	15	1.46	6.67	0.04	166.75	-4.71	-19.38	-14.67
26	15	1.62	5.00	0.04	125.00	-3.99	-19.07	-15.08
20	17	1.37	11.00	2.53	4.35	-5.94	-19.92	-13.97
22	17	1.42	8.33	0.91	9.15	-5.25	-19.62	-14.36
24	17	1.79	26.00	0.59	44.07	-8.07	-20.84	-12.77
26	17	1.97	11.50	0.45	25.56	-6.05	-19.97	-13.91
20	19	1.18	15.00	0.06	250.00	-6.71	-20.25	-13.54
22	19	1.29	6.50	0.07	92.86	-4.64	-19.35	-14.71
24	19	1.43	3.67	0.22	16.68	-3.22	-18.69	-15.46
26	19	1.74	5.00	0.03	166.67	-3.99	-19.07	-15.08

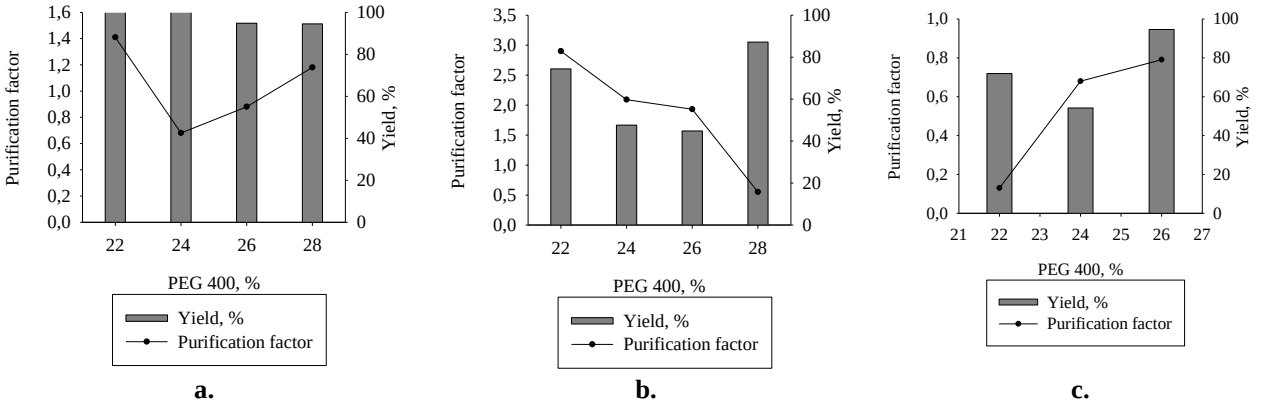

Fig. 2. Lipase purification with ATPS: a. PEG 400/15% potassium phosphate, b. PEG 400/17% potassium phosphate, c. PEG 400/19% potassium phosphate

Purification of lipase using ATPSs

PEG molecular weight can have an important role on the partition behavior of proteins. Enzymes were extracted by the polymer phase because of the polymer–protein interactions, while the accompanying proteins remained in the salt phase. Table 1 shows the main characteristics of lipase purification in PEG 400/potassium phosphate ATPS.

The partition coefficients of the enzyme (K_e) were found to be higher than l in most of the systems, indicating that the lipase is accumulated in the top phase. The partition coefficient of the protein (K_p) is around 1 or less than 1, which shows that the most of the protein is in the bottom phase. That is a prerequisite for a high selectivity of the ATPSs and purification of the enzyme.

The volume ratio (R_v) has values higher than 1 which indicates that the top phase has a higher volume than the bottom phase. Increase of the potassium phosphate concentration led to decrease of the R_v.

Changes of some thermodynamic parameters – enthalpy (ΔH), entropy (ΔS) and the free Gibbs energy (ΔG) of the systems were calculated which are characteristics of the stability of the systems. Free Gibbs energy (ΔG) has negative values in most of the cases which indicates that the partition of the enzyme in the top phase is a spontaneous process.Values of the change in the enthalpy (ΔH) and the entropy (ΔS) reveal that the process was exothermic.

On Fig. 2 lipase yield and purification factor in the top phases of ATPSs with different concentrations of PEG 400 and potassium phosphateare presented. As seen from the chart the highest purification factor was achieved with ATPS 22% PEG 400/17% potassium phosphate. The purification factor was about 3.00 but the yield was lower – about 75%. Highest yield was achieved with 15% potassium phosphate but the purification factor was about 1.00.

Purification of lipase from *Aspergillus carbonarius* NRRL369 with ATPS PEG 4000/potassium phosphate was also examined. As seen from Table 2 in all of the ATPSs with PEG 4000 K_e was higher than 1 and K_p was less than 1. That resulted in high selectivity of the systems (mostly between 25 and 250).

It can also be noticed that the purification factor of the enzyme was much higher when using PEG 4000 (reached 10.18) for ATPSs than when using PEG 400 (up to 2.9). That means that PEG 4000 was more effective for lipase purification than PEG 400. Barbosa *et al*. also confirmed that the increase of the molecular mass of PEG leads to increase of the efficiency of the process [13].

In most cases R_v had values between 1 and 2.

As seen from the thermodynamic parameters lipase partition was spontaneous exothermic process. Negative values of the free Gibs energy revealed that systems were more stable than the ATPSs with PEG 400.

On Fig. 3 protein purification and lipase yield for some ATPSs PEG 4000/potassium phosphate with different concentrations of the two components are shown. As seen from the chart when PEG 4000 was used most of the ATPSs resulted in a purification factor higher than 1.

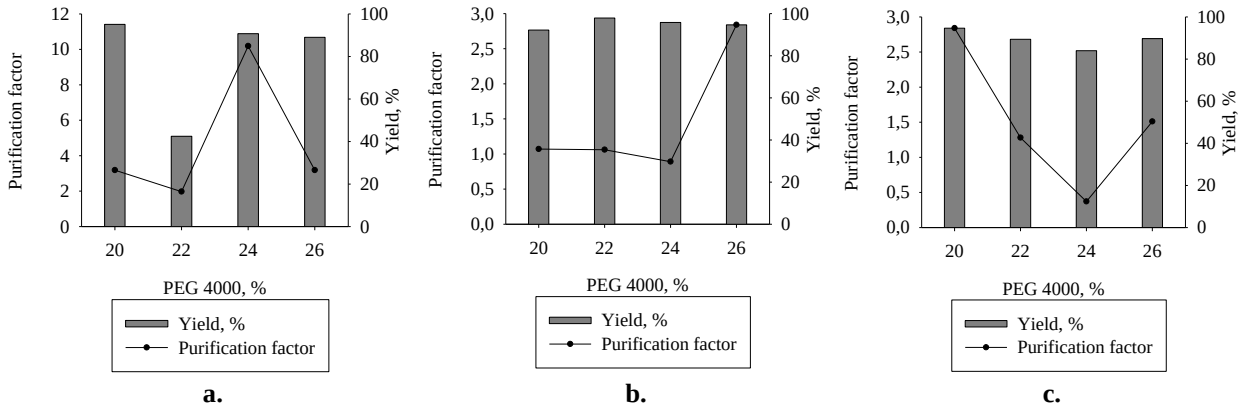

Fig. 3. Lipase purification with ATPS: a. PEG 4000/15% potassium phosphate, b. PEG 4000/17% potassium phosphate, c. PEG 4000/17% potassium phosphate

Table 3. Characteristics of ATPS 24% PEG 4000/15% potassium phosphate/NaCl

NaCl,%	R_v	K_e	K_p	S	ΔG, kJ/mol	ΔH, kJ/mol	TΔS, kJ/mol
0	1.46	6.67	0.04	166.75	-4.70	-19.82	-15.12
2	1.36	5.00	0.06	83.33	-3.99	-19.07	-15.08
4	1.22	4.00	0.10	40.00	-3.44	-19.39	-15.95
6	1.13	7.00	0.04	175.00	-4.82	-19.07	-14.25

The highest purification factor (10.18) was achieved with ATPS 24% PEG 4000/15% potassium phosphate. In this case lipase yield was also high – 90.68%. Because of that this ATPS was chosen for further experiments.

Effect of neutral salts on lipase partitioning

Addition of neutral salts to aqueous solutions provides one of the versatile means by which the selectivity and the yield of target molecules can be manipulated [15]. Changes in the salt type often produce an electrical potential difference between the two phases caused by the preference of one of the ions for one phase relative to the other. The effects of neutral salt were shown in Table 3 and Fig. 4. The APTS with composition 24% PEG 4000/15% potassium phosphate was carried out with NaCl salt at concentration 2%, 4%, 6%, 8%.

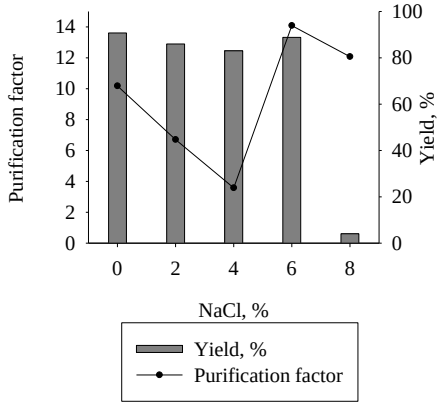

Fig. 4. Lipase purification with ATPS 24%PEG 4000/15% potassium phosphate/NaCl

The results for the influence of NaCl on ATPSs 24% PEG 4000/15% potassium phosphate shows that with the increase of the salt concentration to 6% R_v decreased to 1.13. With this concentration of the NaCl increase of the K_e was achieved while K_p had the same value. Because of that selectivity in the top phase was also increased.

As seen from the negative values of thermodynamic parameters the process was spontaneous and exothermic. Higher negative value of the free Gibbs energy when 6% NaCl was added shows that this system was more stable than the others.

When the concentration of NaCl was higher than 6% that led to crystallization of the salts and inconclusive results.

As seen from Fig. 4 with the increase of the NaCl concentration up to 4.0% the purification factor and lipase yield decreased from 10 to 4. The highest purification factor (14.08) was achieved when 6% NaCl was added to the system (ATPS 24% PEG 4000/ 15% potassium phosphate). In this case lipase yield was 88.81 which was relatively high. With increasing of NaCl concentration above 6% a decrease of the purification factor (12.07) and low lipase yield (14.73%) was achieved. A possible reason for this decrease is crystallization of the salts observed in these conditions.

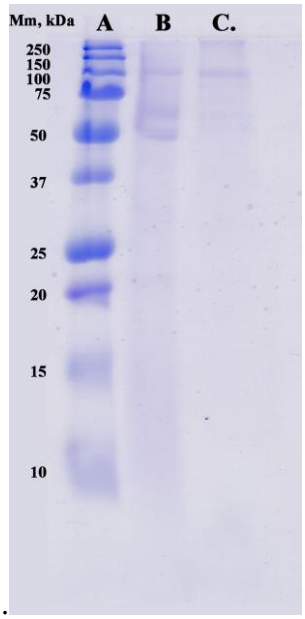

Fig. 5. SDS-PAGE electrophoresis of lipase from A. carbonarius: A. Protein markers, B. Cultural broth. C. Lipase from top phase of ATPS 24%PEG 4000/15%potassium phosphate/6%NaCl

As a result of this study an ATPS 24% PEG 4000/15% potassium phosphate/6% NaCl was chosen for optimal lipase purification and SDS-PAGE electrophoresis was performed (Fig. 5). As seen from the SDS-PAGE only two protein bands were isolated (Mm 100 and 250 kDa) which are possible to be multiple forms of lipase. Compared with the cultural broth (B) by using this ATPS, high purification was achieved.

CONCLUSION

ATPS is an easy, fast and effective method for purification of different biomolecules including enzymes. In this study purification of lipase from *Aspergillus carbonarius* NRRL369 was performed by using PEG/potassium phosphate ATPS. It was noticed that increasing of molecular weight of PEG leads to increasing of purification factor of lipase. As a result an ATPS 24%PEG 4000/15% potassium phosphate/6% NaCl was chosen because of the high purification factor (14.08) and lipase yield (88.81%).

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REFERENCES

1. G.S. Padilha, J.C.C. Santana, R.M. Alegre, B. Tambourgi, *Braz. Arch. Biol. Technol.*,**55**, 7 (2012).
2. 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).
3. A. Salihu, Z. Alam, M.I.A.Karim, H.M. Salleh, *Arab. J. Chem.*, **7**, 1159(2014).
4. C.H. Tan, P.L. Show, C.W. OOi, E.P. Ng, J.C. Lan, T.C. Ling, *Biotechnol. J.*, **10**, 31 (2015).
5. S. Thakur, *IJSER*, **3** (2012).
6. S.M. Basheer, S. Chellappan, P.S. Beena, R.V. Sukumaran, K.K. Elyas, M. Chandrasekaran, *New Biotechnol.*,**28**, 627 (2011).
7. A.M. Goja, H.Yang, M.Cui, C.Li, *J.Bioproces.Biotechniq.*, **4**, 140 (2013).
8. M. Amid, M.Y. Manap, M. Hussin S. Mustafa, *Molecules*, **20**, 11185 (2015).
9. G. Dobrev, B. Zhekova, V. Dobreva, H. Strinska, P. Doykina, A. Krastanov, *Biocatal. Agric.Biotechnol.*, **4**, 77 (2015).
10. J.N. Prazeres, J.A.B. Cruz, G.M. Pastore, *Braz.J.Microbiol*, **37**, 505 (2006).
11. M.M. Bradford, *Anal Biochem*, **72**, 248 (1976).
12. H.O. Johanson, E. Feitosa, A. Pessoa, *Polymers*, **3**, 587 (2011).
13. J.M.P. Barbosa, R.L. Souza, A.T. Fricks, G.M. Zanin, C.M.F. Soares, Á.S. Lima, *J.Chromatogr. B*, **879**, 3853 (2011).
14. U.K. Laemmli, *Nature*, **227**, 680 (1970).
15. M. Anvari, *Biotechnol.Biotechnol. Equip.*, **29**, 723 (2015).

ПРЕЧИСТВАНЕ НА ЛИПАЗА ОТ *Aspergillus carbonarius* NRRL369 С ДВУФАЗНИ ВОДНИ СИСТЕМИ ПОЛИЕТИЛЕН ГЛИКОЛ/КАЛИЕВ ФОСФАТ

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(Резюме)

Изследвано е пречистването на липаза от *Aspergillus carbonarius* NRRL369 с двуфазно водни системи. Построени са бинодалните криви на двуфазни водни системи ПЕГ 400/калиев фосфат и ПЕГ 4000/калиев фосфат и са избрани подходящи концентрации на двата компонента за изследване на процеса на пречистване. За всички двуфазни водни системи са изчислени термодинамичните параметри, за да се опишат процесите. Изследвано е пречистването на липаза с различни двуфазни водни системи. Установено е, че повишаването на молекулната маса на ПЕГ води до повишаване степента на пречистване на липазата. Резултатите показват, че най-подходяща за пречистването на липаза е двуфазна водна система със състав 24% ПЕГ 4000/15% калиев фосфат. Постигнат е добив на липаза 90.68% и степен на пречистване 10.18 пъти. Добавянето на 6% NaCl към двуфазна водна система със състав 24% ПЕГ 4000/15% калиев фосфат повишава степента на пречистване до 14.08 пъти, като крайния добив е 88.81%.

Ключови думи: *липаза, пречистване, двуфазни системи, Asp. Carbonarius*