ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Purification of Lipase From *Rhizopusarrhizus* Using Aqueous Two-Phase System PEG/$(NH_4)_2SO_4$.

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ABSTRACT

In this research purification of lipase from Rhizopusarrhizus by aqueous two-phase system (ATPS) was studied. Phase diagrams of ATPSs PEG400/ $(NH_4)_2SO_4$, PEG4000/ $(NH_4)_2SO_4$ and PEG6000/ $(NH_4)_2SO_4$ 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. The results revealed that for purification of lipase from Rhizopusarrhizus an ATPS 30% PEG4000/ $(NH_4)_2SO_4$ was the most suitable. By using this ATPS high purification factor (5.37) and lipase yield (31.6%) were achieved.

Keywords: aqueous two-phase system, lipase, purification, Rhizopusarrhizus

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ISSN: 0975-8585

INTRODUCTION

Lipases (EC 3.1.1.3) are enzymes with wide share in different industries. These enzymes catalyze the hydrolysis of triacylglycerol and reactions of esterification and thansesterification in organic solvent medium. Following purification, lipases may be used in the fat andoil/chemical industry, for the removal of different lubricants in the textile industry, as industrial detergents, for the resolution of racemic mixtures, in food processing, cosmetics, medical applications, in the pulp and paper industry, for oil degradation and biodiesel production [1, 2, 3, and 4].

Aqueous two-phase systems (ATPS) are a perspective method for protein purification because high yield and purification factor can be achieved. These methods are fast, easy to perform and there is no need of expensive apparatus and materials [1, 5, and 6]. ATPSs contain two liquid phases which are immiscible in concentrations above critical. Commonly used are ATPSs polymer/salt (PEG/K_2HPO_4, PEG/ $(NH_4)_2SO_4$) and polymer/polymer (PEG/Dextran) [6, 7].In order to be used for enzyme purification, conditions for the formation of each ATPS, their physicochemical characteristics and TLL must be studied.

Appropriately designed two-phase aqueous systems are used for isolation and purification of lipase. Zhou et al. [8] compose an ATPS 17%PEG/13%K_2HPO_4 with pH 7.0 and at 4°C for purification of pancreatic lipase. By using this ATPS 94.7% enzyme yield and 4-fold purification was achieved.

Addition of a salt, usually NaCl, to ATPS often improves the results for isolation of microbial lipases.

Application in lipase purification has an ATPS 16% 2-propanole/16% K_2HPO_4 in the presence of 4.5% NaCl. By using these ATPS Ooi et al. achieved 13.5 purification fold and 99% lipase yield[7].

An effective ATPS for purification of lipase from Bacillus was 20% PEG8000/18% K_2HPO_4/6% NaCl with pH 6.0 at 4°C. In this case high purification factor (201.53) was achieved [1].

Zhang and Liu [9] used an ATPS for partial purification of lipase from Trichosporonlaibacchii. Purification factor 5.84 and enzyme yield 80.4% were achieved by using ATPS 12% PEG4000/13% K_2HPO_4/2.0% NaClwithpH 7.0. PEG-rich phase (in which the enzyme was partitioned) was later used for immobilization of lipase with glutaraldechide[9].

In the present study physicochemical characteristics of ATPSs containing PEG/$(NH_4)_2SO_4$ and their application for purification of lipase from Rhizopusarrhizus were studied.

MATERIALS AND METHODS

Microorganism and lipase production

The studied Rhizopusarrhizus strain used in this study was provided by Biovet® Peshtera. Growth medium, inoculums preparation and submerged cultivation conditions were described in previous article [10].

Lipase assay

Lipase activity was measured by spectrophotometric method using p-nitro phenyl palpitate as substrate buffered with Tries-HCl pH 9.0 [11]. 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-nitro phenol per minute under the assay conditions described.

Protein assay

Protein concentration was measured by the method of Bradford [12].

Phase diagram

Bimodal curves were estimated by using cloud-point method [7]. TLLs were prepared by using dry-weight method described by Kaul[13].

Purification of lipase with ATPS

All ATPS were prepared in 15 cm^3 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 6000), and (NH4) 2SO4 and enzyme solution were mixed in concentrations determined by bimodal curve. The tubes were shaken on vortex for 2 min, followed by centrifugation for 20 min at 4000 rpm.

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 [1].

Determination of selectivity, purification factor and yield

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

$$Kp = \frac{Cpt}{Cpb} \quad (1)$$

Where Cpt and Cpb are the concentrations of protein in the top and bottom phases.

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

$$Ke = \frac{At}{Ab} \quad (2)$$

where At and Ab are the enzyme activity in the top and bottom phases.

The volume ratio (Rv) between volumes of top and bottom phases was calculated:

$$Rv = \frac{Vt}{Vb}$$

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

$$Y\% = \frac{At}{A} . 100 \quad (3)$$

where A is the lipase activity of the stock solution.

Selectivity (S) was calculated as:

$$S = \frac{Ke}{Kp} \quad (4)$$

The purification factor in the top phase (PFtop) was defined as:

$$PFtop = \frac{SAt}{SAi} \quad (5)$$

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

ISSN: 0975-8585

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

where A and Cp are the enzyme activity and the total protein [1].

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)[1].

$$\ln Ke = \frac{\Delta H}{R} \cdot \frac{1}{T} + \frac{\Delta S}{R} \quad (7)$$

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

$$\Delta G = -R.T.L.\ (Ke) \quad (9)$$

where T is temperature (Kelvin) and R is the ideal gas constant.

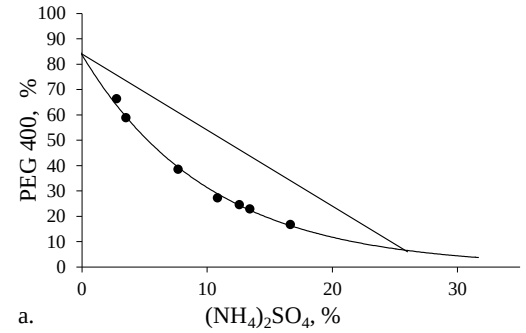
SDS-PAGE electrophoresis

SDS-PAGE was performed in a Cleaver Scientific Ltd; Omni PAGE Electrophoresis System CVS10DSYS, at 20 mA using a method described by Laemmli[14].15% resolving gel was used. Protein samples recovered from the top phase were concentrated and precipitated using 100% TCA solution for removing the salts. The gel was stained with a buffer solution consisting of 0.2% (v/v) Coomassie® Brilliant Blue G-250.

RESULTS AND DISCUSSION

Phase diagrams

Phase diagrams of ATPSs PEG400/$(NH_4)_2SO_4$, PEG4000/$(NH_4)_2SO_4$ and PEG6000/$(NH_4)_2SO_4$ were developed (Fig.1).Bimodal curves characterize the two phase formation of the ATPSs used for purification of lipase from Rhizopusarrhizus. TLL of every phase diagram were also found out. It characterizes the stability of the system – the longer TLL is the system is more stable. As seen from Fig. 1 ATPS PEG 4000/$(NH_4)_2SO_4$ was the most stable. All ATPSs were used for lipase purification.



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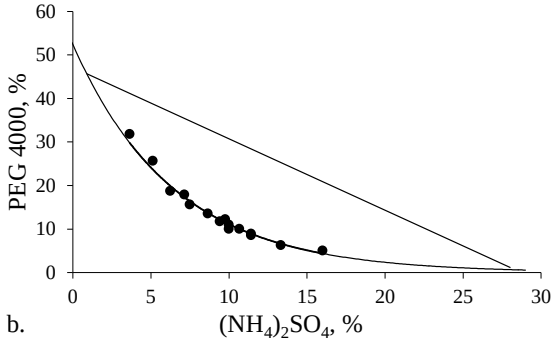

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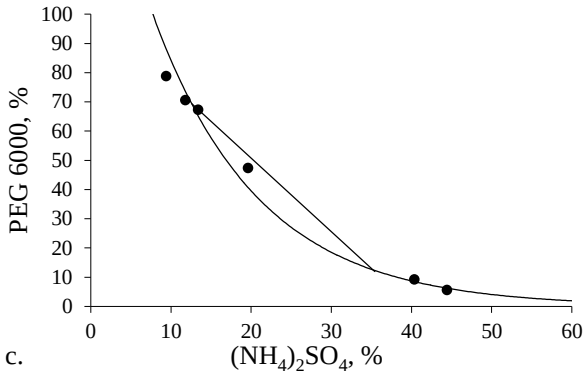

Fig 1: Phase diagrams of PEG/$(NH_4)_2SO_4$ ATPSs: a. PEG400, b. PEG4000, c. PEG6000

Application of ATPSs for purification of lipase from Rhizopusarrhizus

Table 1 shows the main characteristics of lipase purification in PEG 400/$(NH_4)_2SO_4$ATPS.For all of the ATPSs Ke>1 which means that the enzyme was in the top phase. Increasing of PEG 400 concentration led toa decrease in Ke. Low values of Kp reveals low concentration of protein in the top phase which may lead to high purification fold. The volume ratio (Rv) was in the range 1.0 - 2.0 which indicated higher or equal volume of the top phase.

Table1: Characteristics of PEG 400/$(NH_4)_2SO_4$ ATPSs

PEG400 (%)	$(NH_4)_2SO_4$ (%)	Ke	Kp	Rv	S	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ/mol)
22.5	15	50.97	2.50	2.00	20.39	-21.57	-9.74	-0.040
25.0	15	33.43	2.17	2.00	15.38	-21.12	-8.69	-0.042
27.5	15	23.66	3.64	1.85	6.51	-20.75	-7.84	-0.043
30.0	15	35.61	3.81	2.00	9.35	-21.19	-8.85	-0.041
22.5	17	3.95	2.67	1.21	1.48	-18.82	-3.40	-0.052
25.0	17	9.52	2.29	1.33	4.17	-19.77	-5.58	-0.048
27.5	17	9.13	1.83	1.50	4.99	-19.72	-5.48	-0.048
30.0	17	13.07	1.60	1.63	8.17	-20.11	-6.37	-0.046
22.5	19	5.81	50.00	1.09	0.12	-19.24	-4.36	-0.050
25.0	19	4.48	49.00	1.25	0.09	-18.96	-3.72	-0.051
27.5	19	3.95	42.00	1.25	0.09	-18.82	-3.40	-0.052
30.0	19	4.40	46.00	1.04	0.10	-18.94	-3.67	-0.051

Changes of some thermodynamic parameters – enthalpy (ΔH), entropy (ΔS) and the free Gibbs energy (ΔG) were calculated. These parameters characterize the stability of the ATPSs. ΔG had negative value which indicated that the partition of the enzyme in the top phase was a spontaneous process. The values of ΔH and ΔS revealed that the process was exothermic. Similar results were achieved by Nascimentoetal.who studied the partition of gluten in from wheat[15].

Fig. 2 shows the purification fold and lipase yields in the top phases of PEG 400/$(NH_4)_2SO_4$ ATPSs. Although the selectivity of some ATPSs was high, enzyme yield was between 40 and 70 % but purification factor was lower than 1.0. A reason for this may be the low yield and low enzyme activity in the bottom phase. In these ATPSs Kp>1 which indicated that not only the enzyme but also other proteins were present in the top phase. The reason for the activity loss may be an adsorption of lipase molecules between the two phases.

ISSN: 0975-8585

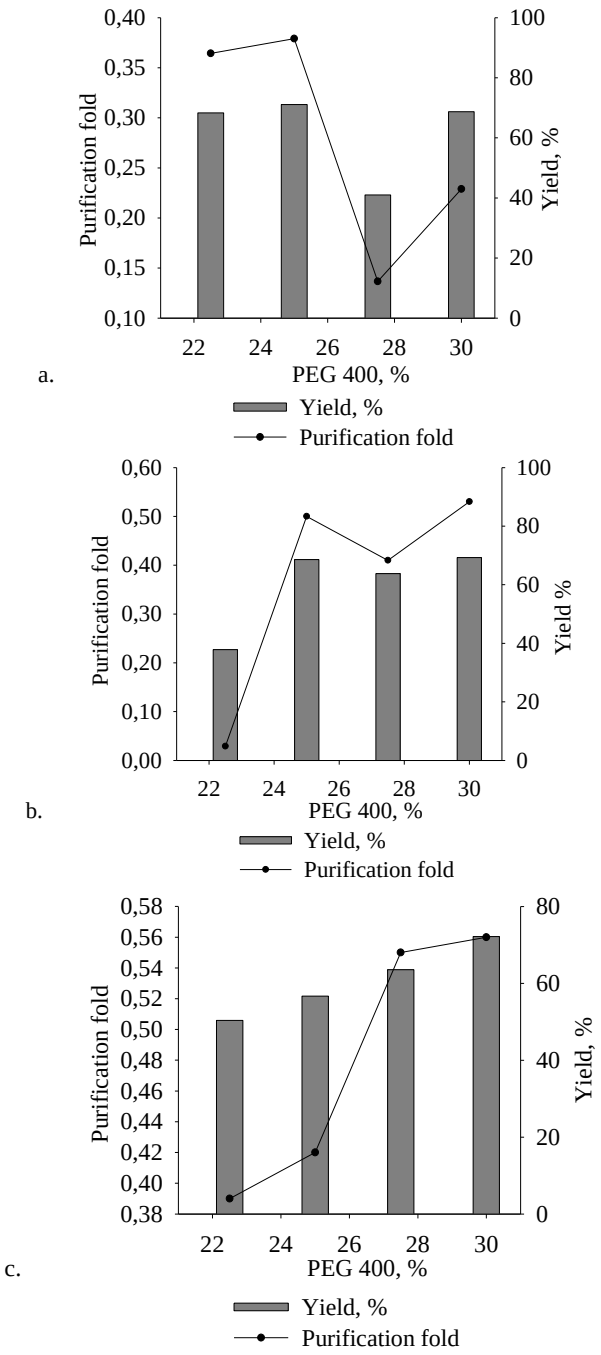

Fig 2: Purification of lipase with PEG400 ATPSs: a. PEG400/15%$(NH_4)_2SO_4$; b. PEG400/17%$(NH_4)_2SO_4$; c. PEG400/19%$(NH_4)_2SO_4$

Table 2 reveals the main characteristics of ATPSsPEG4000/$(NH_4)_2SO_4$. The enzyme was distributed in the top phase (Ke>1.0) while most of the protein was in the bottom phase (Kp<1.0). With the increase of PEG 4000 concentration, Ke also increased. Bassano et al. [16]assumed that in high concentrations of the polymer a compact hydrophobic complex with high affinity to hydrophobic ligands was formed. The presence of hydrophobic domain in the molecule was the reason for lipase to be in the top phase.

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Table2: Characteristics of PEG 4000/$(NH_4)_2SO_4$ ATPSs

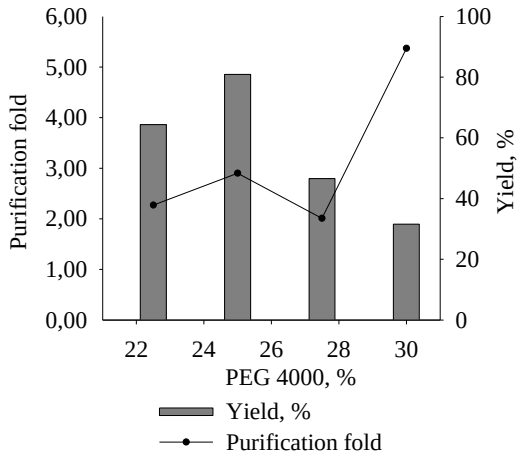
PEG4000 (%)	$(NH_4)_2SO_4$ (%)	Ke	Kp	Rv	S	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ/mol)
22.5	15.0	4.46	1.77	1.03	2.52	-18.95	-3.70	-0.051
25.0	15.0	6.90	0.52	1.23	13.19	-19.42	-4.79	-0.049
27.5	15.0	5.03	0.82	1.32	6.17	-19.08	-4.00	-0.051
30.0	15.0	6.96	0.47	1.49	14.89	-19.43	-4.81	-0.049
22.5	17.0	14.21	0.71	0.91	20.11	-20.20	-6.58	-0.046
25.0	17.0	7.61	0.80	1.05	9.56	-19.53	-5.03	-0.049
27.5	17.0	8.73	1.62	1.14	5.39	-19.67	-5.37	-0.048
30.0	17.0	20.20	1.47	1.32	13.75	-20.58	-7.45	-0.044
22.5	19.0	9.71	0.67	0.80	14.40	-19.79	-5.63	-0.048
25.0	19.0	5.18	1.54	0.88	3.36	-19.11	-4.08	-0.050
27.5	19.0	14.47	8.57	1.00	1.69	-20.22	-6.62	-0.046
30.0	19.0	13.85	9.75	1.10	1.42	-20.17	-6.51	-0.046

Rv was close to 1.0 which mint that both phases had approximately equal volume.

The negative values of ΔG suggested that the process was spontaneous. The process was with enthalpy origin.

In all ATPSs with PEG 4000, purification fold in all cases were above 1.0 (Fig. 3). Highest purification fold (5.37) was achieved in ATPS 30%PEG4000/15%$(NH_4)_2SO_4$. In this case lipase yield was 31.6%. This ATPS was characterized with Ke=6.96, Kp=0.47, S=14.89. Highest lipase yield (about 80%) was achieved by using ATPS 25%PEG4000/15%$(NH_4)_2SO_4$. However in this case the purification factor was much lower – 2.9. It can be noticed that PEG 4000 was more effective for lipase purification than PEG 400. Barbosa et al. also confirmed that the increase of the molecular weight of PEG led to increase in the efficiency of the process [1].

a.



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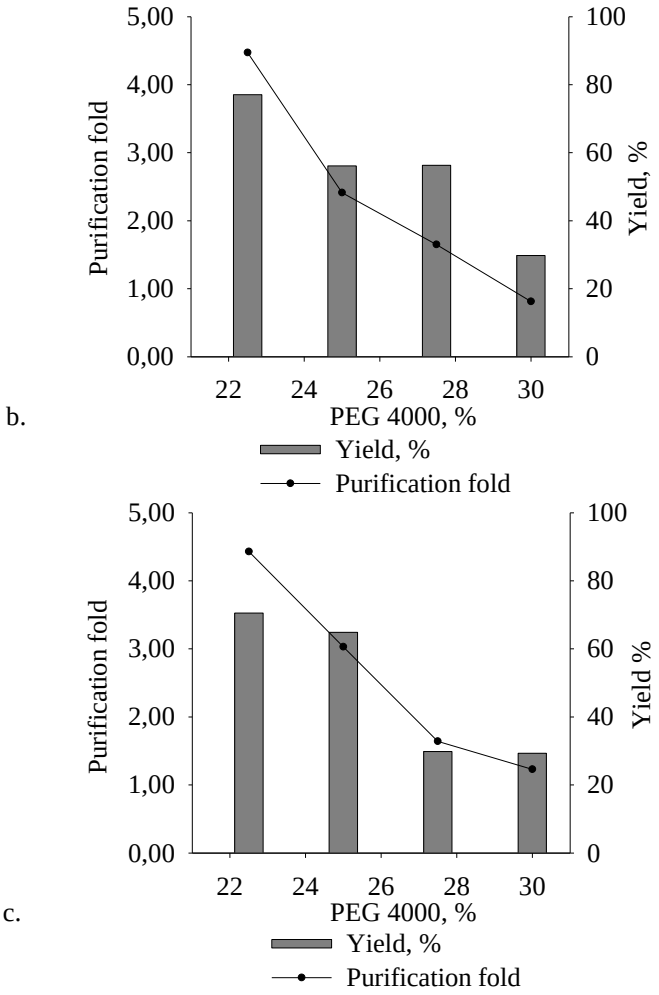

Fig 3: Purification of lipase with PEG4000 ATPSs: a. PEG4000/15%$(NH_4)_2SO_4$; b. PEG4000/17%$(NH_4)_2SO_4$; c. PEG4000/19%$(NH_4)_2SO_4$

In Table 3 the results for some ATPSs containing PEG6000/$(NH_4)_2SO_4$ are presented. In all cases, Ke was close to 1.0 which mint that enzyme activity was present in both phases. However, Kp was higher than 1.0 which indicated that most of the total protein was in the top phase. That led to low selectivity of the systems. Thermodynamic parameters were negative but their absolute values were lower than those in the other ATPSs. That indicated that ATPSs with PEG 6000 had lower stability than ATPSs with PEG400 and PEG4000.

Table3: Characteristics of PEG6000/$(NH_4)_2SO_4$ ATPSs

PEG6000 (%)	$(NH_4)_2SO_4$ (%)	Ke	Kp	Rv	S	ΔG (kJ/mol)	ΔS (kJ/mol)	ΔH (kJ/mol)
22.5	15.0	1.37	0.70	0.75	1.97	-0.78	-0.057	-16.96
25.0	15.0	3.67	1.55	0.94	2.37	-3.22	-0.052	-17.81
27.5	15.0	2.19	1.00	1.00	2.19	-1.94	-0.055	-17.34
30.0	15.0	1.18	2.33	1.25	0.50	-0.41	-0.057	-18.25
22.5	17.0	1.89	1.75	0.80	1.08	-1.58	-0.055	-17.95
25.0	17.0	3.97	2.67	1.31	1.49	-3.42	-0.052	-18.40
27.5	17.0	9.79	1.50	1.20	6.53	-5.65	-0.047	-17.78
30.0	17.0	5.93	1.75	1.25	3.39	-4.41	-0.050	-17.95
22.5	19.0	13.31	3.15	0.80	4.22	-6.41	-0.046	-18.58
25.0	19.0	3.98	12.00	1.00	0.33	-3.42	-0.052	-20.02
27.5	19.0	5.58	11.43	1.00	0.49	-4.26	-0.050	-19.96
30.0	19.0	2.83	1.50	1.25	1.88	-2.58	-0.053	-17.78

ISSN: 0975-8585

Fig. 4 revealed that lipase yield and purification factor were lower when PEG 6000 was used compared with the results with PEG 4000. A reason for this low yields might be the low values of Ke and selectivity, which indicated that the enzyme was partitioned in both phases. Similar results were reported by Zhou et al. [8].As a result of this study an optimal ATPS 30%PEG4000/15%$(NH_4)_2SO_4$ was chosen. In this case 31.6% lipase yield and 5.37 purification fold were achieved.

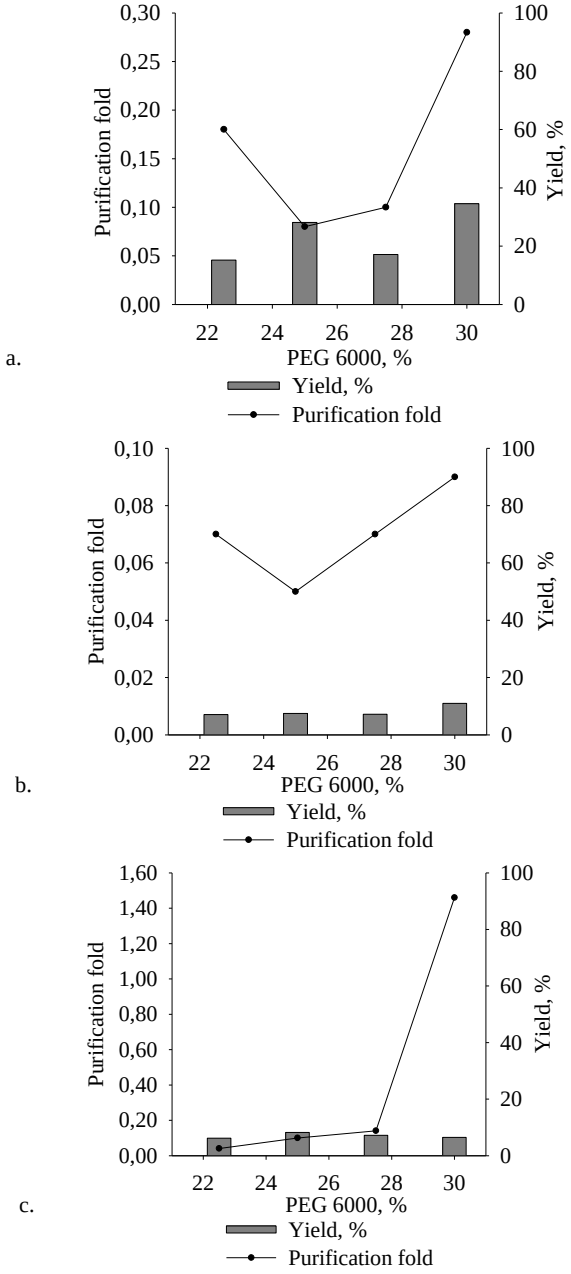

Fig 4: Purification of lipase with PEG6000 ATPSs: a. PEG6000/15%$(NH_4)_2SO_4$; b.PEG6000/17%$(NH_4)_2SO_4$; c. PEG6000/19%$(NH_4)_2SO_4$

By addition of neutral salts to ATPSs the selectivity and the yield of target molecules can be manipulated. Changes in the salt may produce an electrical potential difference between the two phases. Thus, at a certain salt concentration the partitioning of lipase could be driven towards the PEG-rich phase[17].For these reasons the effect of different concentrations NaCl on formation of ATPS 30%PEG4000/15%$(NH_4)_2SO_4$ and lipase purification was investigated(Table 4 and Fig. 5 respectively).Ke was relatively high but decreased with the increase of NaCl concentration. Kp was also high which was the reason for the low selectivity (no more than 5).

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Table4: Characteristics of 30% PEG 4000/15% $(NH_4)_2SO_4$/NaCl ATPSs

PEG4000 (%)	$(NH_4)_2SO_4$ (%)	NaCl (%)	Ke	Kp	Rv	S	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ/mol)
30.0	15.0	0.0	6.96	0.47	1.49	14.89	-19.43	-4.81	-0.049
30.0	15.0	2.0	17.58	5.45	1.25	3.22	-20.43	-7.10	-0.045
30.0	15.0	4.0	15.16	3.20	1.57	4.74	-20.27	-6.74	-0.045
30.0	15.0	6.0	13.73	5.23	1.29	2.63	-20.16	-6.49	-0.046
30.0	15.0	8.0	7.80	9.20	1.25	0.85	-19.55	-5.09	-0.049
30.0	15.0	10.0	5.37	3.60	1.43	1.49	-19.15	-4.16	-0.050

As seen from Fig.5 addition of NaCl led to increase of lipase yield but it also increased the concentration of protein in the top phase, which decreased the purification factor.

In order to track the purification of lipase from Rhizopusarrhizusby ATPS 30%PEG4000/15%$(NH_4)_2SO_4$ an SDS-PAGE electrophoresis was performed (Fig.6). Only one protein band was present, which indicated that one lipase with approximate100 kDa molecular weight was isolated.

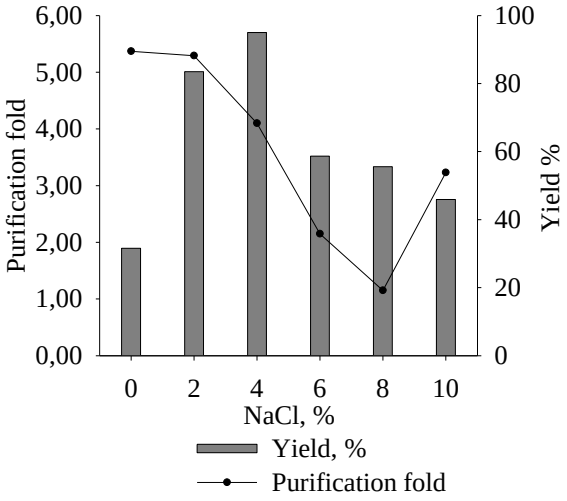

Fig 5: Lipase purification with 30%PEG4000/15%$(NH_4)_2SO_4$/NaClATPS

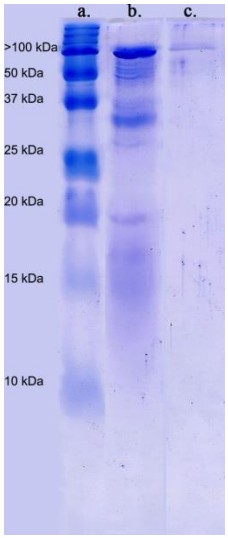

Fig 6: SDS-PAGE of lipase from R. arrhizus: a. Protein markers, b. Cultural broth, c. Lipase from top phase of 30%PEG4000/15%$(NH_4)_2SO_4$ATPS

CONCLUSION

Bimodal curves and TLLs of ATPSs with PEG with different molecular weights and $(NH_4)_2SO_4$were developed. The most stable ATPS was PEG4000/$(NH_4)_2SO_4$. When purification of lipase with ATPSswas studied an ATPS 30% PEG4000/15%$(NH_4)_2SO_4$ was found to be the most suitable. By using this ATPS high purification factor (5.37) and lipase yield (31.6%) were achieved and one lipase with Mm 100 kDa was isolated.

ACKNOWLEDGEMENT

The research was supported by Project№ 05/16-H, University of food technologies, Plovdiv, Bulgaria. The authors are grateful to Biovet® Peshtera for providing us the opportunity to work with the studied strain Rhizopusarrhizus.

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