Aqueous Two-Phase Systems for the Isolation and Partial Purification of Lipases from Soil Bacteria

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ABSTRACT: *Aqueous Two-Phase System (ATPS) is an effective, simple, and single-step technique used for the partial purification of biological molecules. The aim of the study was to develop ATPS technique for the isolation and partial purification of lipases. ATPS composed of polyethylene glycol and ammonium sulfate was used in this study to partially purify lipase from the fermentation broth of a Bacillus strain isolated from local soil. The effect of different percent compositions of PEG 4000, 6000, and 10,000 along with 6.60%, 7.26%, 7.92%, and 8.26% ammonium sulfate was studied on the separation behavior of lipase. The optimal condition for the isolation and partial purification of lipases from soil bacterial was 12.5 % of PEG 10,000 with 7.92% ammonium sulfate. The recovery of lipase was 78.3% in the top phase (rich in PEG).*

KEYWORDS: *Aqueous two-phase system; Polyethylene glycol; Ammonium sulfate; Lipases*.

INTRODUCTION

In the recent era of biotechnology, there is a growing interest in the development of innovative separation and purification strategies that are gentle to preserve the biological activity of biomolecules and also economically viable [1]. Many techniques like high performance tangential flow filtration [2], membrane chromatography [3, 4], affinity precipitation [5, 6], crystallization [7] and high gradient magnetic fishing [8] are currently in use for separation and purification of target molecules. The major drawbacks of these strategies are lower yields, high costs

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and column fouling. Several methods have been described in the literature which can serve as substitute for partial purification chromatographic techniques. Aqueous Two Phase Systems (ATPS) have proved to be such an attractive and alternative technique for the industrial scale production [9]. It is an integrated technique where initial clarification, concentration and partial purification are obtained in single unit operation, thus reducing the number of steps involved in protein purification [10]. ATPS is formed when two or more than two polymers or one polymer and an inorganic salt are mixed together in water above their critical composition [11]. The properties of the phase system such as the type and concentration of salts in the system, concentration and molecular mass of the polymer and pH of the system affect the partitioning of the biomolecules. Similarly, properties of the biomolecules i.e., structure, hydrophobicity and molecular mass also has an important role in the separation of biomolecules in ATPS [12]. Polymer-polymer aqueous two phase system has been replaced by polymer-salt for large scale production because salt is cheaper, less viscous and also requires short time for phase partitioning [13]. The partitioning of various biomolecules in different aqueous two phase system has been reported in literature [14, 15] such as; α-galactosidase [16], xylanase [17], xylose reductase [18], trypsin and α-chymotrypsin [19], β-glucosidase [20] and polyphenoloxidase [14].

Lipases (triacylglycerol acyl hydrolyse, EC 3.1.1.3) are class of hydrolases that hydrolyze triglycerides into diglycerides, monoglyceride, fatty acid and glycerol by acting on the carboxylic ester bonds. Lipases have wide range of applications in detergent, leather, cosmetic, textile, paper and pharmaceutical industry due to their ability to catalyze esterification, interestrification and transestrification reactions in non-aqueous medium [21]. The occurrence of lipases is widely spread in pancreas of humans and pigs. Additionally, plant seed like rapeseed (Brassica napus) and rapeseed (Ricinuscommunis) also consist this enzyme but lipases are most abundantly found in microbial flora [22]. In biotechnology lipases from the microbial origin have got much more attention due to their commercial importance [23].

Partial purification with lower yields have been reported for the recovery of lipases utilizing conventional procedures like precipitation or chromatography [24]. An efficient and cost effective downstream processing technique is required for the commercial scale production of the enzymes [25]. Purification of lipase from Burkholderia pseudomallei with alcohol/salt [26], dextran/PEG [27] and PEG/phosphate [28] based aqueous two phase systems have been performed. Some other investigations reported lipase extraction from Pseudomonas cepacia and Aspergillus niger by the use of a detergent-based aqueous two-phase system [29], and PEG/salt system [30]. ATPS composed of PEG/$NH_4(SO_4)_2$ has also been used for the purification of phenylalanine dehydrogenase from the recombinant strain of bacillus [31]. These studies suggest that there is still a room to further elaborate the effect of different components of the phase composition on the separation behavior of lipases from sources other than described in the literature. This work has been designed for the extraction of lipase from an indigenous bacteria (Bacillus strain) isolated from local soil, utilizing ATPS. Our study is different from the already reported literature due to selection of the microbe and the phases used for the separation of lipase produced from this microbe.

EXPERIMENTAL SECTION

Materials

Polyethylene glycol (molecular weight 4000, 6000 and 10,000) Sodium dodecyl sulfate, Tris, ethanol, methanol and iso-propanol were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium-per-sulfate, sodium hydroxide, methyl red and calcium chloride were purchased from Riedal-de Haen (Seezle, Germany). Tryptone, yeast extract and sodium chloride, were purchased from Oxoid Basingstoke (Hampshire, England). Acrylamide and N, N, methylene-bisacrylamide from Roth (Karlsruhe, Germany). Coomassie brilliant blue G-250, agar, tween 20 and N NNN, were purchased from AppliChem (Darmstadt, Germany). Acetic Acid and phosphoric acid was bought from Lab-Scan analytical sciences (Bangkok, Thialand). Ammonium sulfate were purchased from Merck (Germany) and hydrochloric acid from Scharlau (Spain).

Methods

Media preparation for Bacterial Culturing and Confirmation of lipases (Plate assay)

Submerged culture of lipase producing bacteria (identified as non-pathogenic *Bacillus subtilis* by 16S rRNA)

isolated from local soil (Kohat, Khyber Pakhtunkhwa, Pakistan) was carried out in Luria Bertani (LB) media containing 1% tryptone, 1% NaCl and 0.5% yeast extract. After 48 hours of incubation, the broth was centrifuged at 10,000 rpm for 15 minutes and supernatant was collected and stored in refrigerator for further use.

Sample was tested for the presence of lipase on Tween-20 agar plates prepared by mixing 2% Tween (used as a substrate) and 0.01g methyl red (as an indicator) in distilled water [32] and autoclaved at 121°C for 20min and poured in a plate. After solidification wells were bored and different volumes (10, 15, 20, 25, 30, 35, 40 µL) of supernatant (containing lipases) were loaded into each well.

Formation of Binodal curve and Preparation of Aqueous two phases system

Stock solutions of 25%, 30%, 35% and 40% of PEG (molecular weight 4000, 6000 and 10,000) and ammonium sulfate (2.0 M, 2.2 M, 2.4 M and 2.6 M) were prepared in distilled water. ATPSs were prepared according to binodal curve by mixing an appropriate quantity of PEG, ammonium sulfate, water and enzyme extract. After thorough vertex, the systems were centrifuged at 2500 rpm for 5 mins. The systems were allowed to settle for 20 min to achieve phase equilibrium. After complete phase separation, samples were carefully taken from the top and bottom phases and were checked for total protein contents and lipases activity.

Bradford assay

Bradford assay was performed for the quantitative determination of proteins in the top and bottom phase. Bradford reagent was prepared for the assay according to described protocol (33). Bovine serum albumin was taken as standard. Absorbance was recorded using UV spectrophotometer at 595 nm.

Lipase activity assay

Lipase activity in each phase was calculated by turbidometric method. 100 µl from each sample was taken in test tube and added with 300µl of 120 mM of calcium chloride and 3.6µl of 20 mM of 2% Tween-20. Absorbance was recorded on spectrophotometer at 500 nm per minute over a period of time (10 mins). One unit of lipase was defined as, the change in absorbance per minute [34].

$$\text{Unit} = \frac{\text{Change in Absorbance}}{\text{Minute}} \quad (1)$$

Other parameters were calculated according to the following equations [15, 35, 36].

Phase volume ratio

$$V_r = \frac{V_t}{V_b} \quad (2)$$

Where V_t is the volume of the top phase and V_b is the volume of the bottom phase

Partition coefficient

The partition coefficient is defined as the activity of enzyme in the top phase divided by its activity in the bottom phase and was calculated as

$$K_e = \frac{A_t}{A_b} \quad (3)$$

A_t stands for enzyme activity in top phase and A_b is the enzyme activity in the bottom phase. It is determined quantitatively as unit per milliliter (U/mL)

Specific Activity

Specific activity is the ratio between activity of enzyme in a phase and the total protein content in that phase and was calculated as

$$S_A = \frac{A_i}{P_t} \quad (4)$$

Where A_i is the activity of enzyme and P_t is total protein in that phase. Unit is determined in U/mg.

Purification factor

Purification factor (fold) was defined as

$$P_{F(t)} = \frac{SA_t}{SA_c} \quad (5)$$

$$P_{F(b)} = \frac{SA_b}{SA_c} \quad (6)$$

SA_t and SA_b are the specific activity in top and bottom phases, respectively and SA_C is the specific activity in crude.

Yield (%)

Yield in top and bottom phase were calculated as,

$$Y_t = \frac{100.Vt.K}{Vt.K + Vb} \quad (7)$$

$$Y_b = \frac{100.Vb}{Vt.K + Vb} \quad (8)$$

Where Y_t and Y_b are the yields in top and bottom phases, respectively. V_t and V_b are volumes of top and bottom phases, respectively.

RESULTS AND DISCUSSION

Presence of lipases in the cultured broth

The presence of lipases were confirmed on Tween-20 agar plates. Different volumes (10, 15, 20, 25, 30, 35 and 40 µL) were loaded onto the wells (bored through the pipette tips) of Tween-20 agar plates to check the activity of the enzyme. The plates were incubated overnight and clear zones around each well were observed. This is due to the hydrolysis of Tween-20 by lipases indicating the presence of extracellular lipases in the supernatant. Increase in lipase activity was noted by increasing the volume of supernatant to the wells.

Binodal Curves for polymers (PEG 4000, 6000, 10,000) and salt (ammonium sulfate)

Binodal curve (phase diagram) was constructed for different PEG (4000, 6000 and 10,000) and Ammonium sulfate at different compositions (Fig. 1). The phase formation is necessary before the application of the crude extract. Generally, the area above the curve defines two phases while below the curve is the formation of single phase. The composition should be kept above the curve for better separation of the target molecule. It was noted that PEG molecular weight has a prominent effect on phase formation. The binodal curve tends to approach toward the origin as the molecular weight of PEG increases from 4000 to 10,000.

Effect of PEG 4000 and ammonium sulfate compositions on the partition behavior of lipases

The polymer (PEG 4000) composition has a prominent effect on the partitioning behavior. Increasing the polymer concentration the protein tends to move to the top phase. For example, Table 1, case 1, the partition coefficient for the lower composition of PEG 4000 (12.5%) with ammonium sulfate (6.60%) was 1.16 U/mL. This was high due to the free space phenomena but in this case the percent yield was on the lower side (73.02%). When the composition of PEG 4000 increases from 15% to 17.5% and 20% (keeping salt concentration constant i.e., 6.60%),

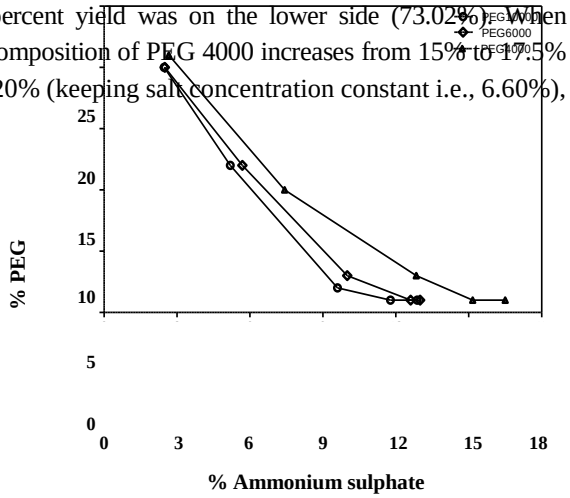

Fig. 1: Phase diagram of PEG (4000, 6000 and 10.000) and Ammonium sulfate represents the initial and final concentration of two phase system on the tie line curve.

a gradual increase in the partition coefficient from 1.11 to 1.14 U/mL was observed.

Similarly, the top phase yield was increased from 73.03% to 74.44%. The increase in the partition coefficient for PEG (15%, 17.5% and 20%, respectively) could be perfectly explained by affinity phenomena. The hydrophobic interactions are developed between the PEG and protein in these systems. Increase in PEG composition allow high number of attachment sites (25). This increase in the attachment sites causes most of the lipases into the top phase. In other ATPS, the partition coefficient decreased with the increase in PEG composition. When all the PEG compositions were tested separately with 7.26% and 7.92% ammonium sulfate, a regular decrease in the partition coefficients and percent yields were observed in cases 2 and 3 in Table 1. This phenomenon could be well explained by volume exclusion effect as described by earlier researchers (37, 38). In case 4 (Table 1) an affinity phenomena was also observed above certain limits of PEG composition (20%) which causes the increase in partition coefficient from 0.79 to 1.14 U/ml and yield in the top phase from 58.51% to 71.06%.

It was also noted that partition coefficient decreases from 1.16 to 1.04 U/ml by increasing the salt concentration from 6.60% to 7.92% but when the composition of salt

in the system reaches to 8.58%, the partition coefficient increases again to 1.12 U/ml (Fig. 2 (A)). This behavior agreed with the finding of Mohamadi et al (39) while recovering recombinant phenylalanine dehydrogenase in PEG/ammonium sulfate ATPS. Under certain conditions the increase in the salt has a constant decreasing effect

Table 1: Partition behavior of lipases in PEG 4,000 and 6000/Ammonium sulfate.

S.NO	PEG (w/w)	$(NH_4)_2SO_4$ (w/w)	PEG 4000						PEG 6000					
			Volume Ratio	Partition Coefficient (U/ml)	Specific activity (U/mg)		Purification factor (fold)		Yield (%)		Volume Ratio	Partition Coefficient (U/mL)	Specific activity (U/mg)		Purification factor (fold)		Yield (%)	
					Top	Bottom	Top	Bottom	Top	Bottom			Top	Bottom	Top	Bottom	Top	Bottom
1	12.50 %	6.60 %	2.3	1.16	1.68	1.51	0.73	0.66	73.02	26.97	2.57	0.89	1.61	2.5	0.70	1.09	69.65	30.34
	15%	6.60 %	2.42	1.11	1.65	1.56	0.70	0.68	73.03	27.13	2.84	1.08	1.96	1.83	0.86	0.80	75.53	24.47
	17.50 %	6.60 %	2.57	1.14	1.74	1.29	0.76	0.56	74.26	25.73	2.33	1.2	2.1	1.7	0.92	0.74	73.68	26.31
	20%	6.60 %	2.52	1.15	1.54	1.39	0.67	0.61	74.44	25.67	2.2	1.16	1.97	1.7	0.86	0.74	71.96	28.1
2	12.50 %	7.26 %	2.5	1.15	1.5	1.25	0.66	0.55	74.93	25	2.47	0.99	2.4	2.4	1.04	1.04	71.05	29.03
	15%	7.26 %	2.18	1.03	1.7	1.67	0.74	0.73	69.27	30.78	2.5	0.96	1.71	1.78	0.75	0.78	71.25	28.86
	17.50 %	7.26 %	2.47	0.93	1.65	1.8	0.72	0.79	69.7	31.36	2.12	1.04	2.04	1.92	0.89	0.84	68.84	31.18
	20%	7.26 %	2.62	1	1.72	1.72	0.75	0.75	72.4	27.6	1.97	1.01	1.8	1.78	0.79	0.78	66.66	33.39
3	12.50 %	7.92 %	2.37	1.04	1.58	1.53	0.69	0.67	71.22	28.79	2.22	1.24	2.47	2.01	1.07	0.88	73.5	26.53
	15%	7.92 %	2.03	1.03	1.5	1.46	0.66	0.64	67.65	32.35	2.33	0.82	1.7	2.04	0.74	0.89	65.67	34.32
	17.50 %	7.92 %	2.35	0.94	1.58	1.74	0.69	0.76	69.02	31.17	2.26	1.28	2.23	1.79	0.97	0.78	74.39	25.62
	20%	7.92 %	2.12	0.96	1.57	1.64	0.69	0.72	67.16	32.92	2.42	1.1	2.04	1.88	0.89	0.82	72.78	27.28
4	12.50 %	8.58 %	2.12	1.12	1.68	1.55	0.73	0.68	70.51	29.62	2.33	0.76	2	2.5	0.87	1.09	64.87	35.13
	15%	8.58 %	1.38	0.94	1.72	1.84	0.75	0.70	56.55	43.56	1.94	0.92	1.54	1.7	0.67	0.74	64.18	35.95

17.50 %	8.58 %	1.77	0.79	1.39	1.82	0.61	0.79	58.51	41.66	1.94	1.03	1.85	1.8	0.81	0.79	69.36	34.69
20%	8.58 %	2.14	1.14	1.65	1.45	0.72	0.63	71.06	29.06	2.35	1.02	1.89	1.83	0.825	0.799	70.61	29.38

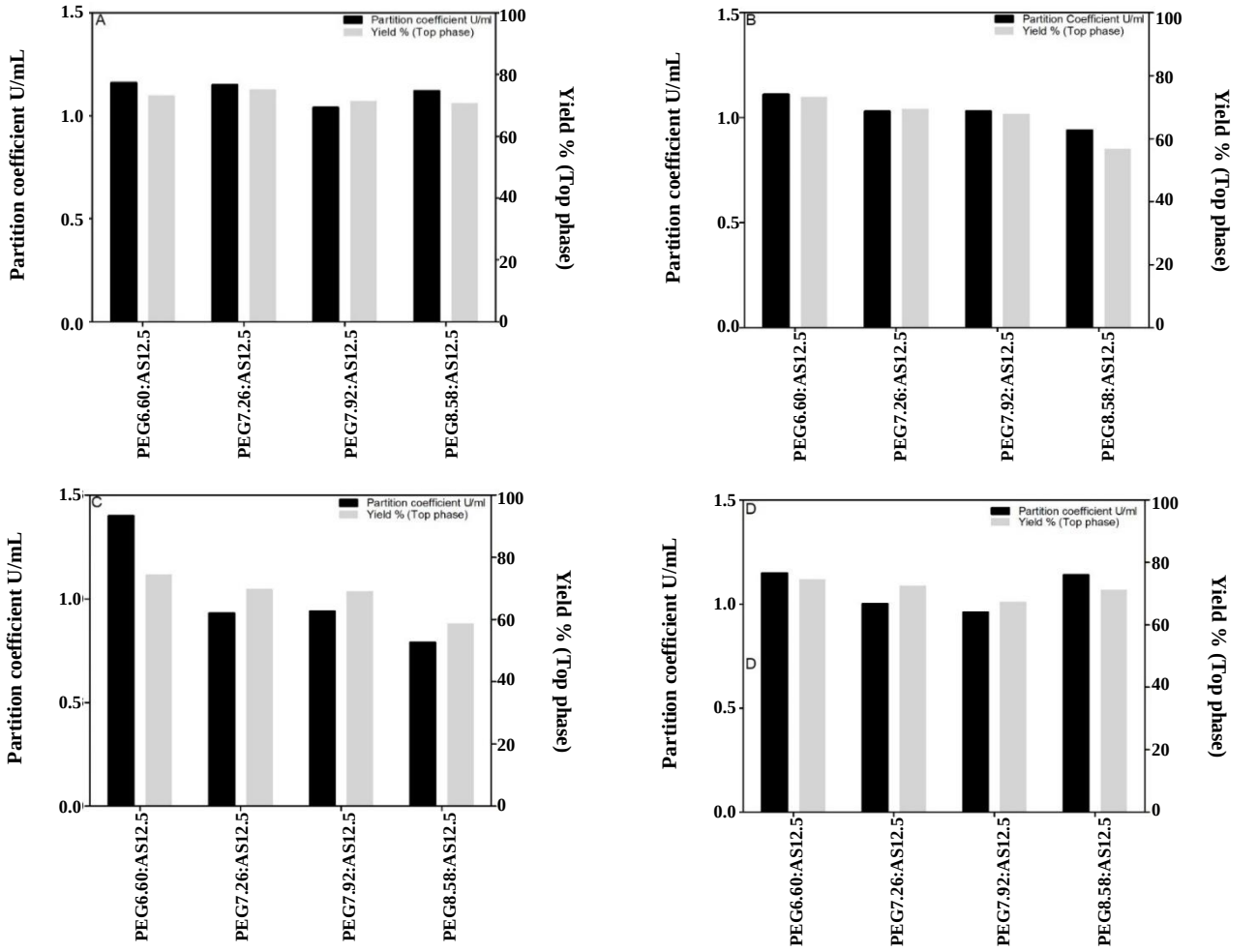

Fig. 2: Effect of ammonium sulfate compositions on Lipases behavior in ATPS composed of PEG (4000) (A) 12.5%, (B) 15%, (C) 17.5 %, (D) 20%. Dark bars describe the partition coefficient and light bars the yield in top phase.

on the partition coefficient For example, changing the salt composition in ATPS containing 15% PEG, the partition coefficient decreased from 1.11 to 0.94 U/ml with increase in salt concentration (Fig. 2 (B)). The same trend was observed when all the salt concentrations were tested against 17.5 % PEG. The results are also plotted in Fig. 2 (C). The same behavior was observed by *Antov et al*. [17] for the purification of xylanase.

Effect of PEG 6000 and ammonium sulfate compositions on the partition behavior of lipases

High molecular weight polymers i.e., PEG 6000 has an inverse effect on the partitioning of the lipase compared to PEG 4000. It was observed that partition coefficient increases from 0.89 to 1.2 U/ml in the top phase by increasing PEG (6000) composition against fixed composition of ammonium sulfate (6.60%) as shown in Table 1, case 1. Beyond certain limit, increase in PEG composition slightly decreases the partition coefficient to 1.16 U/mL. The main reason is volume exclusion effect of PEG which pushed the lipases molecules to the bottom phase. A similar trend was followed by the entire PEG 6000 composition tested against 7.26%

ammonium sulfate where the partition coefficient increases from 0.99 to 1.04 U/ml (Table 1, case 2). Similar findings were reported by *Bassani et al*. [40] where he observed that increasing the PEG composition in the system improves the partition coefficient but declines beyond certain limit. The possible reason of gradual increase in the partition coefficient is the increase in the chain length of PEG which provides more attachment sites to lipases. At highest composition the decrease in the partition coefficient is due to the volume exclusion effect of PEG. The same trend of

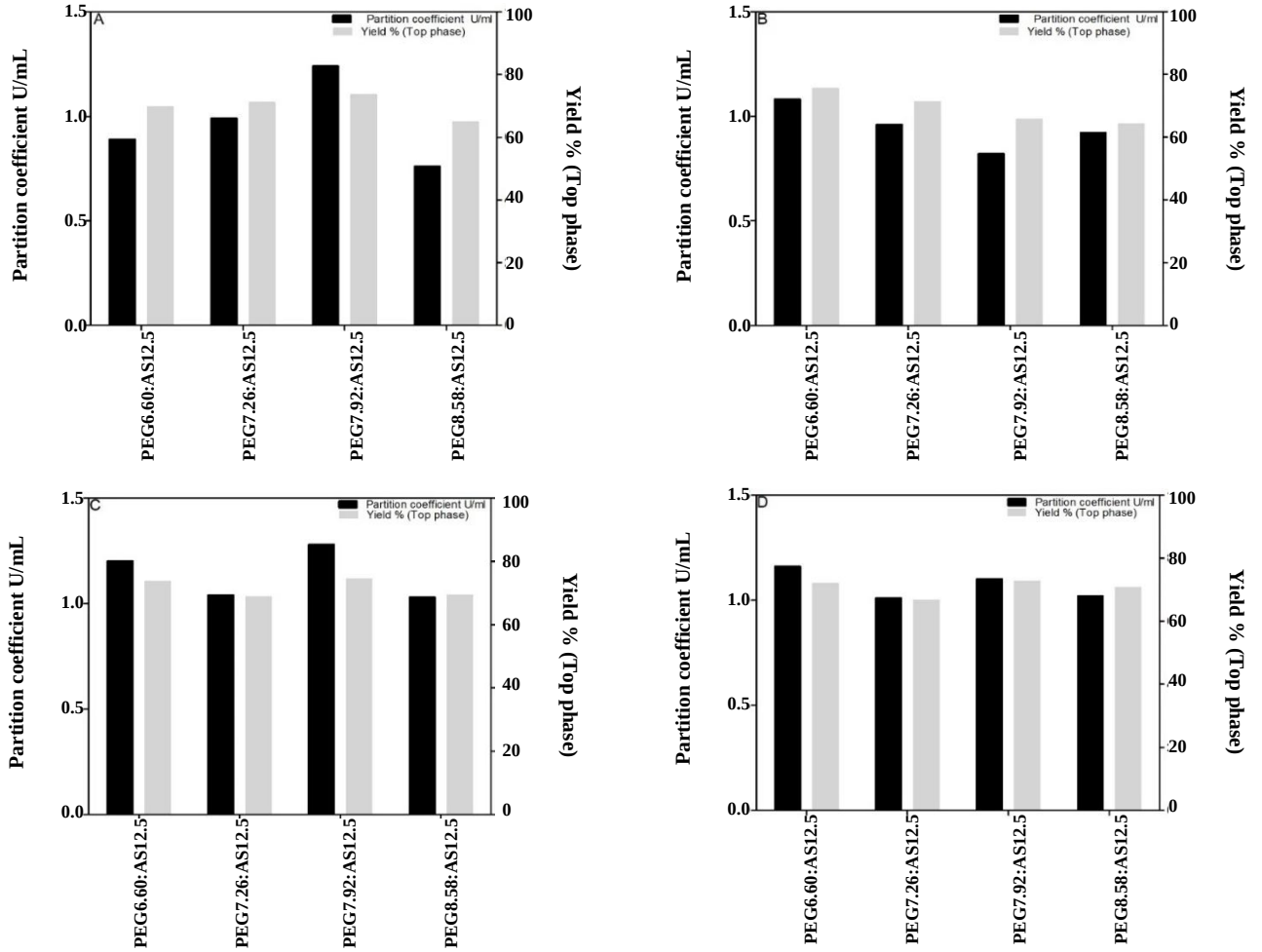

Fig. 3: Effect of ammonium sulfate compositions on Lipases behavior in ATPS composed of PEG (6000) (A) 12.5%, (B) 15%, (C) 17.5 %, (D) 20%. Dark bars describe the partition coefficient and light bars the yield in top phase.

increase and later decrease in the partition coefficient was observed for 7.92% and 8.56% salt concentration against each PEG concentration. The hydrophobic interaction between PEG and the surface of protein increases by increasing the PEG concentration (40). When the difference in hydrophobicity increases between the two phases the strength of hydrophobic interaction also increases between the PEG molecules and proteins.

Ammonium sulfate composition has a fluctuating effect on the partitioning of lipases in PEG 6000/ammonium sulfate aqueous two phase system. When different ammonium sulfate compositions were tested against 12.5% PEG, an increase in partition coefficient was noted from 0.89 to 1.24 U/mL (Fig. 3 (A)). Babu *et al.* (37) also observed the same effect. They observed that increasing the salt concentration the partition coefficient increases. Against 15% PEG, the salt compositions suggested a decrease in partition coefficient from 1.08 to 0.92 U/mL with increase in salt composition (Fig. 3 (B)). The fact may be the volume exclusion effect and the salting out effect. When different compositions of salt in a system were tested separately against 17.5% and 20% PEG it was observed that all the parameter

of the system varies in irregular pattern (Fig. 3 (C, D)). According to Ratanapongleka (13), an increase in salt concentration to a certain limit, change the partition coefficient but beyond that limit no regular relation found that affect the partition.

Effect of PEG 10,000 and ammonium sulfate compositions on the partition behavior of lipases

The composition of PEG 10,000 has a noticeable effect on the separation behavior of lipases. Increasing the PEG composition decreases the partition coefficient.

Table 2: Partition behavior of lipases in PEG 10,000/Ammonium sulfate.

CASE	PEG (w/w)	$(NH_4)_2SO_4$ (w/w)	Volume Ratio	Partition Coefficient (U/ml)	Specific activity (U/mg)		Purification factor (fold)		Yield (%)	
					Top	Bottom	Top	Bottom	Top	Bottom
1	12.50%	6.60%	2.12	1.53	1.76	1.18	0.79	0.51	76.5	23.52
	15%	6.60%	2.49	1.2	1.73	1.63	0.76	0.71	74.24	25.85
	17.50%	6.60%	2.47	1.21	1.59	1.38	0.69	0.62	75.04	24.08
	20%	6.60%	2.54	1.24	1.76	1.44	0.77	0.63	75.96	24.06
2	12.50%	7.26%	2.42	1.26	1.52	1.16	0.66	0.51	75.34	24.66
	15%	7.26%	2.42	1.13	1.79	1.61	0.78	0.70	73.26	26.73
	17.50%	7.26%	2.42	1.04	1.63	1.59	0.71	0.69	71.48	28.34
	20%	7.26%	2.47	0.88	1.43	1.57	0.62	0.69	68.55	31.5
3	12.50%	7.92%	2.49	1.36	2.02	1.5	0.88	0.66	78.3	21.7
	15%	7.92%	2.35	1.29	1.59	1.45	0.69	0.63	75.05	28.06
	17.50%	7.92%	2.26	1.37	1.66	1.24	0.72	0.54	75.69	24.36
	20%	7.92%	2.35	1	1.57	1.59	0.69	0.69	70.2	29.8
4	12.50%	8.58%	2.59	1.28	1.52	1.33	0.66	0.58	75.52	24.64
	15%	8.58%	2.01	1.35	1.83	1.27	0.80	0.55	74.5	25.53
	17.50%	8.58%	2.18	1.26	1.74	1.43	0.76	0.62	73.37	26.65
	20%	8.58%	2.28	1.05	1.5	1.42	0.66	0.62	70.67	29.4

Generally, all the cases in Table 2 present a decrease in the partition coefficient when the composition of PEG increases from 12.5 to 20% against each salt concentration. The main reason is that, an increase in the chain length of polymer results in the reduction of free space and causes volume exclusion effect (37, 38). The effect of partitioning of lipases in PEG 10,000 could be perfectly explained by volume exclusion effect affinity phenomena.

When different ammonium sulfate compositions were tested in ATPS having fixed 12.5% PEG 10,000, it was noted that the partition coefficient decreases from 1.53 to 1.18 U/mL with increase in salt compositions. Ideal separation was achieved in a system with 7.92% and 12.5% PEG with purification factor of 0.882 fold and 78.3% yield in the top phase (Fig. 4 (A)).

The same phenomena were also observed by *Antov et al. (17)* and Barbosa *et al.* (25). When salt compositions were tested against 15% PEG, the partition coefficient first decreased with increase in salt compositions as shown in Fig 4 (B). At higher salt composition (8.58%), the partition coefficient increases again. In other ATPS, by increasing the salt (ammonium sulfate) composition against 17.5% PEG, no regular relation was found Fig. 4 (C). It was noted that all the parameters decrease in a regular pattern with

increase in salt composition when tested against 20% PEG 10,000. The partition coefficient decreases from 1.24 to 1.04 U/mL in these systems (Fig. 4 (D)).

Qualitative test and SDS page

As ATPS is a partial purification strategy so it is understood that the target molecule will certainly have traces of other contaminants. Qualitative test was performed for the crude extract which showed a clear zone around the well applied with different concentrations of the sample

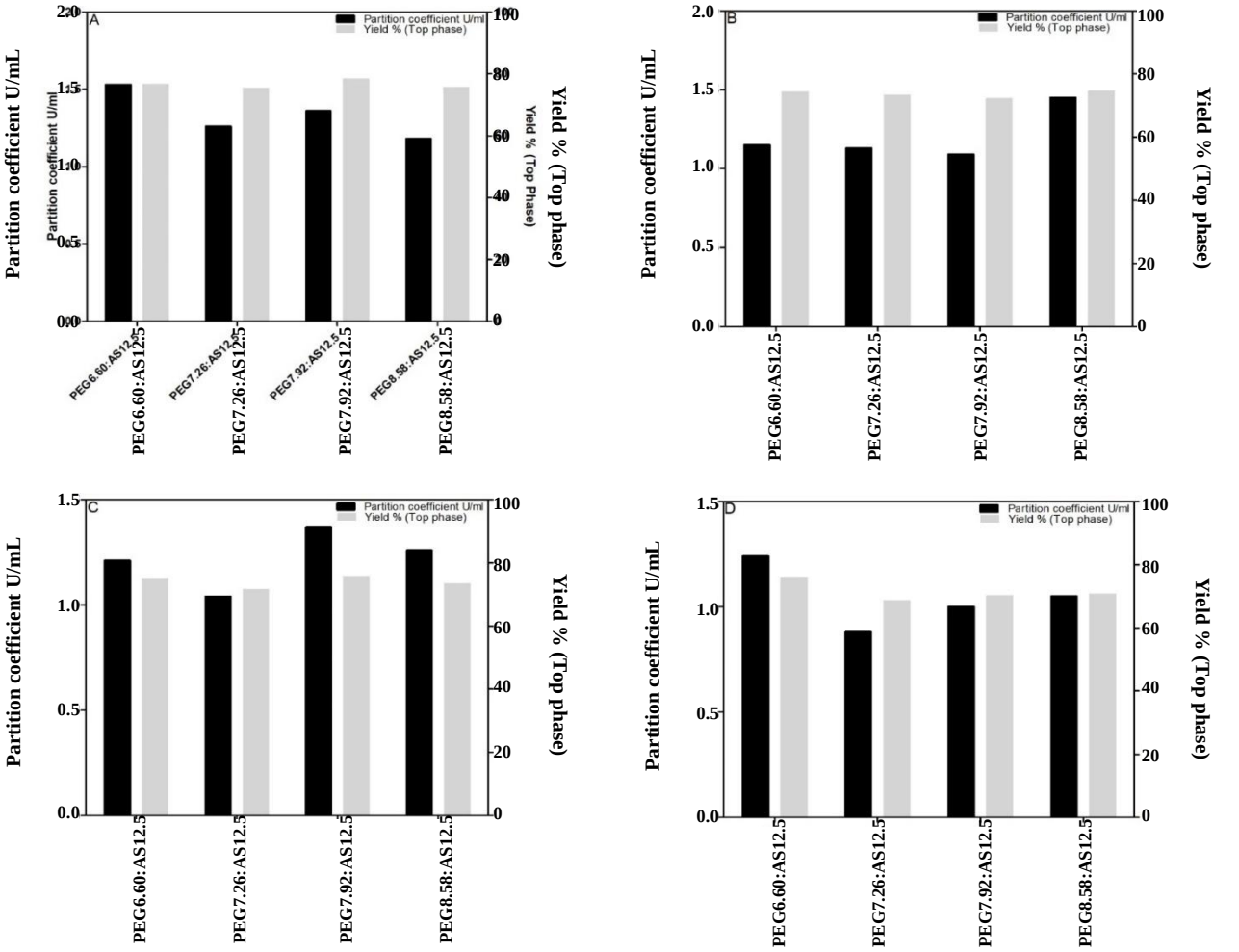

Fig. 4: Effect of ammonium sulfate compositions on Lipases behavior in ATPS composed of PEG (10,000) (A) 12.5%, (B) 15%, (C) 17.5 %, (D) 20%. Dark bars describe the partition coefficient and light bars the yield in top phase.

as shown in (Fig. (5A)). Activity test on tween plate was also performed for the collected fractions of the top and bottom phases from the system which shown maximum purification of the target molecules (12.5% PEG 10,000 and 7.92% Ammonium sulfate) (Fig. (5B)). Samples from the system composed of 7.92% ammonium sulfate and 12.5% PEG (10,000) were also checked on SDS PAGE for purified lipase. The result showed that the most of lipases was purified in the top phase where its yield was 78.3%. Lipases were also detected in the bottom phase but in smaller amount (shown by the activity assay as well). The molecular weight of lipases according to ladder was 43KDa (Fig. 6).

CONCLUSIONS

The isolation and partitioning of lipases from defined bacterial source of Bacillus specie was studied under different compositions of PEG and salt. Results showed higher specific activity and purification factor in ATPS containing PEG 6000 and PEG 10,000. Different compositions of PEG 6000 and 10,000 seems to be the best choice for the isolation and partial

purification of lipases from soil bacteria (Bacillus specie) as compared to PEG 4000. ATPS containing 12.5% PEG 10,000 and 7.92% Ammonium sulfate provides partition coefficient of 1.36 U/mL and 78.3% yield in the top phase has the potential for lipases recovery on industrial scale. This system can be optimized by changing system parameters like temperature, system pH, addition of neutral salt to achieve better yield and purity of lipases on lab and pilot scale.

Fig. 5: Plate assay for lipases (A) Zones after 12 hours of incubation showed the presence of enzyme in crude supernatant applied at different concentration. Well 1: 10 µL, Well 2: 15 µL, Well 3: 20 µL, Well 4: 25 µL, Well 5: 30 µL, Well 6: 35 µL, Well 7: 40 µL. (B) purified enzyme activity in the top and bottom phase of 12.5% PEG 10,000 and 7.92% Ammonium sulfate (performed in duplicate).

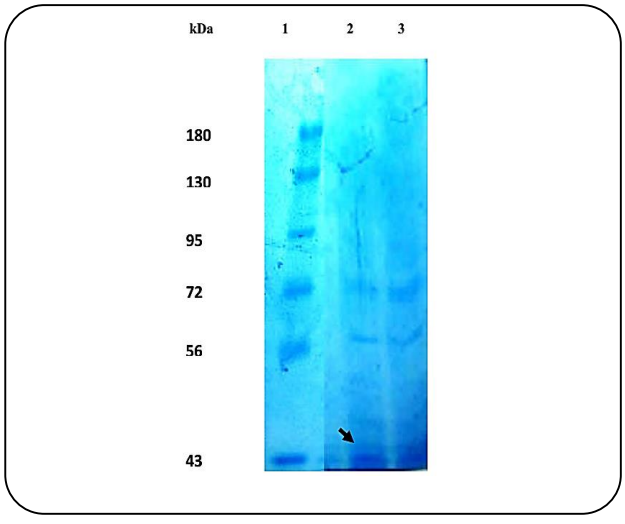

Fig. 6: SDS PAGE for the Presence and Molecular weight determination of lipase, Lane 1 represents; ladder, Lane 2; proteins in top phase, Lane 3; proteins in bottom phase. MW of lipases was 43KDa.

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