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Research Article

Partitioning Studies of Glutaminase in Polyethylene Glycol and Salt-Based Aqueous Two-Phase Systems

The partitioning behavior of glutaminase produced from *Zygosaccharomyces rouxii* in polyethylene glycol (PEG)-salt aqueous two-phase systems (ATPSs) was investigated. ATPSs comprising of different PEG-salts were considered. Binodal data and tie lines generated for the selected systems were analyzed and correlated with Othmer-Tobias and Bancroft equations. Effects of salt type, PEG molecular weight, concentrations of phase components, and tie line length on enzyme partitioning were evaluated.

Keywords: Keywords: Aqueous two-phase system, Glutaminase, Partitioning, Polyethylene glycol, Tie lines

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1 Introduction

Glutaminase (*L*-glutamine amino hydrolase EC 3.5.1.2) is widely applied in pharmaceutical and food industries. In human bodies, there are two glutaminase genes, *Gls* located on chromosome 2 and *Gls2* located on chromosome 12 [1]. Glutaminase catalyzes the hydrolysis of the γ-acyl bond of *L*-glutamine to glutamate and ammonia. It serves as potent anti-leukemic drug, flavor-enhancing agent in the production of fermented food, antiretroviral agent, and biosensor for detection of glutamine [2]. Different purification techniques like protamine sulfate extraction, chromatography, and gel filtration have been employed for the purification of glutaminase [3–5]. However, these conventional purification techniques involve multistep procedures which are time-consuming, expensive, and provide low yields [6]. Hence, an economically viable process for purification of glutaminase is essential. In this regard, an attempt has been made to use aqueous two-phase systems (ATPSs) for extraction and purification.

Aqueous two-phase extraction (ATPE) is an integration of clarification, concentration, and partial purification process and is gaining importance in extraction and purification of biomolecules [7]. It exhibits a number of advantages over other purification techniques such as high water content of the phases (80–90 wt %), high biocompatibility, low interfacial tension, low degradation of biomolecules, good resolution, and simple process scale-up [8]. The polymers associated with ATPSs are known to have a stabilizing effect on biomolecules [9]. ATPE has been made more effective with the introduction of microemulsion phases, affinity ligands, formation of agarose beads, magnetically enhanced phase separation, and use of surfactants as co-solutes [10]. However, protein purification with polymer recycling has been a major advancement for economical application of ATPE methods in industries [11]. ATPE has been exploited for purification of various enzymes like amylase, elastase, human antibodies, pectinase, protease, xylose reductase, penicillin acylase etc. [12].

Moreover, selection of an appropriate phase-forming salt is important to achieve a maximum partitioning of proteins. The generally used phosphate and sulfate salts are able to promote hydrophobic interactions between protein molecules [13]. Citrate salts are biodegradable, nontoxic, and their properties are similar to that of phosphate salts [14]. However, only limited study has been performed on aqueous two-phase partitioning of enzymes using citrate salts. No reports are currently available on partitioning studies of glutaminase using ATPSs.

The partitioning behavior of glutaminase produced from the halophilic yeast *Zygosaccharomyces rouxii* NRRL-Y 2547 at 30 °C in various ATPSs like PEG 2000/salt (potassium phosphate, sodium phosphate, sodium sulfate, trisodium citrate, and tripotassium citrate) was examined in detail. Binodal data and tie lines were generated for these systems and equilibrium phase compositions of the two-phase systems were verified using Othmer-Tobias and Bancroft equations [15]. The partitioning behavior of glutaminase was investigated with varying polymer molecular weight (PEG 2000, 4000, 6000, 8000), salt concentration, polymer concentration, and tie line length (TLL).

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2 Materials and Methods

2.1 Materials

Peptone, yeast extract, malt extract, glutamine, and Nessler's reagent were procured from Himedia (Mumbai, India). Glucose, sucrose, boric acid, ammonium sulfate, trisodium citrate, tripotassium citrate, and potassium phosphate were purchased from Merck Chemicals (Mumbai, India). PEG 2000, 4000, 6000, and 8000 as well as the Bradford reagent were obtained from Sigma Aldrich (USA). Sodium chloride and sodium phosphate were from Rankem Chemicals (New Delhi, India). All chemicals were of analytical grade. Double-distilled water was used for all preparations.

2.2 Production of Glutaminase

Zygosaccharomyces rouxii 3460 was procured from NCIM (National Collection of Industrial Microorganisms), Pune, India. *Z. rouxii* NRRL-Y 2547 was obtained as a gift culture from Northern Regional Research Laboratories type culture collection (NRRL), Peoria, USA. Both cultures were maintained on GPYM agar slants containing 10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 50 g L^{-1} sodium chloride, and 20 g L^{-1} agar [14]. Inoculated slants were grown for two days at 35 °C and pH 7. These slants were stored at 4 °C, and subcultured for every 20 days [16]. Inoculum was prepared in GPYM liquid media containing 10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 50 g L^{-1} sodium chloride. For glutaminase production, the medium of Iyer and Singhal [16] was employed at pH 7. Inoculum volume and carbon source were optimized to maximize the activity [16]. The cells were separated by centrifugation at 8000 × *g* for 8 min. Cell-free fermentation broth was employed for further partitioning studies.

2.3 Preparation of Phase Diagrams

Binodal curves were prepared by titration (cloud point method) in a jacketed glass vessel with 200 cm^3 working volume. The temperature of the glass vessel was maintained at 303.15 ± 0.05 K in a thermostatic water bath (Schott-Geräte CT 52, Germany). To determine the tie lines, a suitable biphasic system was prepared in a centrifuge tube of 15 mL volume by mixing appropriate amounts of salt and PEG. The total weight of the system was made up to 10 g with water. The obtained systems were thoroughly mixed in a vortexer and allowed to settle down in a thermostatic water bath (Schott-Geräte CT 52, Germany) at 30 °C for 24 h. After equilibration, the volumes of top and bottom phases were noted and the individual phases were analyzed for salt and polymer concentrations. The salt concentration in both phases was measured using a flame photometer (1–100 ppm, CT-378, Elico Ltd, India). PEG 2000 concentration in both phases was determined by refractive index measurements [17] (Automatic Digital Refractometer, Atago Co. Ltd, RX-5000*α*) with a precision of ± 0.00004 at 303.15 K. An extensive calibration was initially carried out with different polymer concentrations (W_p = 0.5–20 wt %) and salt (W_s = 0.2–25 wt %) in an aqueous single-phase region at 303.15 K. For dilute aqueous solutions containing a polymer and a salt, the relation between the refractive index (ηD) and the mass fractions of polymer (W_p) and salt (W_s) is given by:

$$\eta D = a_0 + a_1 W_P + a_2 W_S \quad (1)$$

where W_P and W_S are the weight fractions of polymer and salt, respectively, and a_0, a_1, and a_2 are the fitting parameters at 303.15 K (see Tab. 1).

Table 1. Fit parameters for Eq. (1).

ATPS	a_0	a_1	a_2
PEG 2000/KH_2PO_4	1.331	0.1500	0.1409
PEG 2000/NaH_2PO_4	1.331	0.1599	0.1586
PEG 2000/Na_2SO_4	1.331	0.1396	0.1421
PEG 2000/$Na_3C_6H_5O_7$	1.331	0.1537	0.1403
PEG 2000/$C_6H_5O_7K_3$	1.331	0.1518	0.1412

2.4 Partition Study of Enzyme

Biphasic systems were prepared with the known concentration of top and bottom phases. Crude extract (3 g) was mixed with the system, keeping the volume ratio of the system as 1. The mixture was vortexed and equilibrated overnight in a thermostatic water bath at 30 °C for equilibrium and then the phases were separated. Glutaminase and total proteins in the separated phases were analyzed. One international unit of glutaminase was defined as the amount of enzyme capable of producing one μmol of ammonia per minute at 37 °C [18]. The amount of ammonia liberated was quantitatively determined by means of the ammonium sulfate reference calibration curve. Total proteins were estimated by the Bradford method with bovine serum albumin (BSA) as standard.

The activity partition coefficient K_e was calculated as:

$$K_e = A_t/A_b \quad (2)$$

where A_t and A_b represent the enzyme activity (U L^{-1}) in the top and bottom phases.

The partition coefficient of total protein K_p is defined as:

$$K_p = C_{pt}/C_{pb} \quad (3)$$

where C_{pt} and C_{pb} are the protein concentrations (g L^{-1}) in the top and bottom phase, respectively.

Enzyme purification folds, yield, and sensitivity of the system were evaluated as follows:

$$\text{Purification folds (PF)} = \frac{A_t/C_{pt}}{A_i/C_{pi}} \quad (4)$$

where A_t and A_i are the enzyme activity (U L^{-1}) in the top phase and initial extract before partitioning, respectively, and C_{pt} and C_{pi} represent the total protein concentration (g L^{-1}) of

the top phase and initial extract before partitioning, respectively.

$$\text{Yield } (\%) = \frac{100}{1 + \left(\frac{1}{V_r K_e}\right)} \quad (5)$$

where K_e is the enzyme partition coefficient and V_r is the volume ratio.

$$V_r = V_t/V_b \quad (6)$$

V_t and V_b denote the volumes of top and bottom phases (L).

$$\text{Sensitivity } (S) = K_e/K_p \quad (7)$$

where K_e and K_p are the partition coefficients of enzyme and protein, respectively.

The tie line length (TLL) is defined as:

$$TLL \text{ (wt \%)} = \sqrt{\left(W_s^t - W_s^b\right)^2 + \left(W_p^t - W_p^b\right)^2} \quad (8)$$

W_s^t and W_s^b are the salt concentrations in the top and bottom phase (wt %) and W_p^t and W_p^b are the polymer concentrations in the top and bottom phase (wt %).

3 Results and Discussion

3.1 Production of Glutaminase

Yeasts are known to essentially degrade nitrogenous compounds to ammonia and glutamate [5]. In the current study, two different strains of the halophilic yeast *Z. rouxii* (NRRL-Y 2547 and 3460) were applied to produce glutaminase in the modified liquid medium of Iyer and Singhal [16]. With 50 U L^{-1}, *Z. rouxii* NRRL-Y 2547 was found to exhibit a higher activity as compared to *Z. rouxii* 3460. Hence, *Z. rouxii* NRRL-Y 2547 was selected for glutaminase production. Even though Iyer and Singhal [16] described the optimized media, the reported enzyme activity for the same strain in the present work could not be produced. In order to further improve the activity, the inoculum size was varied (1–7 %). It was observed that enzyme activity increases (88 U L^{-1}) up to 4 % of the inoculum size and further decreases with increasing inoculum size (Fig. 1).

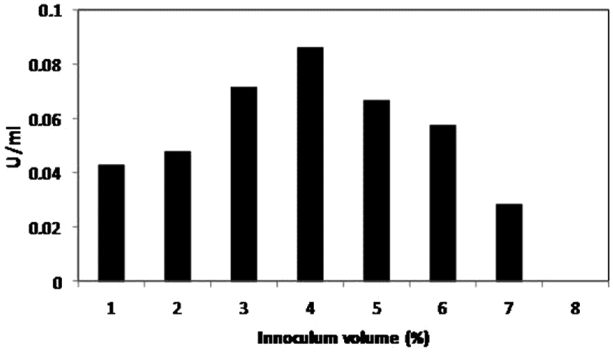

Figure 1. Enzyme activity at different inoculum volumes.

The effect of carbon sources on enzyme production was also studied by replacing sucrose with two alternative carbon sources, namely, maltose and glucose at 4 % of the inoculum size. Maltose was found to stimulate glutaminase production in *Z. rouxii* [19] and glucose was employed as a carbon source during inoculum preparation. The highest glutaminase production of 110 U L^{-1} was obtained with maltose (Fig. 2).

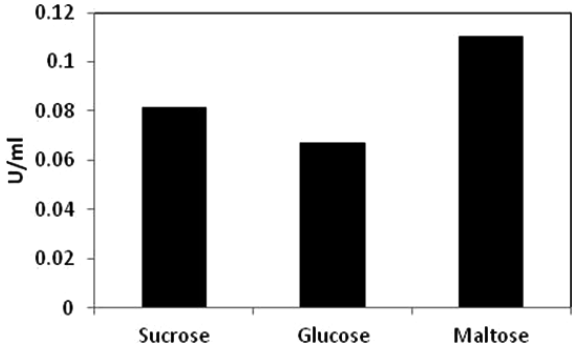

Figure 2. Enzyme activity with different carbon sources.

3.2 Aqueous Two-Phase System: Phase Diagram

The phase diagram consisting of a binodal curve and tie lines provides information on the critical concentration of phase-forming components required to form two phases and the equilibrium characteristics of ATPS [20]. The phase diagrams were generated to design the experiments for partitioning studies. The commonly used salts like sodium phosphate, potassium phosphate, sodium sulfate, trisodium citrate, and tripotassium citrate were selected for protein partitioning studies [12]. The binodal data for the salts with PEG 2000 were generated at 30 °C (Fig. 3), except for the earlier reported system trisodium citrate with PEG 2000 [21]. The binodal curves revealed that the concentration of salts required to form two phases are in the order of $NaH_2PO_4 < Na_2SO_4 < KH_2PO_4 < Na_3C_6H_5O_7 < C_6H_5O_7K_3$.

The interaction of salt cations with water molecules is readily replaced by PEG-cation interactions. The PEG-salt cation interaction depends on the charge of the salt anion. Salts with multivalent anions of high-charge density limit such interactions with the polymer chain, leading to salt-depleted zones and consequently formation of two phases [22]. Water structure as well as making and breaking ability of salts also affect the two-phase formation. The water structure making category of ions includes the cations Li^+, Na^+, NH_4^+, Ca^{2+}, Mg^{2+} etc. and the anions F^-, SO_4^{2-},CO_3^{2-}, PO_4^{3-} etc. The water structure breaking category of ions involves K^+, Rb^+, Cs^+, Cl^-, Br^- etc. According to the Hofmeister series, the order of their ability to change the water structure is $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+}$. The strength of the anions are in the order of $PO_4^{3-} > C_6H_5O_7^{3-} > SO_4^{2-} > H_2PO_4^{2-}$ [23].

Fig. 3 demonstrates that the binodal curve of sodium cation salts shifted towards the origin due to the water structure making ability of the sodium cation, indicating the lesser salt/PEG concentration requirement for the two-phase formation. The

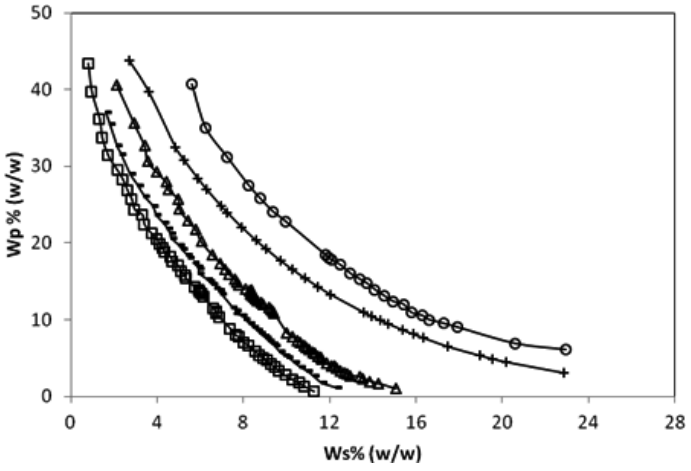

Figure 3. Binodal curves of NaH_2PO_4 (□), Na_2SO_4 (–), KH_2PO_4 (△), $Na_3C_6H_5O_7$ (+), and $C_6H_5O_7K_3$ (○) with PEG 2000 at 30 °C.

biphasic region increased with the decrease in the radii of cations (K^+: 0.133 nm > Na^+: 0.098 nm) [24]. The binodal data of all systems were analyzed and the empirical correlations were developed by modifying the constants of the nonlinear equations reported in [25, 26]. The correlations for all systems along with the constants and fitting parameters are listed in Tab. 2. These equations satisfactorily correlated with the binodal curves of the systems. Moreover, the PEG-salt relationship will be suitable for the commercial application of these systems [21].

Phase equilibrium characteristics of the selected ATPS were studied by preparing two-phase systems without the crude enzyme at different polymer concentrations with constant salt concentration. The individual phase compositions were determined and tie lines were constructed under equilibrium condition (Tab. 3).

The experimental equilibrium data of the tie lines were fitted to Othmer-Tobias (Eq. (9)) and Bancroft equations (Eq. (10)) [15].

$$\left(\frac{1-W_p^{top}}{W_p^{top}}\right) = K\left(\frac{1-W_s^{bot}}{W_s^{bot}}\right)^N \quad (9)$$

$$\left(\frac{W_w^{bot}}{W_s^{bot}}\right) = K_1\left(\frac{W_w^{top}}{W_p^{top}}\right)^R \quad (10)$$

W_p^{top} is the weight fraction of PEG 2000 in the top phase, W_s^{bot} is the weight fraction of salt in the bottom phase, and W_w^{bot} and W_w^{top} are weight fractions of water in bottom and top phase, respectively. K, N, K_1, and R are the fit parameters (Tab. 4). This information was utilized to prepare the ATPSs

Table 2. Fit parameters for binodal correlations from literature.

ATPS	Correlations	a	b	c	d	AARD [%]	R^2
PEG 2000/ KH_2PO_4	$W_P = a + bW_S^{0.5} + CW_S$ [23]	0.799	–2.980	2.290	–	1.990	0.989
PEG 2000/ NaH_2PO_4	$Wp = a + bW_s^{0.5} + cW_s + dW_s^2$ [24]	0.430	–0.299	–4.761	16.83	1.308	0.993
PEG 2000/ Na_2SO_4	$Wp = a + bW_s^{0.5} + cW_s + dW_s^2$ [24]	0.561	–1.214	–2.479	12.51	1.729	0.995
PEG 2000/ $Na_3C_6H_5O_7$	$W_P = a + bW_S^{0.5} + CW_S$ [23]	0.874	–3.122	2.839	–	0.154	0.999
PEG 2000/$C_6H_5O_7K_3$	$W_P = a + bW_S^{0.5} + CW_S$ [24]	–0.0008	0.006	0.008	0.002	0.057	0.990

Table 3. Equilibrium concentrations of ATPSs with partition coefficient of glutaminase (K_e) and % yield.

ATPS	Feed [–]		Top phase [–]		Bottom phase [–]		TLL [wt %]	Yield [%]
	W_p	W_s	W_p	W_s	W_p	W_s		
PEG 2000/KH_2PO_4	0.12	0.1	0.242	0.053	0.010	0.154	25.3	43.27
	0.14	0.1	0.275	0.040	0.010	0.165	29.3	52.73
	0.16	0.1	0.301	0.039	0.010	0.176	32.2	43.27
	0.18	0.1	0.321	0.035	0.013	0.187	34.4	39.76
	0.20	0.1	0.358	0.027	0.009	0.200	39.0	33.82
PEG 2000/NaH_2PO_4	0.07	0.1	0.241	0.030	0.015	0.128	24.6	38.08
	0.10	0.1	0.270	0.025	0.011	0.144	28.5	45.82
	0.12	0.1	0.289	0.023	0.010	0.154	30.8	53.56
	0.14	0.1	0.312	0.018	0.010	0.164	33.5	61.30
	0.16	0.1	0.330	0.015	0.003	0.179	>36.5	48.94

Continued Table 3.

ATPS	Feed [–]		Top phase [–]		Bottom phase [–]		TLL [wt %]	Yield [%]
	W_p	W_s	W_p	W_s	W_p	W_s		
PEG 2000/Na_2SO_4	0.07	0.1	0.230	0.045	0.013	0.123	23.1	30.34
	0.10	0.1	0.268	0.036	0.010	0.134	27.6	53.56
	0.12	0.1	0.296	0.031	0.005	0.145	31.2	61.30
	0.14	0.1	0.326	0.025	0.005	0.155	34.5	69.04
	0.16	0.1	0.366	0.016	0.006	0.165	38.9	57.45
	0.14	0.12	0.39	0.021	0.007	0.175	42.5	56.55
	0.14	0.14	0.44	0.025	0.012	0.193	46.1	47.86
PEG 2000/$Na_3C_6H_5O_7$	0.22	0.1	0.311	0.053	0.070	0.170	26.8	45.82
	0.24	0.1	0.338	0.049	0.048	0.207	33.0	53.56
	0.26	0.1	0.366	0.042	0.027	0.249	39.8	61.30
	0.28	0.1	0.396	0.038	0.014	0.285	45.5	40.42
PEG 2000/$C_6H_5O_7K_3$	0.24	0.1	0.254	0.091	0.004	0.243	29.2	24.36
	0.26	0.1	0.298	0.077	0.002	0.265	35.1	48.37
	0.28	0.1	0.340	0.067	0.002	0.284	40.2	52.73
	0.30	0.1	0.372	0.058	0.003	0.300	44.2	43.27
PEG 4000/Na_2SO_4	0.07	0.1	0.271	0.029	0.008	0.123	28.0	56.55
	0.10	0.1	0.299	0.028	0.009	0.133	30.9	65.24
	0.12	0.1	0.325	0.024	0.007	0.142	34.0	50.04
	0.14	0.1	0.346	0.022	0.006	0.151	36.4	40.05
	0.16	0.1	0.367	0.021	0.007	0.160	38.6	30.05
PEG 6000/Na_2SO_4	0.07	0.1	0.27	0.029	0.010	0.121	27.4	39.18
	0.10	0.1	0.30	0.025	0.009	0.130	31.1	56.55
	0.12	0.1	0.33	0.021	0.007	0.142	34.6	47.86
	0.14	0.1	0.35	0.021	0.006	0.151	37.1	30.05
	0.16	0.1	0.38	0.020	0.008	0.159	39.3	20.06
PEG 8000/Na_2SO_4	0.07	0.1	0.17	0.04	0.002	0.137	19.1	30.49
	0.10	0.1	0.20	0.04	0.006	0.152	22.8	47.86
	0.12	0.1	0.24	0.03	0.004	0.174	27.7	39.18
	0.14	0.1	0.27	0.02	0.003	0.189	31.1	21.80

for partitioning studies with crude enzyme from fermentation broth.

3.3 Effect of Phase-Forming Salts on Enzyme Partition

Phase-forming salts have a direct influence on the environment of the aqueous two-phase system. The partitioning behavior of glutaminase was here examined using different salts (sodium phosphate, potassium phosphate, sodium sulfate, trisodium citrate, and tripotassium citrate) with varying PEG 2000 concentration. The salt concentration was kept constant (10 wt %) for all ATPSs. Salts are known to alter the water structure, thereby modifying their interactions between polymer and salt [27]. There salts also influence electrostatic interactions between proteins and components of APTSs. Fig. 4 illustrates that the K_e of sodium salts was better than that of potassium salts for the same anion. Hofmeister's series suggest that the salting-out ability of sodium ions is better than that of the potassium ions and this could be the possible reason for the better K_e with sodium salts as compared to that of potassium salts. ATPS is a complex system where several factors such as hydrophobic and hydrophilic interactions, hydrogen

Table 4. Fit parameters for Othmer-Tobias and Bancroft equations.

ATPS	Othmer-Tobias equation				Bancroft equation			
	K	N	R^2	AARD [%]	K_1	R	R^2	AARD [%]
PEG 2000/KH_2PO_4	0.185	1.636	0.990	1.243	2.833	0.614	0.991	0.703
PEG 2000/NaH_2PO_4	0.371	1.112	0.995	0.669	2.478	0.899	0.997	0.445
PEG 2000/Na_2SO_4	0.078	1.913	0.993	1.974	3.757	0.542	0.997	0.826
PEG 2000/$Na_3C_6H_5O_7$	0.947	0.533	0.988	0.931	1.381	1.607	0.989	1.503
PEG 2000/$C_6H_5O_7K_3$	0.340	1.893	0.998	0.396	1.828	0.558	0.997	0.247
PEG 4000/Na_2SO_4	0.172	1.387	0.994	0.700	3.620	0.711	0.993	0.535
PEG 6000/Na_2SO_4	0.095	1.720	0.992	1.928	3.798	0.674	0.992	0.582
PEG 8000/Na_2SO_4	0.279	1.555	0.991	1.257	2.228	0.677	0.992	0.833

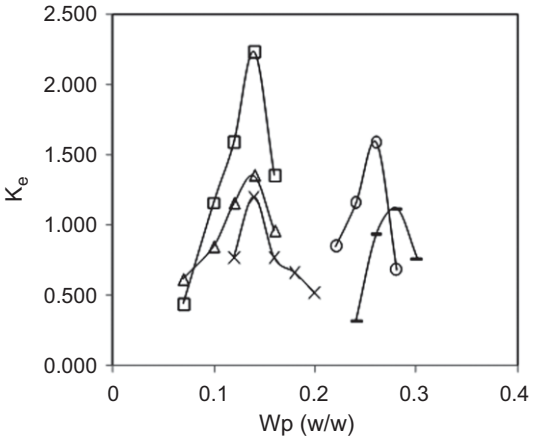

Figure 4. Partitioning coefficient (K_e) of glutaminase with NaH_2PO_4 (△), Na_2SO_4 (□), KH_2PO_4 (×), $Na_3C_6H_5O_7$ (o), $K_3C_6H_5O_7$ (–) with PEG 2000.

bonds, electrostatic interactions, surface properties of proteins, concentration of salts etc. are not absolutely mutually independent [28].

The salt providing maximum K_e and yield was selected for the further partitioning behavior study of glutaminase (Tab. 3). Selected salts should contribute in increasing the hydrophobic interaction between the phases. In this way, hydrophobic interaction between PEG and protein also improves [29]. The PEG 2000/sodium sulfate ATPS was found to be a superior system compared to other ATPSs. Sulfate salts are preferentially excluded from the surface of the proteins and can lead to higher hydrophobic interaction between the top and bottom phases [30]. Maximum K_e was observed in the top phase of the PEG 2000/sodium sulfate system suggesting that partitioning of glutaminase in an aqueous two-phase system is influenced by hydrophobicity. The K_e and yield of glutaminase were obtained as 2.23 and 69.03 %, respectively.

3.4 Effect of PEG Molecular Weight and Concentration on Enzyme Partition

Selection of an appropriate polymer molecular weight is a key factor to obtain maximum protein partitioning in an ATPS. The partitioning behavior of glutaminase at different polymer molecular weights (2000, 4000, 6000, 8000) and sodium sulfate (10 wt %) was studied by varying polymer concentration (7–16 wt %). Fig. 5 indicates that as the polymer molecular weight increased, the partition coefficient of glutaminase decreased. Enzyme glutaminase yields in the top phase and partition coefficients in an ATPS of different polymer molecular weight are reported in Tab. 3.

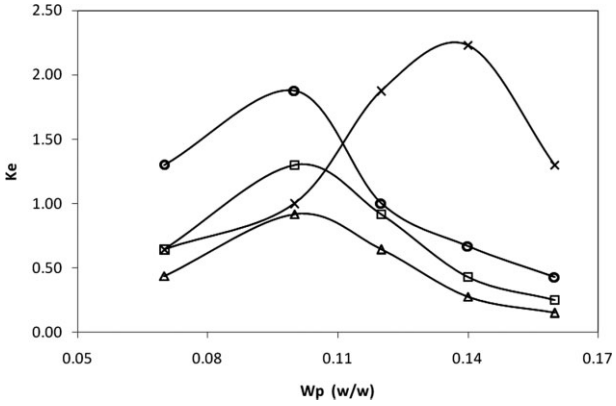

Figure 5. Partitioning coefficient (K_e) of glutaminase with Na_2SO_4 and PEG 2000 (×), PEG 4000 (o), PEG 6000 (□), and PEG 8000 (△).

The overall hydrophobicity is low in an ATPS comprising of a polymer with lower molecular weight as compared to that containing a high-molecular-weight polymer. A polymer of molecular weight 2000 (14 wt %) provided a maximum partitioning of glutaminase with a partition coefficient of 2.23 and yield of 69.03 %. As the polymer molecular weight increases, the interactions between the polymer chain and protein decrease. This may be attributed to the reduction of the overall

hydrophobicity and is known as size exclusion effect [31]. Moreover, in a PEG-salt system the hydrophobicity of proteins plays an important role when compared to the charge of the proteins [32]. However, a very low molecular weight of polymer will drive all the proteins towards the polymer-rich phase resulting in a very poor purification of the targeted protein [33]. Experimental observations reveal that higher polymer concentrations increased the K_e up to a certain extent due to the higher hydrophobic interaction at higher polymer concentration. However, the K_e decreases beyond certain PEG concentration due to less free volume available in the top phase for glutaminase [34].

3.5 Effect of Salt Concentration

Another important factor is the concentration of the phase-forming salt that affects the partitioning of enzymes. The effect of salt concentration (10–14 wt %) on glutaminase partitioning was studied at a constant polymer concentration of 14 wt % for the selected PEG 2000/sodium sulfate ATPS. It was observed that as the concentration of salt increased, the K_p improved from 0.94 to 2.1. Due to the salting-out effect at higher salt concentration, the available volume for the proteins in the bottom phase decreased and pushed the proteins towards the top phase [34]. The yield and K_e were found to be reduced with the increasing salt concentration due to enzyme denaturation at higher salt concentration or precipitation of glutaminase at the interphase. Similar results have been reported in the literature for aqueous two-phase partitioning of invertase and phenylalanine ammonia-lyase [29, 35]. However, for an efficient purification in an ATPS, a high K_e value and a low K_p value are necessary [13]. A maximum K_e of 2.22 and 69.03 % yield with lower K_p (0.94) were obtained with 10 wt % sodium sulfate and 14 wt % PEG 2000 (Fig. 6).

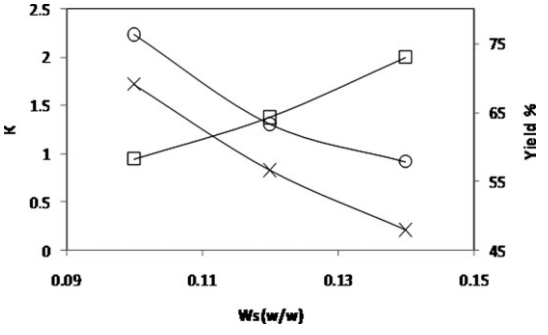

Figure 6. Effect of salt concentration on K_e (o), K_p (□), and yield (x).

3.6 Effect of Tie Line Length (TLL) on Enzyme Partition

Tie lines were obtained from the equilibrium data of different feed compositions. TLLs were calculated (Eq. (8)) for different feed compositions of PEG (2000, 4000, 6000, 8000) and salts (potassium phosphate, sodium phosphate, sodium sulfate, trisodium citrate, and tripotassium citrate). Fig. 7 illustrates the

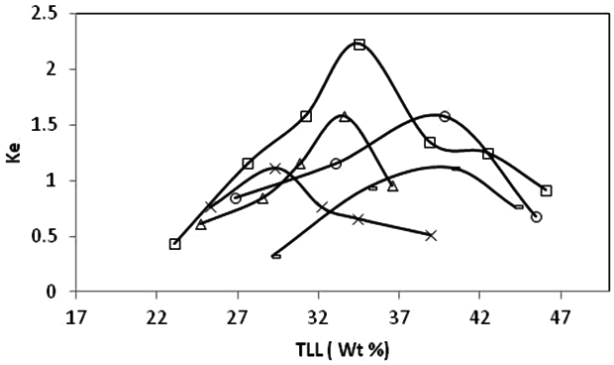

Figure 7. Effect of TLL on K_e. NaH_2PO_4 (△), Na_2SO_4 (□), KH_2PO_4 (×), $Na_3C_6H_5O_7$ (o), $C_6H_5O_7K_3$ (–) with PEG 2000.

effect of TLL on partitioning of glutaminase in ATPSs containing different salts and PEG 2000. Maximum K_e was 2.230 at 34.5 % TLL for the PEG 2000 (14 wt %)/sodium sulfate (10 wt %) system with an enzyme yield of 69.03 % and corresponding purity of glutaminase and selectivity of the system being 1.95 and 2.36, respectively (Tab. 5).

Table 5. Effect of TLL on purity and selectivity of the selected system (PEG 2000/Na_2SO_4).

TLL [wt %]	Yield [%]	PF	Selectivity
23.1	30.338	1.35	0.14
27.6	53.558	1.38	0.43
31.2	61.299	1.64	1.08
34.5	69.039	1.95	2.36
38.9	57.446	1.80	0.99
42.5	56.553	1.72	0.94
46.1	47.864	1.58	0.45

Fig. 8 demonstrates the influence of TLLs on K_e for ATPSs consisting of polymers with varying molecular weight with sodium sulfate. The K_e became higher with increasing TLL up to 34.5 % for the PEG 2000/sodium sulfate system. At a very high TLL, the recovery and K_e decreased because the concentration of polymer and salt in both phases increased and consequently affected the free volume available for different solutes to accommodate in a given phase [36]. It has been reported that a system comprising a polymer with high concentration or high-molecular-weight polymer and high salt concentration resulted in partitioning of biomolecules at the interphase because of the influence of volume exclusion and salting-out effect [37]. Experimental observations revealed that at a very low TLL, K_e was low and a sharp resolution between the phases may not occur due to the minimal difference in physical properties like density [17] which may not favor the partitioning of glutaminase into any one of the phase. The effect of TLL on purity, recovery, and selectivity for the PEG 2000/sodium sulfate ATPS (Tab. 5) revealed that the higher K_e and yield with higher selectivity and purification factor were obtained at a TLL of 34.5 %.

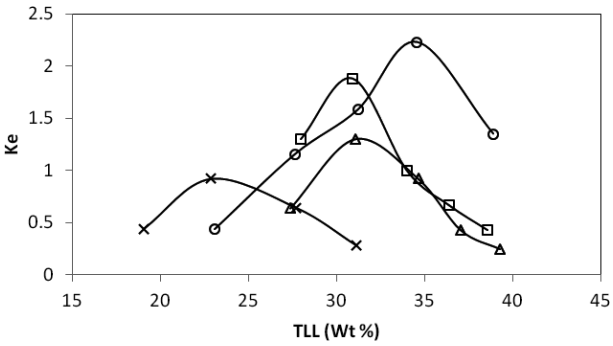

Figure 8. Effect of TLL on K_e. PEG 2000 (o), 4000 (□), 6000 (◇), 8000 (x) with Na_2SO_4.

4 Conclusions

Glutaminase with maximum activity was produced from *Z. rouxii* NRRL-Y 2547 with 4 % inoculum volume and maltose as the carbon source. The binodal curves and tie lines of the ATPSs revealed that sodium salts provided a larger biphasic region and better K_e when compared to potassium salts for the same anions. The K_e was found to decrease with increasing polymer molecular weight and concentrations of phase components. At a very high and very low TLL, the partitioning coefficient and recovery of glutaminase were found to be reduced. A maximum K_e of 2.22, purification factor of 1.95, and sensitivity of 2.36 were achieved with a PEG 2000 (14 wt %)/sodium sulfate (10 wt %) ATPS at a TLL of 34.5 %. Consequently, the PEG 2000/sodium sulfate ATPS may be considered as initial industrial purification process for glutaminase from its fermentation broth.

Symbols used

AARD	[%]	arithmetic average relative deviation
A_b	[U L^{-1}]	enzyme activity in bottom phase
A_i	[U L^{-1}]	enzyme activity in the initial extract
A_t	[U L^{-1}]	enzyme activity in top phase
a_0, a_1, a_2	[–]	fit parameters for Eq. (1)
C_{pt}	[g L^{-1}]	protein concentration in top phase
C_{pb}	[g L^{-1}]	protein concentration in bottom phase
C_{pi}	[g L^{-1}]	total protein concentration of initial extract
K_e	[–]	activity partition coefficient
K_p	[–]	partition coefficient of total proteins
S	[–]	sensitivity
TLL	[wt %]	tie line length
V_b	[L]	volume of bottom phase
V_r	[–]	volume ratio
V_t	[L]	volume of top phase
W_p	[w w^{-1}]	weight fraction of polymer
W_s	[w w^{-1}]	weight fraction of salt
W_s^t	[wt %]	weight percent of salt in top phase
W_s^b	[wt %]	weight percent of salt in bottom phase
W_p^t	[wt %]	weight percent of polymer in top phase
W_p^b	[wt %]	weight percent of polymer in bottom phase
W_p^{top}	[–]	weight fraction of polymer in top phase
W_s^{bot}	[–]	weight fraction of salt in bottom phase
W_w^{bot}, W_w^{top}	[–]	weight fraction of water in top and bottom phase
ηD	[–]	refractive index

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