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Extraction of Bovine Serum Albumin by Aqueous Two-Phase System Using PEG4000/Sodium Citrate and PEG8000/Sodium Phosphate

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Abstract

Aqueous Two Phase System (ATPS) or liquid-liquid extraction is used in biotechnology to recover valuable compounds from raw sources. In Aqueous Two-Phase Systems, many factors influence the Partition coefficient, *K*, (which is the ratio of protein concentration in the top phase to that in the bottom phase) and the Recovery percentage (Rec%). In this research, two systems of ATPS were used: first, polyethylene glycol (PEG) 4000/Sodium citrate (SC), and the second, PEG8000/ Sodium phosphate (SPH), for the extraction of Bovine Serum Albumin (BSA). The behavior of Rec% and *K* of pure (BSA) in ATPS has been investigated throughout the study by the effects of five parameters: temperature, concentration of polyethylene glycol (PEG4000 and PEG8000), the concentration of Sodium citrate or Sodium phosphate, pH, and the addition of sodium chloride as a supporting agent. The recovery percentage of BSA and its partition coefficient are significantly influenced by these factors to various degrees. The most influential variable in this study is PEG concentration for both systems. In addition to the PEG concentration, the stabilizing impact of NaCl is a crucial factor. The interaction between biomolecules and PEG gets more hydrophobic as the PEG concentration is raised. In the first system (PEG4000/SC), the maximum recovery percentage and partition coefficient were 98.99% and 97.69, respectively, at 31°C, PEG4000 concentration 1.5g/10 ml, Sodium citrate concentration 2.7 g/10 ml, pH 10, and 0.5 M NaCl concentration. While in the second system (PEG8000/SPH), the maximum recovery percentage and partition coefficient was 98.93% and 92.12, respectively, at 31ºC, PEG8000 concentration 1.5 g/10 ml, Sodium phosphate concentration 2.4 g/10 ml, pH 10, and concentration of NaCl 0.5 M.

Keywords: *Aqueous two-phase system (ATPS), Protein partitioning, Partition coefficient, Recovery percentage, Bovine serum albumin (BSA).*

1. Introduction

The Aqueous Two Phase System (ATPS) was invented accidentally in 1896 by Martinus Willem Beijerinck, but was not really used until the 1970s by Per-Åke Albertsson [1], [2]. ATPS systems were developed and have been studied a lot in recent years. It is made up of different types of chemicals including, polymer-polymer and polymer-salt systems that can be mixed together, and it is used for a variety of purposes [3].

This process is better than other extraction techniques because it is environmentally friendly, affordable, and can be used to extract many different types of molecules [2]–[5]. Water helps to separate and stabilize the structure of biomolecules in the two phases of Aqueous Two Phase System [3], [5]–[7], while other Liquid-Liquid Extraction (LLE) methods can damage

biological products, since the process conditions and organic solvents are often harsh [4], [5].

Biphasic systems are made of two different materials. They can be either made of two polymers, like PEG and dextran, or a polymer and salt, like PEG and phosphate, sulfate, or citrate. However, there are biphasic systems made of ionic liquids and short-chain alcohols [2], [4]–[6], [8]–[10]. Some researchers have studied different types of ATPS to characterize and understand their ability to separate, extract, and purify biomolecules and particles [11].

Despite the many benefits of using salt ATPS, there is a lack of understanding of how biomolecules partition in these systems, which makes it difficult to predict how they will behave. The concentration of a particular chemical (e.g. phosphate) and the surface characteristics of the target biomolecules (e.g. proteins) are both important factors when it comes to how the biomolecules behave in a system. In addition, the interactions between the biomolecules and system variables (e.g. pH, temperature, etc.) are also important [2], [12], [13].

The target protein in this study is Bovine serum albumin (BSA), a water-soluble protein found in milk [14], [15], a by-product of the cow industry. It is an ideal bioproduct that can be utilized in many bioprocesses since it has no influence on most biochemical reactions and is inexpensive because it can be easily purified in huge amounts from bovine blood [16]. It is created in the liver, and secretory cells allow it to enter milk [17]. About 0.7-1.3% of the total milk protein is BSA, which has a molecular weight of 66 kDA and an isoelectric point (pI) of 4.7–5.2 [14], [15], [17]–[20].

The partitioning of Bovine Serum Albumin between two aqueous phases is a complex process that can be difficult to understand. It is important to know that the partitioning of biomaterial into separate phases is due to the interactions between it and the surrounding molecules, these interactions create a variety of forces that help to create the desired structure [21]–[25]. In order to study the effects of partitioning biomolecules in a liquid mixture, scientists have to conduct many experiments. This can often be expensive, so it is important to make the best choices when doing these experiments. This is because the process of partitioning a substance between different substances is complicated and unpredictable, based on the system variables and the characteristics of the biomolecules involved [26], [27].

In the current study, two of ATPSs were used to examine the partitioning of bovine serum albumin (BSA), named PEG4000/Sodium citrate (SC) and PEG8000/Sodium phosphate (SPH). In this scenario, partitioning behavior of a model protein was studied, and the effect of system factors was analyzed, such as PEG 4000 and PEG 8000 concentrations (1.5-2.4 g/10 ml) each, and phase-forming salts, Sodium citrate (SC) concentrations (1.8-2.7 g/10 ml), Sodium phosphate (SPH) concentrations (1.5-2.4 g/10 ml). Other parameters were, temperature (10-31 ^{o}C) and (17-38 ^{o}C) for PEG/SC and PEG/SPH, respectively, pH (7-10), and NaCl (0.1-0.5 M) for both systems. Protein content for PEG/ Salt ATPSs was (0.01 g/10 ml). The potential interactions of model proteins with other substances may be predicted using their varied physical properties. Moreover, Classical Factorial Design (CFD) was used to inquire deeply into the partitioning behavior of a model protein. This was done as a design experiment to improve our understanding of the proteins' behavior.

The goal of this study is to determine which parameters (temperature, PEG4000 concentration, PEG8000 concentration, phosphate/citrate concentrations, pH, and the addition of NaCl) have the greatest impact on the partitioning of BSA, and to use that knowledge to.

2. Materials and Methods

2.1 Materials

Polyethylene glycol (PEG) was purchased from HIMEDIA Laboratories Pvt. Ltd., India, the molecular weight of the item was 4000 and 8000 g/mol, Disodium phosphate, Na_2HPO_4, (SPH), and Tri-Sodium citrate, $Na_3C_6H_5O_7$, (SC) was acquired from Central Drug House Ltd., India, and Sodium chloride (NaCl) was provided by Alpha Chemika, India. The Coomassie Brilliant Blue G-250 dye and bovine serum albumin (BSA) were bought from HIMEDIA Laboratories Pvt. Ltd., India. A high quality of reagents was used in the analysis. The water utilized in this investigation was double distilled and deionized.

2.2 Methods

2.2.1 Preparation of aqueous two-phase system

It is need to weigh the amount of different system components, Sodium citrate (SC) (1.8-2.7 g/10 ml), and Sodium phosphate (SPH) (1.5-2.4

g/10 ml), to make it work. Also weighing of PEG4000 and PEG8000 of (1.5 – 2.4 g/10 ml) each, to prepare solutions with the desired concentrations Appropriate amount of BSA is dissolved in the solution of Sodium citrate (SC) or Sodium phosphate (SPH), to obtain of simulated solution with concentration of 0.01 g/10 ml of BSA. ATPSs of PEG/SC and PEG/SPH were created based on requirement of binodal curves [28]–[30]. Appropriate pH 9 value was used to create the citrate and phosphate stock solutions.

The two of ATPSs of PEG/SC and PEG/SPH that were investigated to extract the BSA were made in screw tubes. All of two system parts were vortexed and completely blended with a magnetic stirrer for 10 minutes. The samples were spun around in a centrifuge device at 6000 rpm for 20 min so that the different parts of the samples could be separated. To make sure the systems were in equilibrium, they were left to sit at the desired temperature for around 24 hours, see Figure 2.2. A syringe was used to properly separate the phases once phase equilibrium has been reached. Below, is a simplified schematic of how one or two polymers may be mixed together to create an ATPS, see Figure 2.1 [31]. The bottom phases' volumes were finally calculated, and samples were collected for protein concentration analysis [32]–[35].

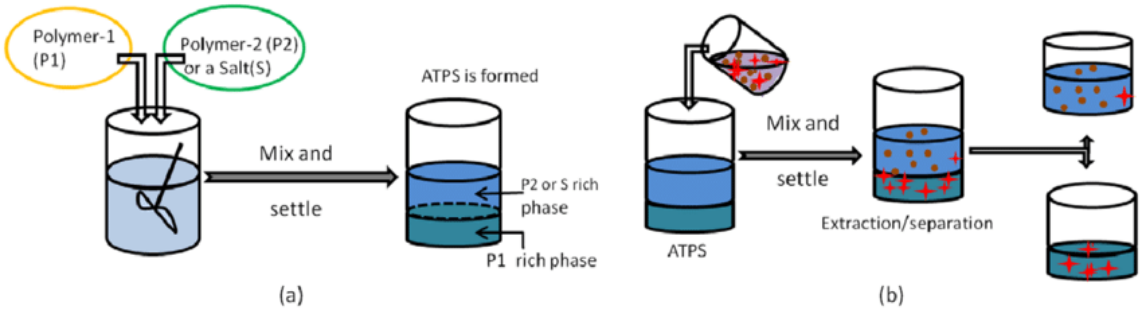

Fig. 2.1. A schematic of an ATPS.

Fig. 2.2. The formation of the two phases in an ATPS after mixing and settling.

2.2.2 BSA concentration determination

To measure the concentration of the protein in a sample, a Bradford technique using Coomassie Blue G250 was used [36] as shown in Figure 2.3. To determine the protein concentration, the samples are taken from the bottom phase and diluted with a known volume of distilled water. Their ultraviolet absorbance was then analyzed using a single-beam spectrophotometer (EMC-11-UV) from EMCLAB or BioPhotometer 6131 from Eppendorf [28, 29, 30] as shown in Figure 2.4. Similar solutions that are free from protein were used as blanks to make the necessary adjustments to prevent interference from PEG and citrate or phosphate. At 595 nm, the optical density was performed at a minimum of three duplicates of each experiment or measurement. The degree of protein separation in the polymer phase is determined by the partition coefficient (K). To ensure the system is working as intended, we also calculated the percentage of recovery using the partition coefficient [31, 32, 33].

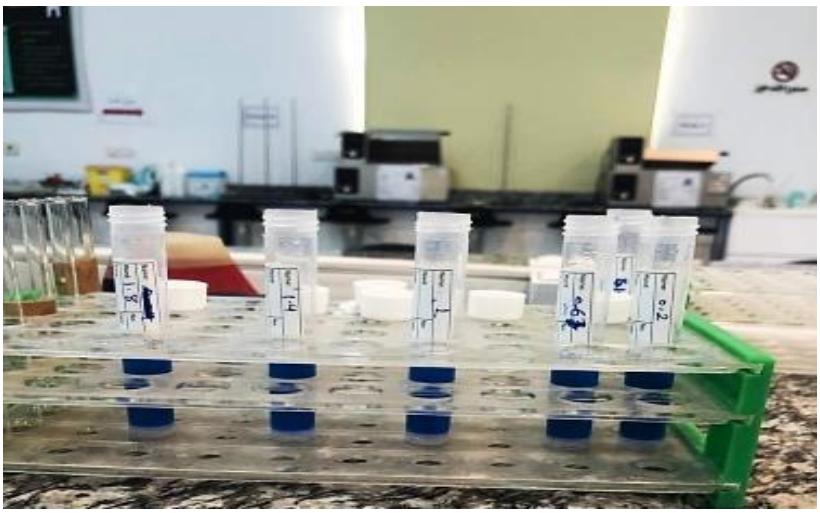

Fig. 2.3. Addition of Bradford Reagent (Coomassie Blue G250) to the collected samples

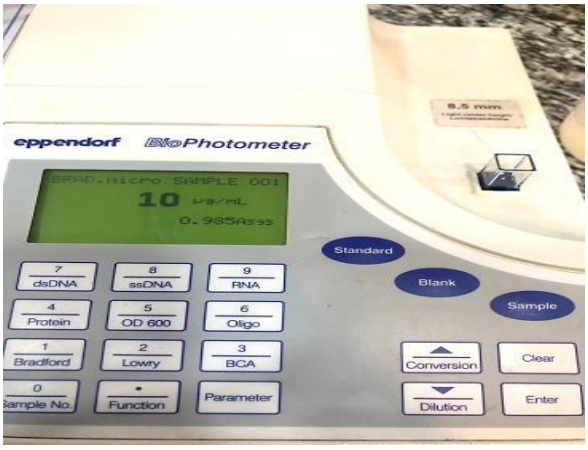

Fig. 2.4. Analyzing ultraviolet absorbance of BSA using a BioPhotometer.

The partition coefficient (K) is a measure of how much protein is left over after being extracted from a liquid. The higher the partition coefficient, the lower protein is left over in the bottom phase, i.e.

$K = C_T / C_B$ … (1)

Where, C_T is the protein concentration in the top layer.

C_B is the protein concentration in the bottom layer [20, 34, 35].

The recovery percentage (Rec%) measures the degree of how well the extraction process is. The following equation was used to calculate the recovery percentage (Rec%):

$$\text{Rec\%} = \left(1 - \frac{C_B}{C_{in}}\right) \times 100\% \quad \dots (2)$$

Where, C_{in} is the initial concentration of protein in the bottom phase [26].

3. Results and Discussion

3.1 Temperature Effect

The effect of temperature on the recovery percentage of BSA and its partition coefficient has been studied when a proper concentration of PEG4000 (1.5 g/10ml), SC (1.8 g/10ml) for PEG4000/SC system, and (1.5 g/10ml) for both PEG8000 and SPH for PEG8000/SPH system were used. Both systems were at pH 9 without the addition of NaCl. Due to the interconnected nature of an aqueous composition's components, such as electrostatic and hydrophobic forces, how temperature impacts how it behaves is extremely complex [32]. When the temperature is elevated, proteins can experience changes in their shape, structure, and stability, so the range of temperature is taken from (10-31 ^{o}C) for the first system (PEG4000/ SC), and from (17-38 ^{o}C) for the second system (PEG8000/ SPH). Figures 3.1

and 3.2 show the effect of temperature for systems PEG4000/ SC and PEG4000/ SPH respectively. It can be seen from Figures 3.1 and 3.2 that the Recovery per cent (Rec%) and partition coefficient (*K*) for protein increases as the temperature rises. This means that the percentage of protein that is recovered after being heated is greater at higher temperatures. In the second system, it was noticed that the Rec% and *K* start decreasing with increasing temperature from (31-38 ^{o}C). When the temperature changes, the liquids in an ATPS will also change. This can make it difficult to mix the two liquids properly, which can lead to problems. Changes in temperature cause the molecules in a substance to move around more, which can affect how easily the substance can be divided into smaller pieces. This is because molecules move more easily through substances that are less viscous, denser, and tension-free [43], [44]. This result agreed with Raja and Murty, 2013, who cleared that the increase in temperature affect the phase composition of the ATPS beside the structure of proteins [26]. Pirdashti et al, 2021, had a different opinion when he found out that the rise in temperature decreases the amount of protein that is recovered in the top phase [29]. Because of temperature rise, the polymer becomes more hydrophobic; as a result, the contacts between the molecules of the polymer and water tend to weaken, and the amount of water in both phases decreases over time. Since scientists know that the temperature in an ATPS experiment can affect the results, they always recommend keeping the temperature consistent.

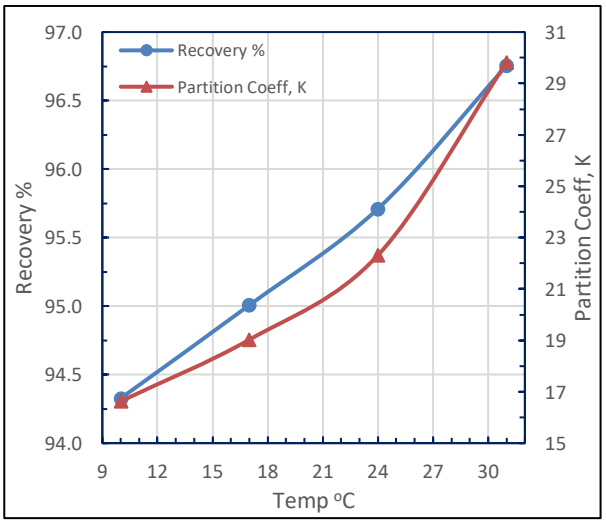

Fig. 3.1. Effect of temp on the Rec% and *K* at conc. of 1.5 g/10 ml for PEG 4000 and 1.8 g/10 ml for SC, pH 9, and without the addition of NaCl.

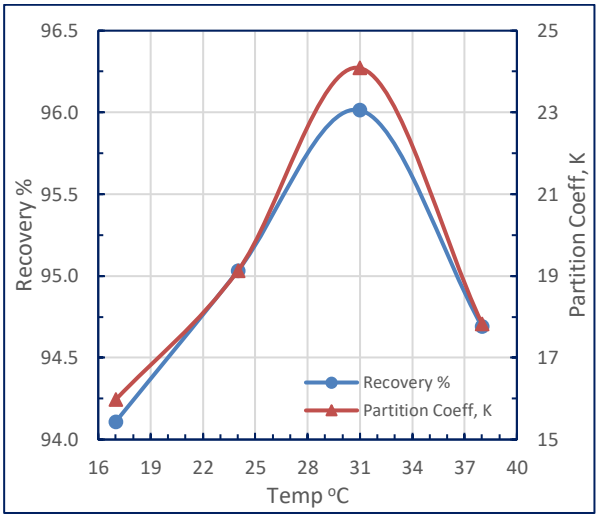

Fig. 3.2. Effect of temp on the Rec% and *K* at conc. of PEG8000 and SPH of 1.5 g/10 ml each, pH 9, and without the addition of NaCl.

3.2 Concentration Effect of PEG4000 and PEG8000

Protein and PEG interact differently; sometimes the interaction between the two is more hydrophobic, which means that the proteins stay apart more easily. Raising the PEG concentration in the system makes it harder for the protein to recover and makes the partition coefficient (K) much lower [45]. In both systems the concentration of PEG4000 and PEG8000 had a significant impact on how quickly and evenly BSA is partitioning, which affects how much of the protein can be recovered. The effect of concentration of PEG for system PEG4000/ SC and system PEG8000/ SPH are shown in Figures 3.3 and 3.4 respectively. For 1.8 g/10 ml of SC and a PEG4000/SC system, higher Rec% and *K* of BSA were found to be 96.98% and 32.16, respectively, when PEG4000 concentration is 1.5 g/10 ml. While for the second system, PEG8000/SPH, higher Rec% and *K* of BSA were found to be 96.06% and 24.40, respectively, when PEG8000 concentration of 1.5 g/10 ml and SPH concentration is 1.5 g/10 ml. The two systems were set to the same temperature of 31 ^{o}C, pH 9 and without the addition of NaCl. It can be seen from Figures 3.3 and 3.4 that as the concentration of Polyethylene Glycol (PEG) increases in the range of (1.5 – 2.4 g/10 ml), the percentage of protein recovered decreases. This result is agreed with Shad *et al*, 2018, who illustrated in their research that when PEG concentration is raised, proteins move from the upper to the lower phase

in ATPS, this is probably because the protein has a stronger preference for water molecules, and the PEG molecule is larger and less hydrophobic than water; which leads to rising the partition coefficient automatically [46]. However, Ramji *et al*, 2011, went with similar finding, as the concentration of polymer in the top layer increases, the partition coefficient decreases since no more space is available for the protein to be partitioned among the different layers [47]. In contrast to what is shown in this work, Faravash *et al*, 2007, [48] and Antelo *et al*, 2015, [49] had a conflicted opinion when they concluded that increasing of PEG concentration levels had a beneficial effect on Rec% and *K* which are increased according to PEG increment.

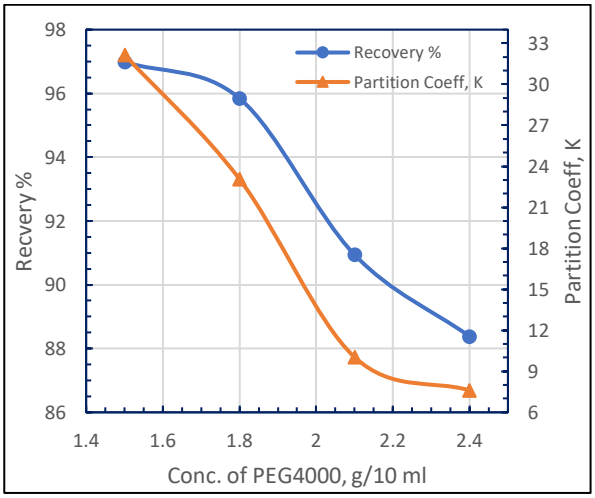

Fig. 3.3. Effect of PEG4000 on the Rec% and *K* at T= 31°C, conc. of SC 1.8 g/ 10 ml, pH 9, and without the addition of NaCl.

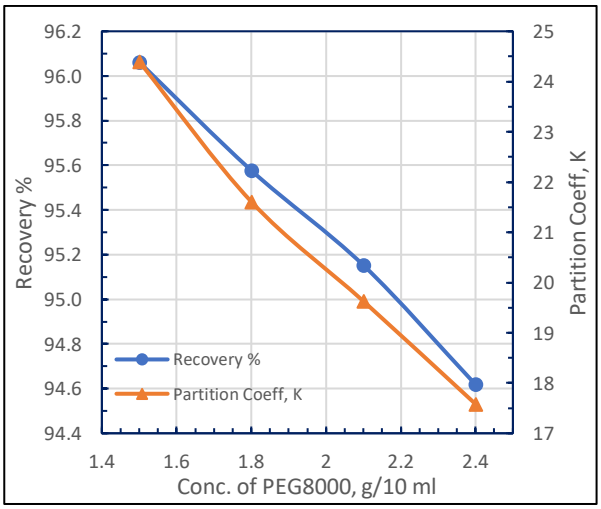

Fig. 3.4. Effect of PEG 8000 on the Rec% and *K* at T = 31°C, conc of SPH 1.5 g/10 ml, pH 9, and without the addition of NaCl

3.3 Concentration Effect of Sodium citrate and Sodium Phosphate

By doing experiments, it was found that the amount of Sodium citrate (SC) or Sodium phosphate (SPH) salt in a solution has an effect on how much protein is extracted in ATPS and how well it is partitioned. According to Baskaran *et al*, 2018, [30], [50], where they found in PEG/salt system that the Rec% and *K* increases when salt concentration increased. By checking Figures 3.5 and 3.6, it is clear to see that the Rec% and *K* are increased when salt is added, this makes the solution more concentrated. This is called the salting-out effect, when something is salted, the salt molecules move up from the bottom to the top and this happens with proteins, too and helps to improve the extraction process and thus the Rec% and *K* rises. It was found by increasing SC concentration from 1.8 g/10 ml to 2.7 g/10 ml for PEG4000/SC system, the Rec% increased from 96.69% to 97.97%, and the *K* increased from 29.24 to 48.20, as shown in Figure 3.5.

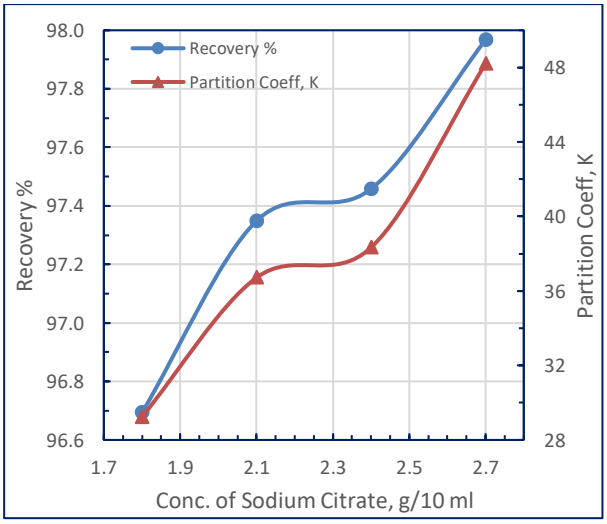

Fig. 3.5: Effect of SC on the Rec% and *K* at T=31°C, conc. of PEG4000 = 1.5 g/10 ml, pH 9, and without the addition of NaCl.

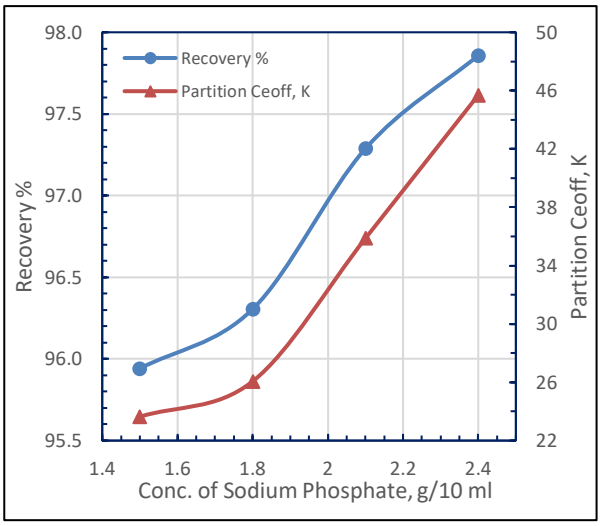

Fig. 3.6. Effect of SPH on the Rec% and *K* at T = 31°C, conc. of PEG8000 = 1.5 g/10 ml, pH 9, and without the addition of NaCl

In PEG8000/SPH system, it is shown that the Rec% increased from 95.94% to 97.86%, and the *K* increased from 23.64 to 45.69, by increasing SPH concentration from 1.5 g/10 ml to 2.4 g/10 ml as in Figure 3.6. This result was convenient to Raja and Murty, 2013, Baskaran *et al*, 2018, Faravash *et al*, 2007, Antelo *et al*, 2015, and Kee *et al*, 2021, [26], [30], [48], [49], [51], and there is no incompatible opinion were found.

3.4 pH Effect

Biomolecules can be partitioned between two phases according to their different charges and surface properties. This can change depending on the pH of the aqueous solution. Protein molecules with a positive net charge will try to pull electrons away from other proteins, creating an electric force. However, if the pH is higher than the pI (which stands for "pH-indicator" or "Potential Isoelectric point"), the electric force is weakened and the proteins will end up with a net negative charge, and if it is lower, it becomes positive and there will be no net charge if pH and pI values are the same [44]. According to reports, when a system has a higher pH, the negatively charged biomolecules will partition more easily and the target biomolecule will tend to be found in the top phase. Because of the positive dipole moment, proteins with a high pH value than their (potential isoelectric point) pI, are more attracted to PEG-rich phases [52], [53]. Figures 3.7 and 3.8 show as the pH of the solution goes up, the partition coefficient (*K*) increases, which means that more protein will be recovered. For PEG4000/SC system, it was found that at pH 10, the Rec% and *K* values are the highest and were 97.86% and 45.69 respectively, as shown in Figure 3.7. Figure 3.8 shows pH changes can have effects on PEG8000/SPH system. The system shows increasing in Rec% and *K* with increasing of pH and higher values of Rec% and *K* were 98.10% and 51.66 respectively, at pH 10. Pirdashti *et al*, 2021, Saravanan *et al*, 2007, Faravash *et al*, 2007, Kee *et al*, 2021, and Johansson 1985, [29], [39], [48], [51], [54] all had good agreement to our investigation, and there is no incompatible opinion were found.

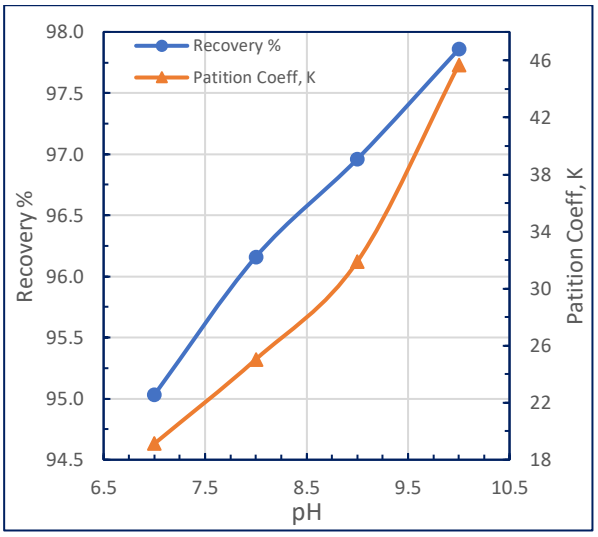

Fig. 3.7. Effect of pH on the Rec% and *K* at T=31°C, PEG4000 conc. of 1.5 g/10 ml, and SC of 2.7 g/10 ml, and without the addition of NaCl

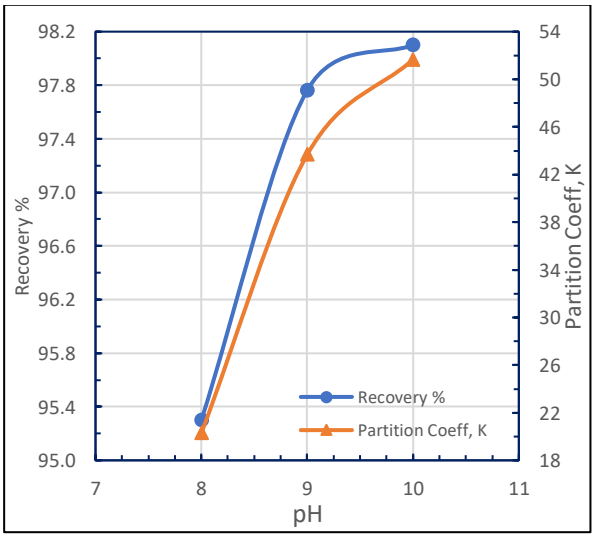

Fig. 3.8. Effect of pH on the Rec% and *K* at T=31°C, PEG8000 conc. of 1.5 g/10 ml and SPH of 2.4 g/10 ml, and without the addition of NaCl.

3.5 Effect of the addition of NaCl.

As a final step in the present work, the effect of NaCl addition has been studied as a way to improve the protein partitioning and the recovery rate of the protein. The partition coefficient in ATPSs is influenced by the addition of salts such as NaCl, KCl [55]. These salts work as partitioning accelerators, ions in these salts have varying degrees of hydrophobicity, and the hydrophobic ions drive the partitioning of the opposite ions to phase with ions that have a higher hydrophobicity and vice versa [44], [45]. The biomolecule transitions from a phase that is salt-rich to one that is polymer-rich due to the salting-out effect [42]. In Figures 3.9 and 3.10, addition of NaCl at concentration range (0.1-0.5 M) to PEG4000/SC and PEG8000/SPH system show an increment in the Rec% and *K*. In figure 3.9, higher values recorded for PEG4000/SC system at (NaCl 0.5 M) was 98.99% for Rec%, and 97.69 for *K*. Figure 3.10 showed that higher values of Rec% and *K* were 98.93% and 92.12, respectively, for PEG8000/SPH system. Raja and Murty, 2013 Settu *et al*, 2015, Faravash *et al*, 2007, Saravanan *et al*, 2007, Ramesh and Murty, 2015, Gündüz, 2000 Perumalsamy and Murugesan, 2007, and Regupathi *et al*, 2013, [26], [28], [30], [39], [41], [56]–[58] all went with similar findings and there is no any incompatible opinion.

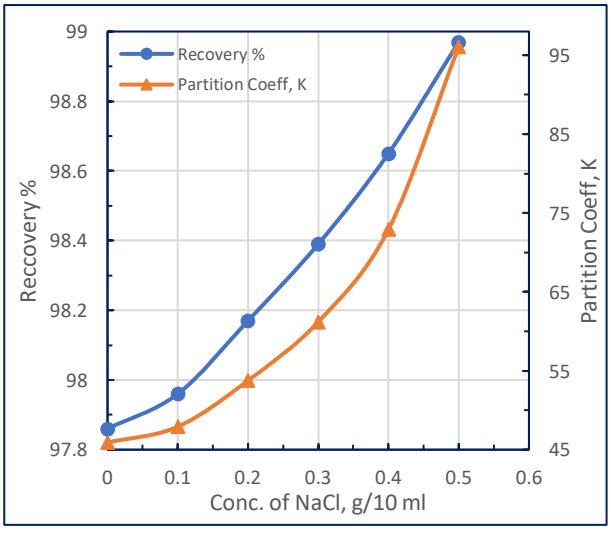

Fig. 3.9. Effect of NaCl conc. on the Rec% and *K* at T= 31°C, conc. of PEG4000 conc. of 1.5 g/10 ml, and SC of 2.7 g/10 ml, pH 10.

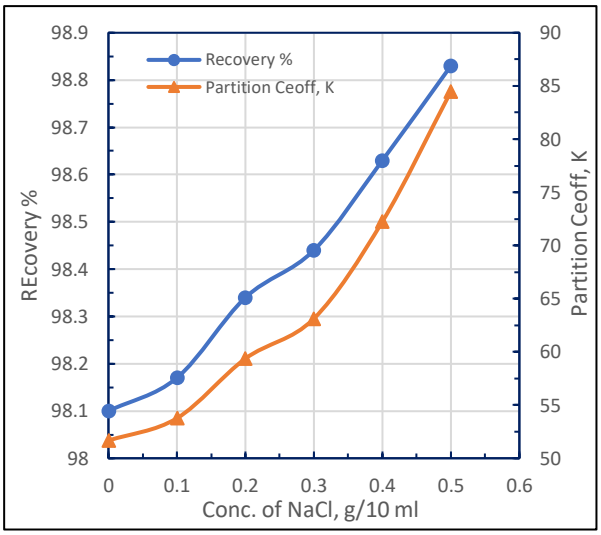

Fig. 3.10. Effect of NaCl conc. on the recovery% and *K* at T = 31°C, conc. of PEG8000 of 1.5 g/10 ml and SPH of 2.4 g/10 ml, pH 10.

4. Conclusions

An aqueous two-phase system (ATPS) is an alternative to traditional methods of extraction, which involve using solvents to extract the proteins. It is a type of method that can be used to recover valuable soluble proteins from aqueous phases. In this study, two systems, PEG4000/sodium citrate and PEG8000/sodium phosphate were used for the extraction of BSA. The two systems have a high degree of efficiency. The ATPS is a simple process that can be used to separate bioproducts. This is a cheaper option than using traditional methods, and it is likely to be more effective in purifying products. The results showed that under certain conditions like concentration of PEG, sodium phosphate/citrate, pH, temperature and NaCl salt addition, it was possible to change the way BSA partitioned. When the quantities of the PEG-solution and SC (or SPH)-solution are equivalent, the BSA recovery percentage reaches 98% in a single stage for the first and second systems, implying that no more stages are necessary.

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استخلاص البومين مصل الأبقار بنظام ثنائي الطورالمائي باستخدام PEG4000 / سترات الصوديوم و PEG8000 / فوسفات الصوديوم

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الخلاصة

تتضمن التكنولوجيا الحيوية استخلاص جزيئات ذات اهمية عالية من المواد الخام باستخدام تقنية استخلاص تسمى نظام ثنائي الطور المائي، وهي طريقة لاستخلاص أنواع مختلفة من الجزيئات كالبروتينات والانزيمات والخلايا أو المواد الأخرى. يستخدم في هذه الطريقة نوعين مختلفين من السوائل لفصل وتنقية الجزيئات وقد وجد بالتجربة بأنها وسيلة فعالة للغاية لاستخلاص وفصل مخاليط من الجزيئات الحيوية. في الأنظمة المائية ثنائية الطور تؤثر العديد من العوامل على معامل التجزئة K (وهو نسبة تركيز البروتين في الطور العلوي إلى ذلك الموجود في الطور السفلي) ونسبة الاسترداد Rec% . في هذا البحث تم استخدام نظامين لاستخلاص البومين مصل الابقار: الأول يتكون من بولي إيثيلين جلايكول 4000 (PEG4000) و سترات الصوديوم والثاني يتكون من بولي إثيلين جلايكول 8000 (PEG8000) وفوسفات الصوديوم . تم ملاحظة بأن نسبة الاسترداد Rec% و *K* لألبومين مصل الأبقار النقي في النظام ثنائي الطور المائي من خلال دراسة تأثير خمسة عوامل هي: درجة الحرارة، تركيز (PEG4000) و (PEG8000) ، تركيز سترات الصوديوم أو فوسفات الصوديوم ، درجة الحموضة ، وإضافة كلوريد الصوديوم كعامل مساعد. وجد ان المتغير الأكثر تأثيرًا في هذه الدراسة هو تركيز PEG لكلا النظامين حيث يصبح التفاعل بين البروتين و PEG أكثر كراهةً للماء مع زيادة تركيز PEG.

في النظام الأول، كانت نسبة الاسترداد القصوى ومعامل التقسيم 98.99٪ و 97.69 على التوالي، عند تركيز (PEG4000) 1.5 جم / 10 مل ، وتركيز 2.7 جم / 10 مل لسترات الصوديوم ، مقارنة بالنظام الثاني الذي سجل نسبة استرداد قصوى ومعامل تقسيم 98.93٪ و 92.12 على التوالي عند تركيز (PEG8000) 1.5 جم / 10 مل، و تركيز فوسفات الصوديوم 2.4 جم / 10 مل. حيث كانت أفضل الظروف عند درجة الحرارة 31 درجة مئوية، الأس الهيدروجيني 10، وتركيز كلوريد الصوديوم 0.5، في كلا النظامين.