Extraction of prodigiosin using aqueous two phase system

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**Abstract.** Aqueous two-phase system is a modified and amazing separation process of bioproducts in downstream since it keeps their properties. This method is able to give high recovery yield and high purity in a single step. Prodigiosin pigment was produced by *Serratia marcescens* in carbohydrate fermentation. It is the first time to extract the prodigiosin by aqueous two-phase system, where two types of system are applied; polymer/polymer and polymer/salt. The parameters studied on the recovery percent and partition coefficient of prodigiosin are temperature, weight fraction of polymer in solution, molecular weight of polymer, weight fraction of salt in solution, and pH. The maximum recovery and partition coefficient of prodigiosin are obtained in the first system (PEG6000/Dextran) at temperature of 20\(^\circ\)C, pH 6, weight fraction of PEG6000 is 0.22, weight fraction of Dextran is 0.15 while in the second system (PEG1500/ammonium sulfate) the maximum recovery and partition coefficient of the prodigiosin are obtained at temperature of 20\(^\circ\)C, pH 5, weight fraction of salt is 0.15, and weight fraction of PEG1500 is 0.24.

1. **Introduction**

The increasing request of natural pigment in food, pharmaceutical, cosmetic, textile, printing and dye industry results to produce bio-colorant from different natural source such as microorganism rather than plants [1]. Pigments that produced from plants have many defects such as changing against light, heat, or adverse pH and low water solubility [2]. On the other hand, the advantages of production of pigment from microorganisms are many such as easy and fast growth in the low cost culture media [3]. Numerous pigments may produce from microorganisms like carotenoids, melanins, flavones, quinines [4]. The numerous types of microorganisms such as bacteria, fungi, algae, yeast are usually present in different colors. The colored microorganisms were separated from various samples and their extracted pigments were applied in different industries [5, 6]. Commonly, protein fractionating is enhanced by Van der Waals, hydrophobic, hydrogen bond, and ionic interactions between the surrounding phase and biomolecules [7].

Aqueous two-phase system (ATPS) has become a proven tool used in separation and purification technology [8]. The application of ATPSs in clarification, partitioning and partial purification of biomolecules and bioproducts had showed the rapid development [9]. The intermixability of polymer mixtures is an exception because most hydrophilic polymer pairs are incompatible in aqueous solutions resulting in two contracting phases in equilibrium with each other [10]. For each polymer containing mostly water and one of the polymers types the phase separation in polymer mixtures is due to the high molecular weight of the polymers together with interactivity between the
segments of the polymers. Theoretically this process has been treated by applying theories on the thermodynamic [11]. The driving force for the demising process in polymer–polymer–solvent systems is the enthalpy associated with the interactions of the components [12], which is opposed by the loss in entropy associated with the isolation of the components through phase separation. Separation based on aqueous two-phase extraction (ATPE) is a committed separation in downstream process for the production of biomolecules products [13]. The using of aqueous two-phase systems has many advantages included but are not limited to easy scalability, ease of continuous operation and a favorable environment for biomolecules [14]. The process development is one of the main challenges related with aqueous two-phase systems [7]. This is in part attributed to the many factors which affect the separation of biomaterials in such systems such as polymer and salt type, pH and charge. Many factors should be considering makes the development of aqueous two-phase systems confrontation since the need to find a hard and efficient separation in a large practical space [15].

Aqueous two-phase systems are easy to use, involving two processes, equilibration and phase separation [16]. Equilibration is rapid, including mixing of the components that constitute the phase system with the material subjected to partitioning, and dispersing the phases to obtain equilibrium of phase compositions and partition [17]. The phase separation under gravity is not fast as in water-organic solvent systems, varying between a few minutes and a few hours because of a rather low difference in the densities of the two phases [18]. The aqueous two-phase system provides a technically simple, easily scalable, energy-efficient, and mild separation technique for product recovery in biotechnology [19]. The interfacial tension is extremely low, between 0.0001 and 0.1 dyne/cm compared with 1–20 dyne/cm for conventional water-organic solvent systems, creating a high interfacial contact area of the dispersed phases and thus an efficient mass transfer [19].

Prodigiosin pigment is commonly extracted by chloroform as suitable solvent [20], but this method has some disadvantages where it is not suitable for medical, industrial only applications. Chloroform is a toxic substance that is not suitable for human consumption [21, 22]. Most of those who know, from previous experience, the sensations produced by ether inhalation, and who have subsequently breathed the chloroform, have strongly declared the inhalation and influence of chloroform to be far more agreeable and pleasant than those of ether [23]. Its perfume is not unpleasant, but the reverse, and the odor of it does not remain, for any length of time, attached to the clothes of the attendant, or exhaling, in a disagreeable form, from the lungs of the patient, as so generally happens with sulphuric ether [23]. Its partial purity reaches 52%. It takes a long time for the separation, from 30 minutes to 6 hours [24].

Instead of above method, aqueous two phase system (ATPS) was used in this research, where its advantage over the previous one is to need small of polymer or salt, decreases pollution environmental, high selectivity and ATPS has low interfacial tension between two phases [19], mass transfer can be easily carried out when the system is stirred. Furthermore, the partition characteristics of biocatalyst and product in an ATPS can be employed as an extractive bioconversion [25], easy of handling sample of large volume, high efficiency of extraction (> 97%) and low cost [8].

The aim of this research is to extract the prodigiosin in aqueous phase without need to the organic solvent and find the optimum operating conditions for this process.

2. Experimental Work
2.1 Biosynthesis of prodigiosin
The production of Prodigiosin is achieved by biosynthesis using suitable media. Serratia marcescens was obtained from Food Contamination Research center/ Ministry of Science and Technology/ Baghdad, Iraq. The isolate was maintained using Luria Bertanni agar. After incubation, the plates and tubes were observed for the growth of pigmented bacteria. Williamson and Salmon method; 2006 was used for the cultivation of Serratia and production of prodigiosin in 1 L flask, which contains (g/L): (Starch, 10; Peptone, 5; CaCl₂.2H₂O, 8.82; FeSO₄.4H₂O, 0.33; MgSO₄.7H₂O, 0.61; MnSO₄.4H₂O, 2). pH was adjusted to 7 prior to autoclaving [26].
Quantification of prodigiosin Red pigment producing bacteria was grown in Nutrient Broth, and King’s B Broth, at 25 °C. The levels of prodigiosin in these conditions were estimated after 72hr. For quantification of prodigiosin produced, bacterial cell absorbance in the Nutrient broth was measured at 620 nm, following which the broth suspensions were subjected to centrifugation at 5000 rpm for 15 min to collect the cell pellet. 10 ml of 95% methanol was added to the cell pellet and centrifuged under the same condition. 95% methanol was used as a blank. Methanolic extract of prodigiosin showed characteristic maxima at 499 nm. Extracted prodigiosin was estimated using the following equation [27, 28].

\[
\text{Prodigiosin unit/cell} = \left( \frac{\text{OD}_{499} - (1.381 \times \text{OD}_{620})}{\text{OD}_{620}} \right) \times 1000
\]

(1)

Where:
\( \text{OD}_{499} \): is the pigment absorbance;
\( \text{OD}_{620} \): is the bacterial cell absorbance
1.381: is a constant

The cells are separated from fermentation broth by centrifugation which is separate material according to different of density [29]. Prodigiosin is an intracellular antibiotic therefore, the cells wall is disrupted using methanol. The methanol is evaporated at 70°C to obtain the prodigiosin pigment in contaminated water with cells waste.

2.2 Recovery of Prodigiosin

Instead of extraction of prodigiosin using chloroform as a solvent as common way, it is extracted using aqueous two liquid phase system (ATPS). Two systems were used; polymer-polymer and polymer-salt. In the first one the polyethylene-glycol PEG6000 and dextran3000 polymers were used. In the second system the PEG1500 and ammonium sulfate salt were used. In the first system the parameters studied are temperature (20 – 50°C), weight fraction of PEG6000 (0.13 – 0.22), and weight fraction of dextran (0.15 – 0.24). While in the second system the parameters studied are pH (5 – 9), weight fraction of PEG1500 (0.15 – 0.24), and weight fraction of dextran (0.15 – 0.24).

To obtain the two phases; dextran or salt is mixed with contaminated water feed which contains pigment for 15 min while the PEG is mixed with distilled water for 15 min also, then 10 ml of the dextran-solution and 10 ml of the PEG-solution are mixed together for 10 min. The all solution is left to settle until the two separated phases are obtained, this will take up about 25-30 min. The upper layer is the PEG phase that draw the pure prodigiosin without contaminated of cell debris and other waste while the lower layer is the dextran or salt phase that lost the prodigiosin.

The recovery per cent of prodigiosin and partition coefficient are calculated using equation 2 and 3 respectively [30]:

\[
\% \text{ Recovery} = \frac{\text{Amount of solute in the extract}}{\text{Amount of solute in the feed}}
\]

(2)

\[
\text{Partition Coefficient (} K \text{)} = \frac{\text{Concentration of solute in the extract}}{\text{Concentration of solute in the raffinate}}
\]

(3)

Where; Solute: is the Prodigiosin
Extract: is the Phase of PEG6000 or PEG1500 that holds the prodigiosin after reaching equilibrium.
Raffinate: is the Phase of Dextran3000 or Salt that lost the prodigiosin after reaching equilibrium.
3. Results and discussions
The concentration obtained of prodigiosin in the contaminated water phase before the extraction by ATPS is 0.433 g/l. The measurement of prodigiosin was achieved using UV-spectrophotometer. Three parameters were studied in each system (polymer/polymer and polymer/salt). The set of experiments are divided in 16 experiments in each system according to the Taguchi method. Table 1 and 2 are showing the number of experiments, their parameters level, recovery per cent of prodigiosin, and partition coefficient ($K$).

**Table 1.** Number of experiments of system-1 with its parameters level, concentrations of Prodigosin in the two phases, % recovery of prodigiosin, and partition coefficient ($K$).

| Run No. | T °C | Dextran Wt fraction | PEG6000 Wt fraction | Conc. of Prodig in Dext g/L | Conc. of Prodig in PEG g/L | % Recovery | K  |
|--------|------|---------------------|---------------------|-----------------------------|-----------------------------|------------|----|
| 1      | 20   | 0.15                | 0.13                | 0.092                       | 0.341                       | 78.73      | 3.77|
| 2      | 20   | 0.18                | 0.16                | 0.112                       | 0.321                       | 74.12      | 3.11|
| 3      | 20   | 0.21                | 0.19                | 0.12                        | 0.312                       | 72.04      | 2.61|
| 4      | 20   | 0.24                | 0.22                | 0.093                       | 0.34                        | 78.52      | 2.3 |
| 5      | 30   | 0.15                | 0.16                | 0.156                       | 0.28                        | 64         | 2.2 |
| 6      | 30   | 0.18                | 0.13                | 0.193                       | 0.25                        | 57.3       | 1.86|
| 7      | 30   | 0.21                | 0.22                | 0.203                       | 0.23                        | 53.2       | 1.23|
| 8      | 30   | 0.24                | 0.19                | 0.213                       | 0.22                        | 50.8       | 1.14|
| 9      | 40   | 0.15                | 0.19                | 0.244                       | 0.189                       | 43.66      | 1.38|
| 10     | 40   | 0.18                | 0.22                | 0.264                       | 0.169                       | 39.1       | 1.26|
| 11     | 40   | 0.21                | 0.13                | 0.28                        | 0.153                       | 35.41      | 1.02|
| 12     | 40   | 0.24                | 0.16                | 0.295                       | 0.138                       | 31.86      | 0.86|
| 13     | 50   | 0.15                | 0.22                | 0.353                       | 0.08                        | 18.47      | 1.04|
| 14     | 50   | 0.18                | 0.19                | 0.367                       | 0.066                       | 15.2       | 1.09|
| 15     | 50   | 0.21                | 0.16                | 0.367                       | 0.066                       | 15.2       | 0.86|
| 16     | 50   | 0.24                | 0.13                | 0.363                       | 0.07                        | 16.16      | 0.68|

**Table 2.** Number of experiments of system-2 with its parameters level, concentrations of Prodigosin in the two phases, % recovery of prodigiosin, and partition coefficient ($K$).

| Run No. | pH | (NH₄)₂SO₄ Wt fraction | PEG15000 Wt fraction | Conc. of Prodig in Salt-phase g/L | Conc. of Prodig in PEG-phase g/L | % Recovery | K  |
|---------|----|-----------------------|---------------------|----------------------------------|----------------------------------|------------|----|
| 1       | 5  | 0.15                  | 0.15                | 0.012                            | 0.421                            | 97.22      | 35.09|
| 2       | 5  | 0.18                  | 0.18                | 0.023                            | 0.41                             | 94.69      | 17.83|
| 3       | 5  | 0.21                  | 0.21                | 0.042                            | 0.391                            | 90.33      | 9.34 |
| 4       | 5  | 0.24                  | 0.24                | 0.102                            | 0.331                            | 76.43      | 3.24 |
| 5       | 6.3| 0.15                  | 0.18                | 0.131                            | 0.302                            | 69.75      | 2.31 |
| 6       | 6.3| 0.18                  | 0.15                | 0.132                            | 0.301                            | 69.52      | 2.28 |
| 7       | 6.3| 0.21                  | 0.24                | 0.142                            | 0.291                            | 67.24      | 2.05 |
| 8       | 6.3| 0.24                  | 0.21                | 0.179                            | 0.254                            | 58.67      | 1.42 |
| 9       | 7.6| 0.15                  | 0.21                | 0.021                            | 0.411                            | 94.9       | 18.6 |
| 10      | 7.6| 0.18                  | 0.24                | 0.121                            | 0.312                            | 72.78      | 2.67 |
| 11      | 7.6| 0.21                  | 0.15                | 0.133                            | 0.30                             | 69.29      | 2.26 |
| 12      | 7.6| 0.24                  | 0.18                | 0.219                            | 0.215                            | 49.53      | 0.98 |
The equations of recovery

\[ Y_1 = 181.88 + 0.047X_1 - 818.06X_2 - 89.55X_3 - 0.49X_4X_5 - 8.43X_6X_7 + 707.83X_8X_9 - 0.004X_{10}^2 + 1431.94X_{11}^2 + 695.84X_{12}^2 \]

\[ Y_2 = 14.016 - 0.345X_1 - 35.783X_2 - 13.02X_3 + 0.31X_4X_5 + 0.147X_6X_7 + 43.06X_8X_9 + 0.003X_{10}^2 + 22.92X_{11}^2 - 6.25X_{12}^2 \]

\[ Y_3 = 4.39 - 24.61X_1 + 1521.21X_2 + 654.62X_3 - 102.92X_4X_5 + 17.6X_6X_7 - 6290.26X_8X_9 + 2.29X_{10}^2 + 283.16X_{11}^2 + 1114.2X_{12}^2 \]

\[ Y_4 = 319.73 - 45.1X_1 - 1234.9X_2 - 230.44X_3 + 64.22X_4X_5 + 44.9X_6X_7 - 370.6X_8X_9 + 1.48X_{10}^2 + 1966.92X_{11}^2 + 16.2X_{12}^2 \]

Where:

- $Y_1$: is the per cent of prodigiosin recovery in PEG6000/dextran system.
- $Y_2$: is the partition coefficient ($K$) of prodigiosin in PEG6000/dextran system.
- $Y_3$: is the per cent of prodigiosin recovery in PEG1500/Salt system.
- $Y_4$: is the partition coefficient ($K$) of prodigiosin in PEG1500/Salt system.
- $X_1$: Temperature °C
- $X_2$: weight fraction of Dextran3000
- $X_3$: weight Fraction of PEG6000
- $X_4$: pH
- $X_5$: Weight fraction of (NH$_4$)$_2$SO$_4$ salt
- $X_6$: Weight fraction of PEG1500

The $R^2$ for equations 4 – 7 are 0.992, 0.993, 0.99, 0.988 respectively.

The Figures 1–10 was drawn depending on the equations of 4 – 7. The drawing of the Figures 1–6 were achieved at constant pH6, stabilized PEG6000 concentration in each Figure and different weight fraction of dextran. While Figures 7–10 was achieved at constant temperature of 20°C, stabilized salt concentration in each Figure and different weight fraction of PEG1500.

### 3.1 Effect of temperature

Depending on the type of the polymer used, the temperature has different effect in each phase system. For instance, at high temperature, it can be form two phases with low concentrations of PEG and salt whereas in case of PEG and dextran system, two phases will easily form at lower temperature [31]. The range of temperature was taken from (20 – 50°C). From Table 1 and 2 it can be seen that the recovery percent and partition coefficient are increased with decreasing temperature because the viscosity and density are changed with temperature, and these results are agreement with Walter and Johansson 1994 [32]. Also, Tjerneld and Albertsson indicated that for polymer/polymer systems, lower temperatures favor phase separation, in general, and the opposite is true for most polymer/salt systems [33]. So further increasing of temperature above this range will be useless. Also, graphically effect of temperature is shown in the Figures 1–3 and Figures 4–6 for recovery per cent and partition coefficient respectively. For the 1st system, if it is focused in the Figures 4–6 the maximum value of partition coefficient is (4.12) where it can be obtained at 20°C, dextran weight fraction of 0.15, PEG6000 weight fraction of 0.22, and pH6 and this is clear in the Figure 5. The larger the value of $K$, the more stable the product which results in better separation. And the maximum value of recovery per cent is 89.8% at the same conditions of partition coefficient as shown in Figure 2. While for the 2nd system, the maximum values of recovery per cent and partition coefficient are 97.9% and 32.5
respectively at a condition of 20°C, (NH₄)₂SO₄ weight fraction of 0.15, PEG1500 weight fraction of 0.24, and pH5 as shown in the Figures 7 and 9.

**Figure 1.** % recovery of Prodigiosin with temp at different concen. of dextran, pH 6 and at constant wt. fraction of PEG6000 (0.13).

**Figure 2.** % recovery of Prodigiosin with temp at different Concen. of dextran, pH 6 and at constant wt. fraction of PEG6000 (0.22).

**Figure 3.** % recovery of Prodigiosin with dextran fraction at different Temp., pH 6 and at constant wt. fraction of PEG6000 (0.22).

**Figure 4.** Partition Coeff. of Prodigiosin with temp at different Concen. of dextran, pH6 and at constant wt. fraction of PEG6000 (0.13).

### 3.2 Effect of polymer concentration

From Figures 1–6, it can be seen that the recovery per cent and partition coefficient are increased with increasing of PEG6000 concentration and with decreasing of dextran concentration. As the concentration of PEG increased that leads to high density, refractive index and viscosity of the phase and finally provide large difference in properties between the phases. The interfacial tension between two phases influenced by concentration of polymer, so it increased by increasing the concentration of polymer. When the molecular weight of PEG reduced the need of its critical concentration that form two phases increased, so the concentration of PEG1500 in the second system increased. Also, the
similar results for PEG1500 are shown in the Figures 7–10. But the different in the recovery percent is not largely, so from the economic view it is preferred to use the minimum amount of polymer. The results obtained are agreement with most results of extraction of bioproducts by ATPS [1, 10, 13, 34]

3.3 Effect of molecular weight of the polymer
In the second system, it is used the lower molecular weight of PEG (PEG1500), Where it is seen that the recovery per cent and hence partition coefficient became larger compare with the 1st system with PEG6000 and that clear from comparison between Table 1 and 2 and from Figures 7–10. However, the results are slightly affected by PEG molecular weight and this is agreement with results of Mujahid et al. 2016 [14] and Oliveira el al. 2002 [35]. The reason of this phenomenon is that an increase in molecular weight of polymer results in an increase in the chain length of the polymer and the exclusion effect, which lead to the reduction in the free volume. So, it is preferred to use the low molecular weight of the polymer. Decreasing the molecular weight of phase forming polymers also decreases interfacial tension [36] and has a hydrophilic end group with shorter polymer chains that reduces the hydrophobicity [37]. However, it is not recommended to use very low molecular weight of polymer in phase forming because the exclusion effect decreases and as a result the polymer can induce all the particles including undesired molecules to the polymer phase [10].

3.4 Effect of \((\text{NH}_4)_2\text{SO}_4\) Salt concentration
When the concentration of salt increased the recovery per cent and partition coefficient decreased as shown in the Figures 7 and 8, and 9 and 10 respectively. Also, it can be seen the salt is more effective than the dextran polymer as well as it is less cost but more environmental effect. When the concentration of PEG increased the concentration of salt required decreased. These results are disagreement with partitioning of proteins and enzymes using PEG/salt that obtained by Arafat et al 2013 [13], Zhao et al 2012 [38], and Karkas et al 2012 [39].

3.5 Effect of pH
The pH of the system influences the partitioning because it may adjust the charge of the solute or it may adjust the ratio of the charged molecules. Moreover, the change in pH influences the phase composition which in turn influences the partitioning behavior. It is clear from Figures 7 and 8 and 9 and 10 that the recovery per cent and frequently partition coefficient is decreased with increasing of pH. These results are convergent with pH effect on lipase extraction using ATPS [40]. These results are close to the partitioning of pectinase of \textit{Polyporus squamosus} [41] and with Partition of trypsin in aqueous two-phase systems [35] in the preferred pH of 6 – 7, but disagreement with partition of tannery wastewater proteins [42] where the recovery efficiency increased with increased pH.
Figure 5. Partition Coeff. of Prodigiosin with temp at different Concen. of dextran, pH 6 and at constant wt. fraction of PEG6000 (0.22).

Figure 6: Partition Coeff. of Prodigiosin with wt.% of Dextran at different temp, pH 6 and at constant wt. fraction of PEG6000 (0.13).

Figure 7. % recovery of Prodigiosin with pH at different wt. % of PEG1500, Temp = 20°C and at constant concn. of salt (15%).

Figure 8. % recovery of Prodigiosin with pH at different wt. % of PEG1500, Temp = 20°C and at constant concn. of salt (21%).
Figure 9. Partition Coeff. of Prodigiosin with pH at different concen. of PEG1500, Temp = 20°C and at constant concen. of salt (15%).

Figure 10. Partition Coeff. of Prodigiosin with pH at different concen. of PEG1500, Temp = 20°C and at constant concen. of salt (24%).

4. Conclusions
From the present work, it can be concluding that the prodigiosin is extracted efficiently by aqueous two liquid system and can be considered an alternative method of the organic solvent extraction particularly when the prodigiosin is used in the medical field. So, the ATPS can be used as a simple, selective, and low cost promising separation technique to purify bioproducts instead of conventional separation methods. The recovery per cent and partition coefficient of the prodigiosin are increased with increasing of PEG concentration, decreased each of temperature, dextran concentration, salt concentration, and pH. The recovery per cent of the prodigiosin exceeds 97% in a single stage when the volumes of the PEG-solution and Salt-solution are equal, that’s mean not furthermore stages are required. Also, the results were better in the PEG1500/(NH₄)₂SO₄ system than the PEG6000/Dextran3000 system.

Further investigation into this aspect of partition is needed, and may lead to ways of making systems more efficient such as using another type of salts like sodium citrate, potassium citrate, and sodium phosphate [43] or adding sodium chloride salt to the ATPS [13, 44].

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