Extraction of Natural Red Colorants From the Fermented Broth of *Penicillium Purpurogenum* Using Aqueous Two-Phase Polymer Systems

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Safety concerns related to the increasing and widespread application of synthetic coloring agents have increased the demand for natural colorants. Fungi have been employed in the production of novel and safer colorants. In order to obtain the colorants from fermented broth, suitable extraction systems must be developed. Aqueous two-phase polymer systems (ATPPS) offer a favorable chemical environment and provide a promising alternative for extracting and solubilizing these molecules. The aim of this study was to investigate the partitioning of red colorants from the fermented broth of Penicillium purpurogenum using an ATPPS composed of poly(ethylene glycol) (PEG) and sodium polyacrylate (NaPA). Red colorants partitioned preferentially to the top (PEG-rich phase). In systems composed of PEG 6,000 g/mol/NaPA 8,000 g/mol, optimum colorant partition coefficient (K_C) was obtained in the presence of NaCl 0.1 M ($K_C = 10.30$) while the PEG 10,000 g/mol/NaPA 8,000 g/mol system in the presence of Na_2SO_4 0.5 M showed the highest K_C (14.78). For both polymers, the mass balance (%MB) and yield in the PEG phase (%η_{TOP}) were close to 100 and 79%, respectively. The protein selectivity in all conditions evaluated ranged from 2.0–3.0, which shows a suitable separation of the red colorants and proteins present in the fermented broth. The results suggest that the partitioning of the red colorants is dependent on both the PEG molecular size and salt type. Furthermore, the results obtained support the potential application of ATPPS as the first step of a purification process to recover colorants from fermented broth of microorganisms. © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 31:1295–1304, 2015
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Introduction

The use of natural colorants is a growing tendency all over the word due mainly to the concern of consumers over the use of synthetic compounds in several industries, such as pharmaceutical, food, textile and cosmetic.[1] Furthermore, natural colorants can not only increase the marketing of products, but also provide to the industrial products biological activities like antioxidant and anticancer activity.[2] Natural colorants can be extracted from plants, insect tissues and microorganisms.[3] Among the latter, filamentous fungi are being investigated as readily available sources of chemically diverse colorants.[4]

Fungi can secure the production of the metabolite concerned, followed by controlled conditions, without the influence of free external factors and seasonal raw material supply and capable of minimizing batch-to-batch variations.[5] Natural colorants produced by fungus *Monascus* are the most studied class. However, *Penicillium purpurogenum* has been reported as a new producer of colorants with polyketides structures.[5,6] The structures of polyketides are known to have unlocalized negative charge ð-electrons, as they often contain polyunsaturated functionality, i.e., ring systems, one or more carbonyl groups, carboxylic acid, and ester or amide functional groups exhibiting UV–vis spectra characteristic.[7,8] N-glutarylmonascorubramine and N-glutarylrubropunctamine are extracellular colorant extracts obtained from the liquid medium of *P. purpurogenum* by Mapari et al.[9]

Efforts have been made in order to reduce the costs of colorants produced by microbial fermentation compared to

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synthetic ones or colorants extracted from other natural sources.[4] Besides the production stage, researches involving the extraction steps of new colorants using different methods are of great interest because traditional colorant extraction methods have many problems, such as long process time, low selectivity and extraction efficiency.[10] Over the last few years, the use of liquid–liquid extraction methods in aqueous systems aiming at molecule extraction (biologically active or not) has been increasing. In general, liquid–liquid extraction consists of a transfer process from a liquid phase solute to another immiscible liquid phase in contact with the first one. Therefore, the addition of hydrophilic polymers, which form the so-called aqueous two-phase polymer systems (ATPPS), is proposed, thus leading to the establishment of two immiscible aqueous phases.[11]

ATPPS separate into two aqueous phases when two polymers (e.g. PEG and dextran) or one polymer and one appropriate salt (e.g. phosphate or citrate) are mixed together and the thermodynamic conditions are established. High water concentration (between 80-90%) in such systems favors the stability of biologically active molecules during the process of separation, in comparison to two-phase systems in organic solvents.[12,13]

The polyethylene glycol (PEG)-sodium polyacrylate polymeric system (PEG/NaPA) forms two phases only in some conditions, such as when the NaPA molecules have to be completely dissociated ($pH > 7.0$).[14] Moreover, a sufficient amount of salt in the system is needed in order to ease highly charged polyelectrolyte (NaPA) compartmentalization in one of the phases. In general, this system presents some advantages: low viscosity, easy scale-up, well-defined clear phases, chance of recyclability, and the possibility of reducing the number of downstream process steps.[14,15]

The NaPA main chain is hydrophobic and its solubility is characterized by the presence of carboxylic groups (anions) in lateral polymer groups. These groups are strongly hydrophilic when charged ($pH > 5.0$), and for this reason, PEG and NaPA separate into two different phases.[14]

Several variables can influence the formation and partition of a solute in ATPPS. Variables include characteristics of the polymers that form the phases (molar mass and concentration), characteristics of the additives (type and composition), pH, temperature, solute characteristics, among others.[16] The biotechnological application of ATPPS is influenced by the ability to develop models and correlations that make it possible to understand how the interaction between the physical and chemical properties of biomolecules and the phases of polymers and/or salts in the partition of these systems occurs.[17] Since ATPS is a technique of quick and easy application several studies to purify and separate contaminants of the target molecules has been performed. Among the molecules studied employing ATPS formed by two polymers or a polymer and a salt can be cited: oxidase ascorbate,[18] proteases,[19] bromelain,[20] laccases,[21] betalain,[22] lutein,[23] polyketides,[24] collagenase,[25] and antibiotics.[26]

In a previous work of our research group, red colorants from fermented broth was successfully recovered using aqueous two-phase systems (ATPS) based on ionic liquids achieving partition coefficient of 24.4 and protein removal of 60.7%.[27] These results showed the potential application of ATPS to recovery colorants from fermented broth and supports further research evaluating other ATPS systems. Thus, aiming at exploring the applicability of ATPPS composed by PEG and NaPA, this work studied the partition of natural colorants from the fermented broth of *P. purpurogenum* DPUA 1275 employing this technique as the first step for purification. To further the understanding about the systems, binodal curves of the evaluated systems were also determined. These systems have potential for use in the extraction of molecules with hydrophilic characteristics, and may also provide an optimal environment for the solubilization of these molecules, include the possibility of concentration the target molecule in one of the phases.

Material and Methods

Materials

PEG polymers with molar mass 1,000 g/mol (PEG1000) and 6,000 g/mol (PEG6000) were purchased from Merck® (New Jersey, NY). PEG with molar mass 10,000 g/mol (PEG10000), sodium polyacrylate (NaPA) with molar mass 8,000 g/mol (NaPA8000) at 45% wt/v and the BCA protein assay kit were acquired from Sigma-Aldrich® (St. Louis, MO). All solutions were prepared in McIlvaine's buffer, pH 8.0, consisting of disodium phosphate and citric acid in water purified by a Millipore Milli-Q system (Bedford, MA). The used glassware was washed in 50:50 ethanol: 1 M sodium hydroxide bath, followed by a 1 M nitric acid bath, rinsed copiously with Milli-Q water, and finally dried in an oven at 70°C for 1 h. All the other reagents were of analytical grade.

Microorganism maintenance

Penicillium purpurogenum DPUA 1275 was provided by the Culture Collection of the Federal University of Amazonas (DPUA), Amazonas, Brazil. The stock culture was maintained on Czapeck Yeast Extract Agar (CYA) tubes. The tubes and plates were inoculated at 30°C for 7 days and subsequently stored at 4°C.

Culture medium and inoculum preparation

CYA was used as the inoculum medium, which had the following composition (g/L in deionized water): K_2HPO_4 (1.0); yeast extract (5.0); sucrose (30.0); agar (15.0); and 10 mL/L of concentrated Czapeck [$NaNO_3$ (30.0 g), KCl (5.0 g), $MgSO_4.7H_2O$ (5.0 g), $FeSO_4.7H_2O$ (0.1 g) and distilled water to 100 mL].[28] The components of the production medium was similar to the one used for the inoculums, except for the absence of agar. Regarding the concentration of sucrose and yeast extract, it was 48.50 and 11.80 g/L, respectively.[8]

For production experiments, 125 mL-Erlenmeyer flasks containing 25 mL of required medium were inoculated with 5 mycelial agar discs punched out with a sterilized self-designed cutter (8 mm diameter) from a stock culture grown at CYA medium in Petri plates for 7 days at 30°C. At the end of the submerged culture (336 h), the fermented broth was filtered as follows. First, employing a filter paper – 80 g/m^2 (Whatman, UK) and later using a 0.45 μm filter acquired from Millipore (Bedford, MA). The supernatant was frozen in an ultra-freezer at −70°C to be used in the partitioning studies. All media were autoclaved at 121°C for 15 min.

Table 1. Yield Obtained in the PEG Phase ($\%\eta_{TOP}$), mass balance (%MB) and the volumetric ratio (R) for the red colorants partition in the PEG 1000, 6000 and 10000/NaPA 8000 polymeric system with 12 (% w/w) of each one of the polymers and in the presence of different salt types (NaCl or Na_2SO_4) and concentrations. The experiments were performed at 25°C

PEG (g/mol)	Salts (M)	$\%\eta_{TOP}$	$\%MB$	R
1,000	NaCl 0.1	87 ± 5	105 ± 1	0.85 ± 0.07
	NaCl 0.5	93 ± 6	105 ± 6	0.87 ± 0.01
	Na_2SO_4 0.1	86 ± 2	96 ± 1	0.84 ± 0.07
	Na_2SO_4 0.5	85 ± 2	96 ± 2	0.84 ± 0.03
6,000	NaCl 0.1	92 ± 3	103 ± 4	0.73 ± 0.01
	NaCl 0.5	90 ± 3	99 ± 2	0.79 ± 0.06
	Na_2SO_4 0.1	98 ± 0	109 ± 0	0.74 ± 0.07
	Na_2SO_4 0.5	98 ± 2	108 ± 1	0.75 ± 0.00
10,000	NaCl 0.1	90 ± 2	107 ± 3	0.76 ± 0.02
	NaCl 0.5	88 ± 1	104 ± 1	0.79 ± 0.06
	Na_2SO_4 0.1	92 ± 1	107 ± 2	0.66 ± 0.02
	Na_2SO_4 0.5	94 ± 3	104 ± 3	0.76 ± 0.02

Table 2. Yield in the PEG Phase ($\%\eta_{TOP}$), mass balance (MB%), and the volumetric ratio (R) for red colorant partition in the PEG 6000/NaPA 8000 g/mol polymeric system in different concentrations of both polymers and in the presence of different salt types (NaCl or Na_2SO_4) and concentrations. The experiments were performed at 25°C

Experiments (composition—w/w)	Salts (M)	$\%\eta_{TOP}$	$\%MB$	R
1 (16% PEG/ 6% NaPA)	NaCl 0.1	78 ± 3	84 ± 3	2.21 ± 0.00
	NaCl 0.5	78 ± 4	87 ± 1	2.27 ± 0.73
	Na_2SO_4 0.1	65 ± 3	90 ± 4	2.31 ± 0.19
	Na_2SO_4 0.5	79 ± 3	87 ± 6	2.14 ± 0.13
2 (14% PEG/ 8% NaPA)	NaCl 0.1	75 ± 2	82 ± 2	1.60 ± 0.12
	NaCl 0.5	72 ± 4	83 ± 1	1.44 ± 0.59
	Na_2SO_4 0.1	80 ± 3	88 ± 3	1.93 ± 0.18
	Na_2SO_4 0.5	75 ± 7	85 ± 3	1.40 ± 0.15
3 (12% PEG/ 10% NaPA)	NaCl 0.1	79 ± 3	87 ± 3	1.05 ± 0.12
	NaCl 0.5	71 ± 5	81 ± 2	0.99 ± 0.16
	Na_2SO_4 0.1	74 ± 2	86 ± 1	0.92 ± 0.30
	Na_2SO_4 0.5	80 ± 2	94 ± 3	0.96 ± 0.00
4 (10% PEG/ 12% NaPA)	NaCl 0.1	78 ± 2	98 ± 3	0.82 ± 0.10
	NaCl 0.5	77 ± 3	90 ± 3	0.75 ± 0.06
	Na_2SO_4 0.1	68 ± 3	81 ± 1	0.74 ± 0.21
	Na_2SO_4 0.5	77 ± 4	92 ± 2	0.70 ± 0.18
5 (8% PEG/ 14% NaPA)	NaCl 0.1	72 ± 1	83 ± 2	0.58 ± 0.05
	NaCl 0.5	71 ± 1	82 ± 2	0.60 ± 0.01
	Na_2SO_4 0.1	63 ± 2	82 ± 2	0.52 ± 0.12
	Na_2SO_4 0.5	78 ± 5	93 ± 8	0.54 ± 0.12

Aqueous two-phase polymer systems

Mapping the Binodal Curves of PEG/NaPA System. Before beginning the partitions studies, the binodal curves were obtained for the systems according to the methodology described by Johansson et al.[29]. Thus, for each phase diagram, stock solutions of PEG (30% w/v), NaPA (45% wt/v) and salts (NaCl and Na_2SO_4 at 0.1 and 0.5 M) were prepared. The stock solutions were mixed in graduated glass tubes (15 mL) with known concentration of each one of the components and centrifuged at 719 x*g* for 1 min. Then the tubes were visually analyzed regarding phase formation. It is possible to get the binodal curve by using titration with three stock solutions. Usually, solutions turn turbid when they are in one of the sides of the binodal curve. However, close to the binodal curve, the lack of turbidity after the mixture is not a good indication of a phase system. So, the systems need to be centrifuged. The experiments were performed at 25°C with an uncertainty of ±0.1°C.

Table 3. Yield Obtained in the PEG Phase ($\%\eta_{TOP}$), mass balance (%MB) and the volumetric ratio (R) for red colorant partition in the PEG 10000/NaPA 8000 g/mol polymeric system in different concentrations of both polymers and in the presence of different concentrations of Na_2SO_4. The experiments were performed at 25°C

Experiment (Composition—w/w)	Na_2SO_4 (M)	$\%\eta_{TOP}$	$\%MB$	R
1 (12% PEG/ 6% NaPA)	0.1	82 ± 5	93 ± 4	1.85 ± 0.11
	0.5	83 ± 2	95 ± 3	1.21 ± 0.13
2 (10% PEG/ 8% NaPA)	0.1	75 ± 4	89 ± 6	1.09 ± 0.12
	0.5	84 ± 0	95 ± 2	0.93 ± 0.08
3 (8% PEG/ 10% NaPA)	0.1	80 ± 5	92 ± 2	0.80 ± 0.00
	0.5	79 ± 1	93 ± 3	0.54 ± 0.02
4 (6% PEG/ 12% NaPA)	0.1	80 ± 5	91 ± 5	0.48 ± 0.14
	0.5	75 ± 2	95 ± 2	0.33 ± 0.08
5 (4% PEG/ 14% NaPA)	0.1	72 ± 4	91 ± 6	0.29 ± 0.00
	0.5	71 ± 1	92 ± 4	0.21 ± 0.07

Partitioning in Aqueous Two-Phase Polymer Systems. ATPPS were prepared in 15 mL graduated glass tubes by adding sodium polyacrylate (NaPA), PEG, saline solution (Na_2SO_4 or NaCl), McIlvaine buffer (pH 8.0) and the fermented broth, resulting in a 10 g total mass system. The system components were added by weighing the appropriate amount in an analytical balance (Shimadzu, model AUX320) and further homogenized in an orbital shaker (Barnstead/Thermolyne, model 400110) at 8 rpm for 20 min at room temperature. It was added 2 g of fermented broth which corresponds to a absorbance of 2.00 Units of Absorbance (UA_{490nm}) in all experiments to minimize the errors. The system was transferred to a controlled temperature bath model 521/2DE (New Ethics, Vargem Grande Paulista, SP, Brazil), previously adjusted at 25°C and kept in rest for 20 min for the separation and balance of the phases. After resting, the samples of the top and bottom phases were carefully collected using Pasteur pipettes. Each partition experiment was made in triplicate and the respective standard deviations and confidence intervals calculated.

Initially experiments were carried out with PEG1000, PEG6000 and PEG10000 and NaPA8000 with 12% (w/w) of each polymer and NaCl and Na_2SO_4 salts, both at 0.1 and 0.5 M concentrations (Table 1). The second set of experiments was made with PEG6000/NaPA8000 and both salts at two concentrations (Table 2) and with PEG10000/NaPA8000/Na_2SO_4 at both concentrations (Table 3). The experimental conditions were selected according to the binodal curve for the respective polymer (Figure 1). All the partitioning experiments were performed at 25°C with an uncertainty of ±0.1°C.

Analytical methods

Red Colorants Production Analysis. The concentration of red colorants was analyzed by measuring the corresponding colorant absorbance.[30] To determine the wavelength at maximum absorption of red colorants, the fermentation broth (pH 8.0) was scanned in the wavelength range between 220–600 nm in 1 cm cuvette at room temperature through UV-spectroscopy (Spectramax, model Plus 384) (Figure 1 from Supporting information). The fermented broth was also analyzed by liquid chromatography aiming to show the existence of distinct colorants (Figure 2 from Supporting information). As can be seen at Figure 2 from Supporting information, it was obtained three fractions of colorants, according the color viewed they were classified as yellow, orange, and red colorant. The fraction containing the red

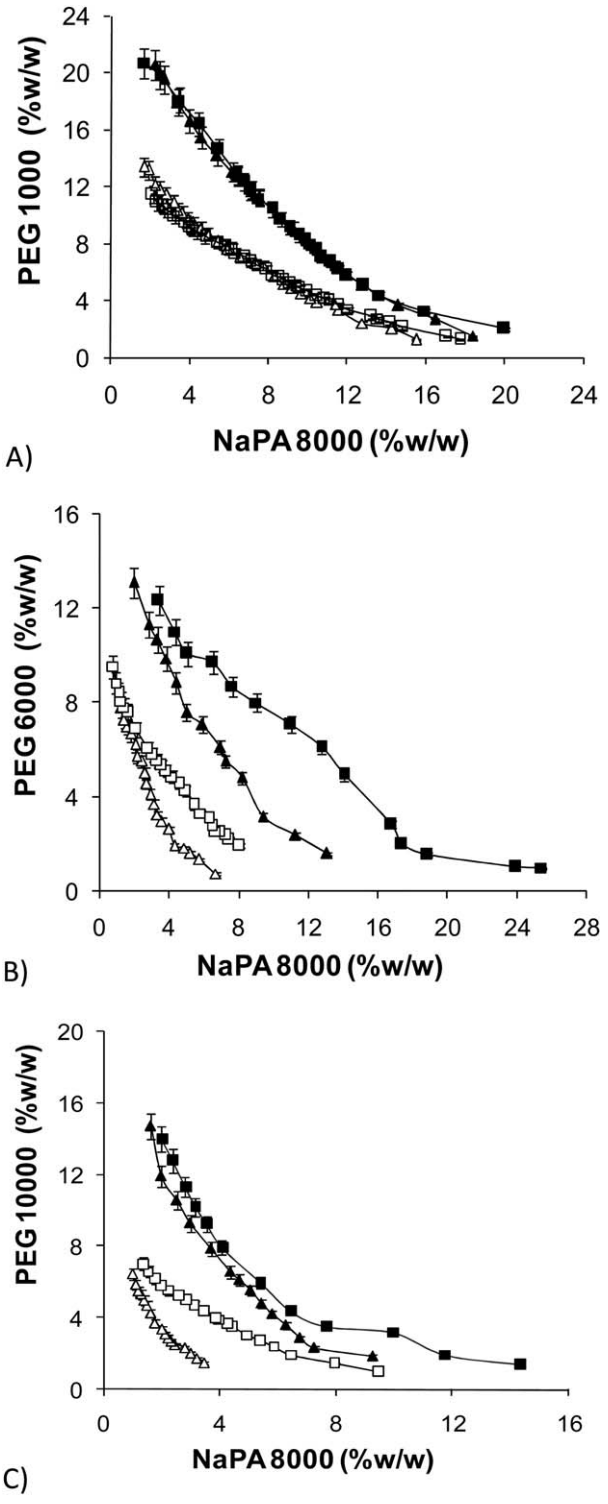

Figure 1. Binodal curve of the PEG 1,000 g/mol (A), 6,000 g/mol (B) and 10,000 g/mol (C) and NaPa 8,000 g/mol system in the presence of NaCl 0.1 (■), NaCl 0.5 (□), Na_2SO_4 0.1 (▲) and Na_2SO_4 0.5 M (Δ).

The system is monophasic below the binodal curves and biphasic above them. The error bars correspond to a 95% confidence interval in the obtained values. The experiments were performed at 25°C.

colorant was scanned, the maximum absorbance peak for these compounds was 490 nm, which is agreement with the results obtained before and the maximum absorbance for red colorants cited in the literature.[3,8,27,30] In this way, the red colorants in each phase were estimated by spectrophotometric analysis by reading the absorbance at 490 nm, which is a methodology characterized to determine colorants, and the results were expressed in terms of Units of Absorbance/mL of fermented broth (UA_{490nm}). To remove the polymers influence in the analysis, a blank essay where the fermented broth was replaced by deionized water was prepared for each system and condition studied. The colorants quantification was performed in triplicate being the final absorbance results reported as the average of the three independent assays performed with the respective standard deviations. Both the scanning and the measurements was carried out through UV-spectroscopy, using a Molecular Devices Spectramax 384 Plus | UV–Vis Microplate Reader.

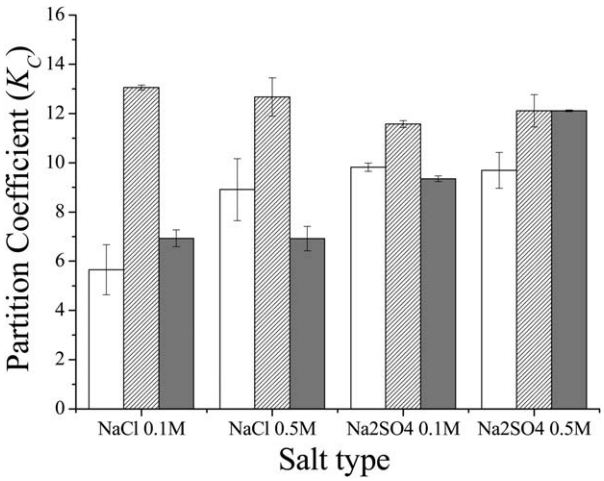

Figure 2. Partition coefficient (K_C) of the system formed by different molar masses of PEG/NaPA 8,000 g/mol in pH 8.0 McIlvaine buffer in the presence of the salts: NaCl 0.1, NaCl 0.5, Na_2SO_4 0.1, and Na_2SO_4 0.5 M. PEG 1,000 g/mol (white bars), 6,000 g/mol (mixed bars) and 10,000 g/mol (gray bars).

Each polymer was used in the concentration of 12% (w/w). The error bars correspond to a 95% confidence interval in the obtained values. The experiments were performed at 25°C.

Total Protein Concentration Determination. Along with the colorants, the *P. purpurogenum* DPUA 1275 also produces proteins in a significant amount.[27] Protein determination was made using the bicinchoninic acid method (BCA),[31] according the methodology described in the kit which was purchased from Sigma-Aldrich®.

Extraction analysis

The extraction was analyzed in terms of partition coefficient of colorants (K_C), recovery in the top phase ($\%\eta_{TOP}$), mass balance (%MB) and volumetric ratio (R), which were determined accordingly to the following equations:

$$K_C = \frac{Abs_{TOP}}{Abs_{BOT}} \quad (1)$$

$$\%\eta_{TOP} = \frac{Abs_{TOP} \times V_{TOP}}{Abs_{INI} \times V_{INI}} \times 100\% \quad (2)$$

$$\%MB = \frac{Abs_{TOP} \times V_{TOP} + Abs_{BOT} \times V_{BOT}}{Abs_{INI} \times V_{INI}} \times 100\% \quad (3)$$

$$R = \frac{V_{TOP}}{V_{BOT}} \quad (4)$$

where Abs_{TOP}, Abs_{BOT}, and Abs_{INI} are the colorants absorbance at 490 nm in the top (PEG—rich phase), bottom

(NaPA—rich phase) and initial, respectively. In this work, the absorbance measurements were used due to the lack of knowledge about the colorants chemical structure. V_{INI} represents the initial volume while V_{TOP} and V_{BOT} represent the volume in the top and bottom phases, respectively.

Selectivity of red colorants separation from the remaining proteins present in the fermented broth (*S*e) was also calculated according Eq. (5).

$$\mathbf{Se} = \frac{\boldsymbol{K_C}}{\boldsymbol{K_P}} = \frac{\frac{Abs_{TOP}}{Abs_{BOT}}}{\frac{P_{TOP}}{P_{BOT}}} \quad (5)$$

where K_P represents the proteins partition coefficient. P_{TOP} and P_{BOT} represent the protein concentration in the top and bottom phases of the systems.

Statistics

The experiments were performed in triplicate and the values obtained are presented as weighted mean, with the respective standard deviations. The significance limit for all statistical analysis was $\alpha = 0.05$, thus resulting in a 95% confidence interval.

Results and Discussion

Binodal curves for the PEG/sodium polyacrylate (NaPA) systems

The mapping of a diagram depicting adequate conditions for the extraction process, such as temperature, pH, and the molar mass of the polymers, is important before beginning work with a new system.[32] Therefore, the binodal curves of the systems composed of PEG and sodium polyacrylate (NaPA) in the presence of NaCl or Na_2SO_4 salts at different concentrations (0.1 and 0.5 M) were determined and are presented in Figure 1. Because the PEG/NaPA systems require a sufficient amount of salt to compartmentalize NaPA through one of the phases,[33] NaCl or Na_2SO_4 were added to the systems. These salts were chosen because they are more frequently used in the application of ATPS.

From Figure 1A, it can be seen that an increase in the concentration of both salts from 0.1 to 0.5 M, shifted the binodal curve to the water-rich region, leading to an increase in the two-phase region. This displacement of the binodal curve is dependent on saturation caused by the salt effect. The salt decreases the entropy force of the polyelectrolyte compartmentalization. Increased addition of salt ions to the system results in a stronger saturation effect. A saturation effect in terms of decreased phase region can occur when the salt concentration is high enough for the polyelectrolyte to act effectively as an uncharged polymer.[14]

For PEG1000 (Figure 1A), there was no significant difference between the salts when working with equal concentrations and therefore, the binodal curves for the same concentration of the salts overlapped. Contrary to this result, the binodal curves obtained for PEG6000 and PEG10000 (Figures 1B and 2C, respectively), showed different curves for NaCl and Na_2SO_4, possibly owing to the hydrophilic force of the SO_4^{2-} ions being relatively higher than that of the Cl^- ions.[14]

The binodal curves for PEG6000 (Figure 1B) at concentrations below 2% (w/w) NaPA8000 at 0.5 M concentration for one of the salts, overlapped. This behavior was not observed with PEG10000 under any of the conditions evaluated. However, for both systems composed of PEG6000 and PEG10000, Na_2SO_4 at 0.5 M shifted the binodal curve to a higher aqueous two-phase region than that observed for systems without Na_2SO_4.

Overall, for all the studied PEG molar masses, an increase in the NaCl and Na_2SO_4 concentration dislocated the binodal curve and caused a difference in the curves at varying concentrations of the same salt. As reported in the literature, the effect of including salts in the ATPPS varies based on the salt and the system itself. Although the salts are distributed almost equally between the phases, there are small differences in the partition coefficients of the different salts. This means that different ions have different affinities for the phases and therefore, create a difference in the electrical potential between them.[14] Furthermore, even though both Cl^- and SO_4^{2-} are cosmotropic anions (known as water-structure-making ions),[34] the presence of two Na^+ ions may have influenced the binodal curve.

Johansson et al.[14] studied the effect of the salt concentration in PEG/NaPA systems. The authors observed a decrease in the entropy as a result of the addition of salt to the system, and the strength of the effect depended on the ionic strength of the ions added. Furthermore, according to the authors, this effect is due to the high entropy gain of a system, considering that numerous contra-ions of the polyelectrolyte are distributed throughout the system. They also reported that a high salt concentration and polymer molecular weight made an increase in the two-phase region possible. The hydrophobic divalent anions promoted a superior increase in the aqueous two-phase region of the PEG-rich phase than in that obtained with the Na_2SO_4, a hydrophilic anion. As previously mentioned, the presence of salt in the ATPPS promoted an increase in the two-region phase, which is in agreement with the literature. Therefore, from the results reported above, in the experimental conditions evaluated for the partitioning studies, it was possible to ensure that the systems would be in two macroscopic phases.

Red colorants partitioning in PEG/NaPA systems

Natural colorants including fungal polyketide pigments are (in most cases) a mixture of several components.[6] Strains of the species belonging to *Penicillium* are known to produce copious amounts of yellow, orange, and red pigments on solid and liquid media or both.[9] Therefore, there are yellow, orange, and red colorants in the fermented broth of the *P. purpurogenum*. Because the red colorants have a higher potential for application in the food industry,[27] it was chosen for evaluation in this study. Although the chemical structure of these three colorants are not yet known, they probably have similar chemical structures since various colorants are produced by microorganism following the same biochemical route and differ only in their aliphatic side chains.[9] Therefore, it would be difficult to achieve their selective separation using the ATPPS, which is a technique with a low purification resolution. In all the conditions evaluated, the selectivity among the red, yellow, and orange colorants was around 1.00 demonstrating their similar affinity for the top phase (data not showed). Therefore, we focused our studies on partitioning the red colorants and determining their protein selectivity. The presence of other colorants did not affect the red colorants' migration.

Red Colorants Partitioning Varying the Type of PEG in Different Salts. We sought to directly evaluate the influence of the salts on PEGs of different molar masses. Therefore, partition studies of the extraction of red colorants produced by *P. purpurogenum* were carried out using PEG1000, PEG6000, or PEG10000 and NaPA8000 with each polymer at 12% (w/w). In addition, the NaCl or Na_2SO_4 salts were used at two concentrations, 0.1 and 0.5 M. The strategy of studying the protein selectivity was used to determine if the ATPPS formed by PEG and NaPA was able to partially separate or concentrate the proteins that are contaminants present in the fermented broth in relation to the red colorants. The results of the partition coefficient (K_C) are presented in Figure 2.

As can be seen, the PEG1000 and NaCl 0.1 M produced the lowest K_C value (5.65). The system formed by PEG6000 achieved the highest K_C compared to the other PEG molar masses evaluated, independent of the salt used. In the condition with the PEG6000 and NaCl at 0.1 and 0.5 M, the values of K_C obtained were 13.06 and 12.67, respectively.

For the PEG10000, contrary to the results obtained with PEG6000, the NaCl resulted in the lowest K_C values. Comparing the K_C values obtained with NaCl 0.1 M, the values achieved were about 45% more inferior for PEG10000 than they were for PEG6000. This result shows the strength of the interaction between PEG and the salt in the red colorants partition. For PEG10000, Na_2SO_4 had an effect that was superior to that of the NaCl in the partition, and the highest concentration of this salt promoted higher K_C values. This increase was close to 75% for NaCl at 0.1 and 0.5 M. The best partition coefficient results were achieved with NaCl for PEG6000 and with Na_2SO_4 0.5 M for PEG10000. Therefore, the high-molar mass polymer promoted a superior interaction between the red colorants and the polymer chain.

Table 1 presents the %MB, volumetric ratio (R), and yield in the PEG phase ($\%\eta_{TOP}$).

The $\%MB$ and $\%\eta_{TOP}$ values obtained were close to 100 and 90%, respectively, showing the good stability of the red colorants under the evaluated conditions, and that the target biomolecule is strongly attracted to the PEG phase in the studied system. These results are in agreement with those of Santos-Ebinuma et al.,[8] who evaluated the effect of PEG and NaPA on the stability of red colorants and found that in the presence of both polymers, the red colorants maintained their color intensity.

In all the experiments, the R values were lower than 1.00. It is known that in a system where both polymers have the same concentration, as was the case in these experiments, they are mutually repellant. These results demonstrated that besides extracting the red colorants in the PEG phase, the system also concentrated them.

In addition to the K_C, $\%MB$, and $\%\eta_{TOP}$, the protein selectivity was also determined. Specifically we determined how much of the proteins present in the fermented broth were separated from the red colorants during this initial extraction stage. The results of selectivity analysis are presented in Figure 3.

The proteins preferably partitioned to the PEG phase with a K_p close to 4.00 for all conditions. Therefore, the best way to evaluate the protein partitioning relative to the red colorants is by determining the selectivity. In terms of selectivity, the red colorants showed a higher preference for the PEG phase relative to the proteins present in the fermented broth. This preference can be explained by difference in the properties of the red colorants and contaminant proteins based on the distinct chemical structures of these compounds. The selectivity was higher for the PEG6000 and NaCl 0.1 M ($Se = 3.05$). For the same salt, this value was 134 and 90% superior in relation to PEG1000 and PEG10000, respectively.

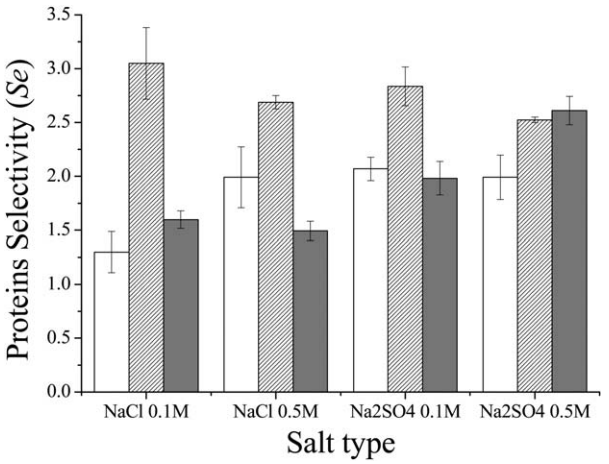

Figure 3. Protein selectivity (*Se*) in the system formed by different PEG molar masses and in the presence of NaPA 8,000 g/mol in pH 8.0 McIlvaine buffer in the presence of the salts: NaCl 0.1 and 0.5, Na_2SO_4 0.1 and 0.5 M.

PEG 1,000 g/mol (white bars), 6,000 g/mol (mixed bars) and 10,000 g/mol (gray bars). Each polymer was used in the concentration of 12% (w/w). The error bars correspond to a 95% confidence interval in the obtained values. The experiments were performed at 25°C.

For the other evaluated salts, PEG6000 also showed the best selectivity varying between 2.52 and 2.84. For the other PEG molar masses and salt concentrations, the *Se* was around 2.00, with the exception of PEG10000 and Na_2SO_4 0.5 M that presented a value that was a little higher ($Se = 2.61$). Although the proteins preferably migrated to the PEG phase, the proteins that are positively charged can strongly interact with NaPA. Therefore, this system can be used as the first step for separating the red colorants from these metabolites present in the fermented broth.

Johansson et al.[14] studied the partitioning of hemoglobin, lysozyme, and glucose-6-phosphate dehydrogenase (G6PDH) proteins using the PEG/NaPA system in the presence of NaCl and Na_2SO_4 salts. Hemoglobin, when extracted at pH 9.0, preferably partitioned to the PEG phase similar to the lysozyme (positively charged), while the G6PDH (positively charged) partitioned to the NaPA phase. Similar results were achieved by Pereira et al.[26] who studied the partitioning of clavulanic acid in the PEG/NaPA system; i.e., the proteins present in the fermented broth of *Streptomyces clavuligerus* preferentially migrated to the PEG-rich phase.

The protein selectivity results coupled with those of the K_C, $\%MB$, and $\%\eta_{TOP}$ show that the PEG6000 with NaCl 0.1 M and PEG10000 with Na_2SO_4 0.5 M had a higher potential for partitioning the red colorants present in the *P. purpurogenum* DPUA 1275 fermented broth. However, these PEG molar masses were evaluated under new experimental conditions using both salts with PEG6000 and Na_2SO_4 with PEG10000.

Partitioning of Red Colorants in PEG6000/NaPA8000 Systems with Different Salts. The experimental conditions were selected based on the binodal curve of PEG6000

(Figure 1B). The mixture points were selected to ensure that all the conditions tested would be within the biphasic region of the phase diagram and to evaluate how all the initial polymeric concentrations can influence the partitioning the of biomolecule. Table 2 presents the $\%MB$, R, and $\%\eta_{TOP}$.

Both the yield values in the $\%\eta_{TOP}$ and the $\%MB$ values were similar for the evaluated conditions. For the $\%\eta_{TOP}$ the lowest value (63.31%) was obtained in experiment 5 (8% PEG/14% NaPA) with Na_2SO_4 0.1 M, and the other extraction conditions were $\%MB = 82.09\%$ and $R = 0.52$. For both parameters $\%\eta_{TOP}$ and $\%MB$, experiment 4 (10% PEG/12% NaPA) generated the highest values, although with NaCl 0.1 M $\%\eta_{TOP} = 88.50\%$ and $\%MB = 98.08\%$. For this condition, the R value was 0.82, indicating the concentration of the target biomolecule in the PEG-rich phase. Overall, Na_2SO_4 0.1 M led to the lowest results of the extraction parameters, comparing across the different experimental conditions.

The PEG/NaPA system induces electroneutral partitioning, and when Na_2SO_4 is included, the colorants can migrate the PEG phase at the same time that the sulfate ion (charge -2) is transferred out from that phase. This change is a thermodynamically favorable process once the salt has a higher repulsion for PEG than the target biomolecule.[33] However, when using Na_2SO_4, inferior K_C results can be obtained because the counter-ion SO_4^{2-} is known to have a strong effect on the PEG salting-out.[14]

It is known that the solubilization of PEG in water is attributed to water molecules forming bonds with numerous or all the molecules around the polyethylene chain.[33] These are hydrogen bonds, which are relatively weak. The salting-out effect occurs when water molecules are preferentially directed to the solvation of salt ions rather than to the polymer molecules.[33]

One main variable analyzed in this study was the partition coefficient, and Figure 4 presents the complete values obtained for this K_C parameter, as well as the associated error.

The salt had a high influence on the partition parameters. For NaCl, the K_C values obtained at 0.1 M were superior to those of the other salts. An increase in NaCl concentration from 0.1 to 0.5 M led to inferior K_C values. However, for Na_2SO_4, the salt influence was contrary to that obtained with the NaCl.

The target biomolecule preferentially partitioned to the PEG phase since the K_C was higher than 1 in several conditions, which can be seen as a contribution from the hydrophobic effect of the partition. Nevertheless, there are also other entropic effects due to the combinatory entropy difference between the phases.[14] In systems with phases that had the same amount of water and polymer concentrations, the phase with the smallest polymer molecule size had the highest partial molar entropy for the partitioned biomolecule. The force associated with the ionic strength can increase the K_C value when there is an increase in the NaPA concentration. Moreover, in the first evaluated condition (16% PEG/6% NaPA) for NaCl 0.1 M, the Kc value obtained was 5.07. Throughout the process of varying the PEG and NaPA concentrations, this parameter increased approximately 2-fold for all salts. Therefore, in experiment 5 (8% PEG/14% NaPA), the K_C value was 10.30.

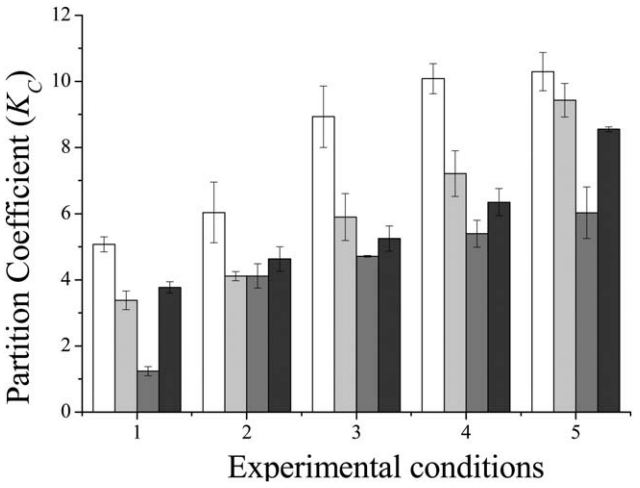

Figure 4. Partition coefficient (K_C) of the PEG 6,000 g/mol/NaPA 8,000 g/mol system in pH 8.0 McIlvaine buffer in the presence of different salt types and concentrations: NaCl 0.1 (white bars), NaCl 0.5 (light gray bars), Na_2SO_4 0.1 (gray bars), and Na_2SO_4 0.5 M (dark gray bars).

The error bars correspond to a 95% confidence interval in the obtained values. The description of each experimental condition (1, 2, 3, 4 and 5) is presented at Table 2. The experiments were performed at 25°C.

According to Da Silva and Loh,[35] the uneven distribution of biomolecules between the two phases of the system results from an intricate and delicate interaction balance between the biomolecule and other species (polymers, buffer, and salt) present in both phases that coexist in balance. These interactions seek to promote a favorable enthalpy that can be obtained in two ways. One way is by the molecular interaction between the biomolecule and the components of each phase, and the other is by the self-energy of the phase. The increase in the NaPA concentration in the system increases the repulsive forces between the colorants and the polymer and, therefore, more free energy is released by the system. Besides enthalpy, the entropic contribution is also related to the partition coefficient, which explains why the red colorants partition to the top phase. It is possible that the red colorants release water molecules when migrating to the top phase, that subsequently interact with the PEG, which favors the system entropy and results in these biomolecules preferring the top phase.

The evaluation of the R in experiments 4 (10% PEG/12% NaPA) and 5 (8% PEG/14% NaPA) showed that the values were <1.00 at all the studied conditions. Therefore, the volume at the top (PEG-rich) was lower than the volume at the bottom (NaPA-rich) was. This observation indicates that the red colorants were not only extracted to the top phase but were also pre-concentrated since its absorbance was higher in a lower volume.

The analysis of the partition coefficients showed an increase in the NaPA concentration and a decrease in the PEG concentration, leading to higher values. This may have occurred because higher concentrations of the charged polymer may have repulsed colorants molecule to the PEG phase, considering that the NaPA is a strongly negative polyelectrolyte at the studied condition and the target biomolecule can be negatively charged. The higher K_C value (10.29) was obtained for the 10% PEG/12% NaPA (experiment 5) and NaCl 0.1 M. At this extraction condition, there was a

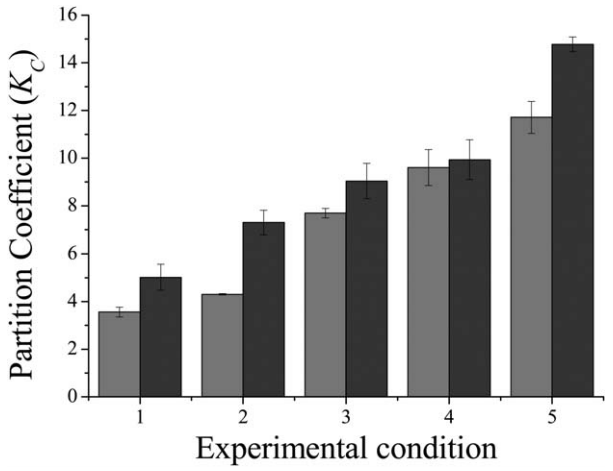

Figure 5. Partition coefficient (K_C) of the PEG 10,000 g/mol/ NaPA 8,000 g/mol system in pH 8.0 McIlvaine buffer in the presence of different salt types and concentrations: Na_2SO_4 0.1 (gray bars), and Na_2SO_4 0.5 M (dark gray bars).

The error bars correspond to a 95% confidence interval in the obtained values. The description of each experimental condition (1, 2, 3, 4, and 5) is presented at Table 3. The experiments were performed at 25°C.

biomolecule concentration in the PEG-rich phase because the $R = 0.58$.

The first PEG and NaPA concentration scanning was carried out using several points in order to analyze the partition coefficient tendency. An increase in this parameter was observed with the increase in the NaPA and decrease in PEG concentrations. Furthermore, for the PEG6000 the NaCl salt was more appropriate for extracting the red colorant from the fermented broth and a small amount of was enough to produce the best result.

Partitioning of Red Colorants in PEG10000/NaPA8000 Systems With Na_2SO_4. For the PEG1000, the Na_2SO_4 salt produced more favorable results than the NaCl, and further experiments were performed under new conditions with this salt only. Table 3 presents the *%MB*, *R*, and $\%\eta_{TOP}$.

In general, higher *%MB* values were achieved with Na_2SO_4 0.5 M that varied from 92 (experiment 5) to 95% (experiment 2). Among the other salt concentrations, the *%MB* varied between 89 and 93%. These results show the good biomolecule stability of NaPA, PEG10000, and the salts. The lowest value obtained for the $\%\eta_{TOP}$ was 71% in experiment 5 (4% PEG/14% NaPA) with Na_2SO_4 0.5 M. However, the highest result was achieved with the same salt concentration in experiment 2 (10% PEG/8% NaPA) with a value of 84%.

Similar to what was obtained with the other PEG molar masses, the increase in the NaPA and decrease in the PEG concentrations increased the K_c values. The best result was obtained in experiment 5 (4% PEG/14% NaPA) with a $K_c = 14.78$ while evaluating with Na_2SO_4 0.5 M. At all the evaluated conditions, Na_2SO_4 0.5 M provided the best results. The *R* between both phases revealed that as from the condition of experiment 3, this parameter was lower than < 1 in all other conditions analyzed, indicating that the PEG-rich phase (top) was more concentrated than the NaPA-rich phase (bottom) was.

For the PEG10000, the results presented in Figure 5 indicate that when the system with a relatively high saline concentration was evaluated, besides the entropic effect, the polymers with high molar masses exhibited salting-out more easily than the smaller sized polymers did. It is worth noting that this is only a supposition when the polymer proportions are not the same when evaluating different PEG molar masses.

Moreover, the decrease in the PEG concentration renders the environment less favorable in terms of entropic force direction. Since the PEG phase becomes more concentrated and hydrophobic, a higher partitioning for this phase could be expected. Nevertheless, the enthalpy effect is partially compensated for by the decrease in the biomolecule partial molar entropy.[14]

The increase in the salt concentration can lead to a series of effects on the PEG/NaPA system charge. Firstly, the NaPA charge is more easily compartmentalized, resulting in higher differences in the NaPA concentration between the phases.[14] Moreover, at high Na_2SO_4 concentrations, the PEG-rich phase tends to become more concentrated, possibly due to the increase in the hydrophobic effect caused by the salt. The entropic force between both phases can be associated with the increase in the polymeric concentration with motive power and, consequently, the red colorants partition.

Esmanhoto et al.[24] studied the extraction of the red colorants rubropunctamin and monascorubramin produced by the submerged culture of *Monascus purpureus* using an ATPPS composed of PEG and phosphate. Different conditions such as the PEG type and concentration, pH, and phosphate concentrations were studied. In this study, the highest partition coefficient values occurred with PEG 6,000 g/mol at 20% and 15% phosphate with a pH varying from 7.0 to 9.0.

Mageste et al.[36] studied the partitioning of a natural cochineal carmine dye in an ATPS prepared with an aqueous polymer solution mixture (PEO 1,500 g/mol) or copolymer (L35) with salt solutions (Na_2SO_4 and Li_2SO_4). The results obtained suggested that the dye partitioning was highly dependent on the nature of the polyelectrolyte and the system pH. The carmine molecules were concentrated in the polymer-rich phase in both studied systems. Moreover, the authors suggest that the enthalpy interaction between the carmine and PEO or the macromolecules of the copolymer L35 probably occurred between the carmine and ethylene oxide units, and this interaction depended heavily on the electrolyte. This salt dependency was attributed to the cation intermediary in the carmine-macromolecule interaction.

Overall, the pattern of the partitioning of a solute depends on the molar mass of the polymer that composes the ATPPS. Usually, if the molar mass of the polymer in the ATPPS increases, the solute concentration decreases in the phase where the polymer is predominant. The main cause of this typical partition behavior is the increase in the macromolecular size, which reduces the contribution of the configurational entropy to The results produced by PEG10000 can be explained by the previously described phenomenon, also called exclusion volume effect, which can be summarized as follows: "the bigger the polymer molar mass is, the smaller the spaces between the molecules in the top phase are and, consequently, they are expelled to another phase". However, for the red colorant produced in the *P. purpurogenum* fermented broth, the force that possibly governs this system is the repulsion between the target biomolecule and NaPA. Furthermore, some sort of interaction occurs between the target biomolecule and the polymeric chain and, therefore, it may

have a higher interaction with the PEG phase and a superior K_C when PEG10000 is used.

The downstream process presents an important challenge to controlling the costs of biomolecule recovery.[37] Therefore, alternative technologies are of great interest for decreasing the costs and improving the purity as well as broadening their application to different fields. The results obtained by Ventura et al.[27] using different liquid ionic-citrate buffer ATPSs were higher than those obtained in this study (partition coefficients of the red colorant, 24.4 ± 2.3 and selectivity S_e, 10.05). However, the recovery of the red colorants in the PEG phase achieved in this study, has potential application because PEG has been used as a solubilizer and stabilizer in the pharmaceutical and biomedical industry.[38] Furthermore, PEG is a polymer that has already been approved for consumption by the US Food and Drug Administration (FDA) because of its nontoxic and biodegradable characteristics.[8,38] Taking into account the results described above, PEG traces can be tolerated in the red colorants, thereby reducing the steps necessary to recover these biomolecules before their application in the pharmaceutical or food industry. However, if it is necessary to separate the red colorants from the PEG, a PEG/salt two-phase system using an appropriate salt such as a phosphate could be performed. In such a system, the red colorants would probably be partitioned to the salt phase and a desalinization process such as dialysis could be performed to remove the salt if necessary. Moreover, because the colorants exhibit a higher affinity for the PEG phase, the superior results can be used to develop new approaches using extractive fermentation aimed at improving the recovery parameters.

Comparing our results with the other reports in the literature, we found that Sheng et al.[39] studied the extractive fermentation of intracellular *Monascus* pigments in a nonionic surfactant micelle aqueous solution. The process involved transferring hydrophobic *Monascus* pigments from a nonionic surfactant to an ionic liquid using a novel hydrophobic ionic liquid–nonionic surfactant–water Winsor microemulsion extraction. This was followed by back-extraction of the ionizable *Monascus* pigments from the ionic liquid by ionic liquid–water two-phase extraction. A yield of nearly 80% was achieved for the complete process of recovery of the *Monascus* pigments. Wang et al.[40] have reported an effective adsorption method for the separation and purification of prodigiosin (bacterial pigment) directly from a culture broth with a high quantitative recovery. The total recovery from this process (83%) was much higher than that obtained with the conventional extraction and silica-gel chromatography process (50%). Celestino et al.[41] studied the extraction of natural colorants from five strains of filamentous fungi using successive partitioning with hexane, ethyl acetate, and butanol. The authors achieved a high purity and obtained the chemical structure of the colorants. Although the liquid–liquid extractions using organic solvents are the techniques most frequently used to recovery natural colorants[7,9,42], it is known that there are some limitations to using organic solvents, particularly for large-scale applications. These limitations include their toxicity, high cost, their impact on the environment, and the fact that organic solvents may lead to irreversible product degradation.[43] Therefore, several technologies have been studied for use in recovery natural colorants from fermented broth; however advances are still necessary to improve these processes and ATPSs are a promising potential technique.

Conclusions

ATPPS efficiently recovered the hydrophilic molecules of the red colorants in the PEG-rich phase of the system ($K_C > 10$) and transferred the hydrophobic contaminants (proteins and other interferents) to the NaPA-rich phase. The best results were achieved with the PEG with a molar mass of 6,000 g/mol using NaCl 0.1 M. Furthermore, the results showed that this processes might be useful for the concentration of biomolecules in the PEG-rich phase. Further investigations with other systems can be performed on these systems. The extraction of the red colorants into the PEG-rich phase is of great interest because traces PEG can remain in the red colorants to be used in future applications, which reduces the number of downstream processes required. Therefore, the results obtained in this study demonstrate the applicability of ATPPS as the first purification step in biotechnology processes to obtain natural colorants from fungi and other microorganisms.

Acknowledgments

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Notation

ATPPS = Aqueous two-phase polymer systems
ATPS = Aqueous two-phase systems
BCA = Bicinchoninic acid method
CYA = Czapeck Yeast Extract Agar
FDA = Food and Drug Administration
MB = Mass balance
PEG = Poly(ethylene glycol)

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