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NATURAL PRODUCTS

# Isolation of natural red colorants from fermented broth using ionic liquid-based aqueous two-phase systems

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**Abstract** There is a growing demand for natural colorants. This is prompting the search for new alternative and "benign" separation systems allowing higher recoveries, extraction yields, and selectivities. This work investigates the use of aqueous two-phase systems (ATPS) based on ionic liquids as extraction processes for the recovery of red colorants from the fermented broth of *Penicillium purpurogenum* DPUA 1275. Several ATPS based on quaternary ammonium and imidazolium were studied in this work aiming at separating the red colorants produced from the remaining colorants and contaminant proteins present in the fermented broth. The results suggest that the red colorants can be isolated by an appropriate manipulation of some of the process conditions, such as the use of quaternary ammonium with short alkyl chains, alkaline media, and short tie-line lengths (extraction point systems with lower concentrations of ionic liquid). These conditions allow large partition coefficients for the red colorants ($K_{red} = 24.4 \pm 2.3$), high protein removal ($60.7 \pm 2.8$ %) and selectivity parameters ($S_{red/prot} = 10.05$).

**Keywords** Aqueous two-phase systems · Ionic liquids · Potassium citrate buffer · *Penicillium purpurogenum* · Colorants isolation · Selectivity

## Introduction

The use of colorants as additives is an important factor in determining a product's acceptability. The world consumption of natural colorants to substitute the synthetic compounds is growing [1], this interest being driven by the problems associated with the safety of industrial products using synthetic colorants in several industries, such as food, cosmetics, and pharmaceuticals.

Natural colorants have been extracted directly from plants or animals, but microorganisms such as fungi [2] are a promising source of these compounds [3]. Furthermore, the production of colorants from microorganisms is associated with high growth rates, high yields, and a large range of potential colorants. This diversity is not only related to the colorants' chemical structures, but also to the color range that may add additional or even new range to the color palette of the existing colorants derived from natural sources [4, 5]. The diversity of tropical and subtropical microorganisms offers a promising range of unknown colorant compounds that might prove useful for different applications. However, as a result of the deficiencies of existing natural food colorants (instability against light, heat, or adverse pH), the demand for more applicable colorants is repeatedly raised by the food industry. Ascomycetous fungi of the genus *Monascus*, for example, have been used as common food colorant producers [2]; however, colorants produced by *Monascus* contain citrinin, and the production of the mycotoxin limits their applicability [6, 7]. In this context, it is of great interest to identify

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alternative microorganism producers of non-toxigenic colorants [8].

Recently, there have been reports [1, 7, 9, 10] considering *Penicillium* strains, namely *Penicillium purpurogenum*, as potential producers of natural colorants, which have a similar chromophore to *Monascus* colorants [9] and are not responsible for the production of any known mycotoxins. In addition, this *Penicillium* strain is capable of producing colorants with significant antimicrobial activities and absence of toxicity against *Artemia salina* [11] under laboratory conditions [5], both in solid and liquid media [10].

Besides the production step, the screening of alternative methods for the colorants extraction and purification is of great interest. Usually, organic solvents are used in their extraction from the fermentation media [4, 12–14]. Their use on a large scale imposes some important constraints due to their high environmental impact, cost, and often their impact upon biomolecules with the formation of irreversible degradation products [15]. Thus, the search and development of more sustainable, efficient, and cost-effective separation and purification processes is crucial to improve the process efficiency and economic viability, keeping the high quality standards required for the market approval [16, 17].

In the search for alternative methods, liquid–liquid extractions performed in aqueous two-phase systems (ATPS) have been investigated for bio-separations [18]. In these systems two water-soluble solutes separate into two immiscible aqueous-rich phases based on polymer–polymer, polymer–salt, or salt–salt solute combinations. Owing to their high water content (ca. 70–90 %) the ATPS favor the stability of biologically active molecules during the separation process when compared with other processes based on organic solvents [19, 20]. ATPS are also able to extract a large range of (bio)molecules [21–26] minimizing a number of the most common environmental problems.

Ionic liquid-based ATPS were firstly proposed by Rogers and co-workers [27] and have since been successfully studied by a growing number of researchers for the separation, concentration, and purification of proteins [24, 28], antioxidants [29, 30], metal ions [31], alkaloids [32, 33], and antibiotics [34, 35]. Unlike polymer-based ATPS they do not suffer from high viscosity [36], the formation of opaque aqueous solutions, and are not limited to a narrow range of polarities [37]. One of the main advantages of the application of ionic liquids (ILs) to ATPS formation is the possibility of manipulating their properties by careful cation/anion design and combination [38].

In this work, the isolation of natural colorants produced by a submerged culture of *P. purpurogenum* DPUA 1275 from the fermentation broth was investigated, considering as the first step of purification the application of ATPS based on two different ILs families. The imidazolium- and quaternary ammonium-based ATPS investigated in this study were prepared using a potassium citrate buffer, as the salt component. This citrate-based buffer was adopted to control the pH and it was chosen because of its biodegradable and nontoxic nature.

## Materials and methods

### Materials

Sucrose and yeast extract were purchased from Synth (São Paulo, Brazil) and Acumedia (Lansing, Michigan, USA), respectively. All the inorganic salts used in the medium preparation were of analytical grade, used as received from Synth (São Paulo, Brazil). The hydrogen chloride used was purchased from Merck (37 wt % pure).

To perform the extraction of the colorants, different aqueous citrate buffer solutions at ca. 50 wt % were used. The buffer solutions were prepared using anhydrous citric acid (99.5 wt % pure) and potassium citrate (99.5 wt % pure) both acquired from Synth (São Paulo, Brazil). The ILs investigated (Fig. 1) were tetraethylammonium bromide $[N_{2,2,2,2}]Br$ (98.0 wt % pure), tetrabutylammonium bromide $[N_{4,4,4,4}]Br$ (≥98.0 wt % pure), and 1-butyl-3-methylimidazolium chloride $[C_4mim]Cl$ (99.0 wt % pure); the quaternary ammonium was supplied by Sigma-Aldrich and the imidazolium purchased from IoLiTec (Ionic Liquid Technologies). All IL samples were dried under moderate temperature (70 °C) and high vacuum conditions (<0.1 mbar), for a minimum of 48 h. The purity of the ILs was additionally confirmed by $^{1}H$ and $^{13}C$ NMR analysis. Ultrapure water that was doubly distilled, passed through a reverse-osmosis system, and further treated with a Milli-Q plus water purification apparatus was used. The bovine serum albumin used to prepare the protein calibration curve was obtained from Sigma-Aldrich (≥96.0 wt % pure).

### Methods

#### *Microorganism, media composition, and fermentation description*

*Penicillium purpurogenum* DPUA 1275 was kindly provided by the Culture Collection of the Federal University of Amazon, DPUA, AM, Brazil. The cultures preserved in distilled water were reactivated in Czapeck Yeast Extract Agar (CYA) medium and maintained at 25 °C for 7 days. The cultures were stocked and maintained at 4 °C. The inoculum was prepared in plates with CYA medium and the cultures were maintained under the same reactivation conditions.

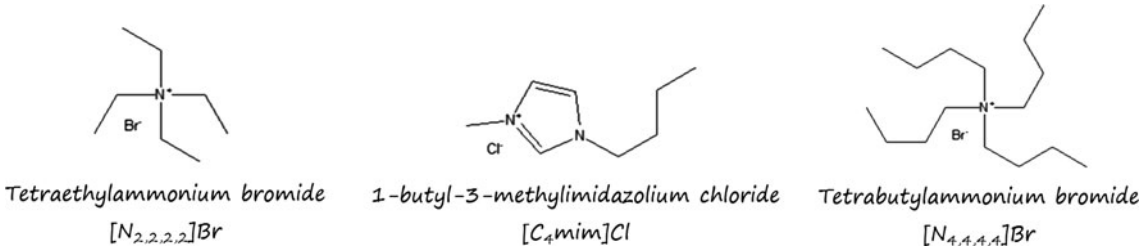


**Fig. 1** ILs used in this work: chemical structure, full name, and abbreviation

For the microorganism maintenance and inoculum preparation, the CYA medium was used with the following composition (g $l^{-1}$ in distilled water): $K_2HPO_4$ (1), yeast extract (5), sucrose (30), agar (15), and 10 ml $l^{-1}$ of concentrated Czapeck solution which comprised (g $l^{-1}$ in distilled water) $NaNO_3$ 3, KCl 0.5, $MgSO_4.7H_2O$ 0.5, and $FeSO_4.7H_2O$ 0.01 [39].

The submerged culture medium used in the fermentation process comprised the same components as the CYA medium, but the concentration of sucrose and yeast extract was 48.5 and 11.80 g $l^{-1}$, respectively.

The fermentations were carried out using mycelial agar discs (8 mm diameter) of *P. purpurogenum* DPUA 1275 punched out with a self-designed cutter from a stock culture grown on CYA medium in Petri plates for 7 days at 30 °C. Finally, five mycelial agar discs were transferred into 125 ml-Erlenmeyer flasks containing the fermentation medium (total of 25 ml). The fermentation was performed in an orbital shaker incubator at 30 °C and 150 rpm for 336 h.

After 14 days of incubation, the fermented broth was filtered and the colorant-rich supernatant was frozen in an ultra-freezer at −70 °C for later use in the extraction studies. The media were autoclaved at 121 °C for 15 min and the pH was adjusted to 4.5 with HCl (5 M).

### *Colorant isolation and protein quantification*

The aqueous systems used for the isolation study of the red colorant were prepared using graduated centrifuge tubes by weighing the appropriate amount of IL and potassium citrate buffer. The potassium citrate buffer (hereafter abbreviated as citrate buffer) was prepared as described in the literature and reported elsewhere [40, 41]. The colorants were added to the extraction systems by dilution in ca. 10 wt % of fermentation broth. To remove the influence of the ILs and citrate buffer in the analysis, a *blank* essay in which the fermented broth was replaced by distilled water was prepared for each system and condition studied. The mixtures were then gently stirred and centrifuged at 6,000 *g* for 5 min. The extraction and *blank* systems were maintained at 25 ± 1 °C, for at least 12 h, to reach equilibrium and allow for the colorant partitioning. Top and bottom phases were then carefully separated, weighted, and centrifuged; the volume of each phase was measured and the colorants quantified. Since the chemical structure of these colorants is not yet known, to determine their maximum wavelength absorption, the fermentation broth was scanned in the entire wavelength range at room temperature by UV spectroscopy, using a Molecular Devices Spectramax 384 Plus | UV–Vis Microplate Reader. The maximum absorbance peaks at 400, 470, and 490 nm, respectively, for the yellow, orange, and red colorants were determined [36]. The colorant quantification was performed in triplicate, the final absorbance results being reported as the average of the three independent assays carried out with the respective standard deviations.

The partition coefficients of each colorant were estimated according to Eq. (1):

$$K_{\text{colorant}} = \frac{\text{Abs}_{\text{Top}} \times \text{df}}{\text{Abs}_{\text{Bot}} \times \text{df}} \quad (1)$$

In this case, the dilution factors (df) and the absorbance results were used, because of the lack of knowledge on the chemical structures of the colorants. Thus, the partition coefficient for each colorant ($K_{\text{colorant}}$) is determined by $\text{Abs}_{\text{Top}}$ and $\text{Abs}_{\text{Bot}}$, which represent the absorbance of each colorant in the respective maximum peak in the top and bottom phases. It should be remarked that all the extraction and *blank* systems are composed of a top phase rich in IL and a bottom phase comprising a citrate buffer-rich phase. The recovery percentages of each colorant for the top ($R_{\text{Top}}$) and bottom ($R_{\text{Bot}}$) phases were determined according to the following equations:

$$R_{\text{Top}} = \frac{100}{1 + \left(\frac{1}{R_v \times K_{\text{colorant}}}\right)} \quad (2)$$

$$R_{\text{Bot}} = \frac{100}{1 + R_v \times K_{\text{colorant}}} \quad (3)$$

where $R_v$ represents the volume ratio between the top and bottom phase volumes. The recovery results are represented by specific abbreviations when different colorants are considered, namely $R_{\text{yellowTop}}$, $R_{\text{orangeTop}}$, and $R_{\text{redTop}}$ for the yellow, orange, and red colorants, respectively.

*Protein extraction*

Along with the colorants, the *P. purpurogenum* DPUA 1275 also produces proteins in a significant amount. However, as briefly discussed in the literature, the methods for protein quantification in the presence of ILs are problematic, because the presence of these ionic compounds is normally associated with changes in the conformational structure of these macromolecules. Thus, to avoid problems in their quantification, the biased spectroscopic method described by Rossmanith and co-workers [42] was applied in this work. The top and bottom phases were firstly diluted (dilution factor of 1:20) for the *blank* and the separation systems and the absorbance values were determined using UV spectroscopy at 280 nm (Table A.1 in Supporting Information). Despite the interference due to the presence of [$C_4$mim]Cl, the use of the *blank* allows the elimination of the negative effect promoted by the presence of this compound (Fig. A.1 in Supporting Information).

Some parameters related to the presence/removal of proteins were then calculated with the aim of accurately evaluating the isolation of the red colorant from the remaining compounds. These parameters are the partition coefficient of the proteins $K_{Prot}$ (Eq. 4), the extraction efficiencies for each phase $EE_{Top}$ (%) and $EE_{Bot}$ (%) (Eqs. 5 and 6), the percentage of protein flocculated, retained in the interface, or accumulated in the bottom of the extraction tubes $Prot_{Int}$ (%) (Eq. 7), and the total protein extraction efficiency $EE_{Total}$ (%) (Eq. 8).

$$K_{Prot} = \frac{C_{Top}}{C_{Bot}} \tag{4}$$

$$EE_{Top}\ (\%) = \frac{C_{Top} \times V_{Top}}{m_i} \tag{5}$$

$$EE_{Bot}\ (\%) = \frac{C_{Bot} \times V_{Bot}}{m_i} \tag{6}$$

$$Prot_{Int}\ (\%) = 100 - \left(EE_{Top} + EE_{Bot}\right) \tag{7}$$

$$EE_{Total}\ (\%) = EE_{Bot} + Prot_{Int} \tag{8}$$

where $m_i$ represents the initial mass of protein added into the extraction systems, $C_{Top}$ and $C_{Bot}$ represent the protein concentration and $V_{Top}$ and $V_{Bot}$ represent the volume of the top and bottom phases, respectively. The calibration curve for the protein was established at 280 nm. The average and respective standard deviations of the parameters calculated are reported, all experiments being carried out in triplicate.

*Selectivity*

Since the main objective of this work was to isolate the red colorant from the remaining components, the isolation of the red pigment was calculated through different selectivity parameters. Thus, the idea was to maximize the selectivity of the red colorant by considering the presence of the contaminant proteins ($S_{red/Prot}$) described by Eq. 9, but also to study the isolation of this colorant from the remaining colorants (Eq. 10).

$$S_{red/Prot} = \frac{K_{red}}{K_{Prot}} \tag{9}$$

$$S_{red/colorant} = \frac{K_{red}}{K_{colorant}} \tag{10}$$

where $K_{red}$ and $K_{Prot}$ represent, respectively, the partition coefficients of the red colorant and proteins, and $K_{colorant}$ corresponds to the partition coefficient of the yellow ($K_{yellow}$) or orange ($K_{orange}$) colorants.

## Results

Since one of the major problems associated with colorant production is the purification step, several efforts have been performed to increase the low selectivity and extraction efficiencies and decrease the long purification process times [43]. In this work, various IL-based ATPS were applied to study the partitioning of three colorants produced by *P. purpurogenum* DPUA 1275 [44], with emphasis on the red colorant recovery owing to its high industrial potential [2, 8, 45]. The ILs tested were based on the quaternary ammonium family because those are considered more compatible with biomolecule structures, and one imidazolium, used for comparison owing to its large spectrum of applicability as the main component of several extraction systems [38]. A potassium citrate buffer solution was also used as the second component of the ATPS. Despite its weaker capacity to promote the ATPS formation when compared with phosphate buffer salts, this buffer has a more "benign" nature toward biomolecules and also a larger pH range of applicability. Our main goal in this work was to evaluate alternative ATPS as the first purification step addressing the isolation of the red colorant from the remaining biomolecules produced by the microorganism.

### Effect of ILs on colorant partitioning and protein removal

Two different quaternary ammonium-based ILs were used in this study aiming at understanding the effect of the alkyl chain length on the colorant partitioning. In addition, the effect of an imidazolium-based IL was also studied for comparison, with the objective of understanding how the cation affects the red colorant partitioning. The results

presented in Fig. 2 suggest that all colorants have a preferential migration towards the top IL-rich phase, which is reflected by the large partition coefficients ($4.3 \pm 0.2 < K_{colorant} < 41.7 \pm 3.0$), represented by the gray bars, and recoveries (full lines) larger than 90 % ($91.0 \pm 0.8\ \% < R_{Top} < 98.0 \pm 0.1\ \%$). The results show that the migration of the colorants can be significantly increased by some structural features of ILs. It seems that the quaternary ammonium-based ILs (an acyclic and non-aromatic cation) have a larger capacity to extract the colorants from the fermentation broth than the imidazolium-based ILs (a cyclic and aromatic cation). Moreover the increase of the alkyl chain length of the IL also seems to enhance the extraction of the colorant. Since the colorant structures are unknown, it is difficult to explain the results on the basis of molecular interactions. From the colorant octanol–water partition coefficients ($K_{ow}$) recently reported [46] (Fig. A.2 in Supporting Information), it is possible to conclude that all colorants are hydrophilic, because their $K_{ow}$ at pH 7 are lower than unity. This means that hydrophilic/hydrophobic interactions alone are not enough to explain the partition coefficients observed for the colorants.

Table 1 shows the results obtained for the protein partitioning in the ATPS investigated. The results suggest that the contaminant proteins also have a tendency to migrate to the top phase, though to a lesser extent than the colorants as can be gauged from their lower protein partition coefficients ($3.1 \pm 0.1 < K_{Prot} < 7.1 \pm 0.3$). Despite the proteins' affinity for the IL-rich phase, it seems to be possible to manipulate the selectivity of these systems, thus achieving the purification of the colorants. However, the proteins' removal in this work is not only obtained by concentrating them in the opposite phase to that of the colorants. In fact, a partial removal is also achieved by their flocculation, which is observed as a white sediment at the interface or/and at the bottom phase, here represented by the $Prot_{Int}$ parameter (Fig. A.3 in the Supporting Information). Thus, the total protein extraction efficiency ($EE_{Total}$) is here described by the total amount of protein flocculated plus the protein in the bottom phase. It should be highlighted that the flocculated protein is considered, because its removal is part of our goal. All the protein parameters presented in Table 1 reflect the tendency of the different ATPS to eliminate the proteins from the colorant-rich top phase ($EE_{Total}$) according to the following sequence:

$$[N_{4,4,4,4}]Br \langle [C_4mim]Cl \langle [N_{2,2,2,2}]Br$$

Despite the higher partition coefficients observed for the $[N_{4,4,4,4}]$Br-based ATPS, it shows a lower capacity to remove the contaminant proteins.

Further investigation aimed at optimizing the IL and salt concentrations and the pH values was thus conducted using the $[N_{2,2,2,2}]$Br-based ATPS because this system shows a higher capacity to isolate the colorants from the proteins, which is reflected by:

1. Higher partition coefficients for the colorants in the top phase ($8.7 \pm 0.7 < K_{colorant} < 9.0 \pm 0.8$)
2. Higher capacity to remove the contaminant proteins ($EE_{Total} = 48.5 \pm 1.9\ \%$)

## Effect of TLL on colorant partitioning and protein removal

The effect of the tie-line length (TLL) was investigated using mixtures of the points P1 with a TLL of 88.6, the point P2 with a TLL of 70.2, and finally the point P3 with a TLL of 77.1 (Fig. A.4 in the Supporting Information). The

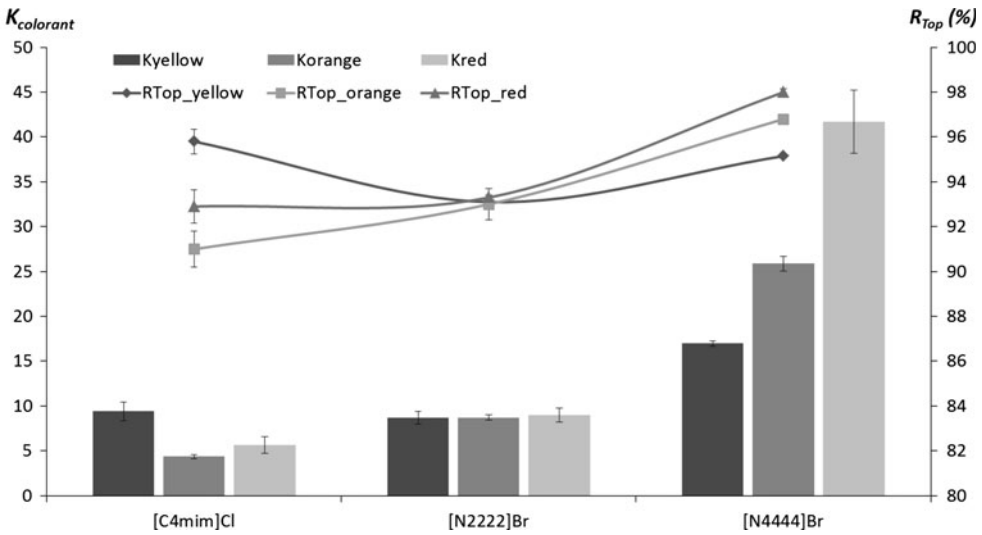


**Fig. 2** Partition coefficients ($K_{colorant}$) and top phase recovery ($R_{Top}$) values for the colorants by the application of different IL + citrate buffer + water mixtures at 298 ± 1 K and pH 7

**Table 1** Effect of different ILs on the partitioning and removal efficiency of the contaminant plus the respective standard deviations (std)

| Ionic liquid | $K_{Prot} \pm$ std | $EE_{Top} \pm$ std (%) | $EE_{Bot} \pm$ std (%) | $Prot_{Int} \pm$ std (%) | $EE_{Total} \pm$ std (%) |
|---|---|---|---|---|---|
| $[C_4mim]Cl$ | 4.64 ± 0.08 | 62.4 ± 5.3 | 6.1 ± 1.2 | 31.4 ± 4.1 | 37.6 ± 5.3 |
| $[N_{4,4,4,4}]Br$ | 7.1 ± 0.3 | 80.2 ± 1.4 | 10.2 ± 1.2 | 9.6 ± 2.6 | 19.8 ± 1.4 |
| $[N_{2,2,2,2}]Br$ | 3.1 ± 0.1 | 51.5 ± 1.9 | 10.8 ± 0.9 | 37.7 ± 2.0 | 48.5 ± 1.9 |

pH 7 was maintained during all the experiments

results depicted in Fig. 3 suggest that the colorant migration is also preferential for the top phase ($6.7 \pm 0.1 < K_{colorant} < 17 \pm 1$) owing to stronger colorant–IL interactions. The lower TLL is reflected by the highest colorant partition coefficients ($8.1 \pm 0.2 < K_{colorant} < 17 \pm 1$). With the objective of understanding these results, we calculated the water mass fraction percentage using the available TL data [40] (Table A.3 in Supporting Information). From these results, it is clear that the mixture point P2 has a more aqueous top phase, which means that the driving force for the preferential colorant migration into the top phase is not only the colorant–IL interactions, but mainly the colorant–water interactions (due to the hydrophilic nature of the colorants described by their $K_{ow}$ values presented in Fig. A.2). The colorant recovery results for the top phase those are higher than 90 % ($90.4 \pm 0.2 < R_{Top} < 95.1 \pm 0.1$).

Table 2 shows the results obtained for the protein removal at the various TLL. In general, the results suggest that the percentage of protein separated from the colorants (i.e., protein in the bottom phase or flocculated) slightly decreases with the TLL. The system with the lower TLL seems to be the best to remove the contaminant proteins with an $EE_{Total}$ of 56.6 ± 1.6 %. Comparing the various results obtained for the parameter $EE_{Total}$, we conclude that the contaminant proteins are more sensitive to the system composition than to the nature of the ionic liquid.

## Effect of pH on colorant partitioning and protein removal

Figure 4 shows the significant effect of the pH upon the colorant partitioning. It can be seen that increasing the pH of the system the $K_{colorant}$ values also increase. pH 8 presents the highest partition coefficients for the colorants ($9.8 \pm 0.5 < K_{colorant} < 16.4 \pm 0.7$) among the pH values studied, with the red colorant reaching a value of $K_{red} = 16.4 \pm 0.7$. This is also reflected by the enhanced colorant recovery achieved in the top phase with increasing pH with $R_{Top}$ changing from 87.2 ± 0.8 % at pH 6 to 96.4 ± 0.4 % at alkaline pH. The protein removal was not significantly affected by the pH variations ($EE_{Total} \approx$ 50 %); thus, the results suggest that the optimal purification will be achieved in alkaline media.

## Selectivity for red colorant isolation

As described before, the red colorant isolation was determined by considering its migration to the top phase and the remaining compounds (contaminant proteins and yellow and orange colorants) to the opposite phase (Tables 3, 4). Thus, the extent of the red colorant isolation is here discussed by considering the different selectivity parameters presented in Table 5, namely the red colorant versus proteins ($S_{red/prot}$) and red colorant versus the yellow

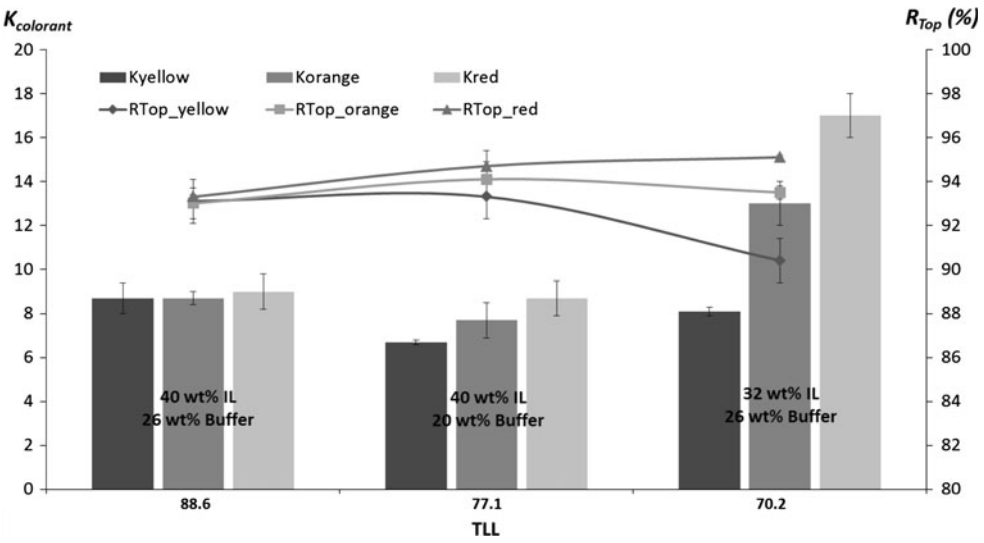


**Fig. 3** Partition coefficients ($K_{colorant}$) and top phase recovery ($R_{Top}$) values (for the colorants using the system $[N_{2,2,2,2}]Br$ + citrate buffer + water and different extraction systems, at 298 ± 1 K and pH 7

**Table 2** Effect of different TLL on the colorant partition coefficients plus the respective standard deviations (std)

pH 7 was maintained during all the experiments

| TLL | $K_{yellow} \pm$ std | $K_{orange} \pm$ std | $K_{red} \pm$ std | Water mass fraction in top phase (wt %) |
|---|---|---|---|---|
| 88.6 | 8.7 ± 0.7 | 8.7 ± 0.3 | 9.0 ± 0.8 | 29.85 |
| 77.1 | 8.1 ± 0.2 | 13 ± 1 | 17 ± 1 | 35.60 |
| 70.2 | 6.7 ± 0.1 | 7.7 ± 0.8 | 8.7 ± 0.8 | 38.44 |

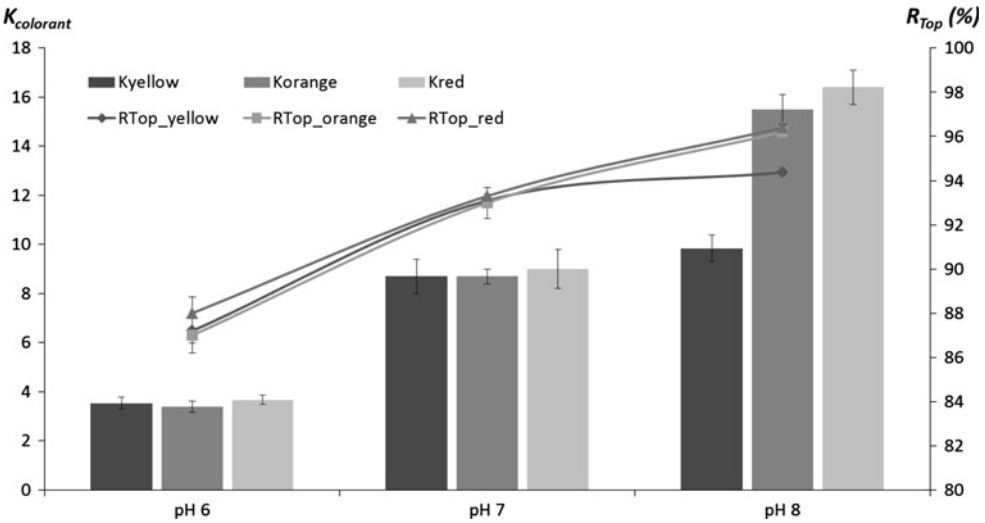


**Fig. 4** Partition coefficients ($K_{colorant}$) and top phase recovery ($R_{Top}$) values for the colorants using the system $[N_{2,2,2,2}]Br$ + citrate buffer + water at different pH values and 298 ± 1 K

($S_{red/yellow}$) and orange ($S_{red/orange}$) colorants. The red colorant was chosen owing to its higher potential for application in the food industry [2, 8, 45]. The first observation stemming from the results in Table 5 is that the selectivity values considering the proteins are always the highest. This can be easily explained by the significant structural differences between the contaminant proteins and the red colorant, and consequently by their distinct properties. Moreover, as previously discussed, the three colorants have a similar hydrophilic nature, which allows us to conclude that their chemical structures can be similar, thereby making their selective separation by the controlling enthalpic effects (i.e., type and interaction strength established between the ATPS components and the colorant) more difficult. However, having in mind the effect of all the different conditions studied, we stress that the selectivity for the red colorant partitioning is favored at higher pH values and lower TLL.

## Optimal extraction point

Considering the optimization tests previously reported showing that the higher selectivity results for the red colorant isolation are obtained by using lower TLL and higher pH values (pH 8), we applied the optimal extraction conditions. The principal parameters were calculated considering not only the red colorant but also the contaminant proteins. These results are reported in Table 6 and confirm that the red colorant isolation is favored by alkaline extraction media and lower TLL values. The results also show that the red colorant and the contaminant proteins are more separated between the two phases as verified by the high selectivity parameter of 10.16 obtained.

## IL- versus polymer-based ATPS

One of the objectives of this study was to evaluate the performance of these ATPS when compared with conventional polymer-based ATPS. Recently [47], the partitioning of the red colorant using different poly(ethylene glycol) (PEG) + sodium polyacrylate (NaPA) + water + electrolyte liquid–liquid extraction systems was investigated. The electrolytes (different inorganic salts) applied in the ATPS formation were used to introduce polarity in both phases, thereby allowing the phase separation. Besides the lower complexity and viscosity of the IL-based systems, these achieve higher partition coefficients of the red colorant ($K_{red} = 24.4 \pm 2.3$ vs. $K_{red} = 13.06$). Consequently, higher selectivity parameters for the red colorant ($S_{red/prot} = 10.05$ vs. $S_{red/prot} = 3.05$) were also achieved using IL-based ATPS, when compared with the polymeric-based ATPS. The success of these novel ATPS based on quaternary ammonium in the isolation of the red colorant is thus demonstrated here.

**Table 3** Effect of different TLL on the partitioning and removal efficiency of the contaminant proteins plus the respective standard deviations (std)

| TLL | $K_{Prot} \pm$ std | $EE_{Top} \pm$ std (%) | $EE_{Bot} \pm$ std (%) | $Prot_{Int} \pm$ std (%) | $EE_{Total} \pm$ std (%) |
|---|---|---|---|---|---|
| 88.6 | 3.1 ± 0.1 | 51.5 ± 1.9 | 10.8 ± 0.9 | 37.7 ± 2.0 | 48.5 ± 1.9 |
| 77.1 | 2.46 ± 0.07 | 45.8 ± 1.1 | 9.1 ± 0.1 | 45.1 ± 1.0 | 54.2 ± 1.1 |
| 70.2 | 3.4 ± 0.1 | 43.4 ± 1.6 | 11.3 ± 0.4 | 45.3 ± 2.0 | 56.6 ± 1.6 |

pH 7 was maintained during all the experiments

**Table 4** Effect of different pH values on the partitioning and removal efficiency of the contaminant proteins plus the respective standard deviations (std)

| pH | $K_{Prot} \pm$ std | $EE_{Top} \pm$ std (%) | $EE_{Bot} \pm$ std (%) | $Prot_{Int} \pm$ std (%) | $EE_{Total} \pm$ std (%) |
|---|---|---|---|---|---|
| 6 | 2.0 ± 0.2 | 47.0 ± 5.7 | 11.6 ± 2.0 | 41.4 ± 7.6 | 53.0 ± 5.7 |
| 7 | 3.1 ± 0.1 | 51.5 ± 1.9 | 10.8 ± 0.9 | 37.7 ± 2.0 | 48.5 ± 1.9 |
| 8 | 3.4 ± 0.3 | 49.5 ± 1.4 | 7.7 ± 0.4 | 42.9 ± 1.0 | 50.54 ± 1.4 |

**Table 5** Selectivity results for the red colorant

| System conditions | $S_{red/yellow}$ | $S_{red/orange}$ | $S_{red/Prot}$ |
|---|---|---|---|
| $[C_4mim]Cl$; TLL 88.6; pH 7 | 0.60 | 1.30 | 1.22 |
| $[N_{4,4,4,4}]Br$; TLL 88.6; pH 7 | 2.38 | 3.62 | 5.84 |
| $[N_{2,2,2,2}]Br$; TLL 88.6; pH 7 | 1.03 | 1.03 | 2.87 |
| $[N_{2,2,2,2}]Br$; TLL 70.2; pH 7 | 2.10 | 1.31 | 5.16 |
| $[N_{2,2,2,2}]Br$; TLL 77.1; pH 7 | 1.30 | 1.13 | 3.53 |
| $[N_{2,2,2,2}]Br$; TLL 88.6; pH 7 | 1.03 | 1.03 | 2.87 |
| $[N_{2,2,2,2}]Br$; TLL 88.6; pH 6 | 1.04 | 1.08 | 1.79 |
| $[N_{2,2,2,2}]Br$; TLL 88.6; pH 8 | 1.67 | 1.06 | 4.82 |

**Table 6** Results obtained for all parameters calculated for the colorants and proteins by the application of the optimal extraction system (pH 8)

| Parameters | $K_{red} \pm$ std | $R_{Top} \pm$ std | $EE_{Total} \pm$ std (%) | $S_{red/Prot}$ |
|---|---|---|---|---|
| Optimal system (pH 8) | 24.4 ± 2.3 | 96.6 ± 0.3 | 60.7 ± 2.8 | 10.05 |

*std* standard deviations

## Conclusions

The purification of a red colorant from the fermentation broth of *P. purpurogenum* DPUA 1275 by the application of different IL–citrate buffer aqueous two-phase systems was studied. In this context, different systems and conditions were investigated, namely the IL and salt concentrations, the IL chemical structure, and the system pH. To study the optimization of the IL and salt concentrations and the pH values the $[N_{2,2,2,2}]Br$-based ATPS was used, because this system shows a higher capacity to isolate the colorants from the proteins. Thus, the partitioning of the red colorant is favored using short TLL and higher pH values. High partition coefficients of the red colorant ($K_{red} = 24.4 \pm 2.3$) and protein removal ($EE_{Total} = 60.7 \pm 2.8$ %) were achieved with the optimal extraction system based on the $[N_{2,2,2,2}]Br$ IL. IL-based ATPS can also be more efficient and significantly improve the isolation capacity (described by higher selectivity values $S_{red/prot} = 10.05$) over the commonly used polymer-based ATPS ($K_{red} = 13.06$ and $S_{red/prot} = 3.05$). Taking into account the results obtained it is possible to envisage the potential application of these systems as a first step towards the purification of natural colorants from fermented broths.

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