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Recovery in aqueous two-phase systems of lutein produced by the green microalga *Chlorella protothecoides*

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Abstract

In this study the potential use of aqueous two-phase system (ATPS) to establish a viable process for the recovery of lutein from the green microalga *Chlorella protothecoides* is evaluated. The partitioning behaviour of lutein, a representative model of natural compounds of commercial interest, was investigated in a polyethylene glycol (PEG)-phosphate system. An evaluation of system parameters including PEG molecular mass, the concentrations of PEG, phosphate and product concentration was conducted, to estimate conditions under which lutein partitions preferentially to the top phase whilst cell debris partition to the opposite phase. The necessary addition of ethanol to the ATPS for the dissolution of lutein affected the phase formation and such effect was evaluated using the change in the volume ratio produced. ATPS extraction comprising Vr = 1.0, PEG 8000 22.9% (w/w) and phosphate 10.3% (w/w), pH 7.0 provided the conditions for the concentration of lutein into the upper phase and the cell debris preferentially to the bottom phase. The use of ATPS resulted in a primary recovery process to obtain lutein with an overall product yield of 81.0 ± 2.8%. The findings reported here demonstrate the potential of ATPS for the further development of a prototype process to recover lutein from *C. protothecoides* as a first step for the generic application of this technique.

Keywords: Aqueous two-phase systems; *Chlorella protothecoides*; Lutein

1. Introduction

With the increasing trend of the market to obtain natural products with bioactive nutraceutical properties, there is considerable interest in the development of efficient and scalable processes to obtain such products. The urgent need to define credible production systems has restricted basic research to establish selective and scalable methods of product recovery that integrate effectively with upstream operations to rapidly yield product in a suitable state for the validation of polishing, formulation and delivery operations. Biotechnological processes represent an attractive alternative to synthetic procedures to produce natural products [1–3]. In this context, the recovery of colour and functional compounds from microbial origin represents a very interesting case. Particularly, in this research lutein produced by *Chlorella protothecoides* was selected as a representative model of this group of natural products of commercial interest. Lutein is one of the most important carotenoids in human serum and foods [4]. Lutein, an intracellular product of *C. protothecoides*, has been widely used for the pigmentation of animal tissues and products, as well as for coloration of foods, drugs and cosmetics. Although the production of lutein has been addressed before, the existing reports addressed mainly the upstream part of the process and the purification by solvent extraction and chromatography [4–7]. However, the recent health benefits associated to carotenes that include the prevention of certain types of cancer and age related macular degeneration [1,8], make the production of lutein from microorganisms an attractive case of study. Nevertheless, the existing protocols [5,6], are characterized by potential high costs associated to the scaling up of the process. Recently, a high-speed counter-current chromatography method for the isolation and purification of lutein has been reported [6]. However, only a small sample size (e.g. 200 mg) could be treated in one run, and the consumption of organic solvents was great. Furthermore, high-speed counter-current chromatography is not easily accessible. Additionally, most processes for commercial recovery of carotenes include a saponification step to increase their solubility in water, and

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large amounts of organic solvents [5], which generates industrial effluents with a harmful environmental impact.

The development of efficient and scalable biotechnological processes is needed for the commercial implementation of prototype processes. An alternative practical approach, exploiting the use of aqueous two-phase systems (ATPS) [9] to address the limitation of the existing protocols for the potential recovery of lutein produced by *C. protothecoides*, is examined in this work. ATPS, assembled from a mixture of polymers (e.g. polyethylene; PEG) and salts (e.g. phosphate or sulphate), result in two-phases for the extraction of bio-molecules. This technology has several potential advantages, including bio-compatibility, ease of scale-up and low cost [3]. In the present research, a practical approach, which exploits the known effect of system parameters such as PEG and phosphate concentration and the nominal molecular mass of PEG upon product partition, was used. This approach was followed to evaluate the feasibility of using ATPS for the recovery of lutein from *C. protothecoides* as the first step in the development of a prototype biotechnological process. Initially, the effect of the necessary addition of ethanol to the ATPS for the dissolution of lutein on the phase formation was evaluated using the change in the volume ratio produced. The research was then conducted using a model system (characterized by the use of a lutein-rich paste) and a complex system (characterized by the use of homogenate from the fermentation of *C. protothecoides*), to obtain different conditions where the target product and the cell debris contaminants partitioned preferentially to opposite phases. These conditions were then used to further investigate the potential process intensification, evaluating the effect of increasing lutein concentration on the system performance.

2. Materials and methods

2.1. Characterization of aqueous two-phase experiments

The binodal curve used in this study were estimated by the cloud point method [10] using poly(ethylene glycol) (PEG, Sigma, St. Louis, MO, USA) of nominal molecular mass of 1000, 1450, 3350 and 8000 g/mol (50% (w/w) stock solution) and di-potassium hydrogen orthophosphate/potassium di-hydrogen orthophosphate (Sigma; 30% (w/w) stock solution). Fine adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide.

2.2. Culture medium and cultivation conditions

C. protothecoides was cultivated in the culture medium described by Shi et al. [7]. The algae were grown under heterotrophic conditions in a batch culture initially in 500 ml Erlenmeyer flasks containing 250 ml medium at 28 ± 1 °C under continuous shaking (180 rpm) in the dark. Further heterotrophic cultivation was performed in a 7 l fermentor (Bio-Flow III, New Braunswick) containing 2.5 l medium. The cultivation conditions were controlled as follows: pH: 6.6 ± 0.1; temperature: 28 ± 1 °C; agitation: 480 rpm; dissolved oxygen concentration: 50% saturation. After harvesting, biomass was recovered by centrifugation (3500 rpm for 5 min; Eppendorf 5415C) and lutein extraction was performed by addition of ethanol (30% wet weight biomass/volume). Cell debris removal was achieved by centrifugation at 3500 rpm for 5 min (Eppendorf 5415C). Cell debris were resuspended in ethanol (30% wet (w/v)) and the resulting solution used to evaluate their partition behaviour in ATPS. The supernatant derived from lutein extraction (referred to as crude extract) was introduced into the aqueous two-phase system previously selected as described below.

2.3. Aqueous two-phase experiments

Aqueous two-phase systems were prepared for convenience on a fixed mass basis using a top-loading balance. Predetermined quantities (see Table 1) of stock solutions of PEG and potassium phosphate were mixed with either a single model (containing a stock solution of purified lutein in ethanol) or a complex model (containing either cell debris suspension or cell debris-free extract from *C. protothecoides* fermentation; referred above as crude extract) systems. Subsequently, di-ionied water was added to give a final weight of 5 g. The stock solution of purified lutein was obtained by extracting 50 mg of lutein-rich paste (Lutein 5% TG Roche; Mannheim, Germany) in ethanol for 15 h at 30 °C under continuous shaking (200 rpm). The final concentration of ethanol in the ATPS was estimated to be around 6% (w/w) (weight of ethanol referred to the total weight of the system). The stock solutions (PEG and phosphate) were mixed and phases dispersed by gentle mixing for 60 min at 25 °C. Complete phase separation was achieved by low speed batch centrifugation at 2000 × *g* for 10 min at 25 °C. Visual estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. The volumes of the phases were then used to estimate the volume ratio (Vr = volume of the top phase/volume of the bottom phase). Samples were carefully extracted from the phases and diluted for analysis and subsequent estimation of lutein partition coefficient (K = concentration of solute in the top phase/concentration of solute in the bottom phase). The system tie-line length (TLL), which represents the length of the line that connects the composition of the top and bottom phases of a defined ATPS, was estimated as described by Albertsson [11]. Results reported are the average of three independent experiments and errors were estimated to be ±10% of the mean value.

2.4. Analytical methods

Total lutein concentration in the commercial paste was quantified using the average extinction coefficient for lutein of $E^{1\%}_{1\,cm} = 2550$ in ethanol and a molecular mass of 569 g/mol. All ATPS fractions were collected and their

Table 1
Systems selected for the evaluation of the partition behaviour of lutein and the effect of ethanol on phase formation

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Volume ratio	Volume ratio (6% (w/w) ethanol)
1	1000	15.6	12.6	28.3	0.95 ± 0.09	2.4 ± 0.2
2		17.6	13.6	36.1	1.03 ± 0.1	2.1 ± 0.2
3		19.8	14.8	38.0	1.09 ± 0.1	2.4 ± 0.2
4		22.2	16.0	49.4	1.08 ± 0.1	2.1 ± 0.2
5	1450	17.6	10.9	34.3	1.03 ± 0.1	3.3 ± 0.3
6		22.0	12.1	47.0	1.1 ± 0.1	3.0 ± 0.3
7		24.9	12.6	53.2	1.1 ± 0.1	3.0 ± 0.3
8		26.1	13.0	55.0	1.07 ± 0.1	3.2 ± 0.3
9	3350	16.9	10.1	33.6	0.9 ± 0.1	2.7 ± 0.2
10		18.7	11.2	39.6	1.0 ± 0.1	2.2 ± 0.2
11		21.0	12.9	45.0	1.1 ± 0.1	2.1 ± 0.2
12		22.1	14.0	48.1	1.0 ± 0.1	2.2 ± 0.2
13	8000	16.1	8.1	27.1	1.0 ± 0.1	3.7 ± 0.3
14		19.0	9.1	40.2	0.9 ± 0.1	3.3 ± 0.3
15		20.0	9.5	45.0	1.08 ± 0.1	3.0 ± 0.3
16		22.9	10.3	49.4	1.08 ± 0.1	3.2 ± 0.3

Systems were selected to evaluate the impact of increasing tie-line length (TLL) and molecular mass of PEG upon the partition behaviour of lutein. The volume ratio was estimated (from blank systems and systems containing ethanol) as described in Section 2.

absorbance readings recorded at 445 nm using a Beckman DU® 650 spectrophotometer (Fullerton, CA). A system blank, without lutein, was prepared for each treatment combination and used as analytical blank for the corresponding phase (top or bottom). Total lutein concentrations were expressed as lutein equivalents using the average $E^{1\%}_{1\,cm}$ of 2550, and a molecular mass of 569 g/mol.

3. Results and discussion

3.1. The effect of ethanol on volume ratio of aqueous two-phase systems

The predictive design of aqueous two-phase processes demands the full understanding of the mechanisms governing the behaviour of molecules in ATPS. However, the lack of knowledge of such mechanism, requires that for each extraction process, once general conditions have been selected on the basis of experience or process limitations (e.g. polymer and salt type) more specific partition conditions (polymer and salt concentration, volume ratio, etc.) need to be empirically established. In the present research before designing the aqueous two-phase process for the recovery of lutein produced by the green microalga *C. protothecoides*, the influence of ethanol addition on the characteristic of ATPS was studied. In these experiments, ethanol was the solvent selected to favour the dissolution of lutein in the ATPS due to its wide use in food and pharmaceutical applications and relative low cost in contrast with other alcohols and organic solvents. In this particular case, the change in the volume ratio (compared with that from a system without ethanol) was used to evaluate the effect of ethanol in the ATPS. The effect of organic solvents on the partitioning of molecules in ATPS have been reported [12,13], revealing that the phase-forming properties can be influenced by the addition of an organic solvent, such as alcohol. These findings were recently applied to enhance the partition efficiency of geniposide in a polymer salt system [13]. However, it is important to consider that, in this paper, the use of ethanol was required to promote the dissolution of lutein to a concentration similar to that found in a fermentation from *C. protothecoides* [7].

Table 1 illustrates the effect of ethanol addition on the volume ratio (Vr) of the ATPS. It is clear that the addition of ethanol caused the Vr to rise for all systems. Regardless of the molecular mass of the PEG, the changes in Vr were more significant in the ATPS close to the binodal (short TLLs). Such situation can be associated to the nature of such systems. Albertsson [11] reported that ATPS located close to the binodal curve exhibited certain sensitivity to changes in system compositions. Subtle changes in the composition of ATPS caused by different factors (as in this case by the addition of ethanol), resulted in great changes in the phase composition and, as a result, in the final characteristics (Vr in this particular case) of the ATPS. In addition, the increase in the Vr of the ATPS loaded with ethanol may be explained by the particular effect of the solvent in the phases of the systems. It has been reported [13] that when complex systems are used, the presence of the solutes from biological suspensions has an impact on the final characteristic of the ATPS (e.g., volume ratio, position of the binodal curve). In the particular case of the volume ratio, it is suggested [14] that the accumulation of the solute to either phase caused the volume of that phase to increase. Thus, the volume ratio is increased by top-phase solvent accumulation or decreased by bottom phase solvent accumulation. It seems that for the ATPS studied the addition of ethanol affected the top phase and as a result the volume ratio of the systems. Thus, the partition behaviour and recovery of the target product loaded to the ATPS with ethanol will be influenced positively if lutein

exhibits top phase preference (where ethanol accumulates preferentially). Once the effect of the addition of ethanol on the ATPS was identified, the influence of system parameters on lutein partitioning behaviour was evaluated.

3.2. Influence of systems parameters on partition behaviour of lutein in PEG-salt aqueous two-phase systems

In order to select the ATPS in which the target product (lutein) and cell debris concentrate in opposite phases (preferentially the target product in the top phase to facilitate further processing), the partitioning behaviour was studied using systems comprising a stock solution of lutein from a paste rich in the product of interest. These model systems did not account for the influence, upon the performance of ATPS, of contaminants such as proteins, lipids, pigments, etc. that may be present in the extraction broth from a *C. protothecoides* fermentation or any other complex source. For the examination of the partition behaviour of lutein, a practical approach that exploits the known effect of system parameters such as tie-line length (TLL) and molecular mass of PEG on biomolecule partition coefficient was used. Thus, sixteen ATPS were selected (see Table 1) based upon experience [3,15]. These systems were characterized by increasing TLL using four different molecular mass of PEG (i.e. 1000, 1450, 3350 and 8000 g/mol) and keeping Vr = 1.0 and pH = 7.0 constant. The partition experiments that used purified lutein in ATPS revealed that this product exhibited a strong top phase preference (data not shown), which imply that the majority of the lutein concentrated in the top phase. The top-phase preference of the lutein resulted in partition coefficients greater than 50 for all the systems studied. Such behaviour was explained by problems associated with the detection of the presence of lutein in the bottom phase, caused by the very low amount of the product concentrated in this phase. As a consequence, it was very difficult to evaluate the impact of system parameters upon the partition behaviour of lutein, by monitoring the biomolecule partition coefficient (K). As a result, it was decided to use the recovery of lutein (expressed relative to the initial amount of lutein loaded to the ATPS) from the top PEG-rich phase as the response variable to evaluated the effect of system parameters on the behaviour of the product in ATPS. For the selection of the operating conditions to concentrate lutein in one phase, the concentration of PEG, phosphate and the molecular mass of PEG were manipulated to maximize recovery in the top phase.

Table 2 illustrates the impact of increasing TLL (for four different molecular mass of PEG) upon cell debris partition coefficient and lutein top phase recovery. As in the case of ethanol addition, the presence of cell debris affected the final Vr of the ATPS. The volume ratio increase by top phase cell debris accumulation or decrease by bottom phase cell debris accumulation. In the current study, ATPS both close and distant to the binodal curve (short and long TLL) for each molecular mass of PEG (1000, 1450, 3350, 8000 g/mol) were selected to examine the behaviour of the volume ratio of the biological systems. In general, the determination of the volume ratio for loaded ATPS requires a clear definition of the top, bottom and any other phase which develop. The latter clearly depends on the nature and complexity of the biological suspension. To simplify the estimation of the volume ratio in the ATPS loaded with cell debris, when the formation of an interface was observed, this was considered as a part of the top or bottom phase according to the initial volume ratio of the non-biological ATPS. Cell debris from *C. protothecoides* exhibited preferentially a bottom phase preference. In these systems a decrease in the volume

Table 2
Influence of increasing tie-line length (TLL) and molecular mass of PEG on cell debris partition behaviour and top phase recovery of lutein in the ATPS

System	Molecular mass of PEG (g/mol)	TLL (%, w/w)	Volume ratio (6% (w/w) ethanol)	Volume ratio (ATPS loaded with biomass)	Cell debris phase preference	Top phase recovery Y_{TOP} (%)
1	1000	28.3	2.4 ± 0.2	2.6 ± 0.2	T	35 ± 2.0
2		36.1	2.1 ± 0.2	2.0 ± 0.2	B	54 ± 4.1
3		38.0	2.4 ± 0.2	2.1 ± 0.2	B	71 ± 3.2
4		49.4	2.2 ± 0.2	2.0 ± 0.2	B	72 ± 3.0
5	1450	34.3	3.3 ± 0.3	2.9 ± 0.2	B	62 ± 3.0
6		47.0	3.0 ± 0.3	2.7 ± 0.2	B	72 ± 2.8
7		53.2	3.0 ± 0.3	3.3 ± 0.3	T	72 ± 3.8
8		55.0	3.2 ± 0.3	3.5 ± 0.3	T	77 ± 2.5
9	3350	33.6	2.7 ± 0.2	2.4 ± 0.2	B	68 ± 2.6
10		39.6	2.3 ± 0.2	2.1 ± 0.2	B	68 ± 3.4
11		45.0	2.1 ± 0.2	2.0 ± 0.2	B	70 ± 2.4
12		48.1	2.2 ± 0.2	1.9 ± 0.1	B	76 ± 2.6
13	8000	27.1	3.7 ± 0.3	4.0 ± 0.1	T	75 ± 2.1
14		40.2	3.3 ± 0.3	3.0 ± 0.3	B	78 ± 2.6
15		45.0	3.0 ± 0.3	2.7 ± 0.2	B	78 ± 2.1
16		49.4	3.2 ± 0.3	2.9 ± 0.2	B	81 ± 2.8

Composition of the systems (1–16) is defined in Table 1. Vr, estimated from blank or loaded with biomass systems containing ethanol was determined after phase separation in graduated tubes. T and B denote top and bottom phase preference for cell debris. The recovery of lutein from the top phase (Y_{TOP}) is expressed relative to the initial amount of lutein content in the stock solution loaded to the ATPS.

ratio was observed compared to that from the cell debris-free ATPS, this implied that cell debris was accumulated in the bottom phase. Top phase cell debris accumulation was observed mainly in ATPS of low molecular mass of PEG (1000 and 1450 g/mol). Although a clear explanation for such behaviour is not currently available, the results obtained facilitate the selection of operating conditions to favour cell debris accumulation into a define phase.

In the case of lutein partition behaviour, it seems that for all the ATPS studied, the product partitioned preferentially to the top phase (lutein was not detected at the bottom phase) regardless the TLL of the system. It seems that increasing TLL caused the top phase recovery of lutein in ATPS to rise for each molecular mass of PEG used (e.g. PEG 1000, 1450, 3350 and 8000 g/mol). Such situation can be associated to the fact that the free volume of the top phase of ATPS rises when the TLL is increased [15]. Thus, the space available to allocate the solute is significantly affected by an increase in the TLL of the system. In the case of PEG 1000 ATPS, increasing TLL caused a significant increment in the top phase recovery of lutein (from 35 ± 2.0 to 72 ± 3.0). Systems comprising PEG 1450, 3350 and 8000 exhibited a similar trend but with less emphasis 62 ± 3.0 to 77 ± 2.5, 68 ± 2.6 to 76 ± 2.6 and 75 ± 2.1 to 81 ± 2.8, respectively (see Table 2). Such partition behaviour may be explained by changes in the free volume [16] and density of the phases [10]. It has been reported that the free volume in the bottom phase decreases when the TLL is increased [16]. As a result, the solutes in the lower phase may be promoted to partition to the top phase. Consequently, top phase recovery of lutein rise when TLL was increased.

For all the ATPS studied, more than 50% of lutein can be potentially recovered from the upper phase. However, system 16 in Table 2 exhibited the best recovery of lutein from the top phase (i.e. $81 \pm 2.8\%$). It is important to mention that the moderate values of top phase product recovery of systems of low molecular mass of PEG (1000 and 1450 g/mol) and short TLL (less than 35% (w/w)) were possibly affected by product accumulation at the interface confirmed by the mass balance (see systems 1 and 5 in Table 2). In PEG 3350 and 8000-salt systems of long TLL, lutein exhibited a more favourable partitioning to the top phase and cell debris to the bottom phase in comparison with that of PEG 1000, and 1450-salt systems. For an extraction system, a PEG 3350 and PEG 8000-phosphate system with long TLL (systems 12 and 16 in Table 2) were selected as the most favourable to maximize the partitioning of the product of interest and cell debris contaminants to opposite phases. It is clear that such result will facilitate the potential application of ATPS processes in the recovery of lutein from *C. prototheocoides*.

3.3. Effect of lutein concentration on PEG-salt aqueous two-phase systems performance

In an attempt to intensify the potential recovery process, the effect of increasing lutein concentration on the performance of ATPS was evaluated. It was decided to examine the impact of an increment on lutein concentration (from 28 to 120 ppm) on the product top phase recovery using selected ATPS characterized by PEG 3350 and 8000 g/mol molecular mass of PEG. Concentration range was selected to simulate final levels (28–120 ppm) in the system that would be obtained by extracting the product from fermentation origin [7]. The ATPS used in this part of the research (i.e. systems 12 and 16 in Tables 1 and 2) were selected on the basis of top phase product recovery from the previous section and bottom phase accumulation of cell debris. Systems 12 and 16 exhibited the best top phase recovery from ATPS of high molecular mass of PEG (3350 and 8000 g/mol). Fig. 1 illustrates the effect of lutein concentration in the system on top phase product recovery. It is clear that top phase recovery of lutein decreased for both ATPS when the concentration of the product of interest (lutein) increased. Such behaviour can be explained on the basis of losses of soluble product caused by lutein accumulation at the interface. In the case of losses of soluble product in the top phase caused by the increase in lutein concentration, PEG 8000-salt ATPS exhibited better performance compared with that of the PEG 3350 system. It is clear that and attempt to intensify recovery process by increasing the amount of biological material that can be processed (simulated by an increase in lutein concentration in the system), resulted in a negative influence to the top phase lutein recovery (Fig. 1).

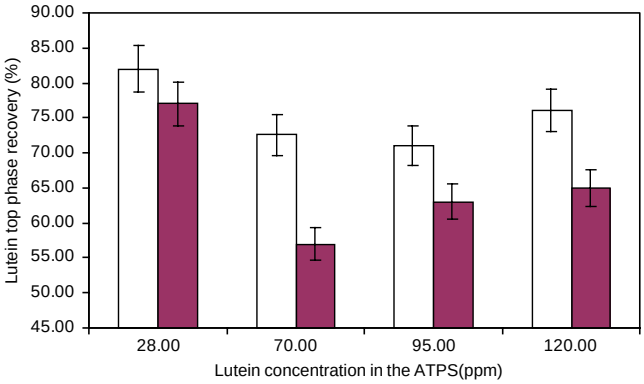

Fig. 1. Influence of increasing lutein concentration on product top phase recovery in PEG 3350 and PEG 8000—phosphate aqueous two-phase systems. The recovery of lutein from the top phase (Y_{TOP}) in PEG 3350 (■) and PEG 8000 (□) ATPS is expressed relative to the initial amount of lutein loaded to the ATPS. PEG 3350 and PEG 8000 ATPS were systems 12 and 16 identified in Table 1, respectively. For all systems, pH was kept constant at 7.0. The ATPS were constructed as described in Section 2. Results reported are the average of two independent experiments and errors were estimated to be ±5% of the mean value.

In general, from the ATPS studied, the system comprising Vr = 1.0, PEG 8000 22.9% (w/w), phosphate 10.3% (w/w) at pH = 7.0 provided the required conditions to concentrate lutein from *C. prototheocoides* in the top phase (i.e. top phase recovery of 81%) and cell debris in the bottom phase. It is clear that such findings will facilitate the potential generic application of the ATPS process to recover

lutein from microbial origin. In the case of top phase product recovery, although a yield of 81% for the ATPS primary step may be acceptable, potential strategies to improve the un-optimised performance of the ATPS process under higher product concentrations could be explored. They may include modifications in the level of ethanol in the ATPS, in order to maximize product solubility prior to subsequent top phase processing. However, at this stage the partitioning behaviour and the mechanism governing the partition of lutein in ATPS cannot be fully explained and further characterization to untangle such phenomena will facilitate the development of a commercial recovery process.

4. Conclusion

This is the first study to report the phenomenon of partitioning of lutein produced by *C. protothecoides* in aqueous two-phase systems. It was shown that tie-line length and molecular mass of PEG influenced the lutein concentration in the top phase. The addition of ethanol to favour the product dissolution increased the volume ratio of the systems. Increasing lutein concentrations (>28 ppm) in the ATPS resulted in reduced product yield. PEG 8000-phosphate ATPS proved to be suitable for the potential recovery of lutein (81%). The operating conditions selected for this particular ATPS system resulted in the partition of lutein preferentially to the top phase and cell debris to the bottom phase. Although the performance of the ATPS selected can be improved by further investigation, the findings reported here demonstrate the potential application of ATPS processes for the recovery of intracellular lutein produced by *C. protothecoides* as a first step for the development of a biotechnological process with commercial application.

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