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Simplified two-stage method to B-phycoerythrin recovery from *Porphyridium cruentum*☆

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Abstract

A simplified two-stage method for B-phycoerythrin (BPE) recovery from *Porphyridium cruentum* was developed. The proposed method involved cell disruption by sonication and primary recovery by aqueous two-phase partition. The evaluation of two different methods of cell disruption and the effect of increasing concentration of cell homogenate from *P. cruentum* culture upon aqueous two-phase systems (ATPS) performance was carried out to avoid the use of precipitation stages. Cell disruption by sonication proved to be superior over manual maceration since a five time increase in the concentration of B-phycoerythrin release was achieved. An increase in the concentration of crude extract from disrupted *P. cruentum* cells loaded to the ATPS (from 10 to 40%, w/w) proved to be suitable to increase the product purity and benefited the processing of highly concentrated disrupted extract. Kinetics studies of phase separation performed suggested the use of batch settlers with height/diameter (H/D) ratio less than one to reduce the necessary time for the phases to separate. The proposed ATPS stage comprising of 29% (w/w) polyethylene glycol (PEG) 1000 g/mol, 9% (w/w) potassium phosphate, tie-line length (TLL) of 45% (w/w), volume ratio (V_R) of 4.5, pH 7.0 and 40% (w/w) crude extract loaded in a batch settler with H/D ratio of 0.5 proved to be efficient for the recovery of 90% of B-phycoerythrin at the top PEG-rich phase. The purity of B-phycoerythrin increased up to 4.0 times after the two-stage method. The results reported here demonstrate the potential implementation of a strategy to B-phycoerythrin recovery with a purity of 3.2 (estimated by the absorbance relation of 545–280 nm) from *P. cruentum*.

Keywords: B-phycoerythrin; Recovery; Aqueous two-phase systems

1. Introduction

With the increasing commercial significance of colouring compounds from microbial origin used in food, cosmetic, detergent and molecular genetics industries, there is considerable interest in the development of efficient and scalable processes to bring such products to market. The reduced protocols reported for the primary recovery of intracellular coloured proteins involve release of the product by mechanical methods and their collection by centrifugation. The resulting homogenate is then fractionated to purify the colouring protein by an excessive number of stages. The entire protocols in most cases are complicated by the need for multiple chromatography steps to obtain a highly purified protein. The multi-step nature of the conventional methods can result in low process yield and high process cost [1]. Consequently, the potential scaling up of these procedures is perceived as economically unviable. To overcome some of the disadvantages attributed to the established purification protocols for coloured proteins, different approaches have been proposed. Such approaches involve the reduction of the chromatography steps [2] and the use of alternative recovery techniques such as aqueous two-phase systems (ATPS) [3]. One-step chromatography method involving precipitation with ammonium sulphate and ion-exchange chromatography has been recently proposed [2] for the particular recovery of R-phycoerythrin from *Polysiphonia urceolata*. In addition, the potential application of ATPS for the recovery of coloured proteins from microbial origin has also been addressed before [3]. In this context, B-phycoerythrin (BPE), a coloured phycobiliprotein found in nature (cyanobacteria, eukaryotic algae, etc.) [4,5], represents a very attractive study case. It has been reported that BPE can be used as a pigment in the food, cosmetic and pharmaceutical industry, and as a

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fluorescent marker [6–9]. The commercial value of highly purify BPE (defined as the absorbance ratio of $A_{545\,nm}$ to $A_{280\,nm}$ greater than 4.0), has been reported as high as $50 USD/mg [10]. This high value makes attractive the attempt to develop an efficient method for the recovery and purification of BPE.

The complexity of the processes for the recovery and purification of BPE has limited its potential practical implementation at commercial level. Such processes are usually characterized for having a high number of stages, low recovery and limitation to easy process scale-up. Previous attempt for the purification of BPE has been documented, for example the protocols reported by Bermejo et al. [8,11,12] involves ion-exchange high-pressure liquid chromatography or expanded bed absorption chromatography, which resulted in an effective way to purify BPE at laboratory scale. Although, some economic constrains may rise because at industrial scale, high pressure liquid chromatography involves high costs related to operation, these protocols represent attractive alternatives to consider for the purification stage of a potential downstream process to obtain pure BPE. In contrast, in the present research, the primary recovery of BPE is addressed using an alternative technique to produce material suitable for further purification.

Aqueous two-phase system is a technique that has proved to have great potential to recover and purify biological compounds [13–17]. A strategy for the primary recovery of BPE from *P. cruentum* using ATPS, ammonium sulphate precipitation and centrifugation was developed in our group [13]. However, the use of ammonium sulphate precipitation, centrifugation and pellet re-suspension were required in order to concentrate the BPE crude extract obtained by an unoptimized cell disruption stage using manual maceration. From this previous work, it was concluded that breakage of the cell wall of the algae *P. cruentum* was not a trivial task. Thus, the need for an alternative cell disruption method to eliminate unnecessary stages was evident. Furthermore, the potential use of ATPS to process high concentrate biomass extracts can also be exploited to optimize the proposed method.

It is clear that the development of a simplified method for the recovery of BPE from *P. cruentum* will benefit the performance of the previously established purification protocols. In this study, based upon a previous report from our group [13], experimental conditions were selected to address the potential process benefits using an alternative cell disruption strategy. Also, potential system intensification was attempted by evaluating the effect of increasing concentration of crude extract from disrupted *P. cruentum* cultures upon ATPS performance. Furthermore, in this work kinetics studies of phase separation under different extraction device geometries are presented, as a first step to establish initial operating conditions to BPE primary recovery from *P. cruentum*.

2. Materials and methods

2.1. P. cruentum culture and cell disruption

P. cruentum was cultivated in the culture medium previously described by Bermejo et al. [8]. The algae were grown in a batch culture (1000 ml Erlenmeyer flasks) at 22–25 °C under natural light conditions, agitation and aeration was provided with an air flow rate of 3.2 cm^3/seg using a peristaltic pump (ELITE 799, Rolf C Hagen Corp, Mansfield, MA, USA). The cells were allowed to grow for 30 days and were harvested by centrifugation (Eppendorf 5415C, Westbury, NY, USA) at 1,000 × *g* for 5 min. After harvesting, cell disruption was carried out using two different methods (i.e. manual maceration or sonication) in order to establish which of those two methods achieved a higher BPE concentration in the resulting crude extract. For manual maceration, the disruption was done using a ceramic pot under refrigeration using a dry ice bath. Wet biomass, glass beads and de-ionised water (4 cm^3/g wet biomass) were added to the ceramic pot and macerated for 10 min/g wet biomass. For sonication the disruption was done using a 50 cm^3 glass test tube and a sonicator (Branson 1510, Branson Ultrasonic Corp., Danbury, CT, USA). The glass tube was added with wet biomass and de-ionised water (4 cm^3/g wet biomass). The sonication time was 10 min/g wet biomass. During the sonication time, contents were mixed periodically to prevent the aggregation of the biomass at the bottom of the tube. In both disruption methods, cell fragmentation was verified using an optical microscope (Olympus CK2, Olympus, Melville, NY, USA). The homogenate obtained as the result of the cell disruption of the *P. cruentum* biomass was referred to as BPE crude extract (regardless the disruption method used), and included the cell debris generated.

2.2. Effect of concentration of BPE crude extract upon ATPS performance

ATPS were prepared for convenience on a fixed mass basis. Predetermined quantities of stock solutions of polyethylene glycol (PEG) and potassium phosphate (Sigma Chemicals, St. Louis, MO, USA) were mixed with different concentrations of BPE crude extract from 10 to 40%, (w/w); wet weight of disrupted biomass/total weight of the ATPS) to give a final weight of 10 g. The system parameters, selected from previous report [13], were: 29% (w/w) PEG 1000 g/mol, 9% (w/w) potassium phosphate, tie-line length (TLL) 45% (w/w), volume ratio (V_R) of 4.5 and pH 7.0. The stock solutions (PEG, potassium phosphate and BPE crude extract) were mixed and phases dispersed by gentle mixing for 10 min. Adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide when needed. Complete phase separation was achieved by low speed batch centrifugation at 1500 × *g* for 10 min. Visual estimates of the volumes of top and bottom phases were made in graduated tubes. The volumes of the phases were then used to estimate the experimental volume ratio (V_R, defined as the ratio between the volume of the top phase and the bottom phase). Samples were carefully extracted from the phases for biochemical analysis. The top and bottom phase recovery were estimated as the amount of BPE present in the phase (volume of the phase × BPE concentration in the phase) and expressed relative to the original amount loaded into the system. Interface BPE recovery was not experimentally determined due to the presence of cell debris in such phase. Results reported are the average of three inde-

pendent experiments and standard errors were estimated to be a maximum of ±5% of the mean value.

2.3. Influence of the system geometry upon ATPS performance and time of phase separation

In order to study the influence of the system geometry upon the recovery, purification factor of BPE and time of phase separation, batch settlers characterized by different geometries expressed as height/diameter (H/D) ratios from 0.5 to 2.5 were selected. For each set of experiments, two different ATPS comprising 29% (w/w) PEG 1000 g/mol, 9% (w/w) potassium phosphate were mixed with BPE crude extract to reach a final concentration of 40% (w/w) in the ATPS. The pH was adjusted to 7.0 when needed. After complete dissolution of the chemicals forming phases, the two phases of the ATPS identified in this work as "centrifuged" were separated by low-speed batch centrifugation at 1500 × *g* for 10 min at 25 °C. Samples were taken from these systems for biochemical analysis and further estimation of BPE recovery and purification factor (defined as the ratio of BPE purity from the phase divided by that from the crude extract). In contrast, once complete mixing of the chemicals forming phases was achieved phases from the "experimental" systems were allowed to separate under gravity (without further mixing) during which a record of the changing volume of the phases formed with elapsed time was kept. Volume of the phases was estimated using graduated settlers. Volume ratio (V_R) of the ATPS was estimated as a ratio of the volume of the top and bottom phases. The phase separation of PEG–potassium phosphate systems under gravity was expressed as the relative volume ratio (volume ratio of the "experimental" ATPS divided by that from the "centrifuged" system) relative to time. Results reported are the average of three independent experiments and the standard errors were estimated to be a maximum of ±5% of the mean value.

2.4. Analytical procedures

The total protein concentration in the samples was estimated by the method of Bradford [18]. The purity of BPE was determined as the relation of the 545–280 nm absorbance (purity of BPE = $Abs_{545\,nm}/Abs_{280\,nm}$). Bermejo et al. [8] reported the use of the 545–280 nm absorbance relation as an estimation of BPE purity, since the absorption spectrum of this protein exhibits a peak at 545 nm. Under this circumstances a ratio greater than 4.0 corresponds to a highly purify BPE (defined as pure commercial BPE; Sigma). The concentration of BPE and the other two coloured proteins produced by *P. cruentum* (Allophycocyanin (APC) and R-phycocyanin (RPC)) was estimated by measuring the absorbance at 565, 620 and 650 nm, and using the equation system reported previously [8,19].

3. Results and discussion

3.1. Cell disruption studies

P. cruentum (as well as other algae) has a cell wall and membrane structures, which confer it high resistance against a wide variety of cell disruption methods. Selection of an appropriate cell disruption method is required to break the cell wall and membrane structures of *P. cruentum* in order to achieve high release efficiency. The potential protocol for the recovery of BPE using ATPS initially designed [13] was characterized by the need of precipitation stages prior to ATPS. Such precipitation stages were introduced in order to avoid handling of a diluted BPE crude extract, obtained after cell disruption by manual maceration. A direct comparison between cell disruption by sonication and manual maceration was performed in order to increase the release of intracellular BPE. Fig. 1 illustrates the concentration of released BPE, allophycocyanin and R-phycocyanin when these two methods of cell disruption were used. It is clear that cell disruption by sonication proved to be superior for the release of intracellular coloured proteins from *P. cruentum* compared with cell disruption by manual maceration. In particular, the release of the product of interest (BPE) by sonication was 5.5 times higher than the one obtained using manual maceration (i.e. 0.22 mg/ml obtained by sonication compared with 0.04 mg/ml achieved by manual maceration). Coloured intracellular proteins are found inside the chloroplasts of the algae attached to the stromal side of the thylakoid membrane [20]. The constant and uniform forces generated by sonication were more effective than the irregular nature of that produced by manual maceration for detaching and releasing these pigments from the thylakoid membrane, and therefore the resulting concentration achieved was considerably higher.

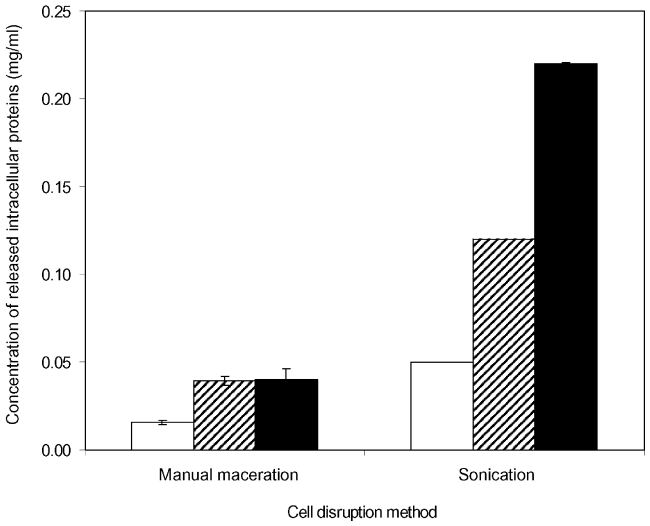

Fig. 1. Effect of methods of cell disruption upon the release of intracellular B-phycoerythrin (BPE), Allophycocyanin (APC) and R-phycocyanin (RPC) from *P. cruentum*. The concentrations of the three main *P. cruentum* intracellular coloured phycobiliproteins; RPC (□), APC (▨) and BPE (■) released are expressed relative to the method of cell disruption used. The concentrations were estimated as described in Section 2.

Interesting, the two selected cell disruption methods gave different patterns in the ratio of BPE to the other two coloured proteins (APC and RPC). Cell disruption by sonication produced a crude extract that exhibited a BPE to APC and RPC ratio greater than the one achieved by manual maceration. In red algae, as well as in cyanobacteria, the phycobiliproteins are assembled

into macromolecular water-soluble light harvesting complexes named phycobilisomes [21]. Phycobilisomes are formed by two structural subunits: a core complex and rod-like segments that are attached to the core complex. While the core complex is formed by phycobiliproteins that absorb in the red region (i.e. APC), the rod-like segments are formed by phycobiliproteins that absorb in the blue/green region of the spectrum (for example BPE and RPC) [5]. Rod-like segments of the phycobilisomes in *P. cruentum* are formed mainly by BPE (phycoerythrin reflects red light and is therefore responsible for the colour of most red algae). The rod-like segments are more exposed to the cytoplasm that the core complex which is attached to the thylakoid membrane. Therefore the cavitations generated by sonication are able to release BPE (that is located in the free end of the rod-like segments) more easily than the other two pigments (APC and RPC). This implies that the core complex is more likely to remain attached to the thylakoid membrane (or thylakoid membrane fragments) when sonication is used instead of manual maceration (which is a disruption method that generates a high mechanical impact over the membrane). As a result, the BPE to APC and RPC ratio achieved by sonication is greater than the one obtained by manual maceration. In the case of BPE purity obtained (purity of BPE = $Abs_{545\,nm}/Abs_{280\,nm}$), both disruption methods provided a crude extract with a purity of 0.8. This implies that even when the methods exhibited a different release patterns for the coloured proteins, the total amount of protein released in relation to the amount of BPE was similar. Using the concentrated BPE crude extract, obtained by sonication, allows eliminating concentration stages prior to ATPS, as the ammonium sulphate precipitation step utilized in the previous protocol reported [13]. Sonication was selected as the method for cell disruption to produce BPE crude extract for the subsequent experimental stages.

3.2. Effect of concentration of BPE crude extract upon ATPS performance

The potential to fractionate heavily loaded biological systems by ATPS has been proved [15,22]. Such biological systems include cells, cell debris, RNA, virus-like particles and proteins. Since an increment in the level of BPE crude extract concentration fractionated via ATPS may benefit the potential intensification of the proposed process, it was decided to examine the impact of highly concentrated BPE crude extract upon ATPS performance. The effect of the concentration of BPE crude extract upon ATPS performance is shown in Table 1. From our previous work [13] it was evident the strong top-phase preference exhibited by BPE in PEG-salt ATPS. Partition coefficient of BPE was difficult to evaluate due to the problems associated to the detection of BPE in the bottom phase. Thus, it was decided to use the top-phase BPE recovery and purity (or purification factor) as the response variables to evaluate the performance of ATPS. For the present work the systems conditions (e.g. molecular weight of PEG, TLL, and system pH) previously established [13] were selected. Large V_R (volume ratio; 4.5) was used to favour the recovery of BPE from the top phase by increasing the free volume available for the target protein to accumulate under increasing crude extract concentration conditions. From Table 1, it is clear that top-phase recovery over 90% was achieved, regardless the concentration of BPE crude extract loaded to the ATPS. Such robust behaviour can be explained in terms of the excluded volume of the top phase of the system [23]. Partition of BPE to the top-PEG-rich phase in ATPS is favoured by using PEG of low molecular weight [13]. Such behaviour has been attributed to the fact that the interaction net of short polymer chains generates a lower excluded volume than the one created by long chains. PEG of low molecular weight (for example, 1000 and 1450 g/mol) helps to overcome saturations problems at the top phase, and therefore BPE is able to migrate at the top phase. Additionally, the ATPS used were characterised by a volume ratio (V_R) greater than one (i.e. $V_R = 4.5$). As the volume of the top phase increases, the free volume available for the BPE to migrate at such phase also increases. An increase in the concentration of BPE crude extract in the system caused BPE purification factor (defined as the ratio between the BPE purity from the top phase and that from the crude extract) to rise (see Table 1). The BPE purity and the purification factor at the top phase increases from 1.3 to 3.2 and from 1.6 to 4.0, respectively (Table 1). When the concentration of BPE crude extract increments (from 10 to 40%, w/w), the amount of BPE presents in the system also increases. The increment in the amount of BPE (a protein with high affinity for the top phase) loaded to the systems favoured the increase of the amount of this target protein partitioned to the top phase. Once the particles with high affinity for the PEG-rich phase have partitioned, molecules with lower affinity can migrate to the upper phase. However, an expected decrease in the free volume available of the upper phase may compromise the further accumulation of the contaminant proteins in this phase [15,16].

Table 1
Influence of the concentration of *P. cruentum* crude extract upon B-phycoerythrin (BPE) recovery and purification factor

	Concentration of crude extract (%, w/w)	Top-phase BPE recovery (%)	Top-phase BPE purification factor
1	10	99.1 ± 3.15	1.6 ± 0.08
2	20	92.6 ± 0.29	2.0 ± 0.02
3	30	91.8 ± 0.22	2.1 ± 0.02
4	40	91.9 ± 0.17	4.0 ± 0.02

The top-phase B-phycoerythrin (BPE) recovery is expressed relative to the concentration of BPE from crude extract loaded into the systems. Concentration of the crude extract represents the total amount of disrupted BPE biomass loaded into the ATPS and it is expressed in wet weight of disrupted biomass relative to the total weight of the ATPS (%, w/w). The BPE purification factor is the ratio between the BPE purity obtained at the top phase of the system and the one from the BPE crude extract. The ATPS used comprised 29% (w/w) PEG 1000 g/gmol, 9% (w/w) potassium phosphate, TLL of 45% (w/w), volume ratio (V_R) of 4.5 and pH 7.0.

3.3. Influence of system geometry upon ATPS performance and time of phase separation

In order to facilitate the practical implementation of lab-scale ATPS protocols at commercial level, the evaluation of system

Table 2
Settlers used to evaluate the influence of the height/diameter (H/D) ratio on kinetics of the phase separation

Geometry	Settler device description	H/D ratio	Cross-section area (cm^2)
1	Glass vessel	0.5	29.0
2	Glass vessel	0.8	20.0
3	Glass vessel	1.2	18.0
4	Glass column	2.5	10.0

The H/D ratio values are the result of the height (H) of the ATPS in the settler divided by the diameter of the settler (D). The cross section-area (interface area) was estimated with the diameter of each settler used.

parameters that provide information relate to the kinetics of phase separation is needed. Exploitation of the potential use of settling equipment for the recovery of biological compounds using ATPS represents a major advantage of the technique for its industrial implementation. It is clear that passive settling to separate immiscible phases is simple in operation and incurs low cost. It has been reported that the settling velocity or phase separation time is strongly influenced by the geometry of the settler used [24,25]. The design or selection of an appropriated geometry of the batch settler is required to facilitate the implementation of ATPS at industrial scale. It is known that in batch settlers, the height (H) and the diameter (D) of the device influences the settling time due to the distance that the droplets must move and the wall effects caused, respectively. In this part of the study, a practical approach that involved the use of height/diameter (H/D) ratio of the settler as a dimensionless parameter was selected to evaluate the performance of ATPS in settlers of different geometries. Such parameter was selected to characterise the influence of the geometry of the settlers (from column to tank or vessel type; H/D ratios from 0.5 to 2.5; see Table 2) on the total time for the phases to separate.

Initially, the influence of the H/D ratio of the batch settlers upon the ATPS performance was evaluated. In this case, the BPE top-phase recovery and the BPE purification factor were not significantly affected by the change in system geometry (H/D ratio; data not shown). Such behaviour can be explained by the fact that changes in geometry (H/D ratio) do not affect the physicochemical properties or parameters of the ATPS (e.g. concentration of polymer and salt, ionic force, pH, V_R, etc.). In contrast, it has been reported that the system geometry have significant influence upon the time of phase separation [24,25]. However, the nature of such effect upon ATPS loaded with BPE crude extract has not been reported before. The influence of the H/D ratio upon the time of phase separation in ATPS loaded with 40% (w/w) of disrupted *P. cruentum* is illustrated in Fig. 2. The solid concentration (40% wet, w/w of the disrupted biomass) was selected based upon the results from the previous section of this work. An increment in the H/D ratio causes the time needed for the phase formation to rise. For the two lower H/D ratios used (i.e. H/D equal to 0.5 and 0.8) a relative V_R of 0.7, which represents 70% of the phase separation efficiency by sedimentation relative to that obtained by centrifugation (used only as a reference), was achieved after

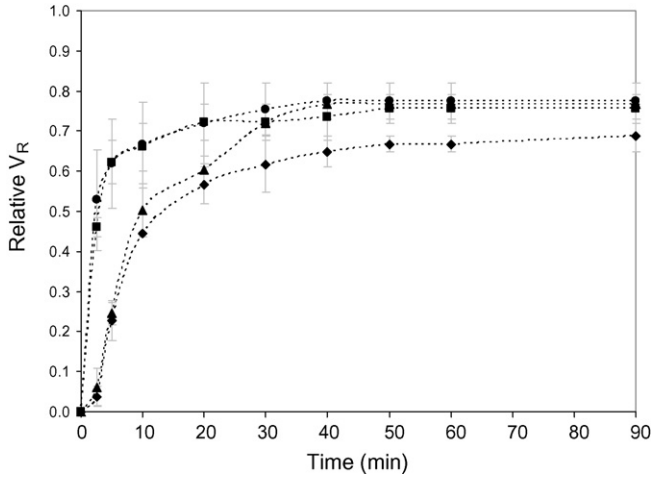

Fig. 2. Influence of the height/diameter ratio of the settler upon the kinetics of phase separation in batch ATPS. Four different height/diameter ratios were used: 0.5 (●), 0.8 (■), 1.2 (▲) and 2.5 (♦). The relative volume ratio (V_R) is defined as the ratio between V_R of the experimental system and the V_R of the centrifuged system as described in Section 2 and it is expressed relative to time. The ATPS used comprised 29% (w/w) PEG 1000 g/gmol, 9% (w/w) potassium phosphate, TLL 45% (w/w), V_R of 4.5, pH 7.0 and 40% (w/w) BPE crude extract load.

20 min. In contrast, for H/D 1.2 and 2.5 a relative V_R of 0.7 was achieved after 30 min and more than 90 min, respectively. This can be explained in terms of the interface area available (cross-section area) in the system (Table 2). Batch settlers with low H/D ratios are characterised by sufficient available interface area (compared with settlers with H/D higher than one) for solids distribution. Solids such as cell debris and other suspended particles present in the BPE crude extract can distribute across the interface (the middle part of settling device characterised by the presence of unseparated phases) and minimize solid accumulation. Thus, it is expected that phase separation under gravity will occur in a relatively short time. In contrast, in batch settlers with higher H/D ratios (e.g. H/D = 2.5) Table 2; the interface area is reduced, and therefore solids accumulate rapidly. Consequently, the time needed for phase separation by gravity is increased. The kinetics of phase separation followed up to 24 h, and the maximum relative V_R achieved was 0.82 for all H/D ratios (data not shown), which represents 82% phase separation efficiency achieved by sedimentation. Cell debris accumulated at the interface, extended to the bottom phase, thus a clear bottom phase was difficult to achieve in these ATPS, and caused an increment in the volume of such phase. Consequently, reaching a 100% phase separation efficiency of the systems separated by gravity was severely limited. The batch settlers with H/D ratio of 0.5 and 0.8 exhibited the best separation performance. These findings suggest the use of settlers with H/D ratios less that 1.0 to minimise the time for the phases to separate. This type of study provides an insight into the kinetics of phase separation necessary to design of appropriate equipment for the required pilot scale of the proposed ATPS process.

A direct comparison of the new proposed protocol for the recovery and purification of BPE from *P. cruentum* with the previous reported protocol involving BPE extract concentration and

cell debris separation stages [13] highlights the superiority of the new approach. Implementation of an alternative cell disruption method resulted in a reduction of the number of unit operations involved in the previous reported protocol by eliminating concentration stages resulting from poor extraction. Cell disruption achieved by sonication would of course be impractical at process scale and would need to be replaced by a mechanical cell disruption method (e.g. bead mill or homogenization). The outline of the new proposed method does not involve an additional cell debris removal stage due to the fact that ATPS was used to eliminate the cell debris. Thus, cell debris removal and BPE primary recovery stages were integrated. It is clear that, for BPE this two-stage method opens the way to potentially evaluate further bioprocess scale up and enhancement.

4. Conclusions

A two-stage method for the recovery of BPE from *P. cruentum* was developed that benefited from a reduced number of stages. It has been shown that cell disruption by sonication was preferred over manual maceration for the release of intracellular BPE, since a five times increase in BPE concentration, release was obtained with a similar degree of purity. An increase in BPE crude extract loaded to the ATPS (from 10 to 40%, w/w) proved to be suitable to increase the product purity and benefitted the processing of highly concentrated disrupted extract. In the case of kinetics of phase separation studies, it was concluded that H/D ratio has no effect upon the purity and the top-phase recovery of BPE when batch settlers were used. It was also concluded that the geometry of the separation device (expressed as H/D ratio) has an effect on the phase separation time. Batch settlers with H/D ratio less than one were particularly suitable to achieve a rapid phase separation. Overall the proposed ATPS process comprising of 29% (w/w) PEG 1000 g/gmol, 9% (w/w) potassium phosphate, TLL of 45% (w/w), V_R of 4.5, pH 7.0 and 40% (w/w) BPE crude extract loaded in a batch settler with H/D ratio of 0.5 proved to be efficient, recovering above 90% of BPE at the top phase (PEG-rich phase) with a purity of 3.2. It is clear that the results reported here, demonstrated the potential implementation of a method for BPE recovery from *P. cruentum* as a first step for the development of a process with commercial application.

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