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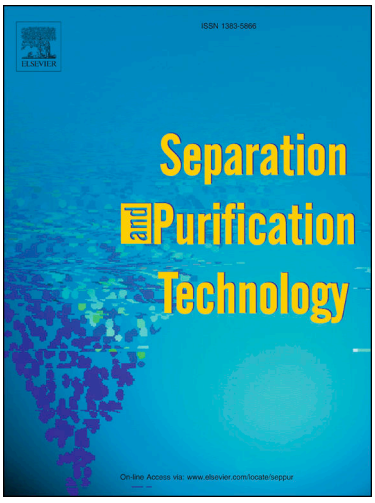

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Economic Analysis of the Production and Recovery of Green Fluorescent Protein Using ATPS-Based Bioprocesses

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Abstract

Green fluorescent protein (GFP) is a useful biomolecule in biotechnology; however, its price makes its widespread application prohibitive. To overcome this issue, recently, the use of aqueous two-phase systems (ATPS) for GFP purification was proposed as an alternative platform to reduce processing costs. Aligned with this goal, this study performed bioprocess modelling coupled with economic analysis using the software Biosolve to evaluate the potential and commercial applicability of ATPS for GFP purification. This work analysed a collection of fourteen ATPS to discriminate through production costs while also incorporating the concept of product purity into the calculations. The two best systems (a PEG-based and an ionic liquid (IL)-based ATPS) were placed in a full bioprocess at different scale models (1 to 100 L) to elucidate the viability of applying ATPS at large scale. Although the results showed that the PEG-based ATPS exhibit the lowest costs (between USD $3.5x10^3.g^{-1}$ at 1 L and USD $0.33x10^3.g^{-1}$ at 100 L), for further developments, the inclusion of an ATPS granting a higher purity is desired for the development of simpler bioprocesses. Therefore, as a third approach in this work, a sensitivity analysis was performed to determine the impact of varying different model parameters (recovery yield, material costs discount and production titre), to elucidate the circumstances under which the IL-based system can overcome the production costs of the traditional PEG-based ATPS. The results indicate that the best cost-effectiveness approach is to improve the production titre (although it can affect all ATPS studied), as an increase from 1.33 to 3.8 g/L is enough for the IL-based ATPS to be less expensive than the traditional system at all analysed scales. This study demonstrates that ATPS can greatly reduce GFP manufacturing costs, which can potentially help to popularize new applications of fluorescent proteins that are currently mostly restricted to research kits due to their high prices.

Keywords: *Aqueous two-phase systems, economic evaluation, green fluorescent protein, bioprocess modelling, ionic liquids*

1. Introduction

Green fluorescent protein (GFP) is a biomolecule that has a widespread use in biotechnology [1, 2]. Most of its applications are as a biomarker and biosensor [1], which demand for high-purity levels. Therefore, and despite of its wide acceptance in biotechnology-related fields, the major drawback for a widespread use is its high cost, for example, it is being sold for around USD 2,000 *per* mg by Biovision Incorporate company [3].

Currently, there is already a profitable field for GFP in the Reporter Gene Assay Kits market (which includes fluorescent proteins and enzymes like luciferase), which achieved a market value of USD 1.6 billion in 2019 [4]. However, considering the exorbitant current prices of GFP (ranging from around 350 to 600 dollars *per* 0.1 mg) [3, 5-7], this application is only commercially viable because it uses very small concentrations of the protein (only around 0.1 mg of GFP *per* kit for 100 assays) [3, 8, 9]. Moreover, other fields of application are emerging, these enclosing innovative products in the medical [10, 11], energy [12] and textile [13] fields, which are still in their infancy, mainly because of the impeditive prices of fluorescent proteins.

There are several reasons that can explain the commercial GFP extremely high prices. As previously stated, the main market of fluorescent proteins today is directed towards assay kits, generally produced by pharmaceutical industries. Although very high, the general price *per* kg of GFP (approx. 2 billion USD per kg) is similar to other pharmaceutical proteins, like recombinant factor VIII and activated factor VII for hemophilia treatment, and recombinant Hepatitis B surface antigen for vaccine production costing from 2 to 10 billion USD *per* kg [14]. This is very distinct from the price of industrial proteins like cellulase and rβ-Glucosidase for ethanol production, which cost ranges from 10 to 37 USD *per* kg [14].

There are many arguments regarding this big difference in price from industrial and pharmaceutical proteins, like the use of the more costly mammalian cells over bacteria and yeast for the expression of the recombinant proteins, the higher degree of purity required, the strict quality controls for production of pharmaceutical-related products, in addition to more R&D costs (usually because of pre-clinical and clinical trials), patenting, and marketing [14]. However, some researchers argue that this is not enough to explain the 8-9 orders of magnitude of difference in price between industrial and pharmaceutical proteins, and that there is a lack of transparency in pharmaceutical industries that prevent an open discussion in the topic to evaluate if the prices are justified [14]. For GFP, this difference is even more evident, particularly since it is mostly produced by bacteria and had no private R&D and patenting

costs, viz. GFP was developed in academia and not under a patent [1, 2]. The unique plausible justification for its exorbitant prices, even if weak, would be related to the high degree of purity required for its commercialization. Hence, simpler and cheaper purification platforms could be an alternative to allow for industries, besides pharmaceutical complexes, to produce recombinant proteins with more competitive prices.

In the last years, some studies are aiming to decrease GFP price by developing new ways to produce it successfully [15, 16]. Mainly two strategies are in place, with the first one focusing on improvements in upstream processes by obtaining high titres using recombinant *Escherichia coli*, the current available commercial product, [17] or tobacco plants [18] to express the protein. Alternatively, there are also studies aiming to reduce costs by improving the recovery and purification techniques in the downstream process as they can represent a large fraction of the production costs [19, 20]. Particularly for biomolecules with medical application, like *in vivo* GFP-based biomarkers/biosensors, a remarkably high purity degree is required to avoid impurities, which can cause an inappropriate immunogenic reaction and harm the patient [21]. Therefore, the purification of pharmaceutical biocompounds is usually complex and based in multi-step platforms [22]. For example, the current strategies for GFP purification involve, mainly, the use of multiple chromatographic units, which are complex and costly [18, 23-27]. Recent studies were successful in the application of alternative methods such as aqueous two-phase systems (ATPS) for the recovery of high purity GFP [28-33] or a combination of ATPS and chromatography [18], which can potentially be applied for more cost-effective large-scale processes.

ATPS are biphasic systems that can be used in liquid-liquid extraction processes for the partition of biomolecules through a series of different physicochemical phenomena, [34] and that can be tuned to selectively isolate a compound in one phase while keeping the contaminants in the other phase [35]. These systems have been proposed as alternatives for the recovery of chemical and biological products, with some outstanding cases, where they can replace conventional unit operations to yield a high purity [36-39]. Current applications include the recovery and/or purification of small molecules, but also proteins, enzymes, antibodies, cell debris and cells (including stem cells), as well as innovative applications in the areas of materials science and electrochemistry [38, 40, 41]. One of the main advantages of ATPS is its potential to reduce the production costs by using relatively inexpensive materials, in contrast to other separation processes. Although several works have been published proposing ATPS for the separation and purification of a wide variety of bioproducts, they have seldom been

used at industrial scale. Practical industrial implementation of ATPS is quite limited and most works reporting it are more than 20 years old [41, 42].

Recently, a collection of fourteen ATPS was successfully screened for the purification of GFP [33]. Briefly, the ATPS analysed were composed of mixtures of polymer/salt, polymer/polymer or polymer/ionic liquid (IL) aqueous solutions, achieving high recovery yields of GFP (close to 100%), with purities ranging from 70% to around 100% of GFP from the total protein content. In that work [33], the selection of the best purification system was solely based on the recovery and purity yields, without any environmental and economic input regarding the ATPS forming components. However, aiming for future implementation at commercial scale, or at least to call the interest from the industrial partners of ATPS-based platforms, an adequate economic analysis should be considered.

The economic evaluation of bioprocesses has been a successful tool employed before to determine the viability and potential applicability of stainless-steel or single-use bioprocesses [43]. Together with bioprocess modelling (through the utilization of specialized software) [44], it allows to perform analysis of processes while they are still at laboratory scale or under development. Software employed with such purpose typically comprises the SuperPro Designer (Intelligen, Inc., Scotch Plains, USA) and a collection of products developed by Aspen (Aspen Technology Inc. Cambridge, USA). Both have been contrasted before highlighting their [44], although these have been specially designed to create chemical and bioprocess models focusing more engineering details. For economic evaluations purposes, the Biosolve Process (Biopharm Services, Buckinghamshire, UK) appears as an interesting alternative, since it provides mainly a model design focused on economically-relevant parameters. The use of bioprocess modelling coupled with economic analyses can help to reduce the amount of experiments needed and focus on work that will ultimately contribute to a better development. Additionally, it is possible to create scenarios that might hinder the cost and their impact in the process, allowing the development of control strategies and preventing future problems. Examples of successful application of this joint modelling approach include contrasts between stainless steel and single-use equipment, batch and continuous fermentation [45-47]. Moreover, ATPS were also studied before by analysing its incorporation into a theoretical process, for example comparing its applicability against other downstream techniques [37, 48, 49].

The objective of the present study is to perform an economic evaluation using bioprocess modelling on the production and recovery/purification of GFP (enhanced GFP, EGFP) through the application of ATPS. This work analysed the database of the fourteen systems previously studied[33], while also incorporating the most cost-effective systems into a complete bioprocess model at different production scales to obtain high purity GFP. A sensitivity analysis was performed to evaluate the impact of potential variations of critical process parameters. This work is expected to serve as a platform for further improvements on the large-scale production of GFP, as well as to direct future researches regarding the effective industrial implementation and/or commercialization of ATPS-based purification platforms.

2. Model Set-up

The model creation was performed in Biosolve Process (Biopharm Services, Buckinghamshire, UK). Following previous insights [50], the construction of the bioprocess model covered the following three main areas: *i*) design of the production scenarios and target output; *ii*) the sequence of unit operations and process parameters; *iii*) and the economic datasets. These areas were interconnected, being filled in no particular order. The experimental results of our previous publication [33] was used as database for the extraction of the inputs to fill the model.

For the design of the production scenarios and target output, the scale range for the fermentation working volume (considering 80% capacity) was selected between 1 and 100 L. In this context, the first analysis was carried for comparative purposes among the collection of 14 ATPS. Therefore, and to compare all systems under the same working conditions, in this initial set, an arbitrary scale with a constant volume of 10 L was selected for all ATPS. The production titre remained constant at 1.33 $g.L^{-1}$, accordingly to what was reported in the experimental base study. Also, sample fed into the ATPS was constant at 10 wt%, as tested before. Together, bioreactor scales (1 to 100 L) and the sample input in the ATPS (10 wt%), will provide ATPS sizes from approximately 100 to 1,000 L (depending on their density), which are some of the largest ATPS reported for real implementation [42].

The next step was to design the sequence of unit operations and process parameters. For that, an initial analysis contrasted the fourteen ATPS (isolated from a complete process, *i.e.* no production of GFP and no removal of phase forming chemicals), by considering only materials and consumables as variables. This approach is adequate for an initial screening, since all ATPS are modelled at the same scale (10 L), and consequently, the operation equipment,

production titre (1.33 $g.L^{-1}$) and feed (10 wt%) remain equivalent. In the second part of the study, an overall theoretical bioprocess centred on ATPS as the main unit was designed (**FIGURE 1**). Briefly, a fermentation operation was designed to produce GFP using *E. coli* cells. Since GFP is produced intracellularly, after the GFP production unit, a centrifuge unit was added to collect the bacterial biomass. A homogenizer was then included to disrupt biomass, followed by an additional centrifuge to remove the cell debris, and recover a clarified broth (raw extract containing GFP). As the main purification stage, an ATPS-based unit was included for the recovery/purification of the GFP. An ultrafiltration/diafiltration (UF/DF) step was added as the last operation unit, for both the removal of phases' components and GFP polishing (*i.e.* GFP is concentrated in a PBS buffer).

All process parameters (**FIGURE 1**), fermentation conditions/results and ATPS compositions/results (**TABLE 1**) were taken from the base experimental study [33]. Theoretical operations (centrifugal separations, homogenization and UF/DF) units were constructed using Biosolve Process defaults (recovery yields, equipment and consumable selection) with some variations. It is important to note that the process times were fixed, while the size of each operation unit was scaled to guarantee the same processing time. It is important to note that, since the main objective was to perform mainly a comparison regarding the incorporation of different ATPS in the whole bioprocess economics, all the other unit operations of the entire process (*e.g.*, disk-stack centrifuges, ultrafiltration apparatus) were maintained, independently of the production scale. Additionally, processing times were maintained at all scales and not ancillary or seed equipment were considered for modeling.

The third pillar is the economic dataset (**TABLE 2**), which is the one that populates the model with the production costs. Cost data in Biosolve comprises 5 topics: capital, materials, consumables, labour, and others (a collection of waste disposal, maintenance and utilities). For capital calculation, Biosolve can calculate the capital to include the associated costs for the construction of a complete production facility. For the present study, this was limited solely to the equipment costs, as the aim is to focus on production. The equipment cost was taken from Biosolve database and, for the analysis of different scales and in order to fulfil intermediate sizes not present at the database, different regressions were then calculated (**TABLE 2**). Approximate values of the materials costs were taken from Sigma-Aldrich® at their largest available size in order to overestimate actual manufacturing prices (which are usually lower due to the economy of scale). Consumables costs were obtained from the Biosolve database. Labour costs, as carried out in previous reports [51], were fixed as 15% of the total production

costs, which have been found to be in an appropriate range across different applications. Lastly, the section "other" costs, which comprises waste disposal, maintenance and utilities, was left to be calculated automatically by the Biosolve software. The cost data presented has been taken from USA websites (for materials) and Biosolve database contains an approximation of costs from worldwide suppliers giving the user an average cost, both reviewed during the year 2019.

A full economic analysis can employ real facility data, but in order to maximize resources, this also needs to be done before the facility is actually build. Hence, despite the lack of data for some operations, this is still a promising test to perform a preliminary study to determine areas of opportunity and decrease future consumption of time and resources. Results collected from economic analysis models usually comprise the production cost *per* unit of product mass (*i.e. per* gram or *per* dose, etc.). In the present work, this is the production costs *per* gram of GFP at the end of the process (either after only ATPS for the first analysis or at the end of the process for the rest of the work), it includes the capital investment (mainly equipment acquisition) and cost of goods (materials, consumables, labor and others), and it is denoted by CoG+CI/g.

3. Results and Discussion

To study the impact of ATPS on GFP recovery/purification, this work designed a series of different scenarios for a thorough analysis. The first analysis contrasted the fourteen ATPS from the base study. From this initial screening, the top two ATPS (lowest CoG+CI/g) were selected and incorporated into the complete process model (**FIGURE 1**). Afterwards, both complete processes were subjected to a sensitivity analysis by varying systematically the recovery yield of each ATPS, a potential discounted cost of materials and consumables, and the production titre.

3.1 Economic Analysis of ATPS Base Data

The assessment of fourteen different ATPS in the base study included a combination of traditional and novel systems, each with different GFP recovery and purity yields (**TABLE 1**). The traditional systems comprise the polymer/buffer-based ATPS (7 and 8 in **TABLE 1**), while the novel ATPS include the polymer/polymer + [Ch]Cl as adjuvant (1 to 6 in **TABLE 1**) and polymer/[Ch]Cl-based ATPS (9 to 14 in **TABLE 1**). In this study, the contrast of the fourteen systems was performed at a fixed 10 L volume scale. Although their production costs are

incomplete, because of the ATPS are screened in a standalone manner, they are still comparable in between them. Results for the first economic analysis are shown in **FIGURE 2**. It should be noted that this screening intended to select the most economic ATPS, but its complete costs are obtained after their incorporation into the full bioprocess (next subsection).

As mentioned before, results obtained from all analyses here comprise the CoG+CI/g. However, one parameter that is not included in the costs in other studies is the concept of the product purity. Indeed, regardless of the purity of the product obtained by any given process, the cost cannot change. However, when designing a full downstream processing plant, purification is critical for the decision-making because certain applications are associated with legislation, which demands to attend standard degrees of purity and product quality.

In this study, the set of ATPS yielded different recoveries (from 69.9 to 100%) and purities (89.2 to 100%) and they do not always match, this means that there are systems that grant a high purity with low recovery, a low purity with high recovery and combinations in between. In the context of process design, this means that having an ATPS-based bioprocess that grants a high recovery but a low purity will require further unit operations to obtain a relatively pure product. The opposite is also true, as a process with an ATPS with high purity (regardless of yield) will require less (or none) additional unit operations to accomplish the desired purity standards of the given bioproduct (in this case, GFP). Since there is a non-linear positive relationship between product purity and price, this creates a great area of opportunity to use ATPS by obtaining high pure products with a relatively low-cost unit operation, and consequently to maximize potential profits.

Therefore, to do a proper contrast, herein the concept of product purity was included into the production costs. Although it is well known that the price is not directly proportional to the purity of the bioproduct, to consider the purity in the production costs, in this work, a simpler approach was performed by dividing the CoG+CI/g by the percentage of purity reported for each ATPS (**TABLE 1**). This means that a high purity ATPS will leave almost unaffected the CoG+CI/g, while a low purity will increase GFP production costs. This strategy is the simplest form to analyse this issue, considering that there is no available data on what other operations are required to improve the purity yields, as well as on how the further operations will work in combination with ATPS.

Results from **FIGURE 2** show that the least expensive ATPS is system 7 (ATPS 7 is composed of PEG-1500 and K_2HPO_4/KH_2PO_4), usually classified as a traditional system. Medium-level

costs were obtained for IL/salt ATPS, and the most expensive were the sodium polyacrylate (NaPA)-based systems. Interestingly, NaPA-based ATPS allowed the highest recoveries for GFP (approx. 100%), while PEG/salt and IL-based systems exhibited recoveries around 90%, but the costs for these last two ATPS types were still lower, even considering the lower recovery yields. Purity, on the other hand, was the highest on IL-based systems, followed by PEG/salt and NaPA-based systems, respectively. Noteworthy, the incorporation of purity changed the economic analysis results. For example, if considering the purity in the CoG+CI/g, the respective costs of ATPS 7 (PEG-1500 + K_2HPO_4/KH_2PO_4), which has a low purity (73.9%), increased and levelled with ATPS 13 (polypropylene glycol (PPG)-400 + cholinium chloride ([Ch]Cl)). Even considering that [Ch]Cl has significantly higher costs than the common phosphate-based salts, the higher purity yields (100%) of the PPG-400/[Ch]Cl ATPS turns it economically attractive. These two systems became the ones with the lowest costs, while the others, despite presenting the lowest initial CoG+CI/g, were regarded as more expensive after factoring purity.

3.2 Economic Analysis in a Complete ATPS-based Bioprocess

After the first economic screening, ATPS 7 (PEG-1500 + K_2HPO_4/KH_2PO_4) and 13 (PPG-400 + [Ch]Cl) were chosen to proceed into the study of the effect of their incorporation into a complete bioprocess. In this evaluation, the incorporation of purity into the CoG+CI/g determination was not considered due to the unknown effect of incorporating additional unit operations in the final EGFP' purification. At this point, the two best systems were selected, these enclosing the "traditional" and the most performant "alternative" ATPS, aiming to evaluate which system can be more affordable mainly in terms of material components. Moreover, this economic analysis was performed for multiple bioreactor scales (1 to 100 L), corresponding to ATPS from 10 to 1,000 L, respectively. The results of both ATPS-based bioprocesses analysis are summarized in **FIGURE 3**. Results presented comprise the CoG+CI/g for both ATPS as an independent unit operation, but also as part of an integrated bioprocess. Full results and its breakdown in cost categories are detailed in **Supplementary Material 3** document.

From **FIGURE 3**, it seems that the complete process will always yield higher production costs, as more complex processes have higher costs associated with them. However, operating at larger scales offers advantages like higher amounts of product are generated, while the

acquisition costs for equipment behave non-linearly, meaning that the CoG+CI/g tends to decrease and stabilize at large scales [50, 52]. This in turn means that a process has a higher potential of becoming economically viable at larger scales. Comparing both ATPS-based bioprocesses, it is observed from **FIGURE 3** that, as the scale increases, the differences between the costs are enhanced. At small scales, the differences are small, being the ATPS 13 (PPG-400/[Ch]Cl) 1.5 times more expensive than the traditional PEG/salt system (ATPS 7). However, with the scale-up, the differences of the process costs also increase, achieving costs up to 6.8 times higher for ATPS 13. The reason behind this is that the materials needed for ATPS 13 preparation, namely PPG-400 and [Ch]Cl, are more expensive and, at larger scales, this contribution adds up, causing a larger difference in CoG+CI/g. This phenomenon is also noted by how much the CoG+CI/g can decrease for each ATPS-based bioprocess. The CoG+CI/g can decrease up to 10 times for ATPS 7, while only 4.2 times for ATPS 13 (when contrasting the production cost at 1 L and 100 L scales for each ATPS). As part of a complete process, the ATPS composed of PEG-1500 and K_2HPO_4/KH_2PO4 (ATPS 7) is a better choice due to lower production costs and the potential to further decrease as the bioreactor scale increases.

These results are highly significant when analysed in the context of real-life production, as product purity is significantly different between the two ATPS-based bioprocesses. If the GFP final product is employed as a highly purified protein for labelling or detection in medical applications, the possible use of ATPS 13 is still relevant as no additional unit operations (beyond those needed for pharmaceutical standards) are needed to increase the product purity (already at 100%). On the other hand, ATPS 7 will need significant work to increase it, as GFP currently stands at a purity of 73.9%, and probably it will require additional and more expensive chromatographic units.

Envisaging the future implementation of ATPS 7-based bioprocess in the purification of GFP, if a high level of purity is needed for whichever application, additional chromatographic operation units will need to be incorporated. In literature, some studies have successfully applied chromatographic operations to purify GFP [18, 27], while others have already addressed the cost implication of incorporating chromatographic operations [53] for purification of proteins. Although there is no economic evaluation of chromatography processes for purifying GFP, the economic evaluation of a bioprocess for production of uricase clearly demonstrated that the inclusion of chromatography generates a large build up in the costs associated to the capital investment and consumables, as more complex equipment is

needed and resins are more expensive than phase-forming chemicals [53]. Therefore, although ATPS 7 is, as an isolated unit operation, cheaper than ATPS 13, it will also demand additional and, possibly, more complex and costly stages to reach the same level of purity reached with ATPS 13. However, considering that other novel applications of fluorescent proteins in the energy and textile fields do not always require > 99% purity of GFP [12, 13], it is also important to demonstrate other viable options for its production aiming to reach less strict markets. Hence, for the traditional applications of GFP in assay kits, which need very high purity levels, ATPS 13 is likely a better option for allowing a simpler production chain, while ATPS 7 can enable more affordable GFP for its novel applications with lower purity requirements.

3.3 Sensitivity Analysis

The application of bioprocess modelling allows to design and test different scenarios, even those that are beyond current status of development work enabling to test the limits of operability. This also helps to cover a wider range of scenarios and determine how far a parameter needs to be modified in order to achieve a desired effect. Ultimately, this can be used as a guideline of where resources and time should be invested for process development and optimization.

In previous sections, it was shown that ATPS 7 (PEG-1500 + K_2HPO_4/KH_2PO_4) seems to be more economically attractive, but if considering that most of current GFP-based applications require a product with high purity level, probably ATPS 13 (PPG-400/[Ch]Cl) has also a significant potential to be implemented in a "real" industrial GFP production plant. ATPS 13 allows to obtain a high purity GFP (100%) after the ATPS unit, while to obtain a GFP with a similar price, the ATPS 7-based bioprocess should include additional unit operations, which consequently will increase the estimated production costs. Additionally, ATPS 7 and 13 represent two major classes of ATPS, "traditional" and "alternative" systems, respectively. One of the advantages of alternative systems is the inclusion of ionic liquids (particularly in this case, the incorporation of cholinum) into their composition, which have resulted to be less damaging to the environment [54]. For these reasons, the sensitivity analysis focused only on the system composed of PPG-400/[Ch]Cl (ATPS 13), intended to elucidate which specific parts of an ATPS-based bioprocess should be adjusted to reduce the GFP production costs, while maintaining the high purity levels. Different parameters were then evaluated to determine which could be improved to make the ATPS 13-based bioprocess economically comparable with the ATPS 7, even without considering the GFP purity added-value issue.

The sensitivity analysis studied three areas of potential improvement, namely: *i*) the recovery yield; *ii*) the discount for the consumable and raw materials; *iii*) the upstream production, particularly the fermentation titre. Results of the sensitivity analysis are shown in **FIGURES 4** and **5**, which include the base scenario and maximum value analysed for each parameter, along with an additional line for ATPS 7 as a benchmark. All possible values in-between were also analysed, but for simplicity, these were not included in **FIGURES 4** and **5**, instead their results can be consulted in the **Supplementary Material 1**.

Firstly, the influence of the recovery yield on the costs was evaluated. Considering that ATPS 13 has a recovery of 92.45% for GFP, it has a limited range for improvement. The yields tested ranged from 92.45% to 100% (with steps of 1%). As shown in **FIGURE 4A**, ATPS 7 is superior at every scale, *i.e.* independently of the recovery yield of the ATPS 13, the traditional PEG/salt system will be always more economic. These results demonstrate that recovery yield is a process parameter that does not need further improvement (denoted by its already high level) and, more remarkably, investment of resources and time for research in case of practical implementation.

On the other hand, and as mentioned earlier, material costs of the ATPS 13 are critical for the high cost of the ATPS-based bioprocesses. Therefore, a reduction of this parameter can play a major role envisaging its future industrial implementation. The material costs' parameter does not have a mechanism for optimization (as other process parameters), but it is easily related with the economy of scales. This dictates that as a process scale becomes larger, the increase in prices for supplies will not increase linearly and will tend to stabilize, being this true for equipment costs and materials price [52]. For the purpose of this study, as the scale of the process increases, the need for materials increase and its bulk price *per* unit of product will decrease. In order to obtain the material costs' impact, for the sensitivity analysis, it was decided to evaluate the effect of having a discount on material costs from 0% to 90% (*i.e.* paying full price from 100% to only 10%), as detailed in **FIGURES 4B** and **4C**. **FIGURE 4B** shows the effect of increasing the discount on CoG+CI/g for ATPS 13 at the different scales, comparing it with ATPS 7 as a benchmark at no discount. It is important to note that it was considered a "no discount" for materials of ATPS 7, because both PEG and phosphate salts are already largely used in industrial processes, which makes their current prices closer to the minimum. **FIGURE 4C** is a logic test to determine which scale/discount ATPS 13 is less expensive than ATPS 7. The logic test represents the contrast between a target value and a benchmark, in this case CoG+CI/g, considering the ATPS 13 as the target, against the

benchmark of the CoG+CI/g for the ATPS 7. Whenever the production cost for ATPS 13 is lower than that of ATPS 7, the final output obtained is "1", if not the response is "0". Given the wide range of production scales, this logic test provides a rapid evaluation and a facile graphical representation of changes in the CoG+CI/g between ATPS 13 and 7.

In contrast to the recovery yield, material discount is a parameter with a larger impact on the complete bioprocess of the ATPS 13 as observed by analysing **FIGURE 4** in two distinct ways. The first is by observing the separation between the base scenarios (recovery yield of 93.45 in **FIGURE 4A** or 0% discount in **FIGURE 4B**) and the maximum value for recovery yield (100%) and materials discount (90%). In **FIGURE 4B** this is wider, which represents a larger impact on the CoG+CI/g from material discount, while in **FIGURE 4A**, results are closer, denoting a minimum impact imposed by recovery yield. A second analysis comes from **FIGURE 4C**, where a logic test inferred that from 1 to 3 L scales with a discount from 60 to 90% of the cost of consumables, the ATPS 13 would become less expensive than ATPS 7. Although this is a desirable result, it is highly unlikely to happen as the effect of the economy of scales needs larger scales to be present (*i.e.* lower scales correlate to low discounts and larger scales with larger discounts).

The last part of the sensitivity analysis assessed the influence of the production titre parameter, which is relative to the concentration of product of interest at the end of the fermentation stage. The production titre, currently at 1.33 $g.L^{-1}$, was tested to increase up to 13 $g.L^{-1}$ (in 0.1 $g.L^{-1}$ steps) for all the production scales (**FIGURE 5**). Similar to the materials costs sensitivity analysis, a logic test was added to determine which results are more favourable than the implementation of the complete bioprocess based on ATPS 7.

It is important to notice that in practice, a fermentation titer of 13 g/L will potentially present inconveniences that will affect the bioprocess and its costs. High titer can be associated with fermentation with prolonged durations, different media feeding regimes or the generation of inclusion bodies [55-57]. The analysis performed here should be taken as a demonstration and evaluation on how big does the titer of ATPS 13 needs to be for it to be less expensive than ATPS 7.

FIGURE 5 shows the behaviour of the CoG+CI/g for each operation scale against the change in production titre. Similar to the analysis for material discount, as the scale increases, the contribution from materials is larger, reducing the potential effect that the titre optimization can have. For example, at 1 L, an increase to 1.6 $g.L^{-1}$ of titre will turn the process based on

ATPS 13 less expensive than ATPS 7 (at base titre). On the other hand, at a 100 L scale, a titre of 3.8 $g.L^{-1}$ would be required to guarantee the same economic viability. These results reemphasized the huge impact of materials contribution on CoG+CI/g of the complete bioprocess using ATPS 13 particularly because of the [Ch]Cl price. Nonetheless, the increase on titre has still a profound effect on CoG+CI/g, as higher titre concentrations are required for higher scales. Moreover, it is important to notice that ATPS 13, at all the scales analyzed here, may be less expensive with titer increase, for material costs discount, only small scales can overcome this, while an improvement for the recovery yield does not provide a less expensive process at any scale.

A different approach to visualize the augment in titer is the increase in overall product mass. At 100 L, the increase in titer needed for ATPS 13 to become less expensive than ATPS 7 is of 2.92 times. This can be translated into an increase in the operating volume of the fermenter and then, potentially, introduce a concentration unit before its incorporation into the ATPS. Additional analyses need to be performed in order to consider the recovery losses of GFP due to this new operation and its associated costs.

An additional conceptual analysis was performed for the titre optimization presented in **FIGURE 6**. It was found that, regardless of the operation scale, the percentage of reduction of the CoG+CI/g remained constant for a given production titre. From **FIGURE 6**, it can be noted that as the CoG+CI/g decrease, every time a higher production titre is required. For example, to achieve a reduction of 60% in the CoG+CI/g, it will require a titre of 3.3 $g.L^{-1}$, while for 70%, it needs 4.5 $g.L^{-1}$, and for 80%, a titre of 6.6 $g.L^{-1}$. Even though it is a 10% reduction step, the first reduction of CoG+CI/g required an increase of 1.2 $g.L^{-1}$, while the second needed more 2.1 $g.L^{-1}$ of the titre (2-fold of that required for a 60% decrease in CoG+CI/g). This sensitivity analysis is essential when the objective is to set up a new bioprocess. The increase of production titre is often a parameter under focus in many works, but an estimation of its potential benefit on overall manufacturing costs should be estimated. Therefore, a proper evaluation of its impact on the bioprocessing can help to reduce time, human effort and resources, focusing their action in other relevant areas of process development.

These results are promising as the incorporation of ATPS 13 (PPG-400/[Ch]Cl) has a higher chance of maintaining a simple bioprocess without the addition of further unit operations for recovery/purification purposes. The sensitivity analysis performed here for three different parameters has provided interesting insights on the path to follow for practical applicability.

Moreover, these results were able to capture the burden of optimising fermentation titre by calculating the needed increase required for a given desired reduction in the CoG+CI/g.

It should be also noted that this system in the 100-L bioreactor, estimated to cost around USD 1,000 *per* g, is 2,000 times lower than the current commercial price of EGFP of USD 2,000 *per* mg [3]. As highlighted above, there is an impressive difference between the cost and price practiced in the pharmaceutical industry, which is not always well-explained due to a lack of transparency in their pricing systems [14]. However, this is another case to support the importance of developing alternative purification platforms, which could be accessible to smaller industries outside pharmaceutical conglomerates. In addition to being simpler and cheaper, they could also increase the availability of recombinant proteins in the market and create competition and price reductions, which is crucial for the popularization of the cited novel applications of fluorescent proteins.

4. Conclusions

The present work constructed a bioprocess model coupled with an economic analysis to produce GFP while using ATPS for its recovery/purification. It was possible to incorporate the concept of product purity into the CoG+CI/g to discriminate relevant results and potential ATPS envisioning their future industrial implementation. The application of bioprocess modelling allowed to construct two types of models: a single unit operation model for ATPS discrimination (limited focus, meticulous analysis) and a complete bioprocess model (complete variables, general focus). Additionally, a sensitivity analysis helped on understanding the impact of different parameters on CoG+CI/g and what parameters should be improved to boost the applicability of IL-based ATPS. It was found that production titre has a profound impact on decreasing CoG+CI/g, but this improvement is highly correlated to the current status of the titre for any process. Oppositely, it was confirmed that the materials discounts had a lower impact on the reduction of the economic costs than the production titre, which is related with the innate high consumption of materials on any ATPS, although the effect is more profound when using more expensive materials, as in the IL-based ATPS.

The production costs calculated in this work range from USD $3.5x10^3$/g at a 1 L scale to USD $0.33x10^3$/g at 100 L. Current prices in the market are approximately USD 2,000/mg (depending on the purity and application). In the end, we are able to confirm the advantage of including ATPS into the recovery/purification process to maintain low production costs, which is critical to highlight the need to consider the additional costs into the calculations performed to properly

evaluate the "real" final production costs. Although the experimental tests are still required to determine practical deployment, these results can potentially be enhanced by incorporating into the model the concepts of recycling ATPS components (decrease of initial cost of materials), and by applying process intensification concepts. This study is an important starting-point to design further ATPS-based experiments, highlighting the importance of the bioprocessing modelling studies as preliminary background for the optimization and adequation (if needed) of process parameters.

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Highlights

- Bioprocess modelling coupled with economic analysis to evaluate the applicability of ATPS.
- PEG-based ATPS are less expensive than IL-based ATPS for GFP purification.
- The cost-effectiveness of IL-ATPS can be improved by increasing production titre.
- Product purity is crucial for decision-making in purification/separation processes.
- Process intensification can make ATPS-based bioprocess feasible for industry.

Table 1. ATPS numbers, compositions, recovery yield and product purity.

ATPS	ATPS Number	Component 1 (wt%)	Component 2 (wt%)	Component 3 (wt%)	Recovery Yield (%)	Product Purity (%)
PEG-600 + NaPA-8000 + [Ch]Cl		***PEG-600***	***NaPA-8000***	***[Ch]Cl***		
	1	15.156	15.226	1.024	91.4	73.9
	2	15.010	15.026	3.042	90.7	73.3
	3	15.561	14.984	5.055	93.0	70.0
	4	20.035	15.211	1.006	103.1	73.1
	5	20.224	15.206	3.085	106.6	70.5
	6	20.133	15.360	5.081	102.8	69.9
PEG-1500 + K_2HPO_4/KH_2PO_4		***PEG-1500***	***K_2HPO_4/ KH_2PO_4***	**-**		
	7	15.020	14.957	-	91.67	73.9
PEG-2000 + K_2HPO_4/KH_2PO_4		***PEG-2000***	***K_2HPO_4/ KH_2PO_4***	**-**		
	8	14.963	14.949	-	92.61	74.8
PEG-600 + [Ch]Cl		***PEG-600***	***[Ch]Cl***	**-**		
	9	38.325	37.907	-	96.3	80.1
	10	39.919	40.081	-	91.4	78.6
	11	41.940	41.955	-	94.9	78.1
PPG-400 + [Ch]Cl		***PPG-400***	***[Ch]Cl***	**-**		
	12	40.642	9.967	-	90.1	93.2
	13	45.548	11.933	-	91.8	100.2
	14	49.973	14.066	-	89.2	97.8

Abbreviations: PEG – polyethylene glycol; NaPA – sodium polyacrylate; [Ch]Cl – cholinium chloride; K2HPO4/KH2PO4 – potassium phosphate buffer; PPG – polypropylene glycol.

Table 2. Economic dataset employed for model construction.

Cost Category	Item	Cost Data (Size on Base 10 L of Scenario)
Capital (USD)	Bioreactor	*Cost = 34,693 x Volume$^{0.4074}$ (1 L)*
	Centrifuge	*Cost = 682,752 x (Flow Rate / 600)$^{0.4}$ (0.5 L.h^{-1})*
	Homogenizer	*Cost = 21,584.62 x (Volume / 10)$^{0.6}$ (0.5 L.h^{-1})*
	ATPS Tank	*Cost = 42.2 x Volume + 3,035.2 (10 L)*
	UF/DF	*Cost = 230,617 (Membrane Size / 5)$^{0.4}$ (0.49 m^2 – Flux 4 L.m^{-2}.h^{-1})*
Materials (USD.kg^{-1})	[Ch]Cl	*143.5*
	Cloramphenicol	*25936*
	Yeast Extract	*1080.4*
	K_2HPO_4	*40.1*
	Kanamycin	*235460*
	KH_2PO_4	*33.2*
	NaCl	*216.1*
	NaPA8000	*622.5*
	PEG1500	*41.5*
	PEG2000	*136.7*
	PEG600	*45.4*
	PPG400	*122.3*
	Tryptone	*2116.6*
Consumable (USD)	UF Filters (m^2)	*4,790*
	Vessel Filters (Bioreactor and ATPS Tank)	*Cost = 0.3058 x Tank Volume + 43.334 (10 L)*
Labour	Labour	*Fixed at 15% of CoG+CI/g at every iteration*
Others	Maintenance, utilities and waste disposal	*4% of Capital cost*

*More technical data available in the **Supplementary Material 2**.

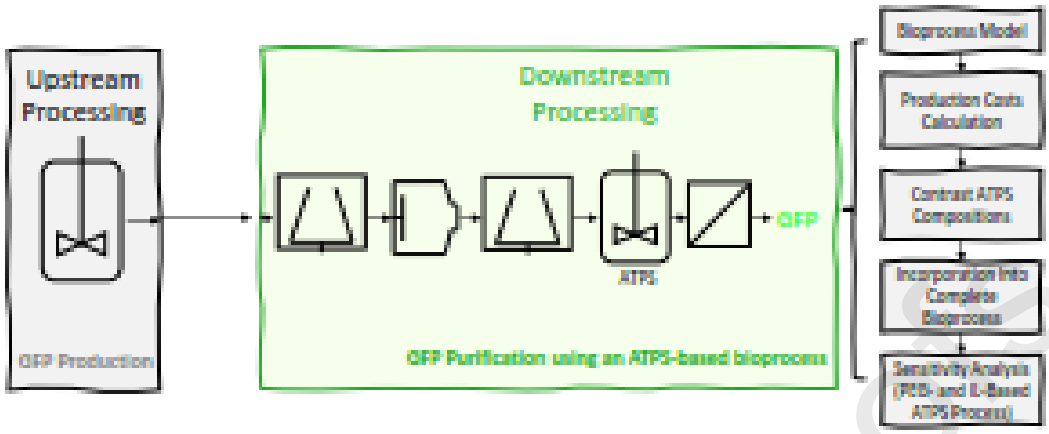

Figure 1

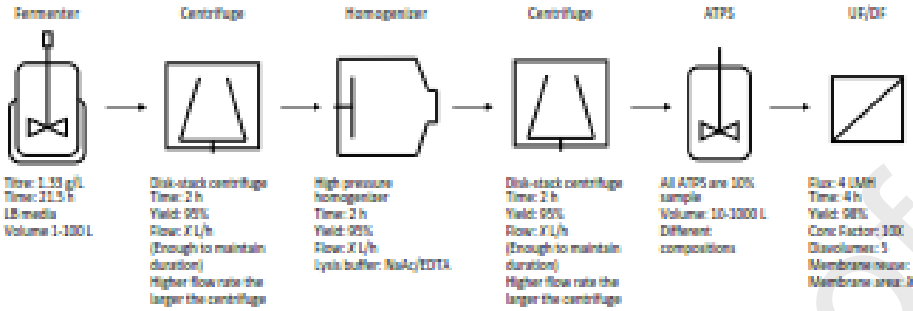

Figure 1. Bioprocess modelled for the recovery/purification of GFP centred on ATPS as the main unit operation. Process parameter shown here were uploaded to Biosolve Process modelling tool. Variables shown in "X" are values that changed accordingly to maintain the same process time while modifying the scale of operation. Compositions employed for ATPS are shown in Table 1. Additionally, considering the duration time of each unit operation and a 300 working days per year, it is possible to calculate batches per year and annual production (no additional special time requirements were considered, such as maintenance, validation, batch losses, etc.).

Figure 2

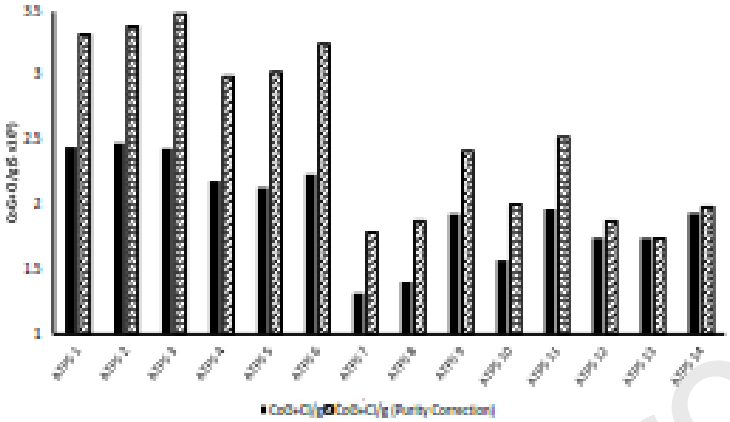

Figure 2. Contrast between the 14 ATPS tested. Results include regular CoG+Cl/g and CoG+Cl/g after purity correction incorporated.

Figure 3

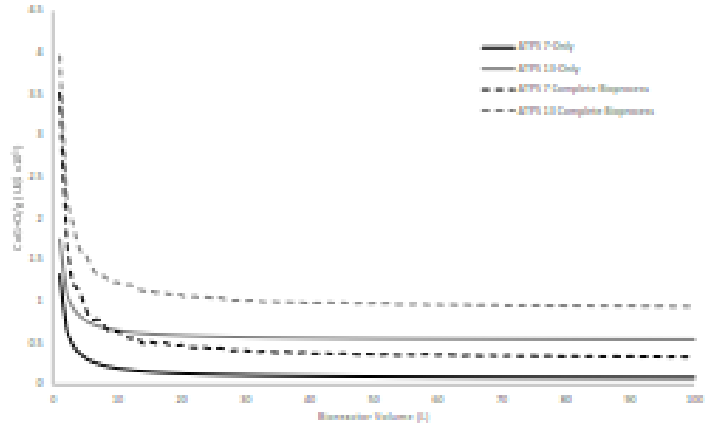

Figure 3. Contrast of the incorporation of ATPS 7 (PEG 1500 + K_2HPO_4/KH_2PO_4) and 13 (PPG/[Ch]Cl) into a complete bioprocess. Results show a higher CoG+Cl/g for each complete process contrasted with their respective ATPS-only model.

Figure 4

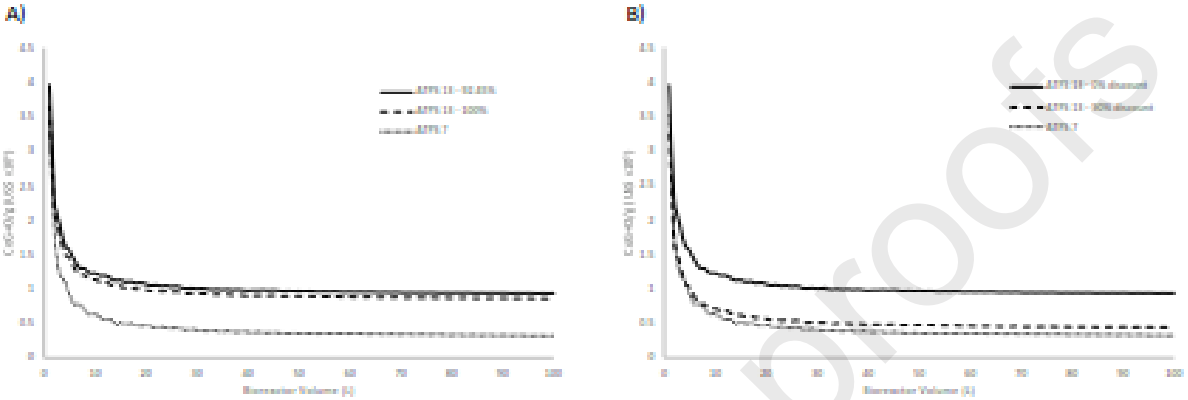

Figure 4. Sensitivity analysis for the recovery yield (A) and materials cost discount (B). Results show in A) the current yield (92.45%) and the highest possible tested (100%). For materials discount (B), data shows a 0% and 90% discount. The recovery yields and discounts in between the ones shown here were also analysed but are included in **Supplementary Materials 1**. C) Show logic test employed to determine scales and discount combination where ATPS 13 (PPG/[Ch]Cl) is less expensive than ATPS 7 (PEG 1500 + K_2HPO_4/KH_2PO_4).

Figure 4

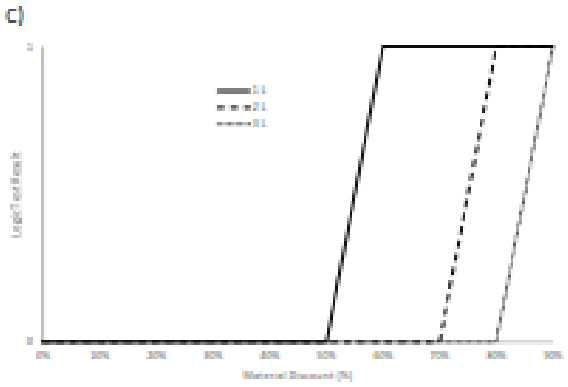

Figure 5

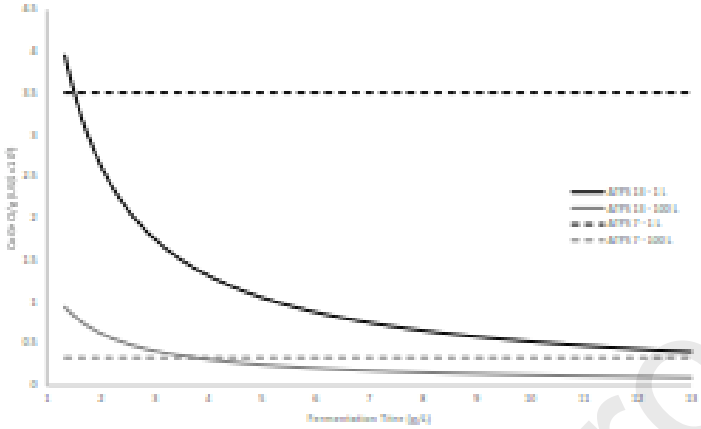

Figure 5. Sensitivity analysis for fermentation titre. Results presented here show the behaviour of ATPS 13 at 1 L and 100 L to the range of titres analysed. As benchmark, the CoG+CI/g for ATPS 7 at 1 L and 100 L at the base titre (1.33 g/L) is added. ATPS 13 at 1 L becomes less expensive than ATPS 7 at 1 L with 1.6 g/L, while at 100 L this situation occurs at 3.8 g/L. The analysis included all the scales in between 1 L and 100 L (in 1 L steps) were analysed and results for their CoG+CI/g are included in **Supplementary Material 1**.

Figure 6

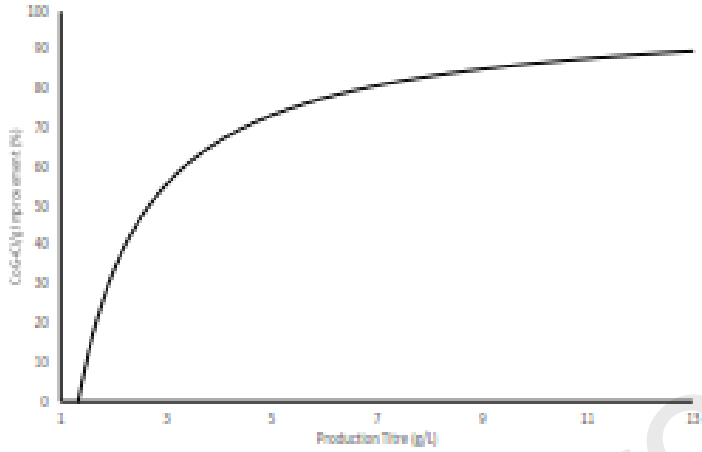

Figure 6. Conceptual analysis of the impact of optimizing production titre on the percentage of improvement of CoG+Cl/g.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Declaration of interests: None