



Versatile recovery of interferon alpha-2b via polymer molecular weight tailoring: From liquid–liquid extraction to three-phase partitioning

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ABSTRACT

In biomanufacturing, downstream processing encompasses the recovery, purification, and polishing of protein therapeutics, representing the major contributor to overall production costs. The development of efficient recovery strategies is therefore critical to enhance product yield and stability, while reducing impurity load and extending the operational lifetime of chromatographic resins and membranes. In this context, this work demonstrates that polymer–polymer aqueous biphasic systems (ABS) and related three-phase partitioning (TPP) strategies constitute effective and versatile platforms for the recovery of interferon alpha-2b (IFN α -2b) from *Komagataella phaffii* culture broths. IFN α -2b, fused to the alpha mating factor to enable extracellular secretion, was successfully produced in *K. phaffii*, reaching maximum titers of 78.1 mg·L⁻¹ upon supplementation of the culture medium with Tween 80. Following upstream production, downstream recovery was investigated using ternary systems composed of water, polypropylene glycol (PPG 400), and polyethylene glycol (PEG) of varying molecular weights (400–6000 g·mol⁻¹). In ABS formed with low-molecular-weight PEGs, IFN α -2b preferentially partitions into the PEG-rich phase, with recovery yields increasing as the PEG molecular weight decreases. Conversely, the use of high-molecular-weight PEGs promotes the formation of TPP systems, in which IFN α -2b is recovered as a precipitate at the interphase, achieving recovery yields as high as 99% with PEG 6000 g·mol⁻¹. These results highlight the pivotal role of polymer molecular weight in dictating phase behavior, protein partitioning, and transition in the separation platform, from liquid–liquid extraction to three-phase partitioning. Overall, this work demonstrates that polymer-based ABS/TPP can be strategically tailored to integrate multiple separation stages, offering a versatile approach for protein recovery in downstream bioprocessing.

1. Introduction

Manufacturing of high-value proteins, including industrial enzymes and therapeutic proteins, is one of the most important areas in industrial biotechnology [1]. Biopharmaceuticals, including therapeutic proteins, have become essential in preventing and treating hard-to-treat or previously untreatable diseases [2]. Data from Fortune Business Insights [3] indicate that the global biopharmaceuticals market was valued at \$666.41 billion in 2025, with protein-based biopharmaceuticals, including monoclonal antibodies, leading new product approvals in both the US and EU [2]. Among the approved therapeutic proteins, interferons (IFNs) have captured particular attention. First approved in 1986, they continue to be used more than three decades later for the treatment of hairy cell leukemia, various cancers, multiple sclerosis, and chronic hepatitis C [4–6]. Although the increasing prevalence of chronic diseases is anticipated to increase the demand for therapeutic proteins

and antibodies [3], the (bio)pharmaceutical industry continues to face the challenge of efficiently producing large amounts of these products [7,8].

Upstream processing is the first step of biopharmaceutical manufacturing, comprising the production of the target product in a suitable host [8]. Downstream processing then begins with clarification via filtration and/or centrifugation, followed by capture, purification, and polishing steps typically using chromatography [8], which can represent up to 80% of total production costs [1]. During upstream processing, *Komagataella phaffii* (*K. phaffii*, former *Pichia pastoris*), a generally recognized as a safe (GRAS) microorganism, has been described as capable of efficiently secreting proteins into the culture medium, removing the need for cell lysis [9,10]. Another advantage is related to the lower amount of endogenous proteins that are co-secreted with the target protein, simplifying subsequent purification steps [10]. In biomanufacturing, advancements in protein upstream processing

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have been driven by improvements in expression systems, engineered host cells, enhanced process control methods, optimized culture media and feed strategies, and more efficient bioreactors [11,12]. However, the improvements in upstream processing, yielding higher product concentrations, have shifted the bottleneck to downstream processing, as existing technologies and equipment are often unable to efficiently handle these more concentrated feed streams [8,11].

Over time, literature has documented that effective recovery strategies (e.g., initial clarification, contaminant removal) can increase product yield and stability while extending resin and membrane lifespan in subsequent purification steps. Bracewell and colleagues [13] highlighted the potential of clarification techniques to act as troubleshooting techniques to extend chromatographic resin lifetime. According to the authors, enhanced clarification leads to higher column capacity, justifying the interest in optimizing clarification approaches to ameliorate damage to chromatographic columns [13]. From a different point of view, selecting a suitable clarification and recovery technique, among which flocculation, filtration, precipitation or liquid-liquid extraction (LLE) can be chosen, is crucial to ensure product stability and high recovery rates [8,14–16].

First proposed by Albertson for the recovery, isolation, and purification of biological products, aqueous biphasic systems (ABS), a well-established LLE approach, represent an effective and versatile tool for the downstream processing of proteins, nucleic acids, and other biomolecules [17]. ABS are ternary systems composed of water and two water-soluble compounds, such as polymers, inorganic salts, ionic liquids, and surfactants. When the components are mixed above a certain concentration, they spontaneously separate into two coexisting phases, each enriched in one of the phase-forming agents [18]. ABS offer several advantages over traditional LLE: they can be designed to be environmentally friendly and biocompatible due to their high water content, are cost-effective, require relatively short processing times, and are more customizable [19].

In addition to ABS, three-phase partitioning (TPP) systems are emerging as a powerful bioseparation technique that integrates concentration and partial purification in a single-step. The main advantage of using TPP over conventional precipitation is that it requires a lower amount of precipitant [20], whereas selectivity is improved due to an increase in the number of phases, and thus degrees of freedom, to which the several biomolecules can partition. Initially described by Dennison and Lovrien [20], TPP is achieved by precipitating target proteins at the interface between an organic liquid phase (usually tert-butanol) and an aqueous solution of an inorganic salt (e.g., ammonium sulfate). Along with the classical TPP concept, modern strategies now incorporate combinations of polymers, ionic liquids, and inorganic salts, which have proven successful in the separation and purification of proteins from different sources [21,22]. ABS-based TPP shares the main advantages of ABS, such as high water content and high versatility to improve the recovery and purity of the target biomolecule [23].

Building on the described evidence, ABS composed of polypropylene glycol (PPG) 400 g·mol⁻¹ and polyethylene glycol (PEG) with molecular weights varying from 400 to 6000 g·mol⁻¹ were investigated in this work as clarification approaches for the recovery of interferon alpha 2b (IFN α -2b) directly from *K. phaffii* culture broth. The obtained results show that by controlling PEG molecular weight and the respective amounts of phase-forming agents in ABS, the therapeutically relevant protein can be recovered in an aqueous phase or as a precipitate, respectively, encompassed within the concepts of ABS or TPP while integrating extraction and precipitation strategies within a single platform. Accordingly, polymer-polymer-based ABS can be considered versatile and tunable multiphase systems, enabling both liquid-liquid extraction and three-phase partitioning. This work discloses a versatile approach for the recovery of a protein-based biopharmaceutical from real culture broths, which can be customized to target protein recovery in either aqueous phases or as a precipitate, depending on the downstream stage and other specific operating requirements.

2. Materials and methods

2.1. Materials

For culture media preparation, the following components were used: yeast extract and peptone were acquired at Biokar Diagnostics (France), yeast nitrogen base (YNB) and agar were purchased at Grisip (Portugal), methanol at Thermo Fisher Scientific (USA), glycerol at Frilabo (Portugal), Tween 80 at Merck Sigma-Aldrich (Germany), and Zeocin at InvivoGen (USA). Polymers used for ABS preparation, namely PPG 400 g·mol⁻¹ and PEGs (400, 600, 1500, 2000, and 6000 g·mol⁻¹), as well as the Novex™ 16% Tris-Glycine Plus Wedge Well Gel and respective running buffers for polyacrylamide gel electrophoresis, were acquired at Thermo Fisher Scientific (USA). Western Blot analysis was carried out using antibodies acquired from Abcam (UK) and Proteintech (Germany), respectively, the rabbit anti-Interferon alpha 2 antibody and the goat HRP-conjugated Affinipure Anti-Rabbit. The enzyme-linked immunosorbent assay (ELISA) assays were performed using a commercial human IFN- α pan ELISA^{PRO} kit acquired from MabTech (Sweden).

2.2. Interferon α -2b biosynthesis

Recombinant expression of IFN α -2b was achieved using the yeast *K. phaffii* X-33 strain modified with the recombinant plasmid pPICZ α B_{IFN} α -2b containing the gene encoding human IFN α -2b fused to the α -mating factor. A similar approach (see the Supplementary Material for further details, including the diagram of the cloning site in the recombinant plasmid – Fig. S1) to that previously used by Srivastava and co-workers [24] was adopted. This involved the expression of IFN α -2b using a mutated form of the α -mating factor, as it has been shown to result in higher levels of IFN α -2b expression [24]. Yeast cultivation was achieved as follows: an aliquot of cells grown at 30 °C in solid YPD medium (1% yeast extract; 2% peptone; 2% dextrose) was used to inoculate 50 mL of buffered minimal glycerol medium (BMGH, 100 mM phosphate buffer pH 6, 1.34 wt% YNB, 4 × 10⁻⁵% biotin, and 1 wt% glycerol) in 250 mL shake-flasks. To promote biomass accumulation, this pre-inoculum was incubated overnight at 30 °C and 250 rpm in an orbital shaker, a point at which the optical density at 600 nm (OD₆₀₀) typically reached 6. Afterward, the culture was centrifuged (1000 xg for 20 min at room temperature) to remove the BMGH medium, and cells were resuspended in 100 mL of buffered minimal methanol medium (BMMH, 100 mM phosphate buffer pH 6, 1.34 wt% YNB, 4 × 10⁻⁵% biotin, and 1 wt% methanol, used as inducer) to induce protein expression. Induction was performed for 120 h, and methanol (1 wt%) was added every 24 h to maintain induction. To improve the secretion levels of IFN α -2b to the culture broth, Tween 80 at concentrations ranging between 0.01% and 10% (v/v) was investigated as an additive to the BMMH induction medium. All culture media were supplemented with 100 μ g mL⁻¹ zeocin and kept in the dark to avoid the degradation of the antibiotic.

2.3. Aqueous biphasic systems

2.3.1. Phase diagrams

To study the partitioning of the target therapeutic recombinant protein, the phase diagrams for the systems composed of PPG 400 g·mol⁻¹ and PEG with varying molecular weights (400, 600, 1500, 2000, 6000 g·mol⁻¹) were determined either in water, or in the presence of induction culture media (BMMH). Since the culture medium is a complex mixture that includes inorganic salts, sugars, methanol, and protein-digested fractions, their presence may influence the phase separation of the system and the respective binodal curves. Each binodal curve was determined by the cloud point titration method at 25 (± 1) °C and atmospheric pressure, as previously described [25,26]. Aqueous solutions of PEG were dropwise added to aqueous solutions of PPG until a cloudy biphasic solution was obtained; subsequently, dropwise

addition of water proceeded until a monophasic region was observed. To verify the influence of the culture media on the binodal curve of each system, the same procedure was adopted, but PEG and PPG were dissolved in the culture media BMMH (including 0.1% Tween 80). This process was carried out under constant stirring, and the system's composition was determined by weight.

The experimental binodal curves were adjusted by eq. 1 proposed by Merchuk et al. [25], following the procedure reported by Freire and co-workers [26].

$$[PPG] = A \exp(B \cdot [PEG]^{0.5} - C \cdot [PEG]^3) \quad (1)$$

where PPG and PEG are the weight fractions (wt%) of PPG 400 g·mol⁻¹ and PEG (400, 600, 1500, 2000, and 6000 g·mol⁻¹), respectively, and A, B, and C are adjusted parameters obtained by least-square regression. The composition of each phase at given mixture compositions (tie-line lengths (TLLs)), was additionally determined, as detailed in Supplementary Material.

2.3.2. Interferon recovery using ABS/TPP

The recovery of IFN α -2b from *K. phaffii* culture broth was investigated using ABS composed of PEG with varying molecular weights (MW) (400, 600, 1500, 2000, and 6000 g·mol⁻¹), PPG 400 g·mol⁻¹, and the culture media comprising the secreted target therapeutic protein (25 wt %). The mixture was then stirred for 1 h under a controlled temperature of 25 °C and further centrifuged (2000 xg for 5 min at room temperature) to accelerate phase separation. Subsequently, the top phase (enriched in PPG) and the bottom phase (enriched in PEG), along with any precipitate formed at the interphase, were carefully separated. IFN α -2b was recovered from both top and bottom phases by ethanol precipitation (ratio 1:4, and suspended in a final volume of 500 μ L) to avoid potential interferences caused by the polymers, and the samples were then analyzed as described in section 2.4.

2.4. Assessment of the total protein profile and IFN α -2b quantification

2.4.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

IFN α -2b and endogenous yeast proteins' expression profiles, as well as their partitioning pattern in ABS/TPP, were monitored using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This analysis was performed according to the method of Laemmli. During the culture process, aliquots were taken every 24 h to routinely monitor the expression levels of proteins. These samples were centrifuged at 2000 xg for 15 min to remove cells, and the culture supernatant was directly analyzed. For the samples resulting from ABS/TPP, precipitates were immediately analyzed following resuspension in 150 μ L. In contrast, the samples from the top/bottom phases underwent an ethanol precipitation step before analysis to eliminate potential interferences in the analysis caused by polymers (Final suspension volume of 500 μ L).

For SDS-PAGE, all samples were diluted (1:1) with loading buffer [4% (w/v) SDS, 20% (w/v) of glycerol, 120 mM Tris-HCl pH 6.8, 0.02% (w/v) bromophenol blue and 310 mM of DTT] and denatured at 95 °C for 5 min. Finally, the samples were injected in an Invitrogen NovexTMTris-Glycine Gel with a polyacrylamide concentration of 16% and run for 1 h 30 min at 100 V in a ThermoFisher Scientific Mini Gel Tank. Protein staining was achieved by incubation with BlueSafe (NZYtech, Lisbon) under mild agitation according to the manufacturer's instructions.

2.4.2. Bicinchoninic acid assay

For the determination of the total protein content during partitioning in ABS/TPP, the bicinchoninic acid assay (BCA) was applied using the PierceTM BCA Protein Assay Kit from ThermoFisher (USA). The protocol provided by the manufacturer using Bovine Serum Albumin (BSA) was applied.

2.4.3. Enzyme-linked immunosorbent assay

The IFN α -2b expression levels in *K. phaffii* culture medium, as well as the amount of precipitated IFN α -2b in the ABS/TPP approaches, were determined using the commercial kit IFN- α ELISA^{PRO} kit (MabTech, Sweden). The recovery yield (Y, %) of precipitated IFN α -2b was determined considering the amount of IFN added to the systems (eq. 2):

$$Y (\%) = \frac{W_{\text{IFN precipitated}}}{W_{\text{IFN initial sample TW 80 0.1\%}}} \times 100 \quad (2)$$

where $W_{\text{IFN precipitated}}$ and $W_{\text{IFN initial sample TW 80 0.1\%}}$ correspond, respectively, to the mass (in μ g) of IFN α -2b precipitated in the investigated ABS/TPP, and in the initial sample (supplemented with TW 80 0.1%) added to the ABS.

Additionally, the purification factor (PF) was determined using eq. 3:

$$PF = \frac{\frac{[\text{IFN}]_{\text{precipitated}}}{[\text{Total protein}]_{\text{precipitated}}}}{\frac{[\text{IFN}]_{\text{initial sample}}}{[\text{Total protein}]_{\text{initial sample}}}} \quad (3)$$

where [IFN] and [Total protein] precipitated correspond to the concentration of IFN α -2b and total protein precipitated (suspended in 150 μ L) in the investigated ABS/TPP; on the other hand, [IFN] and [Total protein] initial sample indicate the concentrations of IFN α -2b and total protein in the initial sample added to the ABS.

2.4.4. Dot-blot and western-blot analyses

Western-blot and dot-blot were performed to identify immunoreactive IFN α -2b. For dot-blot, a nitrocellulose membrane (Amersham Protran 0.45 μ m) was initially activated with 50 mM Tris, 380 mM glycine, and 20% (v/v) methanol, and then semi-dried until 20 μ L of the samples were directly injected into the membrane. To initiate the western-blot protocol, proteins were transferred from an unstained SDS-PAGE gel (obtained as described above) to a nitrocellulose membrane over 60 min at 10 mA in a specific buffer [50 mM Tris, 380 mM glycine, and 20% (v/v) methanol], using an Invitrogen Mini Gel Tank system from ThermoFisher Scientific (USA). Following these initial steps, the same protocol for membrane blocking, antibody probing, and membrane revelation was followed for both techniques. Membranes were blocked with Tris-buffered saline with Tween 20 buffer (TBS-T) (pH 7.4) supplemented with 5% (v/v) non-fat milk for 1 h at room temperature. Afterward, they were probed with primary antibodies recognizing human IFN α -2b (1:1000 fold-dilution in TBS-T) over 1 h at room temperature. After three consecutive washes with TBS-T, membranes were incubated for 1 h at room temperature with HRP-labeled anti-rabbit/goat IgG secondary antibody (1:5000 fold-dilution in TBS-T). Signal detection was performed with ECL substrate (Advansta Inc., CA, USA) according to the manufacturer's instructions, and images were acquired with the ChemiDocTM XRS system (Bio-Rad, USA).

3. Results and discussion

This work addresses the use of polymer-polymer ABS and related TPP systems composed of PPG 400 g·mol⁻¹ and PEG with varying molecular weights for the clarification and direct recovery of IFN α -2b from *K. phaffii* culture broth. The engineered expression vector comprising the target gene fused to the α -mating factor enables *K. phaffii* to secrete the recombinant protein into the culture broth, which *per se* facilitates protein recovery.

In the upstream processing, different concentrations of Tween-80 were screened as culture media additives, being observed that the levels of secreted recombinant IFN α -2b were positively impacted. IFN α -2b recovery was then studied in the designed PPG-PEG-based ABS/TPP. It was observed that the mixture point influences the IFN α -2b recovery yield, and that flexible and versatile multiphase systems fitting within the concept of liquid-liquid extraction or TPP can be designed by controlling the MW of PEG.

3.1. Interferon α -2b biosynthesis

Biosynthesis of IFN α -2b was accomplished using *K. phaffii* X-33 modified with a recombinant plasmid containing the sequence encoding for human IFN α -2b and mutated α -mating factor, allowing protein secretion to the culture broth (see Supplementary Material, sections 1.1 and 1.2). Since the production of the target protein in *K. phaffii* was driven by the alcohol oxidase promoter, methanol was used as inducer. The culture medium was supplemented with methanol every 24 h to ensure consistent protein expression throughout the 120 h of expression.

Previous literature reports have shown that culture media supplementation with Tween-80 enhanced the levels of secreted proteins from yeast, namely human granulocyte colony-stimulating factor (hG-CSF) [27], recombinant human growth hormone [28], and antibody Fab fragments [29]. Park and colleagues [27] highlighted the role of Tween 80 as an effective protein stabilizer, efficiently preventing multimerization of hG-CSF secreted from *Saccharomyces cerevisiae*. Using *Pichia pastoris*, Padmanabhan and co-workers [28] showed that supplementation with Tween 80 enhances the secretion levels of growth hormone. Similarly, Ferrer and colleagues [29] demonstrated that the secretion of Fab fragment antibodies is enhanced using Tween 80, which may involve alterations in fluidity of the membrane lipids that facilitate the translocation of soluble proteins, overall enhancing their rate of secretion. Based on these promising observations, the effect of Tween 80 on the secretion levels of IFN α -2b was investigated in this work.

Aliquots were regularly recovered at different stages of the induction period and analyzed by SDS-PAGE to confirm the expression of the desired protein in the culture broth in the presence and absence of BMMH supplementation with Tween 80 0.01% (v/v), whose results are provided in Fig. 1.

As shown in Fig. 1, a prominent band with MW around 17 kDa, corresponding to IFN-2b [30], begins to be identified after 72 h of culture in BMMH. In contrast, when Tween 80 is added as a culture media additive, the protein can be detected earlier, at 48 h of induction. Furthermore, based on the electrophoresis profile and band intensity

after 120 h, we can infer that adding Tween 80 to the culture medium not only enables earlier detection of IFN α -2b, but also increases the overall content of the target protein secreted to the culture broth. Considering the positive effect of the polysorbate Tween 80 in enhancing the secreted levels of the target protein, the next assays focused on determining the optimal concentration as a culture media additive. The respective results are provided in Fig. 2.

As shown in Fig. 2, the concentration of Tween 80 added to the culture media exhibits a significant effect on the levels of the target protein secreted into the culture media. In general, increasing the concentration of Tween 80, from 0.01 to 0.1%, enhances the levels of secreted IFN-2b. However, an additional increase in the concentration of Tween 80 to 10% (v/v) does not result in significant increments in the concentration of IFN α -2b. Instead, it can be observed in the SDS-PAGE gel the presence of a contaminating protein band, appearing at ~140 kDa.

Overall, it is evident that Tween 80, as a culture media additive, has a favorable effect on the levels of secreted IFN α -2b. At a concentration of 0.1% (v/v) of Tween 80, the ideal balance between membrane permeabilization and IFN secretion is obtained, after which contamination with endogenous proteins starts to occur, as well as interferences caused by the high concentration of Tween 80. Therefore, for the next assays, *K. phaffii* was cultivated in BMMH medium supplemented with 0.1% (v/v) Tween 80.

3.2. ABS phase diagrams

After setting up the culture conditions allowing for IFN α -2b secretion to the culture media, the next step encompassed investigating the application of polymer-polymer ABS/TPP to recover the target protein. Having this goal in mind, we proceeded to the determination of phase diagrams of ABS composed of PPG 400 g·mol⁻¹ and PEG 400, 600, 1500, 2000, and 6000 g·mol⁻¹ in water, and in the culture media – BMMH. Since the global aim is to directly recover the target protein from the culture media, which is a complex mixture that includes inorganic salts, sugars, methanol, and protein-digested fractions, their presence may influence the phase separation of the system and the respective binodal curves. Therefore, the determination of the phase diagrams for both conditions provides a better understanding of the behavior of the systems and how protein partitioning will be influenced.

Fig. 3 displays the phase diagrams for ABS composed of PPG 400 g·mol⁻¹ and PEGs of varying molecular weights (400, 600, 1500, 2000, and 6000 g·mol⁻¹). These diagrams are shown in an orthogonal representation, with the amounts of water (A) or culture media (B) not being represented on an axis.

From Fig. 3A, it is evident that PEG molecular weight (MW) strongly affects biphasic system formation when water is used as the third phase-forming component. The closer the binodal curve lies to the axes, the lower the concentration of phase-forming components required to generate an ABS. Accordingly, and until 15 wt% PEG, the ability of the studied systems to phase-separate follows the trend: PEG 600 < PEG 400 < PEG 1500 < PEG 2000 < PEG 6000, which is generally in agreement with the trend reported by Millain *et al.* [31]. These results can be attributed to the increase in PEG hydrophobicity with increasing molecular weight. As the polymer MW increases, the water-exclusion effect becomes more pronounced, making two-phase formation more entropically favorable. For phase diagrams prepared with the phase-forming agents dissolved in culture medium (cf. Fig. 3B), a different behavior was observed, and the trend is as follows: PEG 400 < PEG 1500 < PEG 600 < PEG 6000 < PEG 2000. Notably, in the culture-medium diagrams, the binodal curves shift to the left, indicating an expansion of the biphasic region. This behavior likely arises from the salts present in the culture medium (e.g., phosphate buffer), enhancing the salting-out effect. Here, the culture medium acts as an adjuvant for phase formation, promoting the development of the biphasic region and facilitating phase separation. As a result, the effect of the MW of PEG

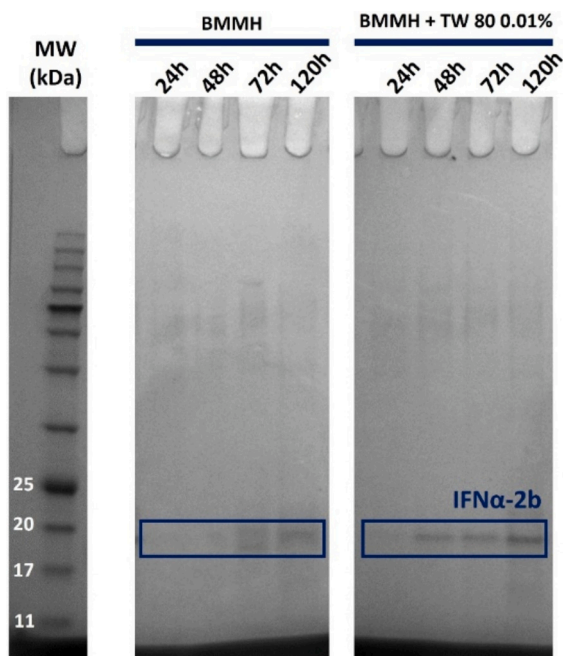


Fig. 1. SDS-PAGE analysis under denaturing conditions of culture broth resulting from the growth of *K. phaffii* X-33 modified with the plasmid pPICZαB-IFN α -2b in the presence and absence of 0.01% (v/v) Tween 80 as BMMH additive. Aliquots were regularly recovered at different stages of the induction period (24 to 120 h).

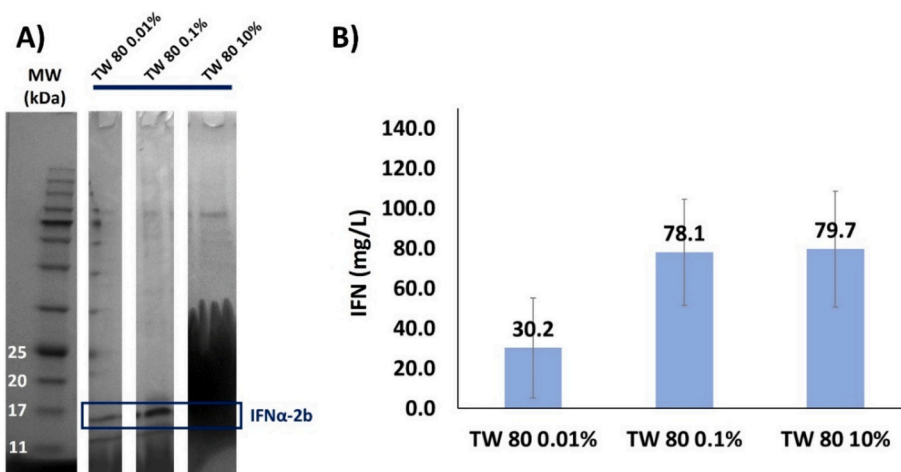


Fig. 2. SDS-PAGE analysis (A) and IFN α -2b concentration determined by ELISA (B), resulting from the 120 h cultivation of *K. phaffii* in BMMH media containing different concentrations [(0.01, 0.1 and 10% (v/v))] of Tween 80. IFN α -2b concentrations correspond to the average of three independent experiments.

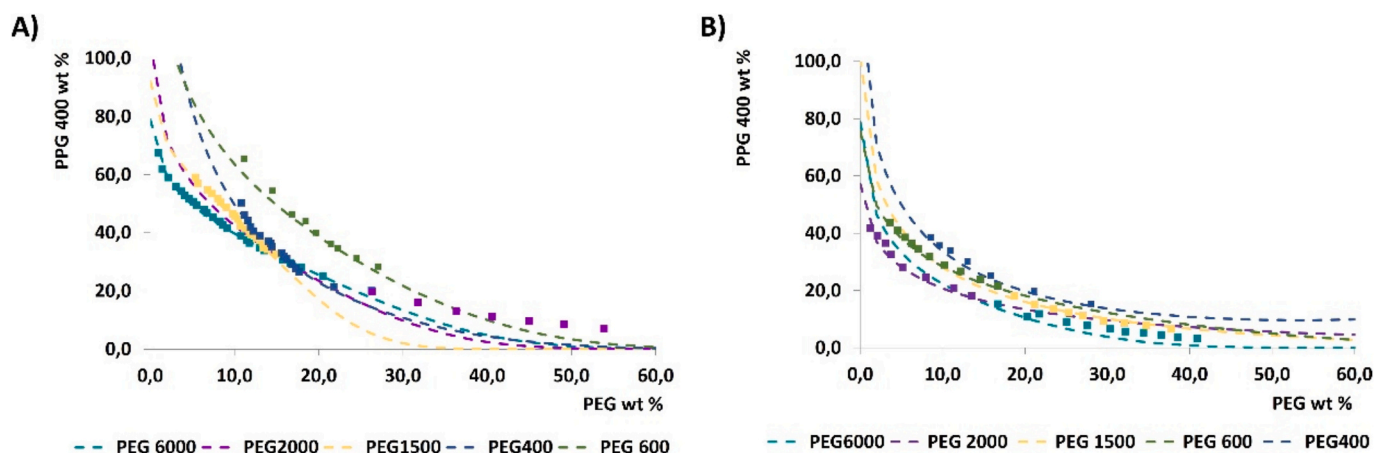


Fig. 3. Phase diagrams at 25 ($\pm$ 1) °C and atmospheric pressure for systems composed of PPG 400 g·mol⁻¹ and PEGs of varying molecular weight (400, 600, 1500, 2000, and 6000 g·mol⁻¹): A) Water as the third phase-forming component; B) BMMH culture media as the third phase-forming component.

becomes less dominant, thus justifying the different trend from that observed when phase diagrams were determined in water.

Overall, the polymer concentration required to form a biphasic system decreases as PEG molecular weight increases, and when salt is present, viz. when culture media is used as solvent. Consequently, systems prepared in culture medium and/or with higher-MW PEG require lower polymer concentrations to achieve phase separation. Details on the fitting of the experimental data by eq. (1) and the determination of the respective tie-line (TLs) and lengths (TLLs) are shown in the Supplementary Material (Table S1).

3.3. Recovery of interferon α -2b from *K. phaffii* culture media using ABS/TPP

ABS and TPP approaches provide simple, mild and scalable platforms to separate proteins, and may allow rapid fractionation while preserving protein stability and activity. Thus, polymer-based ABS/TPP were investigated in this work for the recovery of IFN α -2b from *K. phaffii* culture broth. PEG and PPG were chosen as phase-forming agents since they have been used in medical applications [32], thus having a more biocompatible character when compared with other ABS components. Even though IFN α -2b structure was not evaluated after the extraction process, PEG precipitation has been previously applied to recover proteins from different sources [16,33], overall providing encouraging

evidence regarding its mild character over proteins while confirming its suitability for protein extraction processes.

Considering the phase diagrams previously determined (see Fig. 3), three mixture points for PEG 400/600/1500/2000/6000 g·mol⁻¹ and PPG 400 g·mol⁻¹-based ABS were selected to study the partitioning profile of IFN α -2b, namely: 30–30 wt%, 20–35 wt% and 35–20 wt%, respectively, for the concentrations of PEG and PPG. Initially, the samples (top and bottom phases, as well as the precipitate at the interphase when present) from all mixture points were analyzed for the total protein content by the BCA protein assay (see Fig. S2 of the Supplementary Material for quantitative data regarding the mass of total proteins (μ g) in each ABS phase and precipitate). Since only residual amounts of proteins were detected in the top phase enriched in PPG, and hence not detectable by SDS-PAGE (data not shown), only the bottom PEG-rich phases and the precipitates were systematically analyzed by SDS-PAGE and dot-blot. The respective results are shown in Fig. 4.

According to the SDS-PAGE analysis, a single and well-defined band with a molecular weight close to 17 kDa is detected, corresponding to IFN α -2b. Additionally, precipitate formation was observed in all the systems investigated (Fig. 4), thereby placing this approach within the ABS-based TPP framework.

Protein partitioning using ABS is influenced by several factors, ranging from the protein's intrinsic characteristics (e.g., isoelectric point and molecular weight) to the concentration and hydrophobicity of the

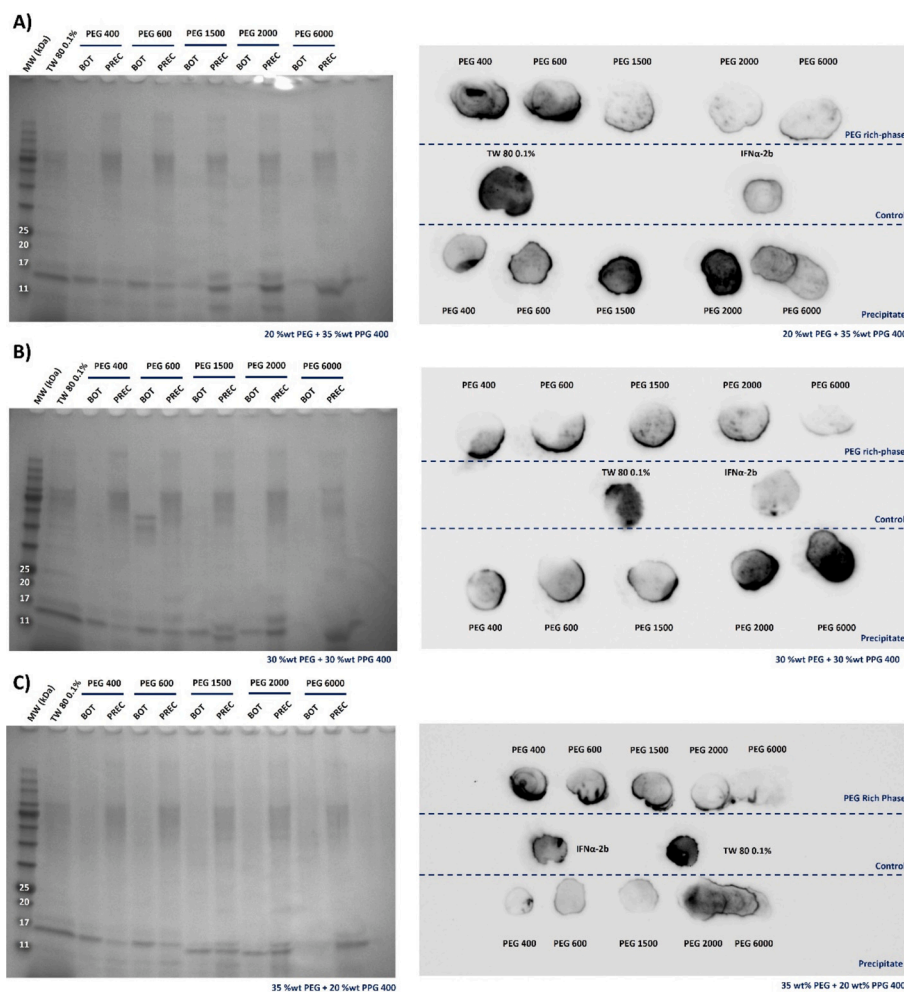


Fig. 4. SDS-PAGE (left) and dot blot (right) analysis for the PEG-rich phase and precipitate obtained for ABS composed of PEG of varying molecular weights (400, 600, 1500, 2000, and 6000 g·mol⁻¹) and PPG 400 g·mol⁻¹ after fractionation of *K. phaffii* culture broth expressing IFNα-2b. A, B, and C represent, respectively, the following mixture points (PEG-PPG): 20–35 wt%, 30–30 wt%, and 35–20 wt%. BOT represents the PEG-rich phase, while PREC represents the precipitate. IFNα-2b and TW 80 0.1% correspond, respectively, to a commercial protein standard (Abcam, UK) and the initial culture broth.

polymers [33]. Additionally, increasing the polymer molecular weight, thus increasing its hydrophobicity, is expected to decrease the protein solubilization and accordingly enhance protein precipitation. The same behavior is expected by increasing the polymer concentration. The SDS-PAGE and dot-blot analysis (Fig. 4) show that the extension of the IFNα-2b precipitation depends on both the MW and concentration of PEG. High MW PEGs lower the IFNα-2b solubility in the respective rich phase, leading to increased precipitation levels of the target protein. However, if the PEG-rich phase has a lower MW, and thus a higher content of water, protein solubility increases, and IFNα-2b tends to preferentially partition to the PEG-rich phase. The same behavior occurs for lower PEG concentrations.

Considering the overall hydrophobicity of these systems, residual amounts of protein precipitate even in systems composed of PEG with lower MW (e.g., PEG 400 g·mol⁻¹) and low concentrations of PEG in the phase due to the presence of the highly hydrophobic PPG 400 g·mol⁻¹. Nevertheless, among the PEGs in study, it is noticeable that from PEG 1500 g·mol⁻¹ upwards, the target protein has a greater tendency to precipitate than to be recovered in the aqueous, PEG-rich phase. To benchmark the ABS-TPP approach reported in this work, IFNα-2b precipitation from *K. phaffii* was performed using 30 wt% PEG 2000 g·mol⁻¹ (see Supplementary Material Fig. S3). However, the target protein remains undetected in the suspended pellet after centrifugation, indicating inefficient recovery and precipitation, and thus the relevance of ABS-TPP to perform its clarification and recovery from the

fermentation broth.

Along with SDS-PAGE, dot-blot (Fig. 4) was employed to monitor the partition profile of the target protein in the designed systems. The signal corresponding to IFNα-2b was detected both in the aqueous phase enriched in PEG and in the recovered precipitates. This indicates that the epitope responsible for the interaction with the antibody raised against IFNα-2b remained structurally stable, further confirming the preservation of immunoreactivity during the recovery process. These observations were also confirmed by western blot, as visible in Fig. 5. For either PEG-rich phases or precipitates, a single band corresponding to immunoreactive IFNα-2b with the expected molecular was obtained.

3.4. Purification factor of IFN α-2b recovered using ABS/TPP

Considering the information presented in the previous section, the global purification performance of ABS/TPP approaches was assessed based on the determination of IFNα-2b levels in precipitates. Fig. 6 shows the amount of recovered IFNα-2b expressed as the recovery yield (Y, %), whereas Fig. 7 depicts the purification factors of the target protein recovered as a TPP interphase in the several systems studied.

Based on the results shown in Fig. 6, and at the same mixture composition (35 wt% PEG + 20 wt% PPG), preferential partitioning of the target protein to the PEG-rich phase was observed for low-molecular-weight PEG systems (PEG 400/600/1500 g·mol⁻¹), with only 22–62.9% of the protein being precipitated. In contrast, increasing

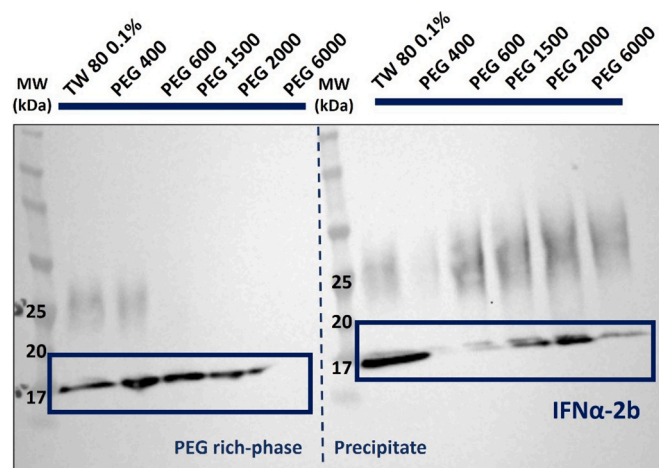


Fig. 5. Western Blot analysis of the PEG-rich phase (left) and precipitate (right) for ABS composed of PEG 400/600/1500/2000/6000 g·mol⁻¹ 30 wt% and PPG 400 g·mol⁻¹ 30 wt%. TW 80 0.1% represents the culture broth cultivated with tween 80 0.1% (v/v).

the PEG molecular weight to 2000 and 6000 g·mol⁻¹ markedly enhances IFNα-2b precipitation, with precipitated fractions rising to 70–99%. As expected, the IFNα-2b recovery yield in the TPP interphase is enhanced as the MW of PEG increases and as the concentration of both polymers increases. Under these circumstances, the amount of water decreases, thus decreasing protein solubility in the PEG-rich phase and favoring protein precipitation. In protein precipitation by polymers, the “exclusion effect” (excluded volume effect) [33] becomes dominant; when steric repulsion effects start to dominate, proteins in aqueous solution will have lower solubility and tend to precipitate [34]. Overall, higher-molecular-weight PEGs enhance precipitation by causing stronger excluded-volume and dehydration effects. Their larger polymer chains occupy more volume in solution, reducing the amount of water and free space available to maintain the protein hydration shell. This promotes protein–protein interactions, decreases protein solubility, and favours precipitation at the interphase in TPP. For low MW PEGs, such as PEG 400 and 600 g·mol⁻¹, the excluded volume effect is less pronounced, allowing the recovery of a significant fraction of IFNα-2b in the aqueous,

PEG-rich phase. Noticeable, despite the relatively low MW (~17kDa) of IFNα-2b that will result in a lower exclusion effect, high levels of the target protein precipitate (99%) when increasing the MW of PEG up to 6000 g·mol⁻¹. However, it should be acknowledged that since IFNα-2b levels have only been determined in precipitates, a complete mass balance across all phases (ABS top and bottom phases, and the interphase) could not be performed, precluding a more accurate assessment of the recovery yields of the developed platform.

Based on the levels of both IFNα-2b and total protein in the interphase of TPP systems (Supplementary Material, Fig. S2), the IFNα-2b purification factors (PF) were determined, allowing to infer if the TPP process is indeed selective for IFNα-2b. The results obtained are shown in Fig. 7.

From Fig. 7, for most of the conditions addressed, the PF is above 1, indicating that the samples from ABS-TPP are enriched in IFNα-2b. Particularly for the mixture point composed of 35 wt% PEG 400/600/1500/2000/6000 and 20 wt% PPG 400 g·mol⁻¹, the IFNα-2b PF increases with increasing the PEG MW. In addition to have a more selective precipitation of IFNα-2b over other proteins, the enhanced PF obtained is also explained by the high amount of the target protein that is precipitated, in agreement with Fig. 6.

Overall, the obtained results demonstrate that polymer molecular weight and polymer concentration are key parameters governing both phase behavior and selectivity in ABS-TPP systems, directly impacting the recovery yield and purification factor of IFNα-2b. The observed increase in the PF with increasing PEG molecular weight and concentration of the polymers, coupled with high recovery yields through selective ABS/TPP interphase precipitation, highlights the potential of these systems to integrate primary recovery and partial purification into a single step. This dual functionality is particularly relevant for biopharmaceutical downstream processing, where process intensification and cost reduction are critical. By enabling a tunable transition between liquid–liquid extraction and three-phase partitioning within the same platform, this approach opens new avenues for the design of flexible and scalable purification strategies. Moreover, it lays the foundation for extending these concepts to other recombinant proteins and complex biological matrices and/or to the development of continuous processes, fostering the development of more efficient, sustainable, and economically viable bioprocesses. The IFNα-2b PF obtained in this work are lower than those previously reported using ABS composed of PEG 600 –

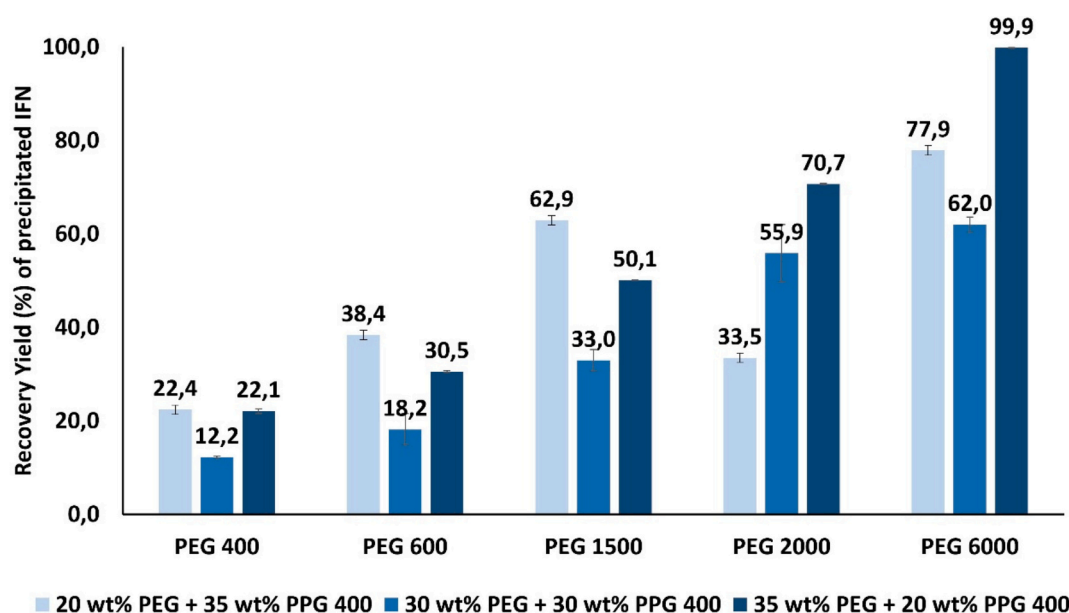


Fig. 6. Determination of the recovery yield of IFNα-2b by ELISA, in percentage, precipitated using ABS-TPP approaches composed of PEG 400/600/1500/2000/6000 g·mol⁻¹ and PPG 400 g·mol⁻¹ with the following mixture points (PEG-PPG): 20–35 wt%, 30–30 wt%, and 35–20 wt%.

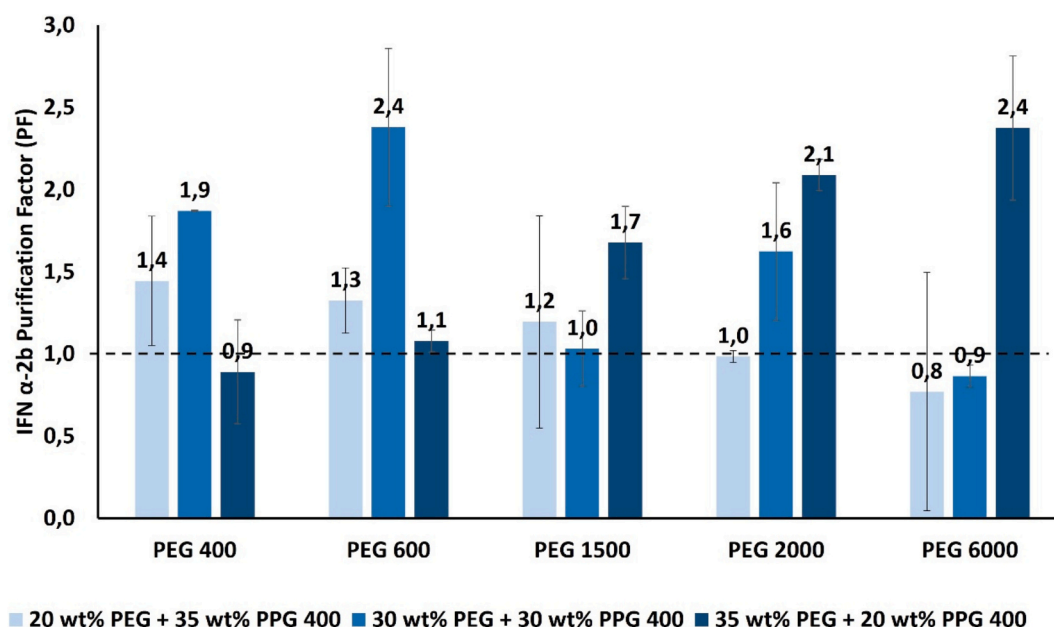


Fig. 7. Purification factor (PF) of IFN α -2b in the interphase of ABS/TPP systems composed of PEG 400/600/1500/2000/6000 g·mol⁻¹ and PPG 400 g·mol⁻¹ with the following mixture points (PEG-PPG): 20–35 wt%, 30–30 wt%, and 35–20 wt%.

PPG 400 with ionic liquids as adjuvants (PF = 6.77) [30] or 2-propanol – ammonium sulfate with sodium chloride as adjuvant (PF = 16.24) [35]. However, it should be taken into consideration that the sources of IFN α -2b are different, respectively, *Escherichia coli* inclusion bodies [30] and periplasm [35].

Although outside of the scope of this work, these processes hold potential for scaling up, particularly for the liquid-liquid approach based on lower MW PEGs, which renders the phases less viscous and potentially more amenable to scale-up with high IFN recovery in the aqueous, PEG-rich phase. The feasibility of scalable and continuous process operation using ABS is noted in the literature [19,36].

4. Conclusions

This work demonstrated the effective recovery of IFN α -2b from *K. phaffii* culture broth using polymer–polymer ABS and related TPP strategies, enabling the biopharmaceutical recovery either in aqueous PEG-rich phases or as interphase precipitates. At the upstream level, supplementation of the culture medium with low concentrations (0.1%) of Tween 80 significantly enhanced protein expression, reaching titers of 78.1 mg·L⁻¹. Downstream recovery was then successfully achieved using ABS/TPP composed of PPG 400 and PEGs with varying molecular weights (400–6000 g·mol⁻¹), highlighting the pivotal role of polymer molecular weight and polymer concentration in controlling phase behavior and protein partitioning. Phase separation is also favored in the presence of the culture media, as experimentally verified through the determination of the respective phase diagrams. Overall, ABS/TPP comprising low-molecular-weight PEGs favored the partitioning of IFN α -2b into the PEG-rich phase, maximizing recovery yields in the aqueous phase. In contrast, higher-molecular-weight PEGs promoted the excluded volume effects, leading to selective IFN α -2b precipitation (PF of 2.4) with high recovery yields (99%).

This work demonstrates that polymer–polymer ABS can be finely tuned to achieve both efficient recovery and clarification of protein therapeutics. By modulating PEG molecular weight and polymer concentration, it is possible to recover the target protein either in an aqueous phase or in a solid interphase, thereby integrating extraction and precipitation strategies within a single platform. Accordingly, polymer–polymer-based ABS can be considered versatile and tunable

multiphase systems, enabling both liquid–liquid extraction and three-phase partitioning. Overall and in comparison with high-resolution purification techniques (e.g., chromatography), the ABS/TPP platform reported in this work represents a cost-effective approach, offering a good cost-benefit balance by providing high protein recovery, good concentration, and partial impurity removal using inexpensive reagents and simple equipment, contributing to reducing the number, size, or cost of the following purification steps.

This integrated approach offers significant potential for process intensification in downstream bioprocessing, opening new opportunities for the development of scalable, cost-effective, and adaptable purification strategies potentially for recombinant proteins and other biopharmaceuticals.

CRediT authorship contribution statement

Leonor S. Castro: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Mara G. Freire:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Augusto Q. Pedro:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.154497>.

Data availability

Data will be made available on request.

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