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Aqueous micellar systems containing Triton X-114 and *Pichia pastoris* fermentation supernatant: A novel alternative for single chain-antibody fragment purification

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ABSTRACT

The main goal of this work was to evaluate the feasibility of using aqueous micellar two-phase systems (AMTPS) of Triton X-114 to purify a single-chain antibody fragment (scFv) directly from yeast fermentation supernatant. The coexistence curves of aqueous micellar two-phase systems, highly loaded (up to 90% wt/wt) with the biological feedstock, were determined. Besides, the effect of several additives such as inorganic salts and affinity ligands on the phase separation behavior was investigated. The obtained coexistence curves demonstrated that the assayed surfactant/yeast broth solutions were able to separate into two phases at temperatures lower than 24 °C. This information was then utilized to assay scFv partitioning and purification. In general, proteins present in the yeast broth, including the scFv, partitioned to the top, micelle-depleted phase due to its hydrophilic character. When affinity ligands were used, an opposite behavior was observed for scFv due to the uneven partitioning of ligands toward the micellar-rich phase. The best purification performances were attained for the system consisting of 4% wt/wt of Triton X-114, 60% wt/wt of yeast fermentation supernatant and the synthetic ligand Fabsorbent™ F1P HF, with a recovery percentage of 88% and a purification factor of 2. These results demonstrate the potential applicability of these systems for designing early steps for scFv purification directly from yeast broth. New perspectives are now open for the use of this methodology for recombinant antibody fragments purification.

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1. Introduction

In the last years, single-chain antibody fragments (scFv) have gained importance in the pharmaceutical industry because of the several advantages that these antibodies present respect to full-sized immunoglobulins, e.g. easy manipulation, high permeability and the fact that their immunocomplexes can be quickly cleared from the human body [1]. In fact, thousands of examples of scFv and derivatives are currently in development or are undergoing clinical testing [2,3].

At present, most of scFv molecules are produced by microorganisms, such as *Pichia pastoris*, and purified directly from the fermented broth [4–6]. However, the efficient purification of the target molecule requires several unit operations (e.g. centrifugation, filtration, precipitation and different chromatographic techniques) due to the complexity of culture medium (high viscosity, high density, mixture of biomolecules) [7]. Since the use of multi-steps purification protocols generally leads to high time consumption and low product recovery [7], is imperative to find and evaluate new extractive/purification techniques which could solve some actual technological limits.

Liquid–liquid extraction in aqueous two-phase systems (ATPS) is a methodology which has been widely applied to purify biomolecules directly from biological feedstock [8–11].

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Nomenclature

AMTPS	aqueous micellar two-phase systems
ATPS	aqueous two-phase systems
BCA	Bicinchoninic Acid
BGCM	Buffered Glycerol Complex Medium
BSA	Bovine Serum Albumin
C^0	protein concentration (scFv o TP) (mg/L) in the clarified broth
$C_{(T/B)}$	protein concentration (scFv o TP) (mg/L) in top (T) or bottom (B) phase
CB	Cibacron Blue F3GA
CP	cloud point
ΔCP	CP at condition A – CP at condition B (°C)
HF	Fabsorbent™ F1P HF
IMAC	immobilized metal affinity chromatography
K	partition coefficient
MB	mass balance
P%	total purity
PBS	phosphate buffered saline
PEG	polyethylene glycol
PF	purification factor
PMSF	phenylmethanosulfonylfluoride
scFv	single-chain antibody fragment
TP	total proteins
V^0	volume of added yeast supernatant (mL)
$V_{(T/B)}$	top (T) or bottom (B) phase volume (mL)
%wt/v	grams of specific compound in 100 mL of total system/solution
%wt/wt	grams of specific compound in 100 grams of total system/solution

Particularly, aqueous micellar two-phase systems (AMTPS) have become a popular methodology because of its advantages of high enrichment factor, high recovery, simplicity and low cost [12,13]. This technique is based on the ability of some surfactants to form two immiscible aqueous phases, a micelle-rich phase and a micelle-poor phase, over certain temperature, defined as cloud point (CP) [14]. The first use of AMTPS as separation methodology was reported by Watanabe and Tanaka for the concentration of zinc ions [15]. Afterwards, Bordier et al. demonstrated the efficiency of Triton X-114 AMTPSs to separate proteins based on their hydrophobicity [16]. At present, this surfactant is the most widely used AMTPS forming component due to its low cloud points (24–33 °C) [17]. Besides, the use of AMTPS has been extended to extract and purify different molecules such as aromatic hydrocarbons, viruses, metal ions, as well as whole cells [13,18,19].

In this context, the main goal of this work was to evaluate the feasibility of using aqueous micellar two-phase systems (AMTPS) of Triton X-114 to purify scFv molecules directly from yeast fermentation supernatant. The effect of several variables such as fermented broth loading, addition of salts and ligands on scFv partition and purification was investigated. In addition, phase diagrams of broth loaded aqueous micellar systems were initially characterized in order to adequately select the working temperature and to detect changes in phase compositions that would affect partition behavior.

2. Materials and methods

2.1. Yeast growth and scFv production

A single-chain variable fragment (scFv) producing *P. pastoris* (SMD 1168 Invitrogen: Δpep4::URA3 ΔKex1::SUC2 his4 ura3 His⁻Mut^T) was used in this work [20]. The genetically modified yeast was kindly provided by the Department of Clinical and Toxicological Analysis of the University of Sao Paulo (SP), Brazil, and maintained at −80 °C in 50% glycerol (v/v) before use. Buffered Glycerol Complex Medium (BGCM), see Table 1, was used to start *P. pastoris* culture. The yeast growth was initialized for 16 h in a 250-mL orbital shaker (250 rpm) at 20 °C. Afterwards, the inoculum was transferred into a 3-L bioreactor and grown for 96 h at 20 °C in BGCM medium. 1% v/v of inductor (methanol) and 1 mM of the proteases inhibitor phenylmethanesulfonylfluoride (PMSF) were added every 24 h maintaining the pH at a constant value of 6.80. The final fermented broth was then centrifuged for 10 min at 2000 rpm in order to remove yeast cells. The supernatant was storage at −4 °C before use.

2.2. Mapping the coexistence curve of the Triton X-114/yeast broth

To better understand protein partitioning behavior in aqueous micellar two-phase systems, phase separation temperature and surfactant concentration in the two coexistent phases must be known. Thus, the coexistence curves of the assayed systems should be mapped out as a previous step to develop liquid–liquid extraction (Supplementary Fig. 1 shows the features of a typical AMTPS coexistence curve) [12,14].

The coexistence curves of aqueous solution containing Triton X-114 (Sigma–Aldrich; St. Louis, MO, USA) and yeast broth supernatant were performed by the method described by Watanabe and Tanaka [15]. Thus, solutions of different surfactants compositions (from 0 to 10% wt/wt) were prepared in McIlvaine buffer pH 7.00 (1.38 mM citric acid and 5.3 mM disodium phosphate) in 10 mL-glass tubes (total system mass of 3.0 g). The systems were mixed at 8 rpm for 1 h at 8 °C in order to obtain a homogeneous clear system. Afterwards, the solutions were transferred into a thermo-regulated bath where the temperature was step-wise raised (temperature increment = 0.1 °C). The temperature at which the solution first became cloudy was taken as the cloud point. The same procedure was repeated by replacing McIlvaine buffer for yeast fermentation supernatant to obtain different loadings (30, 60 and 90% wt/wt). The pH was kept constant at 7.00. The observed values of CP were then plotted as a function of the corresponding surfactant concentration. The procedure was repeated three times to warrant reproducibility. The effect of several additives such as NaCl (5%wt/wt), $MgSO_4$ (5%wt/wt), Cibacron Blue F3GA (Polysciences Inc) (0.05 10^{-3}% wt/wt) and Fabsorbent™ F1P (Prometic Biosciences) (0.008% wt/wt) on CP was also evaluated.

All the used glassware was firstly washed in a 50:50 ethanol/1 M sodium hydroxide bath, then, washed in a 1 M nitric acid bath, and finally rinsed copiously with Milli-Q water and dried in an oven at 70 °C.

Table 1
Composition of Buffered Glycerol Complex Medium (BGCM).

Components	Composition% wt/v
Yeast extract	1
Peptone of casein	2
YNB medium + 10% wt/wt of NH_4SO_4	1.34
Biotin	$4 \cdot 10^{-5}$
Glycerol	1
Casamino acids	2

2.3. Protein partitioning

Each buffered systems, with a total mass of 10.0 g, were prepared in graduated test tubes (15 mL). To accomplish that, Triton X-114 surfactant (4% wt/wt) was mixed with McIlvaine buffer at pH 7.00 and fermentation supernatant at increasing concentrations (30, 60 and 90% wt/wt). The resulting solutions were mixed at 8 °C for 1 h. Subsequently, the solutions were placed in a thermo-regulated device at 24 °C. Solutions were maintained at that temperature for 3 h to attain partitioning equilibrium. Samples from both upper and lower phases were then taken for the determination of scFv and total protein concentration. The effect of inorganic salts (NaCl and $MgSO_4$, 5%wt/wt), and ligands (Cibacron Blue F3GA, 0.05 10^{-3}% wt/wt, and Fabsorbent™ F1P, 0.008% wt/wt) on the extraction performance was also assayed.

2.4. Recombinant antibody fragment and total proteins assays

The quantity of single-chain antibody fragment (scFv) was determined by immobilized metal affinity chromatography (IMAC). Samples from both, yeast broth and phase systems, were passed through the chromatographic column. Bottom phases were previously diluted at least four times in PBS buffer at pH 7.40 to avoid surfactant interference. The chromatographic procedure was performed according to the manufacturer's instructions (GE Healthcare, Munich, Germany) [20]. After elution, scFv concentration was estimated by using the Bicinchoninic Acid method (BCA; Pierce, Rockford, IL, USA). Bovine Serum Albumin (BSA) was used as standard. Total proteins concentration was determined with the same methodology. All experiments were run in triplicate and the medium effect was discounted.

2.5. Extraction performance parameters

The partition coefficient (K) of scFv and total proteins was calculated as follows:

$$K = \frac{C^T}{C^B} \tag{1}$$

where C^T and C^B represent scFv or total proteins concentrations in the top (T) phase (micelle-poor) and bottom (B) phase (micelle-rich), respectively.

ScFv recovery percentage ($Y_{scFv\ (T/B)}$%) was calculated at the phase it preferentially partitioned (T or B) according to the following equation:

$$Y_{scFv(T/B)}\% = \left(\frac{C_{scFv(T/B)} * V_{(T/B)}}{C^0_{scFv} * V^0}\right) * 100\% \tag{2}$$

where $C_{scFv(T/B)}$ represents scFv concentration in the phase and $C_{scFv}{}^0$ represents scFv concentration in the clarified fermented broth. $V_{(T/B)}$ and V^0 are, respectively, the volumes of the phases and the volume of added yeast supernatant.

ScFv purification factor ($PF_{scFv(T/B)}$) was determined as follows:

$$PF_{scFv(T/B)} = \frac{C_{scFv(T/B)}/C_{TP(T/B)}}{C^0_{scFv}/C^0_{TP}} \tag{3}$$

where C_{TP} and $C_{TP}{}^0$ represent total proteins (TP) concentration in the phase and in the fermented broth, respectively.

ScFv total purity (P_{scFv}%) in either the phase or the broth supernatant was calculated according Eq. (4):

$$P_{scFv}\% = \left(\frac{C_{scFv}}{C_{TP}}\right) * 100\% \tag{4}$$

where C_{scFv} and C_{TP} represent scFv and total proteins concentrations in the sample.

The mass balance (MB) was calculated according the following expression:

$$MB = \left(\frac{C_{(T)} * V_{(T)} + C_{(B)} * V_{(B)}}{C^0 * V^0}\right) * 100\% \tag{5}$$

where $C_{(T)}$, $C_{(B)}$ and C^0 are scFv or total protein concentrations in top phase, bottom phase and in the crude extract, respectively. $V_{(T)}$, $V_{(B)}$ and V^0 are the respective volumes in each case.

3. Results and discussion

3.1. Defining the coexistence curves of Triton X-114/yeast broth systems

The coexistence curves of aqueous micellar two-phase systems of Triton X-114/*P. pastoris* fermentation supernatant were mapped out varying the concentration of surfactant and yeast supernatant in McIlvaine buffer pH 7.00 as described in Section 2.

As can be noticed from Fig. 1(A and B), the presence of fermentation supernatant resulted in a drastic decrease of phase separation temperature (ΔCPs up to −14 °C), being this decrease proportional to the concentration of the added yeast broth.

Although phase separation basically depends on surfactant structure and concentration [21], the presence of additives such as inorganic salts, biopolymers, fatty acids, aliphatic alcohols, and phenols is known to strongly affect the cloud point (CP) [22–24]. So, as expected, most of yeast broth components may have contributed to CP lowering [24,25]. Alternatively, some broth components could have played a role as phase forming molecules. In this sense,

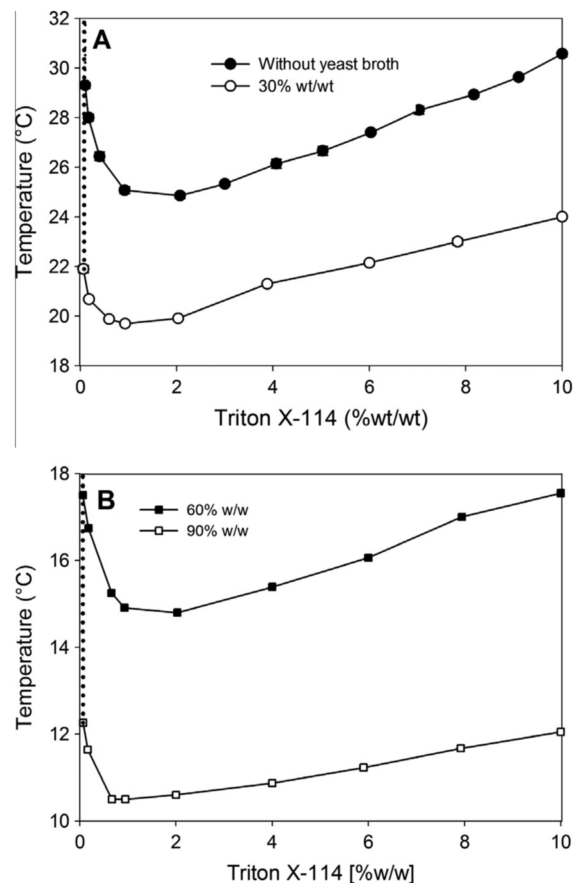

Fig. 1. Effect of clarified yeast broth percentage on the coexistence curves of Triton X-114/McIlvaine buffer system, pH 7.00. (A) Without and with 30% wt/wt of yeast broth. (B) 60 and 90% wt/wt of yeast broth.

Rito-Palomares and co-workers [26] have pointed out that the biopolymers present in biological suspension caused the binodial curves of PEG/Salts systems to move toward lower component concentrations. A similar conclusion was attained by Selvakumar et al. [11,27], who deeply investigated the influence of biomass in binodial curve position and phase volume ratio. Although similar analysis was not performed yet using Triton X-114 and a high concentration of *P. pastoris* broth, at least up to our knowledge, the results presented in Fig. 1 agree with other author works in which lower CPs are found when adding different biological feedstock to surfactant solutions [28,29].

3.2. Effect of additives on the cloud point of Triton X-114/yeast broth systems

Protein partitioning in aqueous micellar two-phase systems (AMTPS) basically depends on its hydrophobicity, thus limiting the use of AMTPS for the separation of hydrophilic proteins from the hydrophobic ones [16,30]. The addition of salts (NaCl and $MgSO_4$) and affinity ligands (Cibacron Blue F3GA (CB) and Fabsorbent™ F1P HF (HF)) was assayed in order to enhance the liquid–liquid extraction selectivity [31,32]. CB was selected since it is frequently used in protein purification due to its low cost and good stability [31,33]. HF ligand is a synthetic compound, cross-linked to an agarose bed, which has been designed to specifically capture and purify antibody fragments including monovalent antibody fragments (e.g. Fab, scFv), engineered antibody variants, and single-domain antibodies [34,35].

Figs. 2 and 3 show the obtained phase separation temperatures of micellar solutions containing yeast fermentation supernatant (30% wt/wt) and different additives. As expected, the presence of salts markedly lowered the CP values (Fig. 2). This behavior is usually attributable to a "salting out" effect which result in the reduction of the micellar "hydration shell" – layer of water molecules that surrounds micelles-, a closer interaction among micelles and therefore, a favored phase separation. [24,36]. The presence of affinity ligands also shifted down the phase separation temperatures (Fig. 3), but in a slighter extent than the salts did. Similar results were observed at 60 and 90% of yeast broth (data not shown).

3.3. Protein partitioning

Once the phase separation temperatures were obtained, the influence of yeast fermentation supernatant concentration, the addition of salts and affinity ligands on scFv partitioning behavior was investigated. Solutions of Triton X-114 4% wt/wt and a temperature of 24 °C were selected as working conditions to ensure phase separation in all the assayed systems. According to previous works, scFv structure was demonstrated to be unaffected by the selected surfactant at room temperature [37].

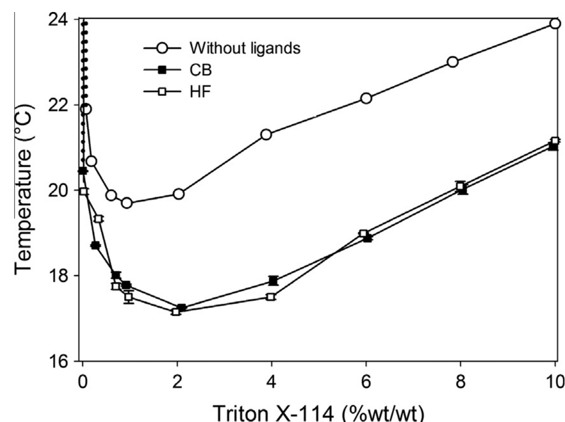

Fig. 3. Effect of Cibacron Blue F3GA (CB) ($0.05 \cdot 10^{-3}$% wt/wt) and Fabsorbent™ F1P (HF) (0.008% wt/wt) on the coexistence curves of Triton X-114/McIlvaine buffer micellar systems. Clarified yeast broth percentage: 30% wt/wt. Medium pH: 7.00.

3.3.1. Effect of broth loading percentage

The effect of broth loading on protein partition is shown in Fig. 4. As it can be seen, total proteins (TP) partition coefficients resulted to be >1 under all the assayed conditions. This agrees with the fact that most of the fermentation supernatant molecules are expected to be water-soluble [38,39]. As far as the effect of yeast broth concentration is concerned, it was observed that when the percentage of biological feedstock is increased (from 30 to 90%wt/wt), TP presented a slightly higher affinity for the micelle-poor (top) phase, which is evidenced by an increase in their *K* values (see Fig. 4). According to the excluded-volume theory [45], when the difference between surfactant concentration in the top and bottom phases becomes higher, hydrophilic biomolecules will partition to the micelle-deplete phase in order to meet larger free volume [14,40,41]. This behavior is attained, for example, when a coexistence curve is lowered by an additive (see Supp. Fig. 2). In this context, as a higher yeast broth loading leads to a higher difference in phase surfactant concentrations (see Fig. 1), it is

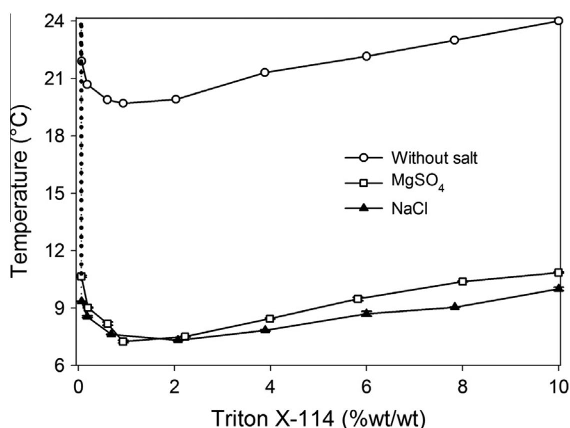

Fig. 2. Effect of electrolytes (5% wt/wt) on the coexistence curves of Triton X-114/McIlvaine buffer micellar systems. Clarified yeast broth percentage: 30% wt/wt. Medium pH: 7.00.

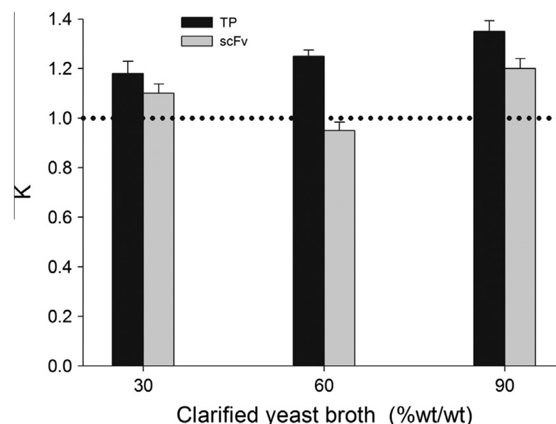

Fig. 4. Effect of clarified yeast broth percentage on total protein (TP) and single-chain antibody fragment (scFv) partition coefficient (*K*). Medium pH: 7.00. Temperature: 24 °C.

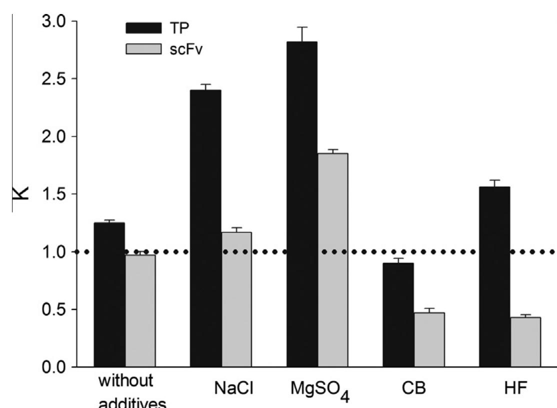

Fig. 5. Effect of salts (NaCl and $MgSO_4$ at 5% wt/wt) and ligands (Cibacron Blue F3GA (CB) at $0.05{\cdot}10^{-3}$% wt/wt and Fabsorbent™ F1P (HF) at 0.008% wt/wt) on total protein (TP) and single-chain antibody fragment (scFv) partition coefficient (K). Clarified yeast broth: 60% wt/wt. Medium pH: 7.00. Temperature: 24 °C. $V_{(T)}/V_{(B)}$ = 1.5.

reasonable that TP partition will be favored toward the micellar-poor phase [38].

A similar trend was observed for scFv partitioning in systems loaded with 30 and 90% wt/wt of fermentation supernatant (see Fig. 4). The slight decrease in scFv partition coefficient (from 1.1 to 0.95), when a 60% wt/wt of clarified broth is added, can be attributable to both, the protein precipitation at the interface or a mechanism of protein partitioning [8] which includes effects other than the exclusion.

3.3.2. Effect of additives

As it was previously discussed (Section 3.2), when inorganic salts are added to aqueous micellar systems, the hydration shell of micelles becomes thinner and the protein solubility in this medium decreases. Consequently, most of water-soluble proteins are driven to the micelle depleted phase [38,42].

Fig. 5 shows that both, total proteins (TP) and scFv partitioned mainly to the micelle-poor (top) phase when NaCl and $MgSO_4$ were used. This behavior evidences the water soluble character of most of proteins present in the broth supernatant, including the scFv molecule. Moreover, it agrees with our previous works which demonstrated that scFv relative surface hydrophobicity was similar to that of hydrophilic model proteins [37].

A different pattern was observed when the ligand effect on K of TP and scFv was analyzed (Fig. 5). Firstly, in presence of CB both, total proteins and the recombinant antibody were directed toward the phase were the dye preferentially partitioned (bottom phase). These results are in agreement with other authoŕs works which demonstrated that Cibacron Blue can interact with different proteins in a semi-specific manner [33,43]. Oppositely, when HF was used, only scFv was partitioned to the micelle-rich (bottom) phase while TP were slightly directed to the opposite phase. That behavior shows that HF can be used to selectively separate scFv from the other proteins present in the yeast broth [34].

Table 3
Effect of salts (NaCl and $MgSO_4$ at 5% wt/wt) and ligands (Cibacron Blue F3GA (CB) at $0.05{\cdot}10^{-3}$% wt/wt and Fabsorbent™ F1P (HF) at 0.008% wt/wt) on scFv purification parameters. Yeats broth: 60% wt/wt. Medium pH: 7.00. Temperature: 24 °C. $V_{(T)}/V_{(B)}$ = 1.5.

	scFv Concentration (mg/L)	PF	P%	Y%	MB%
Without additives	124[a]	1.1[a]	18[a]	69[a]	93
NaCl	93[a]	0.6 [a]	11[a]	56[a]	79
$MgSO_4$	104[a]	0.8 [a]	13[a]	63[a]	71
CB	137[b]	1.2[b]	19[b]	51[b]	90
HF	271[b]	2.0[b]	32[b]	88[b]	92

[a] Calculated in top phase.
[b] Calculated in bottom phase.

3.3.3. ScFv purification parameters

In order to evaluate the separative capacity of the assayed systems, scFv purification parameters were also calculated. In this context, purification factor (PF), total purity percentage (P%), recovery percentage (Y%) and mass balance (MB) were determined for the antibody fragment (Tables 2 and 3).

As shown in Table 2, no significant changes were observed at working with broth loading of 30 and 60% wt/wt. However, a marked decrease in scFv recovery was obtained for a broth loading of 90% wt/wt. This behavior could be attributable to the precipitation of scFv at interface in agreement with the low mass balance presented in Table 2. Protein precipitation at the interface is commonly observed when certain system components reach their solubility limit [8]. Moreover, this phenomenon is more frequently attained when working with complex samples such as yeast broth [27].

The scFv purification parameters, obtained when using different additives, are shown in Table 3. For this purpose, 60% wt/wt of yeast fermentation supernatant was selected since it was the highest loading that presented minimal protein precipitation (see Table 2). Table 3 shows that the addition of both, NaCl and $MgSO_4$, decreased the purification factor and the recovery percentage of scFv. The low PF obtained in presence of salts can be explained by the fact that TP partitioned more unevenly toward the top phase than scFv (Fig. 5). Besides, the recovery decrease could be attributable to scFv precipitation at the interface [44], agreed with the low MB shown in Table 3.

When using HF and CB as additives, improved purification parameters for scFv extraction were obtained. This confirms the advantage of using affinity ligands to improve the selectivity of liquid–liquid extraction [32,45]. The best purification parameters were observed in presence of HF, with a recovery percentage of 88% and a purification factor of 2. These results are similar or even better than those obtained from other single-step purification methodologies. Table 4 [7] shows that recoveries lower than 85% were reported for scFv purification with packed-bed chromatography and precipitation with polyelectrolytes [46,47]. Only affinity-based expanded bed adsorption presented higher scFv recoveries, however, this methodology provides a final product with low scFv concentration (15 mg/L) thus requiring a subsequent concentration step. In regards to scFv purification factor, our results are

Table 2
Effect of clarified broth percentage on scFv purification parameters. Medium pH: 7.00. Temperature: 24 °C.

Yeast broth[a]	scFv Concentration (mg/L) [b]	PF[b]	P%[b]	Y%[b]	MB (%)	$V_{(T)}/V_{(B)}$
30	57	0.9	14	63	92	1.4
60	124	1.1	18	69	93	1.5
90	111	1.0	16	48	61	2.3

[a] Expressed as % wt/wt of the total system mass.
[b] Calculated in top phase.

Table 4
Reported antibody fragments purification parameters (adapted from Ref. [7]).

Methodology	scFv Source	scFv Concentration[a] (mg/L)	Yield percentage	Purification factor
Affinity-based packed bed adsorption	*S. frugiperda*	400	15	2.02
Ion-exchange packed bed adsorption	*P. pastoris*	3660	84	2.30
Hidrophobic-based packed bed adsorption	*P. pastoris*	1200	33	1.15
Affinity-based expanded bed adsorption	*E. coli*	15	93	–
MAL-Ni(II) precipitation	*E. coli*	19	80	21
Protanal LF precipitation	*E. coli*	–	30	–

[a] In final product.

comparable to those obtained from other methodologies. Exceptionally, the precipitation with affinity macroligands ($MAL\text{-}Ni_{(II)}$) allows a PF of 21, however, low final scFv concentration is also achieved by this technique.

4. Conclusion

In this work, the feasibility of using aqueous micellar two-phase systems (AMTPS) to purify scFv directly from yeast fermentation supernatant was investigated for the first time. The study of phase separation behavior of AMTPS consisting of Triton X-114 and a high percentage of yeast broth was investigated as a previous step to develop extractive methodologies. The obtained coexistence curves demonstrated that the assayed surfactant solutions were able to separate into two phases at temperatures lower than 24 °C, thereby decreasing the potential protein denaturation by high temperatures. Besides, phase separation behavior as well as protein partitioning resulted to be highly sensitive to the addition of different additives such as salts and ligands, suggesting that the work conditions must be rigorously adjusted in order to reach the desirable systems. The best purification performances were attained for the system consisting of 4% wt/wt of Triton X-114, 60% wt/wt of yeast fermentation supernatant and the synthetic ligand HF, with a recovery percentage of 88% and a purification factor of 2.

Even though complementary experiments should be carried out to optimize the obtained purification performances, the results here reported have demonstrated the potential of the assayed systems to be used in concentration and first-step purification of scFv from a fermentation broth. A comparative analysis of purification performances vs. simplicity, cost and time consumption, shows the scFv extraction with AMPTS possess a favorable benefit/cost balance among other methodologies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2014.05.045.

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