A Study on Lipid Removal from Yeast Homogenate by Chemical Pretreatment

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ABSTRACT: *Lipids discharged into the yeast homogenate following cell disruption process have deleterious impact on the performance of chromatography columns during downstream processing. In this study, removal of lipids from Pichia pastoris crude extract was investigated using various chemicals at varied concentrations. After treatment of the sample with 0.05 M borax and 1.5% PEI, a lipid reduction of about 52% and 79% was respectively obtained in the flocculated protein. Addition of PEG 6000 to the sample at a final concentration of 20% led to 57% lipid removal. A lipid reduction of 67%–70% was observed when protein precipitation performed by 20%–40% ammonium sulfate. The efficiency of acetone and diethyl ether for lipid reduction was greater than that of isopropanol, and substantial lipid removal (95%–100%) was demonstrated subsequent to feedstock treatment with 0.5%–5% acetone and diethyl ether. After conducting detergent lipid dissolution experiments, it was revealed that non-ionic detergents (Triton X-100 and Tween 20) at a concentration of 0.1% were able to eliminate 100% of lipids in the feedstock. Overall, these findings suggest that such chemical treatment strategies may be considered to be used for pre-treating process stream and facilitating related research and applications in downstream processing.*

KEYWORDS: *Yeast homogenate; Lipid removal; Fouling; Flocculation; Salt and polymer precipitation; Solvent and detergent lipid dissolution.*

INTRODUCTION

With the advent of recombinant DNA technology in the early 1970s, the biotechnology industry was fundamentally transformed. Modern biotechnologies have had myriad applications in various areas including

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fermentation, microbial physiology, metabolic engineering, enzyme engineering and recombinant protein production [1-3]. One of the main achievements of modern biotechnology has been the ability to manufacture recombinant therapeutics, some of which previously derived from conventional sources such as plasma or organs, with eliminating the risk of contamination to pathogens in the product [4]. To date, numerous protein pharmaceuticals have been developed by recombinant DNA technology and approved by the Food and Drug Administrations [5]. For instance, yeast-cell derived hepatitis B vaccine (containing recombinant hepatitis B surface antigen, rHBsAg) is now commercially available as a good replacement for the first generation of the plasma-derived vaccine [6, 7]. Amongst yeast, *Pichia pastoris (P. pastoris)* is the leading host of choice for production of the heterologous proteins due to its ability for carrying out N-linked glycosylation in endoplasmic reticulum (ER) that mimics mammalian glycosylation [3, 8, 9]. In the production process of the recombinant proteins and subsequent to the cells harvest, disruption and product release steps, the target protein molecules are accompanied by other biomolecules (including host cells proteins, nucleic acids, carbohydrates and lipids) which are called product-related contaminants [6, 10]. Chromatographic purification of various proteins and biopharmaceuticals is an integral part amongst downstream operations [11-14]. The released lipids in the process feed stream, in particular, can have negative effect (i.e., lipid fouling) on chromatography columns utilized for purification of target protein throughout downstream processing [10, 15, 16]. It has been reported that the fouling occurred by lipids in the chromatography column has not been completely irreversible following application of standard Clean-in-Place (CIP) conditions, and removing of lipids from the column by harsher CIP operation can adversely influence the ligand and as a result reduce the binding capacity of the column in subsequent purification cycles [10]. Lipid accumulation in a chromatography column can influence the binding properties of the resin ligands by creating lipid layer covering the surface of the adsorbents which subsequently changes the ligand density and type in the resins, complicates product elution from the chromatography resins and eventually makes the purification process inconsistent throughout successive chromatography cycles [10]. Subsequent to column capacity reduction and binding quality alteration occurred by accumulation of lipids in the column and application of harsh CIP conditions, the frequent column replacement becomes necessary which eventually makes the downstream processing considerably expensive.

Given these challenges, exploration for finding procedures aiming at lipid removal from the process feed stream prior to chromatographic purification step seems necessary. To date very limited number of investigations have been performed to remove lipids from lipid-rich feedstocks. Triton X-100, at a final concentration of 0.4% v/v, is the only chemical used to prepare low lipid feed, and polystyrene beads (XAD-4) have been usually applied to remove Triton X-100 prior to target protein purification by column chromatography. However, it has been shown that Triton X-100 can significantly remain in the feed due to inefficiency of XAD-4 in the presence of lipids [10, 16]. The lack of extensive methods for lipid removal from the yeast homogenate was the driving force for doing this research. The processes including protein flocculation, protein precipitation by polyether compound and lyotropic salt, and lipid dissolution combined with protein precipitation were scrutinized. Throughout these procedures, the efficiency of different kinds of chemicals at various concentrations, including borax (disodium tetraborate), polyethylene glycol (PEG), polyethylenimine (PEI), anionic and nonionic detergents (sodium deoxycholate, Triton X-100 and Tween 20), polar and non-polar solvents (acetone, diethyl ether and isopropanol) and ammonium sulfate for lipid removal from *P. pastoris* crude extract was evaluated.

EXPERIMENTAL SECTION

Materials

P. pastoris crude extract (i.e., mechanically disrupted yeast cells in 300 mM Tris-HCl buffer containing rHBsAg) and rHBsAg active pharmaceutical ingredient (rHBsAg API) were obtained from Pasteur Institute of Iran. Acetone, ammonium sulfate, diethyl ether, disodium hydrogen phosphate, disodium tetraborate 10-hydrate (borax), dithiothreitol (DTT), glycerol, glycine, hydrochloric acid, isopropanol, phosphoric acid, polyethylene glycol (PEG) 6000, silver nitrate, sodium chloride, sodium dihydrogen phosphate and sulfuric acid were purchased from AppliChem GmbH (Ottoweg, Darmstadt, Germany). Ammonium persulfate, barium chloride 2-hydrate, bromophenol blue, chloroform, sodium deoxycholate, sodium dodecyl sulfate (SDS), Triton X-100 and Tween 20

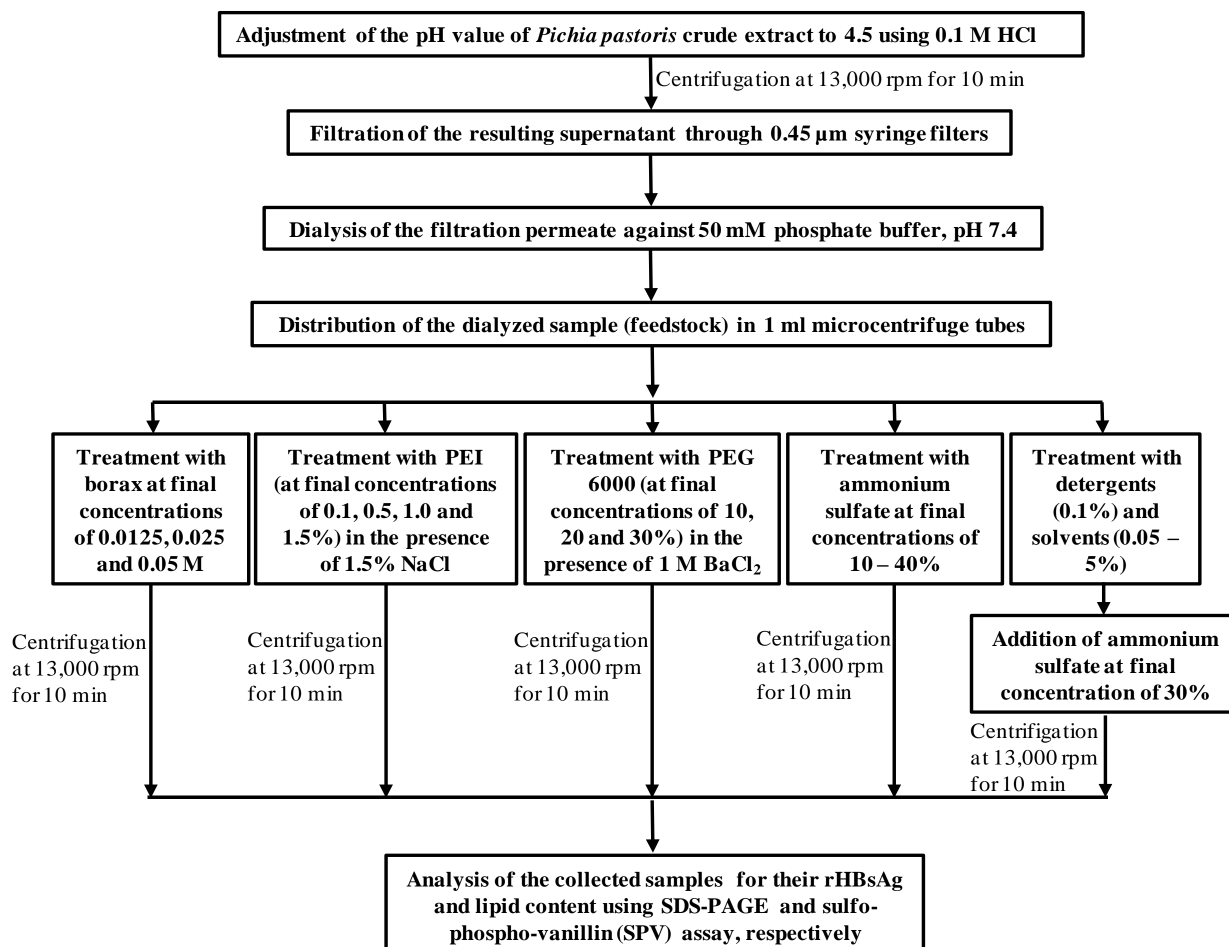

Fig. 1: Flowchart of the methodology

were obtained from Life Biolab GmbH (Im Neueheimer Feld, Heidelberg, Germany).

Methods (Fig. 1)

Preparation of the feedstock from P. pastoris crude extract (homogenate)

The pH value of the yeast crude extract was adjusted to 4.5 using 0.1 M hydrochloric acid and the solution was then centrifuged (at 13,000 rpm for 10 min) and filtered through 0.45 μm syringe filters (FilterBio, Nantong City, Jiangsu P.R China). The filtration permeate was dialyzed overnight against 50 mM phosphate buffer (pH 7.4) and distributed in microcentrifuge tubes (1 mL in each tube). These microcentrifuge tubes containing feedstock were used in the following experiments.

Treatment of the feedstock with borax

In order for having final borax concentrations of 0.0125, 0.025 and 0.05 M in the feedstock, various volumes of 0.1 M borax stock solution (prepared in 50 mM phosphate buffer, pH 7.4)) were added to 1 mL feedstock in the microcentrifuge tubes, at 25°C. After 1 h, the tubes were centrifuged using Hettich centrifuge (Tuttlingen Germany) at 13,000 rpm for 10 min and the supernatants and pellets were collected and stored at 4°C.

Treatment of the feedstock with PEI

Different volumes of 5% (v/v) PEI stock solution (prepared in 50 mM phosphate buffer, pH 7.4) were added to 1 mL feedstock containing 1.5% sodium chloride to reach final PEI concentrations of 0.1, 0.5, 1.0 and 1.5%. The microtubes were kept at 25°C for 1 h and then centrifuged at 13,000 rpm for 10 min. The supernatants and pellets were collected and stored at 4°C.

Treatment of the feedstock with the PEG 6000

A hundred microliters of 1 mM $BaCl_2$ was added

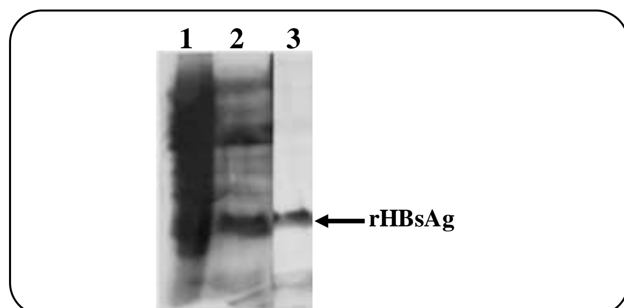

***Fig. 2:** SDS-PAGE analysis of supernatant and pellet samples following the adjustment of P. pastoris crude extract pH on 4.0 (acid precipitation). Lanes: 1– pellet; 2– supernatant; 3 – rHBsAg active pharmaceutical ingredient (rHBsAg API)*

to each of the microcentrifuge tubes containing the feedstock. Various amounts of PEG 6000 was weighed and added to the tubes to achieve PEG final concentrations of 10, 20 and 30%. The tubes were placed at 25°C, overnight, and then centrifuged at 13,000 rpm for 10 min. The supernatants and pellets were collected and stored at 4°C.

Treatment of the feedstock with ammonium sulfate

Different amounts of ammonium sulfate were weighed and gradually added to the feedstock in the microcentrifuge tubes, with gentle shaking, to obtain final salt concentrations of 10, 20, 30 and 40%. The tubes were kept at 4°C for 1 h and next centrifuged at 13,000 rpm for 10 min. The supernatants and pellets were collected and stored at 4°C.

Treatment of the feedstock with detergents and solvents

Ionic and non-ionic detergents (sodium deoxycholate, Tween 20 and Triton X-100) at a final concentration of 0.1% and solvents (acetone, isopropanol and diethyl ether) at the final concentrations of 0.05 - 5% (v/v) were added to the feedstock in the microcentrifuge tubes. After 1 h incubation of the tubes at 25°C, ammonium sulfate was added to each tube to reach a final concentration of 30% (w/v) and the tubes were kept at 4°C, overnight. The tubes were eventually centrifuged at 13,000 rpm for 10 min and the supernatants and pellets were collected and stored at 4°C.

Analytical methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 12% gels at 150V/20 mA for 2 h using Laemmli electrophoresis buffer (containing 0.025 M Tris base, 0.19 M glycine, 0.1% SDS). Prior to electrophoresis, all samples were diluted 1:1 with the sample buffer (containing 0.3% SDS, 0.1 M DTT, 1% bromophenol blue and 20% glycerol) and boiled for 3 min. Silver staining method was used for detecting proteins following gel electrophoresis.

Sulfo-Phospho-Vanillin (SPV) assessment of total lipids

Total lipids in the samples was quantified base on a modified colorimetric method of *McMahon et.*al, 2013 [17]. For this purpose, various concentrations of cholesterol ranged between 50 and 200 mg/mL were prepared in chloroform and used as standards in SPV assay. Samples and standards (10 µL) were set up in triplicate. Concentrated sulfuric acid (200 µL) was then added to each tube and the mixtures were heated at 95°C for 20 min. The reaction mixtures were allowed to cool to room temperature. Ten microliters of the mixture in each tube was added to a well of the microtiter plate, and 250 µL of phospho-vanillin reagent (0.2 mg/mL vanillin, prepared in 85 % aqueous phosphoric acid) was next added to each well of the plate. The plate was covered, placed in dark for 20 min at room temperature and read at 530 nm using a Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA). The average absorbance calculated for each series of standards and samples was subtracted from the control (i.e., 10 µL of sulfuric acid plus 250 µL of phospho-vanillin reagent) value and the lipid quantity in each sample was measured using the standard curve.

RESULTS AND DISCUSSION

Following *P. pastoris* crude extract pH reduction (acid precipitation) step, the collected samples were analyzed using SDS-PAGE. The silver staining of the gel (Fig. 2) showed that most protein contaminants were left in the pellet and the supernatant contained the target protein when compared to the pure rHBsAg (i.e., rHBsAg active pharmaceutical ingredient, API) as control. This shows that the acid precipitation step eliminated most of the concomitant protein. The resultant supernatant was dialyzed against 50 mM phosphate buffer (pH 7.4) and used for further lipid removal experiments.

The mechanism of flocculation with borax has been explained elsewhere. Boron oxyanions, including planar trigonal and tetrahedral forms, are generated in the solutions of borax and react with 1, 2 cis-diols to produce cyclic esters. The tetrahedral form also generates a spirane-type complex at high diol to borate ratios and elevated pH

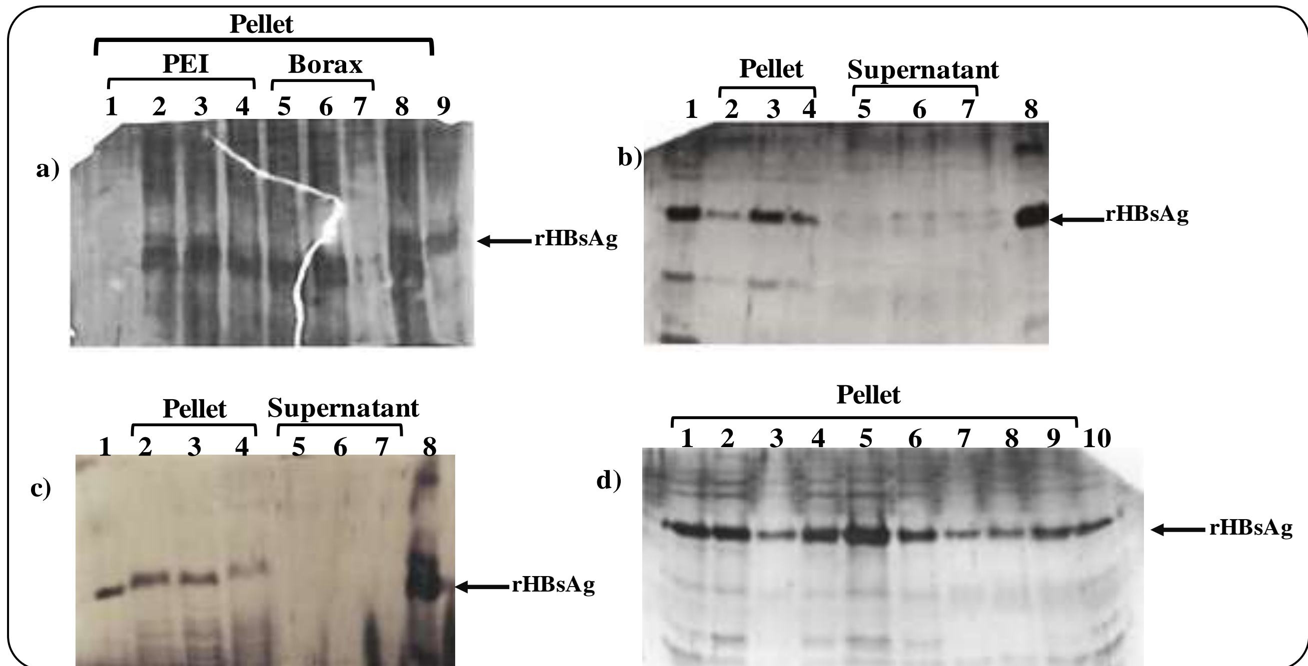

Fig. 3: SDS-PAGE analyses of pellets and/or supernatants resulted from the treatment of the acid precipitation supernatant sample (lane 2, Fig. 2) with: a) PEI and Borax (Lanes: 1 – 0.1% PEI; 2 – 0.5% PEI; 3 – 1% PEI; 4 – 1.5% PEI; 5 – 0.05 M Borax; 6 – 0.025 M Borax; 7 – 0.0125 M Borax; 8 – 0.05 M Borax plus 1.5 % PEI; 9 – before treatment), b) PEG 6000 in the presence of 1 mM $BaCl_2$ (Lanes: 1 – before treatment; 2 & 5 – 10% PEG; 3 & 6 – 20% PEG; 4 & 7– 30% PEG; 8 – rHBsAg API), c) ammonium sulfate (Lanes: 1 – before treatment; 2 & 5 –20% ammonium sulfate; 3 & 6 – 30% ammonium sulfate; 4 & 7 – 40% ammonium sulfate; 8 – rHBsAg API), d) detergents and organic solvents (Lanes: 1 – 0.1% sodium deoxycholate; 2 – 0.1% tween 20; 3 – 0.1% Triton X-100; 4 – 0.5% isopropanol; 5 – 5% isopropanol; 6 – 0.5% acetone; 7 – 5% acetone; 8 – 0.5% diethylether; 9 – 5% diethylether; 10 – rHBsAg API)

values is assumed as the cause of flocculation [18, 19]. Boronate ester formation is completely cleaved under strongly acidic conditions [20]; therefore, such flocculation is hindered under acidic conditions and lowering pH has been actually used for reversing the flocculation with borax [18, 21]. Based on these descriptions, the specific cross-linking of polyhydroxy compounds (such as carbohydreate - containing biomolecules) by boron oxyanions can be used for selective separation purposes. According to this selective crosslinking mechanism, borax has been previously used for the aim of cell debris flocculation, and the treatment of yeast homogenate with borax solutions has produced large flocs being separated in a short time at low centrifugal forces [18, 19, 21-24]. Borax, at a concentration ranging from 0.02 M to 0.1 M, has been added to yeast homogenates for flocculation of yeast cell debris and preparation of clarified yeast homogenate [18, 19]. Borax treatment has failed to remove nucleic acid and lipids from the calcified homogenate [18]. Throughout borax-mediated clarification process, it has been shown that glycoproteins (such as invertase) could form a complex with the tetraborate anion via their carbohydrate moiety and be removed from the supernatant on centrifugation; as a result, glycoprotein loss has been considered as a problem when using borax to flocculate and remove cell debris [19]. Yet, there has been no report on the application of borax for the cross-linking and particular separation of polyhydroxy - containing proteins, following conventional (i.e., borax-independent) yeast homogenate clarification. In the current study, borax at various concentrations was added to the feedstock sample (originated from *P. pastoris* crude extract) and pellets were separated from supernatants by centrifugation, subsequent to the treatments. A greater volume of sediment could be seen with the naked eye upon addition of borax at the concentrations of 0.025 and 0.05 M. Electrophoretic analysis of the solid samples also showed that the feedstock samples treated with 0.025 and 0.05 M borax contained a considerable amount of rHBsAg (Fig 3a). These results demonstrated the effect of borax concentration

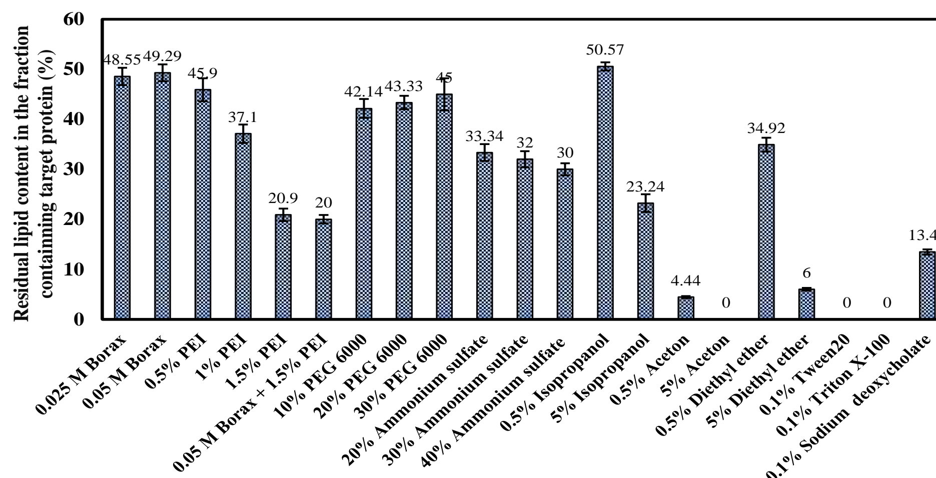

Fig. 4: Sulfo-phospho-vanillin (SPV) assessment of total lipids in the samples containing target protein after treatment with various chemicals at different concentrations

on the flocculation and sedimentation of rHBsAg.

Quantification of total lipid in the pellets obtained after treatment of the feedstock samples with borax at the concentrations of 0.025 and 0.05 M showed that the residual lipid content in the samples was 49.29% and 48.55, respectively, which indicated almost similar performance of these concentrations of borax in terms of total lipid reduction in the samples (Fig 4). To our knowledge, no investigation on target glycoprotein protein flocculation by borax and simultaneous lipid reduction in the sediment fraction has been done previously.

Polyethylenimine (PEI) or Polymin P is a cheap and safe reagent and its removal from proteins can be managed by ionic strength control [25]. PEI is a highly branched basic cationic polymer, originated from polymerization of ethyleneimine, which is positively charged at pH values lower than 10.8. Flocculation of anionic materials (such as nucleic acids, lipids and colloidal particles) by polycationic PEI occurs via polymer bridging and charge neutralization [18, 26, 27]. In the present study, PEI (molecular weight 10,000) was added at various concentrations (ranged between 0.1 and 1.5%) to the feedstock samples. The pellets of the samples treated with PEI contained considerable amount of rHBsAg (Fig. 3a) and quantification of residual lipid content in these samples showed a lipid reduction up to 79% using 1.5% PEI (Fig. 4). In other words, up to 79% of the initial feedstock total lipid was remained as soluble in the supernatant and separated from the flocculated rHBsAg during the treatment with 1.5% PEI. Concurrent use of PEI with borax did not increase the effect of PEI on lipid removal from the flocculated rHBsAg. The effect of varied concentrations of PEI with various molecular weights (from 600 to 100,000) on borax – preclarified Baker's yeast (*Saccharomyces cerevisiae*), *Escherichia coli* and *Pseudomonas putida* homogenates, for flocculation and removal of cellular contaminants in the presence of 3% – 5.84% sodium chloride, has been previously investigated [18, 25]. Dissimilar to our aim, flocculation of lipid content with a value up to 97% has been targeted in such studies. Therefore, the ionic strength of the flocculation medium seems to be a factor determining the selectivity for separation of target proteins from lipids.

Polyethylene glycol (PEG) is a water-soluble synthetic polymer which is manufactured by polymerization of ethylene oxide molecules [28]. PEG polymers can form hydrogen bonds using their oxyethylene ($-CH_2CH_2O-$) groups [29], and the amount of water molecules in the formation of hydrogen bonds depends on the numbers of oxyethylene units in the PEG molecule. Based on the proteins steric exclusion mechanism, it can be imagined that PEG polymers act like an inert solvent sponge reducing solvent availability which then results in an increase in the effective protein concentration and precipitation [30-32]. As a net positive or negative charge of a target protein is developed at pH values far from its isoelectric point, which results in more resistance to protein precipitation, the addition of divalent cations or anions can neutralize the predictable net charge on the target protein and consequently ease its precipitation in the

presence of PEG at such pH values [33]. In the present study, the precipitation of rHBsAg from *P. pastoris* feedstock by various concentrations of PEG 6000 was investigated in the presence of 1 mM $BaCl_2$, at pH 7.4. rHBsAg was successfully precipitated using 20% PEG 6000 (Fig. 3b). Quantification of total lipid in the pellet obtained after this treatment showed that the residual lipid content in the sample was about 43%, indicating 57% total lipid reduction in the original feedstock sample (Fig. 4). In the similar works done by others on protein precipitation using PEG, the amount of residual lipid in the precipitated protein fraction has not been determined.

Extremely soluble in water, ammonium sulfate is an organic salt used for protein salting out. Charged and hydrophobic zones of protein surfaces result in both electrostatic interactions between molecules and structuring of water molecules around the hydrophobic regions of the surface. Adding salts, such as ammonium sulfate, into protein solution leads to compression of the electric double layer (i.e., Stern and Gouy – Chapman layers) around the surface of protein molecules and reduction of zeta potential, which results in disruption of the hydration barriers between protein molecules. This allows interactions between hydrophobic patches exposed in neighboring protein molecules, and subsequent collision and precipitation of proteins [34]. The precipitation by ammonium sulfate results from a reduction in solubility rather than protein denaturation; therefore, the precipitated protein can be solubilized using buffers. However, the success of salting out depends on the hydrophobicity of the protein, and less hydrophobic proteins tend to remain soluble even at high salt concentrations. Based on the aforementioned salting out mechanism, in the recent study ammonium sulfate was added to *P. pastoris* feedstock at various concentrations ranged between 10% and 40%. Following centrifugation, sedimentation was observed in the samples treated with the salt at concentrations of 20% and above, and SDS-PAGE analysis of these samples demonstrated rHBsAg precipitation (Fig. 3c). The quantification of total lipid in the samples showed a lipid reduction of 67%-70% in the feedstock using 20%-40% ammonium sulfate (Fig. 4). In the similar works done by others on protein precipitation using ammonium sulfate, the amount of residual lipid in the precipitated protein fraction has not been determined.

Detergents are amphipathic molecules consisting of a polar head and a long non-polar hydrocarbon tail. On the basis of their head group, detergents are classified into four categories including anionic, cationic, non-ionic and zwitterionic [35]. Detergents can disrupt lipid - lipid and lipid - protein interactions [36]. Also, nonpolar or weakly polar organic solvents, namely fat-solvents, can notably dissolve lipids [37]. The efficiency of non-polar and polar solvents (acetone, diethyl ether and isopropanol) as well as non-ionic and anionic detergents (Triton X-100, Tween 20 and sodium deoxycholate) for lipid removal from *P. pastoris* feedstock was also investigated in this study. For this purpose, subsequent to the addition of these detergents and solvents to the feedstock, rHBsAg was precipitated by 30% ammonium sulfate (Fig. 3d), and the residual lipid was quantified in the sediments. According to the results presented in Fig. 4, subsequent to treatment of the feedstock with sodium deoxycholate, Triton X-100 and Tween 20 (at concentration of 0.1%), 87% - 100% reduction in the lipid content of ammonium sulfate – precipitated protein was accomplished. Amongst detergents, Triton X-100 (at a final concentration of 0.4% v/v) has been widely used to produce low lipid feed for column chromatography, and its removal from the feed has been done by using polystyrene beads (XAD-4). However the inefficiency of XAD-4 in the presence of lipids in the feed has been demonstrated [10, 15].

In the treatment experiments with solvents, most of lipid content (ca. 95%) in the feedstock was eliminated using 0.5% acetone or diethyl ether, and 100% lipid removal was observed when these solvents were utilized at a concentration of 5%. The efficiency of isopropanol was lower than acetone or diethyl ether, and a feedstock lipid content reduction of 50% and 76% was respectively obtained by 0.5% and 5% isopropanol. To our knowledge, solvents have not been investigated so far for removal of lipids from cell homogenates. Ammonium sulfate protein precipitation done in the present research would leave detergents and solvents in the solution and help their reduction in the precipitated protein.

CONCLUSIONS

In the conventional downstream processing for production of biologicals, particularly ER-associated ones being manufacture in yeasts, a high amount of the host lipids are discharged into the process stream containing target protein. This lipid discharge can reduce the performance

of chromatography columns in terms of dynamic binding capacity, pressure drop, number and consistency of operational cycles, during subsequent target protein purification step. Therefore, removal of lipids from the feed stream is crucial. In the present study, it was demonstrated that high amounts (57 - 100%) of lipids in the yeast homogenate can be removed by using low concentrations of various chemical treatments including borax, PEI, PEG 6000, detergents and solvents. Following lipid dissolution with detergents and solvents, ammonium sulfate precipitation of the target protein would leave chemicals in the solution and help their reduction in the precipitated protein. The safety, low cost and high lipid removal efficiency of such chemicals at low concentrations as well as the possibility of their removal from target protein fractions offer that these chemical methods may be considered for pretreatment of yeast cell homogenates to facilitate chromatographic separations *via* considerable reduction of host lipids and their fouling impact.

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