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Original Article

Improvements in the Downstream Processing of rhGH production in *Pichia pastoris*

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Abstract

Production of human proteins in *Pichia pastoris* has significant advantages. However, there is still need for improvement of various stages of its downstream processing like clarification and purification. In fact downstream processes are usually the most critical part of production of biotech products. This work aimed to evaluate the effect of two steps added to the downstream processes of human growth hormone (hGH) production in *Pichia pastoris*. Firstly, the effect of clarification, with activated carbon, on capture of hGH by ion exchange chromatography (IEC) was investigated. For this purpose, a clarification process using activated carbon was used to remove process contaminants like pigments. The clarified sample was applied to the IEC column and the recovery of hGH, following IEC, was assessed using SDS-PAGE, Bradford protein assay, and area under the curve (AUC). The obtained results showed that the AUC values were 2.81 and 5.61 for the with- and without-treatment samples, respectively. Protein recovery of clarified sample with activated carbon was 541 mg in comparison with 328 mg for the sample without treatment. The yield of IEC was also improved from 50.46% to 83.23% following treatment with activated carbon. Secondly, the effect of three concentrations of ammonium sulfate in the binding buffer on resolution of hGH upon elution on hydrophobic interaction chromatography (HIC) was investigated. Biological activity was used as the main criterion for evaluation of purified hGH using HIC. The obtained results indicated that by increasing the concentration of ammonium sulfate form 1 to 3 mol/L, resolution of hGH was improved, as the purified fraction using 3 mol/L of ammonium sulfate showed a specific activity of 3.1IU/mg. So, the results of the present study demonstrated that activated carbon is a promising candidate for efficient clarification of recombinant hGH and for improving the efficiency of the capture step. Therefore, it can be considered by biotech companies as a cost-effective and sustainable clarification procedure of recombinant proteins from high cell density cultures. This study also revealed that 3% ammonium sulfate has a positive effect on the separation of hGH variants with the desired biological activity.

Keywords: *Pichia pastoris*, hGH**,** Downstream processing, Ion exchange chromatography, Hydrophobic interaction chromatography, Clarification, Optimization

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1. Introduction

Human growth hormone (hGH) also known as somatropin is a protein hormone secreted by the pituitary gland. [1]. It consists of 191 amino acid residues (22kD) which are folded into a four-helix bundle structure with two intramolecular disulfide bridges [2, 3]. It has a wide range of therapeutic applications in the treatment of hypo-pituitary dwarfism, skin burns, bleeding ulcers, bone fractures, and AIDS [4, 5]. Until 1985, GH was obtained through extraction from human cadaveric pituitaries and administered to children suffering from hormone deficiency [6]. The administration of cadaver-derived hGH was halted when its association with Creutzfeldt-Jakob disease was proved [7, 8]. With the development of recombinant DNA technology, the hGH gene was cloned in 1979 and recombinant hGH was approved for clinical use in 1985, eliminating the need for pituitary-derived preparations and avoiding the risk of transfer of human pathogens [4, 9]. Furthermore, the recombinant human growth hormone (r-hGH) produced by *E. coli* and yeast expression systems [10-13], have high biological activity identical to its human counterpart [14, 15].

The yeast *P. pastoris* is now an established protein expression host mainly applied for production of recombinant proteins [7, 8]. This yeast offers many of the advantages usually considered desirable for protein expression, including: GRAS (Generally Recognized as Safe) status, simple molecular manipulation, high expression level of recombinant proteins, ability to promote post-translational modifications, such as folding, the efficient secretion of extracellular proteins and growth to high biomass levels [9, 10, 11]. Despite many existing strategies in downstream processing of recombinant hGH, there is still margin for significant improvement in various stages of its production process like removal of process contaminants, simpler and more efficient purification schemes, increased yield, and overall cost effectiveness.

Usually, recombinant proteins produced during fermentation of *P. pastoris* are secreted and soluble proteins can be directly recovered by clarification of the culture media which may contain impurities such as host cell proteins and DNA, pigment and pyrogenic components as well as fermentation media ingredients [16, 17]. One of the most unfavorable impurities resulting from employing *P. pastoris* is pigments, produced normally during methanol induction phase. The pigmentation has a considerable impact on hGH purification, since the pigments may bind to the target proteins and reduce the loading capacity and effective life span of the capturing matrix. This leads to reduced yields and purity [18-20].

A typical protein purification scheme includes the broad steps of clarification,

capture, intermediate purification, polishing, and formulation [21]. Hence, preparation of a clarified sample of soluble protein and removal of particulate matter such as pigments, which are not compatible with chromatographic purification, is a critical step in downstream processing. A variety of methods are available for clarification of recombinant protein in *Pichia pastoris* mainly including centrifugation and filtration. In the present study, the effect of clarification with activated carbon on capturing recombinant human growth hormone by ion exchange chromatography was investigated. Clarification process using activated carbon is a simple and effective procedure which may reduce process related impurities such as pigments and increase the efficiency of downstream purification stages.

Moreover, to optimize the intermediate purification step, the effect of three concentrations of ammonium sulfate on selectivity of hGH on hydrophobic interaction chromatography (HIC) was also investigated. These experiments were performed with the method of one-factor-at-a-time (OFAT) in which only one variable was analyzed at a time while keeping the others fixed.

2. Materials and Methods

2.1. Microorganism, Inoculum and Media Preparation

P. pastoris GS 115 strain Mut^+ carrying hGH cDNA under the control of AOX1 which has high capability to secret the target protein into the fermentation broth was streaked from glycerol stock onto YPD-agar, containing Yeast extract (10 g/L), dextrose (20 g/L), peptone (20 g/L) and agar (20 g/L), and incubated for 48 h at 30 °C [22]. A single colony of *Pichia pastoris* was inoculated into BMGY medium containing 10 g/l yeast extract, 13.4 g/L YNB, 20 g/L peptone, 4×10^{-5} g/L biotin, 10 g/L glycerol and 0.1 M potassium phosphate buffer [23] and incubated at 30°C in a shaker incubator at 150 rpm orbital shaking, until the optical density (OD_{600}) reached a value of 1-2.

2.2. Fermentation Conditions

The culture obtained was used as inoculum for a 13L fermenter containing 3 liter of basal salts medium which consisted of (g/l): glycerol (40); K_2SO_4 (18); $MgSO_4.7H_2O$ (14.9); KOH (4.13); $CaSO_4$ (0.9) and 27 ml H_3PO_4, plus 4.0 ml of a trace metal stock solution that consisted of (g/L): $CuSO_4.5H_2O$ (6); KI (0.09); $MnSO_4.H_2O$ (3); H_3BO_3 (0.02); $MoNa_2O_4.2H_2O$ (0.20); $CoCl_2$ (0.5); $ZnCl_2$ (20); $FeSO_4 \cdot 7H_2O$ (65); biotin (0.2) and H_2SO_4, 5.0 (ml/l) [21]. The 13 L bioreactor (Infors, Switzerland), had a working volume of 3L and included temperature, pH, foam, stirring rate, feed inlet rate and dissolved oxygen control systems. Dissolved oxygen (DO) concentration was maintained above 20% air saturation at 400-700 rpm, using air and, when needed, enriching the inlet air with pure oxygen passing through a digital mass flow controller. Temperature was maintained constant at 30.0±0.1°C throughout the entire bioprocess. In the first two phases, the pH was held at 5.0±0.2 and then in the production phase was lowered to 3.0±0.2. The pH was maintained at the relevant values, by adding 25% ammonia solution.

2.3. Clarification Process Step

Pigment-related contaminants generated during methanol induction phase are one of the major challenges of heterologous protein production using the *P. pastoris* expression system. For clarification, the fermentation broth was centrifuged at 14000 RPM for 30 min (Sigma 8K10, Germany). Then, the pharmaceutical grade activated carbon (Norit®, CN_1) at concentration of 1% (W/V) was added to the supernatant. The mixtures were incubated for 30 min at 8-12 ˚C with constant stirring. After incubation, the mixtures were centrifuged at 13,000 rpm for 10 min to precipitate the activated carbon, and the supernatant was filtered using 0.22 µ filter (Stricup™, Millipore). The clarified supernatant was diafiltered against 10 volumes of 20mM tris buffer using Millipore ProFlux M12 tangential flow filtration system and Pellicon® 2, 100 kDa, ultrafiltration cassette.

2.4. Protein capturing by Ion Exchange Chromatography

Anion exchange chromatography was conducted with a XK 50/60 Column (GE healthcare, Sweden) packed with DEAE Sepharose Fast Flow Resin in an AKTA purifier 100 FPLC (GE healthcare, Sweden). The supernatant (about 1500 ml) was loaded onto a column that was equilibrated with 50 mM Tris-HCl Buffer (pH 8.2). The column was washed in equilibrating buffer and eluted with a linear gradient of 80 to 300 mM Tris-HCl Buffer (pH 8.2) at a flow rate of 25 ml/min. The eluate was monitored at 280 nm and the active fractions (approximately 500 ml) were collected. Subsequent to IEC, total protein concentration, hGH purity, and recovery were evaluated using Bradford protein assay, SDS-PAGE, and Unicorn software, respectively.

2.5. Intermediate Purification of hgh with Hydrophobic Interaction Chromatography

HIC usually follows an ion exchange step for intermediate purification of recombinant proteins. In this study, the effect of varying the concentration of salt in the binding buffer on the purification of hGH was investigated. The binding buffer containing 1.0, 2.0, and 3.0 mol/L of ammonium sulfate was subjected to a XK 50/40 column packed with Phenyl Sepharose Fast Flow. The column was washed and equilibrated in buffer B (50 mM tris, 2 mM EDTA, 1.5 M NaCl pH 8) and 3M ammonium sulfate as eluent. The volume of pooled fraction was about 900 ml.

2.6. Analytical Methods

2.6.1. SDS–PAGE Analysis

To determine the purity and recovery in IEC of r-hGH, SDS–PAGE was performed in a Mini-Protean® 3 cell gel apparatus (Bio-Rad, USA) according to the method of Laemmli [24]. The samples were dissolved in 1X sample buffer and incubated at 100 °C for 5 min. About 25 µl of each sample was loaded onto a gel comprising a 15% resolving and a 5% stacking gel with 0.75 mm thickness at a constant voltage of 100 V. After running, the gel was stained with Coomassie Brilliant Blue R250

and the intensity of hGH band with/without activated carbon clarification was compared.

2.6.2. Determination of Total Protein

Determination of total protein concentration was conducted by the Bradford assay using a UV/Vis spectrophotometer (PerkinElmer, USA), at 595 nm [25]. Bovine serum albumin (BSA) was used as the standard protein in the 100-1000 µg/ml range. A standard curve was developed using a series of BSA concentrations. All the samples were prepared in duplicate by appropriate dilution with distilled water so that the absorbance would fall in the linear range of the standard curve. Total protein concentration of each sample with/without activated carbon clarification step was then estimated from the constructed standard curve.

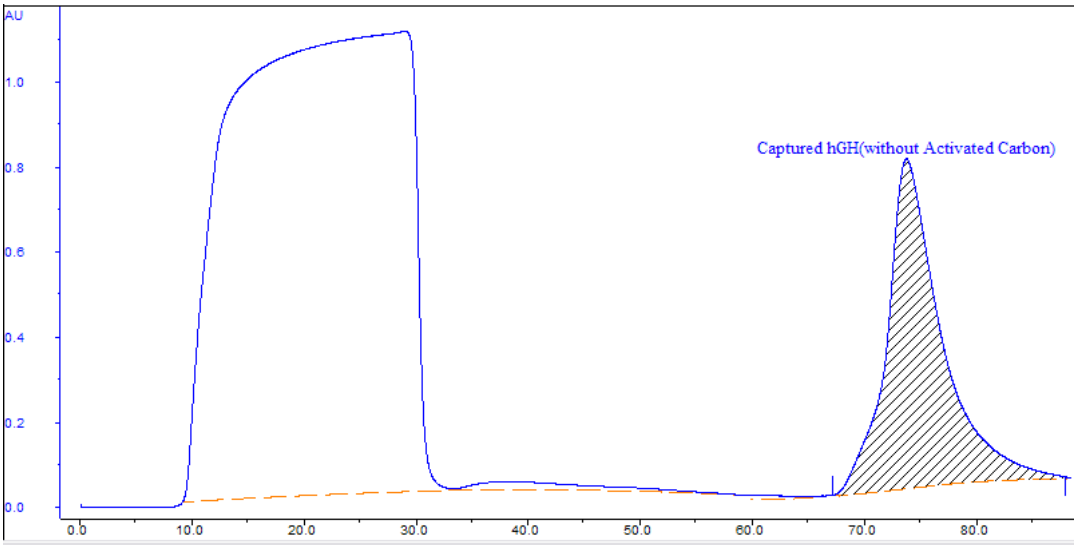
2.6.3. Hgh Cell-Based Assay

Biological activity of hGH in HIC fractions were evaluated by Nb2-11 cell proliferation assay. The rat T-lymphoma cell line, Nb2-11 cells (European Collection of Authenticated Cell Cultures, Cat No: 97041101, UK) were maintained in Fischer's medium (Wellgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Biowest, Nuaille´, France), 10% horse serum (Gibco/Invitrogen, Carlsbad, CA), and 1% penicillin-streptomycin (Gibco/Invitrogen) in a 37°C humidified incubator containing 5% CO_2. Cell proliferation was determined using MTS assays [20]. Briefly, cells were harvested, rinsed in culture medium without FBS, and then incubated for 48 h in 96-well plates at 20,000 cells/ml (100 µl/well) in the presence of various amounts of hGH. Following incubation, 20 µl of the MTS reagent (Promega, WI, USA) was added to each well, and cells were incubated for 2 h. The absorbance was recorded on a microplate reader (Bio-Rad, CA, USA) at a wavelength of 490 nm. Cell numbers were determined using a standard curve plotted from a linear relationship between cell number and absorbance [26]. According to somatropin monograph in European Pharmacopeia, specific activity of hGH should not less than 2.5 IU per mg of protein [27].

3. Results and Discussion

3.1. Effect of Activated Carbon Clarification on hgh Capture

Following fermentation, the culture medium was clarified using 1% activated carbon for the removal of pigments. Then, IEC was conducted to capture hGH. In order to evaluate the efficacy of activated carbon on the capture step, first, the obtained chromatogram from IEC with activated carbon clarification was compared with that of without treatment. The comparison included calculation of the AUC values of the samples with and without activated carbon treatment with Unicorn software. As shown in figures 1 and 2, the area under the curve (AUC) value for the untreated sample was 2.82, whilst that of the clarified sample was 5.62. Furthermore, as indicated in table 1, protein recovery of the clarified sample with activated carbon was 541 mg in comparison with 328 mg for the sample without treatment. The yield of



A: IEC 1 rhGH 93 08 20001:10_AIR131UV@07.PEAK2

No	Peak name	Area (AU*min)	Height (AU)
1	Captured hGH(without Activated Carbon)	2.8162	0.590
2			
3	Total number of detected peaks		5
4	Total area (AU*min)		7.8613
5	Area in evaluated peaks (AU*min)		2.8162
6	Ratio peak area / total area		0.358240
7	Total peak width (min)		17.53

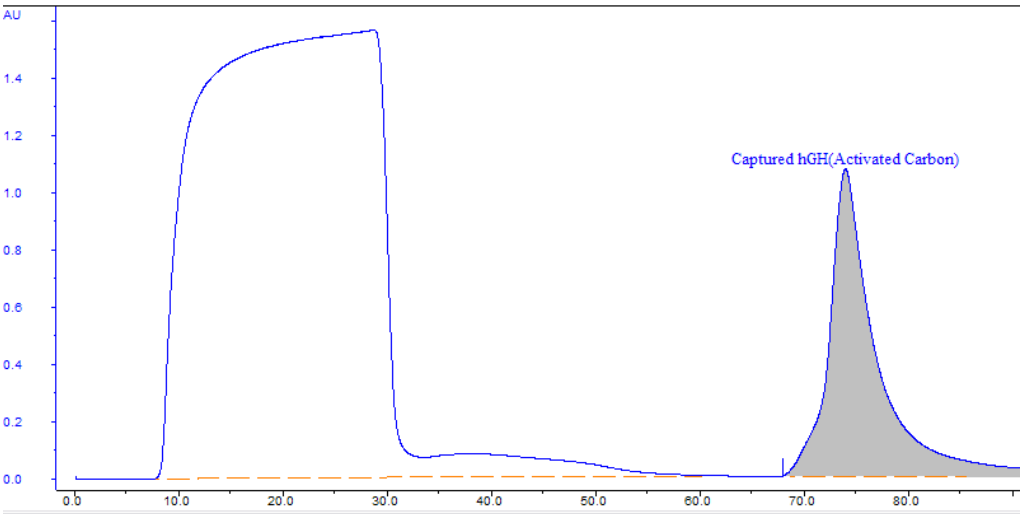
Figure 1. IEC chromatogram of hGH without activated carbon treatment.

Table 1. the analytical results of IEC main fraction with/without activated carbon clarification

Condition	Total protein (Before IEC)	AUC	Protein Recovery (mg)	Yeild (%)
Clarification without activated carbon	650 mg	2.82	328	50.46
Clarification with activated carbon		5.62	541	83.23%

IEC was also improved from 50.46% to 83.23%.

Secondly, the effect of the activated carbon clarification on IEC was evaluated using SDS-PAGE. The obtained results (figure 3) confirmed the AUC values (figures 1 and 2) as the intensity of the hGH band with activated carbon treatment is much higher than that of without treatment.



A: IEC 1 rhGH 93 07 29001:10_AIR131UV@07,PEAK1

No	Peak name	Area (AU*min)	Height (AU)
1	Captured hGH(Activated Carbon)	5.6184	1.073
2			
3	Total number of detected peaks		5
4	Total area (AU*min)		37.7838
5	Area in evaluated peaks (AU*min)		5.6184
6	Ratio peak area / total area		0.148698
7	Total peak width (min)		23.37

Figure 2. IEC chromatogram of hGH with activated carbon treatment.

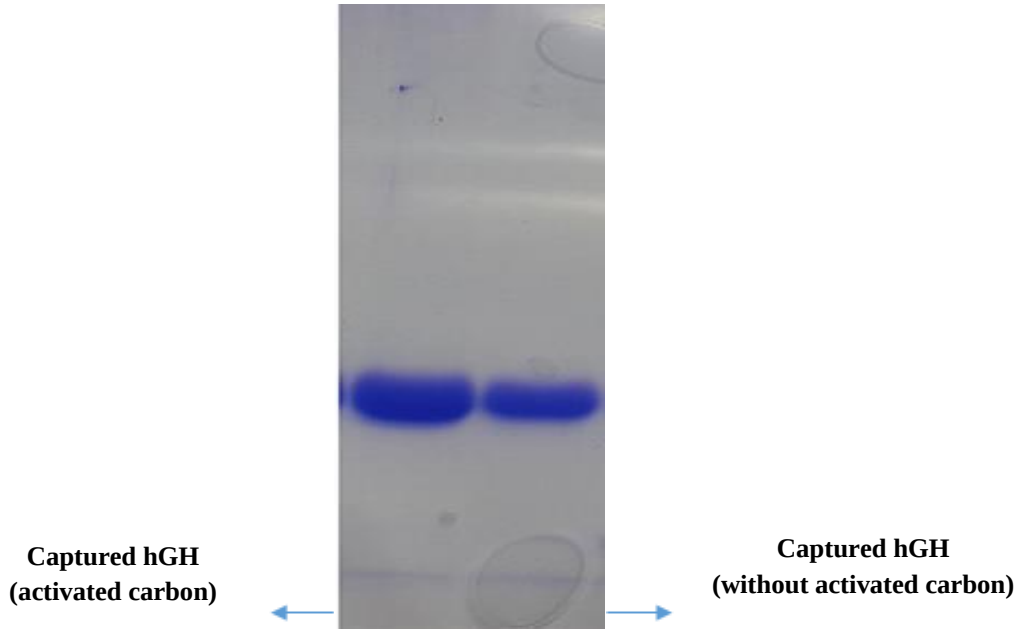


Figure 3. SDS-PAGE analysis of captured hGH with/without activated carbon treatment.

Thirdly, total protein concentration of IEC main fraction was determined with Bradford protein assay. Table 1 shows the summarized results of protein concentration. According to the

Table 2. Biological activity analysis of HIC main peaks.

Ammonium sulfate concentration	Biological activity of hGH(IU/mg)	
	Peak 1	Peak 2
1%	2.1	-
2%	2.25	2.6
3%	2.0	3.1

obtained results, protein recovery in the IEC main peak of untreated/treated condition was 328 and 541 mg, respectively.

3.2. Effect of Ammonium Sulfate Concentration on Intermediate Purification of hGH

HIC is well-suited for the intermediate step in a purification scheme. The hydrophobic behavior of proteins is difficult to predict, hence, binding conditions must be optimized to obtain good resolution of the bound protein upon elution. In this study the effect of ammonium sulfate concentration (1.0, 2.0 and 3.0 mol/L) in the binding buffer on selectivity and resolution of hGH was investigated. The obtained chromatograms are shown in figures 4-6. The obtained results clearly indicate that by increasing the concentration of ammonium sulfate form 1 to 3 mol/L, resolution of hGH was improved to an acceptable level as confirmed by separation of two peaks (figure 6). Biological activity was considered as a major criterion for the evaluation of HIC main peaks at different concentrations of ammonium sulfate. The specific activity of hGH obtained from 1% ammonium sulfate was about 2.1 IU per mg of protein, which would not meet the acceptance criteria of minimum 2.5 IU/mg. On the other hand, the biological activity of main fraction (peak 2) collected with 2% and 3% ammonium sulfate was 2.6 and 3.1 IU/mg. In addition, the biological activity of peak 1, collected with 2% and 3% ammonium sulfate was about 2 and 2.25 IU/mg, respectively.

Pichia pastoris is an outstanding host for high-level expression of heterologous proteins. Most of the proteins produced in these systems are expressed under the control of the methanol induced alcohol oxidase I (AOX1) promoter [28]. High level expression of recombinant proteins in *P. pastoris* is associated with generation of pigment-related contaminants during methanol induction phase of fermentation [18]. These pigments could bind to the resin of most chromatographic media such as DEAE Sepharose, reducing the loading capacity and seriously affecting the column lifespan. The pigments in the culture supernatant could also complicate the purification procedure because they are hard to quantify and analyze, as some of these pigments also bind to some of the proteins in the preparation [19, 20]. On the other hand, human growth hormone is a highly hydrophobic protein that can interact with hydrophobic surfaces of other proteins. Moreover, *Pichia* generates a large amount of AOX crystalloids, resulting in a strong possibility that growth hormone interacts with these crystalloids by multiple interactions

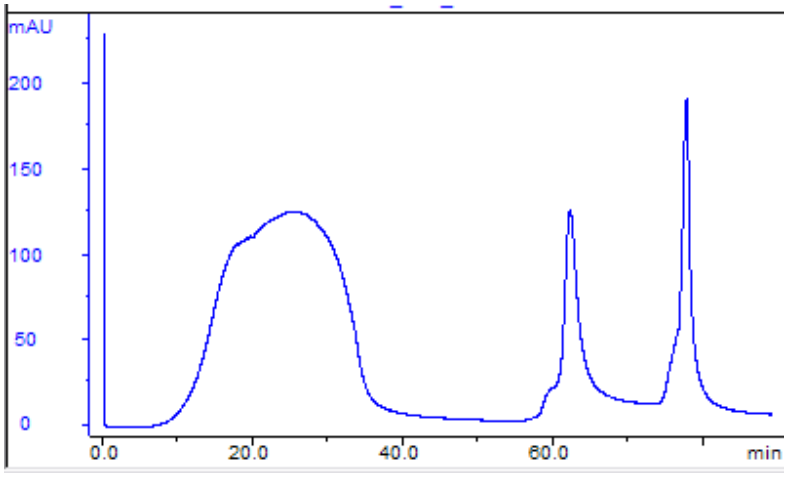


Figure 4. Sample applied in 1 mol/L ammonium sulfate

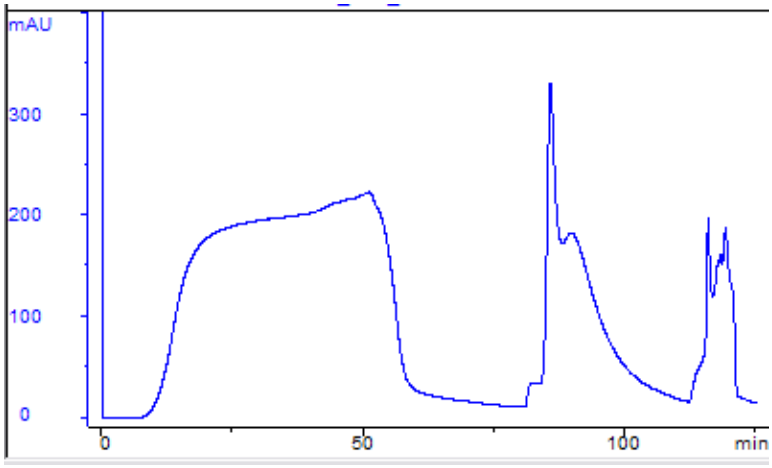


Figure 5. Sample applied in 2 mol/L ammonium sulfate

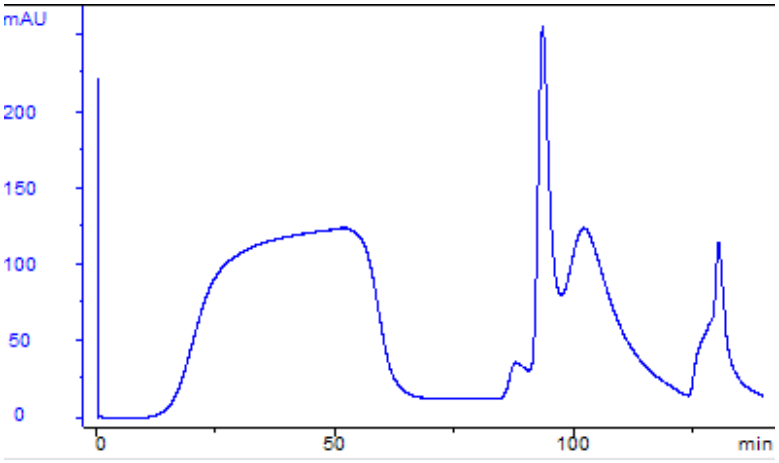


Figure 6. Sample applied in 3 mol/L ammonium sulfate

which change the hydrophobic and charge properties of this protein [18]. Therefore, in this study, several methods were employed to remove as much pigments as possible.

The clarification step is a demanding operation, and is particularly difficult when processing high cell density cultures of microorganisms. High speed, large-scale centrifugation, and microfiltration are the most common processes used to obtain clarified protein solutions for packed-bed chromatography [29]. However, complete removal of the pigments is usually not attained using these traditional procedures, imposing the need for alternative clarification strategies.

One of the aims of the present study was to evaluate the effect of a simplified and optimized clarification process to reduce pigment-related impurities as much as possible, thereby increasing the loading capacity and the yield of the capture step. The results revealed that a clarification method with a certain grade of activated carbon could be used to selectively remove pigments from culture supernatant and improve capturing in ion exchange chromatography. The comparative study showed that clarification with activated carbon could improve the protein recovery from IEC by about 1.6 fold, as the yield of IEC reached 83.3%.

Hydrophobic interaction chromatography (HIC) is a widely used technique for the purification of recombinant proteins [30]. The technique is an outstanding complement to ion exchange chromatography particularly where multiple protein isoforms exist. HIC can be utilized for selective separation of closely related proteins with similar recognition sites which are not distinguishable by other chromatographic techniques. Separation of recombinant proteins by HIC is based on an interaction between the hydrophobicity of the medium, the nature and composition of the sample, hydrophobic residues distribution in proteins, and the salt type and concentration used in the binding buffer [31]. Although HIC is a powerful method for purification of proteins, successful choice of media and conditions can be challenging. Most HIC experiments should be optimized in terms of binding and elution conditions. The type and concentration of the salt in the binding buffer for the adsorption of proteins onto the HIC media is of supreme importance as this can affect the selectivity and capacity of the adsorbent for the target protein. Hence, in the present study, the impact of ammonium sulfate concentration was investigated by varying the salt concentration of the binding buffer. The results obtained indicated that ammonium sulfate at a concentration of 3% could separate hGH with desired biological activity, since the fraction collected with this concentration showed a biological activity of 3.1IU/mg. It seems that increasing hydrophobicity of hGH is associated with more biologically active variants. The presence of 1% ammonium sulfate in the binding buffer could not, however, purify hGH efficiently and the activity of the fraction collected at this concentration was about 2.1 IU/mg. On the other hand at a concentration of 2% ammonium sulfate, the resolution of hGH main peak was not adequate for efficient separation of the active hGH from other variants.

4. Conclusion

The results of the present study demonstrated that clarification of hGH with 1% activated carbon could remove pigment-related contaminants generated during *Pichia pastoris* fermentation. Hence, preparation of a clarified sample of soluble protein and removal of particulate matter using 1% activated carbon could improve the yield of hGH capture step with IEC. Moreover, this study supported the effectiveness of inclusion of 3% ammonium sulfate in the binding buffer of HIC for the purification of hGH variants with desired biological activity.

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