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Expression and purification of recombinant human granulocyte colony-stimulating factor (rG-CSF) from *Pichia pastoris*

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ABSTRACT Recent advances in biotechnology have sparked global interest in developing biosimilar drugs, particularly those containing physiologically active proteins, such as growth factors and cytokines. The methylotrophic yeast *Pichia pastoris* can produce and secrete fully active heterologous proteins with strong secretory capacity and low levels of native proteins and has the ability to achieve high cell densities. In this study, a yeast-based system was used to express and purify recombinant human granulocyte colony-stimulating factor (rG-CSF). Cultures were induced every 12 h for 48 h to express rG-CSF, and parameters such as cell density, media pH, and cell dry weight were observed. Cell density increased along with the corresponding secretion of rG-CSF during the induction period, as determined by Western blot assay, while the pH of the media remained stable. Ammonium sulfate at different saturation levels was used to precipitate the recombinant protein, with the highest total protein content determined spectrophotometrically at 29.6 µg/mL. Ni-NTA resin with affinity column purification was used to purify the recombinant protein. The purified protein showed rG-CSF with a molecular weight of approximately 18 kDa based on SDS-PAGE analysis and immuno slot blot assay detected in purple. Overall, the study results indicated that the production and purification of rG-CSF was successful, although optimization was required. The long-term goal of this research is to discover alternative methods and sources for producing biosimilars of the therapeutic protein rG-CSF, which can be utilized in the pharmaceutical industry to support health programs, particularly cancer treatment.

KEYWORDS G-CSF; *Pichia pastoris*; Production; Purification; Recombinant

1. Introduction

Granulocyte colony-stimulating factor (G-CSF) is a protein that stimulates the myeloid cell lineage, drives the differentiation of committed progenitor cells into mature neutrophil granulocytes. G-CSF has several significant effects, including preserving the viability of progenitor cells and their mature progeny, inhibiting the maturation process, preventing apoptosis, promoting cell division, and identifying lineage affliction (granulocytes or macrophage monocytes) (Mehta and Corey 2021). In its native form, G-CSF is O-glycosylated with a molecular weight of 19 kDa (Lazarus and Gale 2021). G-CSF levels in healthy individuals are typically undetectable or below 100 pg/mL (picograms per milliliter) of blood. However, under stressful conditions such as infection or high-dose chemotherapy, G-CSF levels can increase to over 2,000 pg/mL (Link 2022). Chemotherapy-induced neutropenia can lead to complications, such as febrile neutropenia or other infections that reduce granulocyte reserves in the bone marrow. Exogenous G-CSF can speed up progenitor cell differentiation and proliferation, reducing the neutropenia phase by increasing the availability of neutrophil replenishment (Theyab et al. 2021). When the risk of febrile neutropenia is equal to or greater than 20%, the American Society of Clinical Oncology (ASCO) guidelines recommend the use of G-CSF as an adjuvant therapy for tumors and cancer as a primary prophylaxis measure (Aras et al. 2020; Rahi et al. 2021; Link 2022). Various types of therapeutic rG-CSF have been developed, including a non-glycosylated form (filgrastim), glycosylated form (lenograstim), N-terminus mutated form (nartograstim), and pegylated form (pegfilgrastim and lipegfilgrastim). Numerous investigations have consistently found no significant difference between glycosylated and non-glycosylated forms in recovery from neutropenia (Mehta and Corey 2021). Mammalian cells and *Escherichia coli* expressed glycosylated and non-glycosylated form of rG-CSF, respectively (Yang et al. 2019). Therefore, *Pichia pastoris* has emerged as an alternate yeast host for rG-CSF production.

Pichia pastoris is a common cell factory that produces recombinant proteins and is generally regarded as a safe (GRAS) microbe (Cai et al. 2022). There were many reports that have utilized *P. pastoris* as recombinant expression system (Pan et al. 2022). It was initially developed in the 1980s as a heterologous protein expression system utilizing the potent and tightly controlled AOX1 promoter. Currently, more than 5,000 proteins have been successfully cloned and expressed using *P. pastoris* (Pan et al. 2022). This methylotrophic yeast offers significant physiological benefits compared to other frequently utilized host cells (Cai et al. 2021). These benefits include proliferation in simple media with high cell densities (>150 g dry cell weight/liter) and the secreting proteins with substantial yields under bioreactor conditions (Pan et al. 2022). Other advantages include a strong expression system, genetic stability, and well-developed secretion system for sending proteins to the external environment facilitated by Kex2 as a signal peptidase (Safder et al. 2018). Driven by the AOX1 promoter, *P. pastoris* produces more than 10 g/L of recombinant protein, equivalent to 30% of the total cell protein. According to a literature survey, *P. pastoris* is more commonly used as a yeast expression system for producing heterologous proteins than *Saccharomyces cerevisiae* due to its efficient platform for recombinant protein production (Pan et al. 2022). Although the operation of the *P. pastoris* expression system is straightforward and follows a well-defined method, heterologous protein production is lower under normal conditions, especially when complex proteins are expressed. Therefore, optimizing the recombinant protein production is necessary to achieve the most significant expression process (Karbalaei et al. 2020; Pan et al. 2022).

GS115 is a widely used strain of *P. pastoris* for protein expression, especially in industry and medicine. It contains two genes that encode the alcohol oxidase (AOX) enzyme, particularly AOX1 and AOX2. Most *P. pastoris* vectors use a strong and tightly controlled AOX1 promoter to increase heterologous protein expression (Pan et al. 2022). Methanol, glycerol, and glucose are commonly used as carbon sources for *P. pastoris* to produce recombinant proteins (Ergün et al. 2021). Methanol can be used as the only carbon source for cell development because it triggers the PAOX1 enzyme, which can be rigorously blocked by glucose or glycerol. Stated differently, it dissociates the phase of protein synthesis from cell development (Pan et al. 2022). The standard process for expressing heterologous proteins in *P. pastoris* consists of two distinct stages: the growth phase, in which the carbon source is glycerol or glucose, and the induction phase, in which methanol is used as the inducer (Ergün et al. 2021).

Our preliminary research optimized the growth of *P. pastoris* cells in both transformants and non-transformants by inducing culture for 72 h at 25 °C and 30 °C with 0.5% methanol every 12 h (data not shown). We found that cell density slightly increased at 48 to 60 h and was relatively constant at 60 to 72 h post-induction. There was no significant increase in growth as it did at below 48 h post-induction. The optimum growth temperature is 30 °C. Based on the data, we produced *P. pastoris* cells at 30 °C with an induction time of 48 h. We also precipitated proteins with saturation degrees of ammonium sulfate 40% up to 80%. We used immobilized metal affinity chromatography (IMAC) to purify rG-CSF due to the high affinity of his-tag fused to rG-CSF against metal ions and its strong binding to the IMAC column. Ni^{2+} is most often an ion used for recombinant protein his-tag purification. This technique is most commonly used to purify recombinant proteins and can usually provide relatively pure recombinant proteins directly from a crude lysate or culture medium.

Pichia pastoris expression system is widely used in the biotechnology industry due to several advantages over other eukaryotic and prokaryotic expression systems. These advantages include rapid growth rate, high cell density fermentation, independence from complex medium or culture conditions, genetic manipulability, post-translational modification capabilities, having less glycosylation than other yeasts such as *Saccharomyces cerevisiae*, and minimal levels of host protein secretion. *P. pastoris* strains that are commercially used include GS115, X-33, PichiaPink™, and KM71. Two protease-deficient strains commonly used are SMD1168 and SMD1168H (Safder et al. 2018). This study focuses on evaluating the expression and purification of rG-CSF using GS115 strain. The main goal of this research was to develop a biosimilar of the physiologically active rG-CSF therapeutic protein, which could have potential applications in the biopharmaceutical industry.

2. Materials and Methods

2.1. Expression of rG-CSF

Pichia expression vector pPICZα and GS115 host strains were used to produce recombinant proteins (Fuad et al. 2009; Yuliawati and Fuad 2018; Pratiwi et al. 2020; Yuliawati et al. 2020). The resultant plasmid pPICZα-*CSF3syn-his6* was transformed into *P. pastoris* GS115 strain, and the non-transformed was used as a control. Transformants contain a synthetic *CSF3syn* gene (525 bp) encoding human G-CSF. Transformation of *P. pastoris* GS115 strain by electroporation method using Gene Pulser Xcell™ (Bio-Rad) following the manufacturer's instruction. As much as 80 µL of GS115 cells were mixed with 50–100 µg of plasmid pPICZα-*CSF3syn-his6* (in 5–10 µL sterile water) in a sterile 1.5 mL tube. The mixture was transferred to a 0.2 cm electroporation cuvette and placed on ice. Cuvette was allowed on the ice for 5 min before applying a single pulse under precise conditions using Gene Pulser Xcell™ (Bio-Rad). Promptly add 1 mL of 1 M sorbitol ice-cold to the cuvette, transfer the contents to a sterile 1.5 mL tube, and incubate at 30 °C without agitation for 1 to 2 h to enable the transformation process to occur. Subsequently, 50–200 µL of the transformed cells were spread on YPD agar media containing 100 µg/mL zeocin,

and then the plates were incubated at 30 °C for 3–10 d until colonies formed. Finally, the selection and purification of 10–20 colonies by streaking for single colonies on fresh YPD plates containing 100 µg/mL zeocin.

Yeast extract peptone dextrose (YPD) contains 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar were used for cell cultivation. Buffered glycerol-complex medium (BMGY) contains 1% yeast extract, 2% peptone, 0.1 M potassium phosphate (pH 6.0), 1.34% yeast nitrogen bases (YNB), 4×10^{-5} % Biotin, and 1% glycerol was used for growth cells and biomass production. Buffered methanol-complex medium (BMMY) was used as induction media to produce heterologous proteins by replacing glycerol as a carbon source with 0.5% methanol. A single colony of transformants GS115/pPICZα-*CSF3syn-his6* and the non-transformed were inoculated into 2 mL BMGY media (preculture) and incubated in a shaking incubator (175 rpm) for 24 h at 30 °C. Then, the preculture was transferred to 23 mL BMGY media and grown in a shaking incubator (175 rpm) for 24 h, maintained at 30 °C. Furthermore, the inoculum was transferred to 225 mL BMGY media, and incubation continued until the OD_{600} reached 2–6 (approximately 20–24 h). The cells were harvested by centrifugation at 6,000 rpm for 20 min.

Production of rG-CSF was initiated when cells were induced with methanol. All harvested cells from BMGY media were resuspended in 250 mL BMMY media (OD_{600} approximately ± 3). Induction cultures were carried out by adding 0.5% methanol every 12 h and incubating in a shaking incubator (175 rpm) for 48 h at 30 °C. After induction time had been completed, the supernatant was collected by centrifugation at 6,000 rpm for 20 mins. OD_{600}, media pH, and dry cell weight were measured at 0, 12, 24, 36, and 48 h post-induction.

2.2. Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 3.9% stacking gel and 15% separating gel was performed using the Mini-Protean II apparatus (Bio-Rad). PageRuler™ prestained protein ladder low molecular (Bio-Rad) was used as a marker. Protein was transferred from SDS-PAGE to a nitrocellulose membrane by electroblotting using the Mini Trans-Blot® Cell (Bio-Rad). Immunodetection was achieved using anti-GCSF, polyclonal antibody (Santa Cruz) as primary antibody, and antirabbit IgG peroxidase conjugate (Promega) as secondary antibody. The band was visualized by an NBT-BCIP staining kit (Promega).

2.3. Ammonium sulfate salting-out and determination of total protein concentration

Supernatant 48 h post-induction was precipitated by ammonium sulfate salting-out. In a 50 mL supernatant, different amounts of ammonium sulfate were added until the solution reached saturation degrees of 40%, 60%, and 80%. Ammonium sulfate was added slowly with a magnetic stirrer and incubated overnight at 4 °C. Suspensions were separated by centrifugation at 10,000 rpm for 10 mins at 4 °C; then, the precipitated protein was dissolved with 1 mL phosphate-buffered saline (PBS) pH 7.4. Furthermore, the protein solution was dialyzed for 2 d using a Spectra/Por® 3 dialysis membrane (Spectrum) with a molecular weight 3500 daltons cut-off. During this stage, the volume of protein solution increases by varying amounts. Therefore, PBS pH 7.4 was added to achieve an equal final volume (5 mL) for each treatment. Total protein was measured using the colorimetric method. This method was determined spectrophotometrically at 562 nm wavelength using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Bovine serum albumin (BSA) was used as a standard curve made in 6 concentrations: 10 µg/mL, 12.5 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL, and 30 µg/mL. The total protein concentration was calculated by comparison with a standard curve.

2.4. Purification of rG-CSF

Precipitated protein with a saturation degree of ammonium sulfate 80% was purified using an affinity column purification technique with 2 mL resin of nickel-nitrilotriacetic acid (Qiagen). The resin was cleaned two times in sterile distilled water and two times in a binding buffer containing 0.16 M phosphate, 4 M NaCl, and 10 mM imidazole at pH 7.4 (GE Healthcare). Precipitated protein (4 mL) was pipetted into the column and left on the rotator for 6 h at 4 °C to ensure intensive contact between protein and Ni^{2+} metal ion. Next, the resin was washed three times in a washing buffer containing 0.16 M phosphate, 4 M NaCl, and 20 mM imidazole at pH 7.4 (GE Healthcare). Elution buffer containing 0.16 M phosphate, 4 M NaCl, and 500 mM imidazole at pH 7.4 (GE Healthcare) was pipetted onto the column and left for 5 min. Elution was performed six times with 200 µL of elution buffer, and each fraction was collected separately. All fractions were stored at 4 °C for analysis.

2.5. Analysis of purified rG-CSF

Purified protein was visualized using SDS-PAGE with 3.9% for stacking gel, 15% for separating gel, and 1.5 mm thickness according to the Laemmli method (U K Laemmli 1970). PageRuler™ prestained protein ladder low molecular (Bio-Rad) was used as a marker. A mixture of purified protein and reducing sample buffer (Thermo Scientific) was heated at 95 °C for 10 min. Next, 35 µL of this mixture was loaded into each well. Electrophoresis was performed using a Mini-Protean II apparatus (Bio-Rad). After electrophoresis, the gel was stained with PhastGel® Blue (Pharmacia) and washed until blue bands with transparent backgrounds were obtained.

Purified rG-CSF was detected using the immuno slot blot assay. Protein was transferred to the nitrocellulose membrane using a vacuum or pressure technique. Nitrocellulose membrane and filter paper were soaked with tris buffered saline (TBS) and arranged on the tray of the slot blot apparatus. Purified protein and control as much as 100 µL were pipetted into each slot or well and then connected to the vacuum pump. The vacuum pump was turned

on to transfer the protein to the nitrocellulose membrane. Recombinant G-CSF on nitrocellulose membrane was detected using anti-GCSF, polyclonal antibody (Santa Cruz) as primary antibody, and antirabbit IgG peroxidase conjugate (Promega) as the secondary antibody. The band was visualized by an NBT-BCIP staining kit (Promega).

3. Results and Discussion

3.1. Cultural conditions for rG-CSF expression

In this study, we cultured recombinant GS115/pPICZα-*CSF3syn-his6* cells under the control of the AOX1 promoter in two phases. In the first stage, *P. pastoris* cells were grown in BMGY complex media with glycerol as a carbon source to achieve a high biomass before producing heterologous proteins. In the second stage, we expressed the recombinant protein by transferring all harvested cells from BMGY to BMMY medium containing methanol as an inducer and carbon source. It is well established that the production of secreted proteins is highly correlated with the cell density. This correlation is a crucial aspect of many biological processes and has been investigated in numerous scientific studies (Li et al. 2007; Wollborn et al. 2022). Zhu et al. (2021) investigated the effect of carbon sources on human serum albumin (HSA) expression in *P. pastoris*. The results showed that HSA production was inhibited by glucose, although higher cell density was achieved. Unlike glycerol, which had a slightly lower cell density, it did not inhibit HSA production. In addition, glycerol was superior to sorbitol and sucrose in increasing the biomass. During the induction phase, *P. pastoris* converted methanol to formic acid. Methanol metabolism causes two problems: high oxygen requirements and decreased growth rate (Zhu et al. 2021). A co-feeding strategy is a technique employed to enhance cell growth during the induction phase without repressing recombinant gene expression. It is known that most carbon sources repress the AOX1 gene except for methanol, which allows for optimal cell growth prior to gene expression (Juturu and Wu 2018; Wollborn et al. 2022). Co-feeding also decreases methanol consumption and heat generation (Ergün et al. 2021). Co-feeding strategies, combining methanol with either glycerol or sorbitol, have been proven to be wise choices (Zhu et al. 2021).

TABLE 1 Cultural conditions of *P. pastoris* GS115/pPICZα-*CSF3syn-his6* during induction.

Induction Time (Hours)	Cell Density OD_{600}	Cell Dry Weight mg/mL	Media pH
0	3.6	1.3	6
12	9.3	3.3	6
24	11.1	4	6
36	14.2	5.1	6.5
48	17.8	6.4	6.5

Starting cell density and dry weight at the induction phase (0 h) were 3.6 and 1.3 mg/mL, respectively, while at the end of the induction phase (48 h), they were 17.8 and 6.4 mg/mL, respectively (Table 1). Figure 1a showed that as time passed, cell density (inoculum density) increased along with a corresponding secretion of rG-CSF. Recombinant G-CSF was first detected at 24 h post-induction and increased at 48 h post-induction with a molecular weight of approximately 18 kDa, as determined by Western blot assay (Figure 1b). The protein levels increased every 12 h. This research verifies that starting cell density during induction substantially affects the increased production of rG-CSF. Nurdiani et al. (2021) compared two starting cell densities at $OD_{600} \approx 1$ and $OD_{600} \approx 10$ in a shake flask, with induction parameters: 0.5% methanol (v/v) as inducer, duration 72 h, media pH 6.0, and temperature 30 °C on the expression of human insulin precursor (IP) in *P. pastoris*. They found that starting cell density ≈ 10× would increase IP secretion ≈ 12×. The effect of inocu-

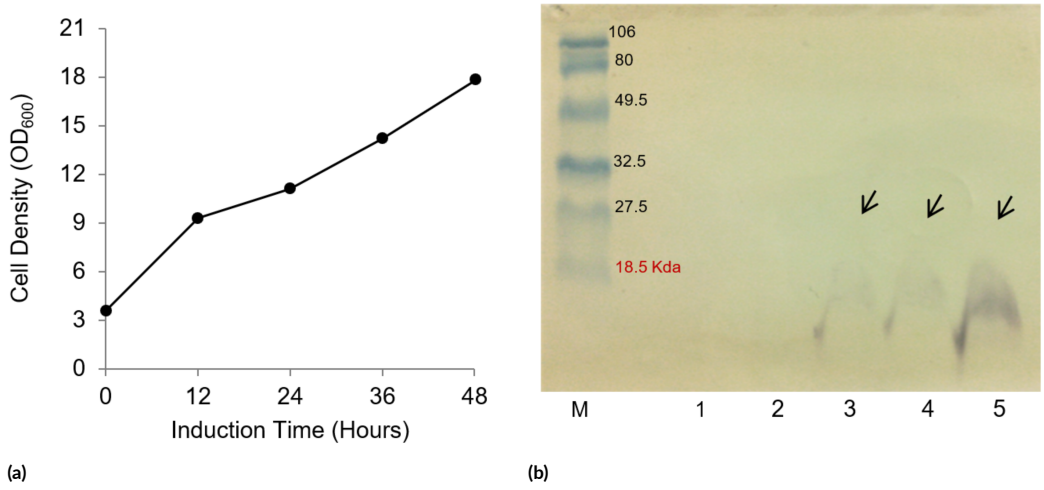

FIGURE 1 Accumulation during induction time (A) cell density of *P. pastoris* GS115/pPICZα-*CSF3syn-his6*. (B) rG-CSF expression with a molecular weight 18 kDa determined by Western blot assay. Lane M: marker protein PageRuler™ prestained protein ladder low molecular (Bio-Rad), Lane 1–5: 0, 12, 24, 36, 48 hours post-induction.

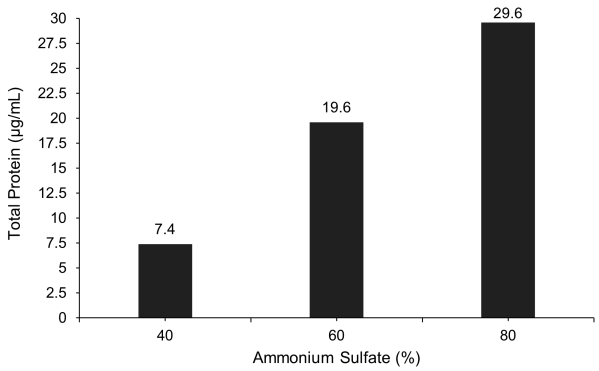

FIGURE 2 Total protein content from *P. pastoris* GS115/pPICZα-*CSF3syn-his6* at different saturation degrees of ammonium sulfate.

lum density on scFv expression in *P. pastoris* was studied by Shi et al. (2003) in a baffled flask with cell densities ranging from 10 to 100. They discovered that inoculum densities below 40 OD_{600} nm U/mL significantly increased scFv accumulation, whereas inoculum densities above 40 only slightly increased scFv accumulation despite a roughly 2-fold increase in the total number of cells within 48 h post-induction. Therefore, very high cell density was ineffective for producing recombinant proteins, especially in a shake flask. In addition, too high cell density has increased the concentration of extracellular proteases and may have a negative impact on cell physiology, thereby reducing protein production.

Media pH during induction is crucial for cell growth, protein formation, protein stability, and limiting protease activity in the *P. pastoris* expression system (Shi et al. 2003; Li et al. 2007). In our research, there was only a slightly change in the pH value of the culture media from 6.0 to 6.5 during induction. In order to produce recombinant *P. pastoris*, BMMY is commonly used as the induction media to express secreted proteins, particularly when the media pH can affect the activity of the protein. Buffered media such as BMMY helps to control and maintain a stable pH condition, reduce protease activity, induce expression, and enhance nutrient absorption by cells. The stability of the media pH and all components of BMMY had prominent effects on the growth and expression of recombinant *P. pastoris* (Shi et al. 2003; Li et al. 2007; Pan et al. 2022). Shi et al. (2003) found that the highest cell growth during induction was achieved at pH 6.0–7.0, although scFv production reaches its peak at pH 7.5–8.0. Lee (2018) study found that the optimal pH range for expressing recombinant xylanase enzymes from *P. pastoris* was between 5.0–7.0. The xylanase enzyme activity was highest at pH 6 (3683 mU/mL), whereas it was lower at pH 7.0 (1826 mU/mL) and pH 5.0 (2569 mU/mL). It is important to control the pH value of the culture media during the induction phase to ensure protein stability and limited protease activity. The optimal pH value depends on the properties of each protein (Li et al. 2007).

3.2. Measurement of concentrated protein

Furthermore, the harvested supernatant 48 h post-induction was precipitated with saturation degrees of ammonium sulfate 40%, 60%, and 80%. We determined the total protein concentration spectrophotometrically at a wavelength of 562 nm using a colorimetric method (Gomes et al. 2022). The principle of determination is proteins can reduce Cu^{2+} to Cu^{+} in alkaline conditions (biuret reaction), resulting in a purple color. The amount of Cu^{2+} reduced is proportional to the amount of protein in the solution. BSA (Thermo Scientific) was used as a standard curve to obtain the equation y = 0.0257x + 0.0096 with R^2 = 0.9976. The total protein concentration with saturation degrees of ammonium sulfate 40%, 60%, and 80% resulted in 7.4 µg/mL, 19.6 µg/mL, and 29.6 µg/mL, respectively.

Bian et al. (2014) concentrated and isolated human plasminogen kringle 5 (HPK5) as a histidine-tagged fusion protein expressed in *Escherichia coli* BL21 (DE3). They found 60% as the optimum saturation degree of ammonium sulfate to obtain the total protein yield and the purity of recombinant HPK5. Schagen et al. (2000) used ammonium sulfate to recover and concentrate recombinant adenoviruses (rAds). They found that ammonium sulfate precipitation of rAds from a cell-culture medium is a simple and fast technique to increase the yields of rAds. Ammonium sulfate is frequently utilized as a salting-out agent owing to its high solubility, which allows for high ionic strength solutions, low price, and availability of pure material. Almost all commercial proteins commonly use ammonium sulfate to concentrate proteins. This biotechnology technique is particularly significant in purifying proteins from crude mixtures and concentrating aqueous solutions without causing denaturation (Baker et al. 2019).

3.3. Analysis of purified rG-CSF

Purification was performed on precipitated proteins with saturation degrees of ammonium sulfate 80%. SDS-PAGE revealed that filgrastim, used as a control, was found with a size of approximately 18 kDa (Figure 3). A targeted protein band of approximately 18 kDa, similar to filgrastim and Western blot findings, was visible in the elution fraction lane. However, non-targeted protein bands were also detected. Although rG-CSF was successfully purified by affinity chromatography with Ni^{2+} metal ions, further purification was necessary to eliminate non-targeted proteins. It may occur because non-targeted proteins have polyhistidine sequences that bind to the Ni^{2+} metal ion (Figure 3).

Single-step purification of recombinant protein substances his-bind with Ni^{2+} ions is often a method of choice, like some other reports (Meng et al. 2019; Cheng et al. 2023). The recombinant protein attached to the polyhistidine (6×His) tag will bind reversibly to the Ni^{2+}-nitrilotriacetic acid (NTA) surfaces in this technique. However, proteins not carrying the 6×His tag will be excreted with the flow-through buffer or washing buffer.

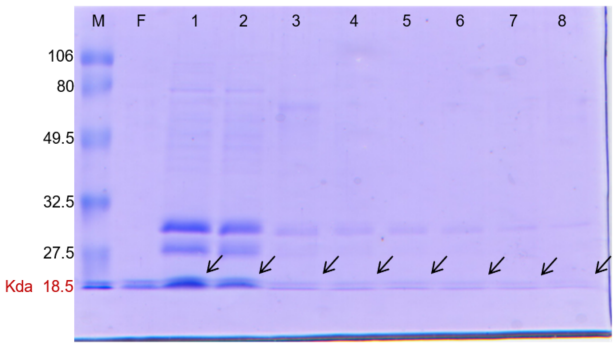

FIGURE 3 SDS PAGE analysis of recombinant protein purification. M: marker protein PageRuler^{TM} prestained protein ladder low molecular (Bio-Rad), F: filgrastrim as a control, 1: precipitated protein from *P. pastoris* GS115/pPICZα-*CSF3syn-his6* at saturation degree of ammonium sulfate 80% (crude), 2: flow-through, 3: washing, 4–8: elution fractions.

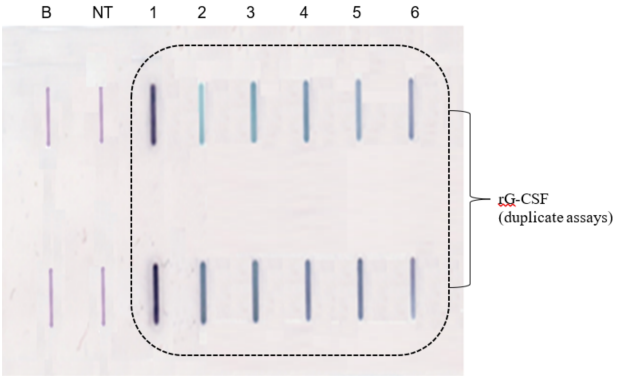

FIGURE 4 Immuno slot blot assay of rG-CSF purification. Lane 1: precipitated protein from *P. pastoris* GS115/pPICZα-*CSF3syn-his6* at saturation degree of ammonium sulfate 80% (crude), 2: flow-through, 3: washing, 4–6: elution fractions. As a controls: lane B = induction media and lane NT = precipitated protein *P. pastoris* GS115 non-transformed at saturation degree of ammonium sulfate 80%.

Polyhistidine tags have a strong affinity for metal ions, such as Ni^{2+}; therefore, a substance with a stronger affinity for Ni^{2+} is required to break the bond. Imidazole with a high concentration in elution buffer has a stronger affinity for metal ions, which replaces the 6×His tag so that the recombinant protein will be released in the elution buffer (Tovar and Odunuga 2019; Sembiring et al. 2022).

However, sometimes more than single-step purification is needed to achieve purified protein. Further purification using size exclusion chromatography (SEC) or ion exchange chromatography (IEC) technique is necessary to overcome this problem. Peng et al. (2019) expressed the recombinant serine protease domain of factor XII in *P. pastoris* carried out a two-step protocol using immobilized Ni-NTA affinity chromatography and continued with Superdex75 gel filtration (size exclusion chromatography). Wang et al. (2008) expressed the recombinant human Zbtb7A (zinc finger and BTB domain-containing 7A) in *P. pastoris* strain GS115, carried out a two-step protocol using Q-Sepharose ion exchange chromatography followed by His-Bind affinity chromatography to purify recombinant Zbtb7A.

According to the immuno slot blot assay, the induction media and precipitated protein *P. pastoris* GS115 non-transformed at saturation degree of ammonium sulfate 80% as a control appeared in a faint purple shade. Precipitated protein from *P. pastoris* GS115/pPICZα-*CSF3syn-his6* at saturation degree of ammonium sulfate 80% (crude fraction) appeared in a deep purple shade, indicating that the concentration of rG-CSF in the solution was high. The flow-through, washing, and elution fraction lane also appeared in purple with the same intensity, suggesting that partially rG-CSF was not bound to Ni-NTA resin during purification. In this case, possibly the amount of Ni-NTA resin used was insufficient to bind all rG-CSF in the sample optimally. Another possibility was that impurities and non-targeted proteins inhibit the binding between rG-CSF and Ni-NTA resin or inappropriate concentrations of imidazole in the elution buffer. However, overall results in this study indicate that the purification of rG-CSF was successful, even though optimisation is necessary to achieve the maximum concentration and purity of rG-CSF.

Immunodetection was performed with a polyclonal anti-GCSF antibody (Santa Cruz) as primary antibody and antirabbit IgG peroxidase conjugate (Promega) as secondary antibody. The complex was detected using an NBT-BCIP staining kit (Promega). Recombinant G-CSF was detected by Western blot and slot blot assay. Western blotting is a highly effective procedure commonly used to precisely determine the presence, abundance, and mass of proteins. This crucial application hinges on the specific interaction of antibodies with the target antigen/protein in the sample (Sembiring et al. 2022). This research also used slot blot assay to identify rG-CSF in fractions of purification. Slot blot assay is a quick method for detecting proteins using anti-GCSF antibodies, and it doesn't require sample pretreatment such as protein denaturation or SDS-PAGE. Although slot blot assay does not reveal the molecular weight of the protein, it does offer the advantage of providing semi-quantitative information based on colour intensity (Takata 2023). This feature makes the method valuable for researchers who need to quantify proteins in addition to using other techniques.

4. Conclusions

P. pastoris GS115/pPICZα-*CSF3syn-his6* showed increased cell density and rG-CSF expression with a molecular weight of approximately 18 kDa as determined by Western blot assay performed every 12 h post-induction. The highest total protein content was 29.6 µg/mL, precipitated with 80% ammonium sulfate saturation. Purification using affinity chromatography demonstrated successful binding of rG-CSF by Ni-NTA resin, with a size of approximately 18 kDa as confirmed by SDS-PAGE analysis. The protein was detected in purple by immuno slot blot assay.

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Authors' contributions

AMF designed the study and provided inputs to the experimental results. ERS carried out the laboratory work and wrote the manuscript. AMF and ERS analyzed the data. HS corrected the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

All authors declare that there are not any conflicts of interest.

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