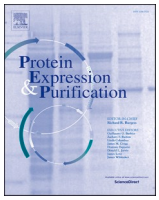
Protein Expression and Purification 218 (2024) 106441

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



Research Article

High performance production process development and scale-up of an anti-TSLP nanobody

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ARTICLE INFO

Keywords:
Nanobody
Anti-TSLP
Pichia pastoris
Expression
Production

ABSTRACT

Nanobodies (Nbs) represent a class of single-domain antibodies with great potential application value across diverse biotechnology fields, including therapy and diagnostics. Thymic Stromal Lymphopoietin (TSLP) is an epithelial cell-derived cytokine, playing a crucial role in the regulation of type 2 immune responses at barrier surfaces such as skin and the respiratory/gastrointestinal tract. In this study, a method for the expression and purification of anti-TSLP nanobody (Nb3341) was established at 7 L scale and subsequently scaled up to 100 L scale. Key parameters, including induction temperature, methanol feed and induction pH were identified as key factors by Plackett-Burman design (PBD) and were optimized in 7 L bioreactor, yielding optimal values of 24 °C, 8.5 mL/L/h and 6.5, respectively. Furthermore, Diamond Mix-A and Diamond MMC were demonstrated to be the optimal capture and polishing resins. The expression and purification process of Nb3341 at 100L scale resulted in 22.97 g/L titer, 98.7% SEC-HPLC purity, 95.7% AEX-HPLC purity, 4 ppm of HCP content and 1 pg/mg of HCD residue. The parameters of the scaling-up process were consistent with the results of the optimized process, further demonstrating the feasibility and stability of this method. This study provides a highly promising and competitive approach for transitioning from laboratory-scale to commercial production-scale of nanobodies.

1. Introduction

Thymic Stromal Lymphopoietin (TSLP), a member of the interleukin-2 (IL-2) cytokine family [1], plays an important role in the development and persistence of airway inflammation and can drive the release of downstream type 2 cytokines, which lead to inflammation and asthma symptoms. TSLP is recognized as an effective therapeutic target for asthma [2]. Anti-TSLP antibodies, which can specifically bind to human TSLP and block its interaction with the receptor complex, effectively prevent immune cells targeted by TSLP from releasing proinflammatory cytokines, thereby preventing exacerbation and improving asthma control. Currently, Amgen/Astrazeneca's Tezspire (tezepelumab) is the only globally approved TSLP-targeting antibody, reflecting the broad market potential of anti-TSLP antibody drugs.

Nanobodies (Nbs) are derived from the heavy-chain variable domain of IgG2 and IgG3 antibodies expressed in Camelidae, lacking the CH1 domain and light chain. Nbs represent the smallest antibody fragments (12–15 kDa) capable of maintaining the specificity and binding affinity of the original whole antibody. Nbs have great potential in improving the physical and chemical properties of traditional monoclonal antibodies (mAbs), while retaining a high degree of recognition within the nanomolar range [3]. As a result of these improvements, Nbs have emerged as a compelling alternative to traditional mAbs [4].

Nbs have been produced in various expression systems, including prokaryotic cells, yeasts, fungi, insect cells, mammalian cell lines, and plants [5]. Among these systems, yeast expression systems, particularly *Pichia pastoris* (now known as *Komagataella phaffii*), have gained significant popularity and become an important pillar in biotechnology, especially for the production of heterologous proteins [6]. The yeast system holds a distinct advantage due to its high degree similarity to advanced eukaryotic expression systems like CHO cell lines [7]. Notably, this yeast system is cost-effective, provides relatively fast expression times, and supports cotranslational and posttranslational processing. Combining the benefits of prokaryotes and mammalian cells, ease of genetic manipulation, rapid growth on inexpensive media, and eukaryotic features, the *Pichia*-expression system is often referred to as the "best of both worlds". These unique characteristics have made *P. pastoris* a highly preferred host organism for application in biotechnology, pharmaceutical industry, and research endeavors [8]. The production of Nbs in *P. pastoris* was first achieved by Rahbarizadeh et al. [9]. They successfully expressed two anti-MUC1 Nbs, achieving

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https://doi.org/10.1016/j.pep.2024.106441
Received 16 October 2023; Received in revised form 25 January 2024; Accepted 3 February 2024
Available online 16 February 2024

Abbreviations

Nbs	Nanobodies
PBD	Plackett-Burman design
TSLP	Thymic Stromal Lymphopoietin
mAbs	monoclonal antibodies
HCPs	host cell proteins
HCD	host cell DNA
DO	dissolved oxygen
SEC-HPLC	size exclusion chromatography-high-performance liquid chromatography
AEX-HPLC	anion exchange chromatography-high-performance liquid chromatography
EC50	half maximal effective concentration

concentrations ranging from 10 to 15 mg/L by optimizing the expression conditions. Similarly, Ezzine et al. obtained anti-AahI Nbs with a concentration of 17 mg/L [10]. Enhancing the Nb production and reducing the production cost is a challenging task that must be solved in the development of Nb-based drugs.

Nbs expressed by *P. pastoris* always has high purity, good thermal stability, and brilliant pharmacokinetics [11]. However, chromatography is usually required to control the content of aggregates, charge isomers [12], host cell proteins (HCPs), host cell DNA (HCD) and other impurities in a low range to avoid affecting the safety and effectiveness of drugs, and maintain high yield to control and reduce the cost. Currently, Nbs are lacking in specific capture tags, which resulting in unstable platform processes and difficulties in capture and subsequent purification, including challenges in removing impurities such as charge isomers, HCPs, and HCD, which limit the druggability of Nbs. Additionally, many tagged Nbs need to remove their tags eventually, which increases the difficulty in achieving target purity and may introduce new impurities, potentially affecting the efficacy and safety of drugs [13].

The chromatography plays a crucial role in the production of Nbs, with ion exchange chromatography, hydrophobic chromatography, or other single-mode chromatography being the primary choices, similar to the purification of mAbs. While these single-mode chromatography effectively remove most impurities, their capacity to eliminate aggregates and charge isomer is insufficient [14]. Advances in chromatographic techniques have led to the widespread application of multimodal chromatography, offering an effective way to streamline chromatographic steps. It combines ion-exchange and hydrophobic interactions, enhancing the selectivity and sensitivity of chromatographic resin. In numerous cases, multimodal chromatography has demonstrated significant advantages in the purification of Nbs [15].

Herein, we present an efficient expression and purification process for a humanized anti-TSLP Nb (Nb3341) at 7L scale, along with the scaling-up process to a 100L scale. Consistency production process was achieved at 100L scale with a titer of 22.97 g/L. To the best of our knowledge, this represents the highest reported production level of Nbs by *P. pastoris* in the existing literature.

2. Materials and methods

2.1. Strains and growth conditions

E. coli XL10-Gold was used as a host strain to amplify the plasmid, and *P. pastoris* was chosen for the secreted expression of recombinant proteins under the induction of methanol. The *P. pastoris* strain used herein was constructed by transformation of the host strain *P. pastoris* X-33 with a Nb3341 expression plasmid (pPICZαA). All yeast strains were stored as frozen stocks at −80 °C in YPD medium supplemented with 15% (v/v) glycerol. *E. coli* XL10-Gold was cultured at 37 °C in LB medium with zeocin (25 μg/mL). Meanwhile, *P. pastoris* X-33 was cultured at 30 °C in YPD liquid medium. Yeast Extract Peptone Dextrose Medium (YPD), Buffered Glycerol-complex Medium (BMGY) and Buffered Methanol-complex Medium (BMMY) were prepared according to the instructions for the cultivation of yeast from the EasySelect Pichia Expression Kit user manual (Invitrogen).

2.2. Affinity determination and activity detection

The kinetics of Nb3341 binding to TSLP antigen were performed by biofilm interferometry (BLI) with a Fortebio's Octet RED96 instrument (ForteBio, Menlo Park, CA, USA). Briefly, the diluted Nb3341-biotin (3 μg/mL) were coupled to streptavidin biosensors and then incubated with a series diluted TSLP, followed by dissociation in PBST. The binding curves were fit in 1:1 binding model by Octet Data Analysis software 9.0. The association and dissociation rates were monitored and the equilibrium dissociation constant (Kd) was determined.

To determine the activity of Nb3341, series diluted Nb3341 were incubated with 4 μg/mL TSLP-biotin in TSLPR protein-coated plate for 1 h. Next, SA-HRP was added to the plate followed by TMB addition. The absorbance at 450 nm was read by the microplate reader (Bio-Rad, Hercules, CA, USA), and the half maximal inhibitory concentration (IC_{50}) was determined.

2.3. Transformation and selection of recombinant P. pastoris

The expression vector pPICZαA was linearized with *Sac* I prior to transformation. *P. pastoris* X-33 strain was transformed using the electroporation transformation method, as described in the pPICZαA, B, C user manual (Invitrogen). Zeocin-resistant colonies were isolated from YPD plates with zeocin concentrations of 500 μg/mL, 200 μg/mL, and 100 μg/mL, respectively. 10 randomly chosen single clones from each zeocin-resistant plate were inoculated into 5 mL YPD medium. These colonies isolated from YPD plates with 500 μg/mL zeocin were coded as T-1 to T-10, those from YPD plates containing 200 μg/mL zeocin were coded as T-11 to T-20, and those from YPD plates containing 100 μg/mL zeocin were coded as T-21 to T-30. After incubation at 30 °C, 250 rpm overnight, the clones were collected by centrifugation (1500 g, 5 min, RT) respectively, and resuspended in BMMY medium. The cells were incubated in 24 well plate at 24 °C for 48 h. 2.0% (v/v) pure methanol was added to a 24-well plate every 24 h to maintain the induction. At the end of induction phase, the culture supernatants were collected by centrifugation (4000 g, 5 min, RT). Nb3341 expression levels in the cells were detected by Fortebio's Octet RED96 instrument (ForteBio, Menlo Park, CA, USA).

2.4. Relative gene copy number detection of recombinant P. pastoris

The relative gene copy numbers of T-1, T-12 and T-22 (sample) based on T-23 (calibrator) were determined using the real-time PCR assay, performed on the CFX Connect™ Real-Time System with CFX Maestro Software (Bio-Rad, Hercules, CA, USA). Genomes were extracted from

clones with varying yields using the PureLink™ Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instruction. Genomic DNA concentrations were measured with a spectrophotometer at 260 nm (Nanodrop). PCR was conducted in a 40 μL reaction mix, consisting of 30 ng of genomic DNA, 20 μL of 2 × SYBR Green-based real-time PCR (Absin, Shanghai, China), and 0.5 μM primers. The amplification conditions were as follows: an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 1 min. Each sample was amplified in triplicate. The relative gene copy number of recombinant *P. pastoris* was calculated by the $\Delta\Delta C_t$ method according to Real-time PCR handbook (Thermo Fisher Scientific). With this method, the C_t values for the gene of interest (Nb3341) in both the test samples are adjusted in relation to a normalizer (Glyceraldehyde-3-phosphate dehydrogenase, GAPDH) gene C_t from the same two samples. The relative copy number is calculated as follows:

C_t (Nb3341) - C_t (GAPDH) = ΔC_t sample

C_t (Nb3341) - C_t (GAPDH) = ΔC_t calibrator

$\Delta\Delta C_t = \Delta C_t$ sample - ΔC_t calibrator

Relative gene copy number of sample = $2^{-\Delta\Delta Ct}$

2.5. Plackett-Burman design

To optimize the expression level of Nb3341, Plackett-Burman design (PBD) was carried out to screen for significant factors influencing the titer of Nb3341 by shake flask cultures. T-1 clone was grown at 250 rpm with a culture volume of 50 mL. Seven factors, denoted as A, B, C, D, E, F and G, represented inoculation proportion, culture temperature, culture pH, culture time, induction temperature, induction pH and methanol feed, respectively. This study explored the influence of 7 variables. The Plackett-Burman experiments design for the seven factors was formulated using the Minitab Statistical Software. The variables represented at high and low levels were coded as +1 and −1, respectively. The specific factors and levels of Plackett-Burman design are detailed in Table 1.

2.6. Fermentation process development of recombinant P. pastoris by 7 L bioreactor

The effect of induction temperature, methanol feed and induction pH on the production of Nb3341 was investigated using high cell density fermentation in a 7 L bioreactor. 2 L of BMGY (40 g/L glycerol instead of 10 g/L glycerol) medium was inoculated with 100 mL of inoculum seed. Operating conditions included a temperature set at 30 °C, pH fixed at 6.0 by automated addition of 25–28% NH_4OH, dissolved oxygen (DO) maintained at 20%, and a constant airflow of pure air at 4 L/min. Increasing the agitation with a range of 500–850 rpm and feeding oxygen when the DO was less than 20%. Glycerol (50% v/v, containing 12 mL/L PTM_1) was fed at a speed of 12 mL/L/h after the consumption of all the glycerol from the batch growth phase (approximately 18.5 h), aimed to increase the cell biomass under limiting conditions for approximately 3.5 h. When the wet cells weight reaches 180–220 g/L, the glycerol feed was switched to the methanol feed (100% v/v, containing 12 mL/L PTM_1). The constant rate of methanol feeding culture continued for approximately 181 h. Meanwhile, induction temperature was fixed to 28 °C, 24 °C or 20 °C, methanol feed rates were adjusted to 7.5 mL/L/h, 8.5 mL/L/h or 9.5 mL/L/h and induction pH was established at 7.5,6.5 or 5.5, respectively. Other fermentation operations were carried out according to Pichia Fermentation Process Guidelines (Invitrogen). During the fermentation process, fermentation broth was sampled at different fermentation time and centrifuged by 12,000 rpm for 5 min. The supernatants of different fermentation time were stored at −20 °C until analysis. The fermentation was terminated at approximately 203 h. The fermentation solution underwent centrifugation at 7500*g* for 15 min, followed by filtration through a 0.45 μm filter. Subsequent purification processes were applied and the titer of supernatants was tested using Fortebio's Octet RED96 instrument (ForteBio, Menlo Park, CA, USA).

Table 1
Seven factors screening using a Plackett-Burman design.

Factor Code	Variables	High (+1)	Low (−1)
A	Inoculation proportion	10%	5%
B	Culture temperature	32 °C	28 °C
C	Culture pH	6.6	6.0
D	Culture time	48 h	24 h
E	Induction temperature	28 °C	24 °C
F	Induction pH	6.6	6.0
G	Methanol feed	2%/day	1%/day

2.7. Scale-up fermentation at 100 L scale

The fermentation process was scaled up from 7 L to 100 L, following the principle of geometric similarity to maintain consistent or proportional height-to-diameter ratio. Throughout the scale-up, essential process parameters were kept unchanged, while the initial culture volume was increased from 2 L to 33 L to facilitate a successful scale-up process. The inoculum was first grown overnight at 30 °C in a shake flask containing 80 mL YPD. Subsequently, the cultures were transferred to a 7 L fermenter with 2 L YPD and was further incubated at 30 °C for 11 h. The 100 L fermenter (Gaoji, Shanghai), containing 33 L medium, was inoculated with 1.65 L culture. The temperature was maintained at 30 °C and the pH was controlled at 6.0 using 25–28% NH_4OH. The DO was maintained around 20% and the airflow of pure air was held constant at 66 L/min. Agitation levels were adjusted within the range of 300–450 rpm, and additional oxygen was introduced when the DO was less than 20%. The induction pH was set to 6.5. Other fermentation control and sampling operations remained consistent with the procedures described above. The fermentation process concluded at 203 h. The fermentation liquid was clarified according to the above method. Supernatants collected at different fermentation time were tested by ForteBio and SDS-PAGE.

2.8. Purification process development

2.8.1. Diamond Mix-A and Capto Adhere

The multimodal anion exchange chromatographic purification was carried out using the EzScreen column × 4.5 mL (Bestchrom), powered by AKTA Pure. The columns were washed and equilibrated with buffer A (Phosphate/Citrate buffer, pH7.0). Fermentation supernatants of Nb3341 were filtered with 0.2 μm filter and loaded on the column at the resin capacity of 26.5 mg/mL. To remove unbound proteins, the column was washed with buffer A until the A280 signal approached zero after sample loading. Elution was performed with a linear gradient ranging from 0% to 100% buffer B (Phosphate/Citrate buffer, pH3.0) to collect the target protein. The experiments were performed at room temperature. Diamond Mix-A (Bestchrom, Shanghai) and Capto Adhere (Cytiva, America) were compared following the same chromatographic procedure as described above. The purity of the collection was detected by size exclusion chromatography-high-performance liquid chromatography (SEC-HPLC) and anion exchange chromatography-high-performance liquid chromatography (AEX-HPLC). SEC-HPLC analysis utilized Waters Acquity Arc system with AdvanceBio SEC 130A Columns (Agilent Technologies, Palo Alto, CA, USA), while AEX-HPLC analysis was performed using Thermo Fisher Ultimate 3000 system with ProPacTM WAX-10 Columns (Thermo Scientific, Rockford, IL, USA).

2.8.2. Diamond MMC and capto S ImpAct

The Diamond MMC and Capto S ImpAct were employed with the EzScreen column × 4.5 mL (Bestchrom), powered by AKTA Pure, after multimodal anion exchange chromatographic purification. The columns

were washed and equilibrated with buffer C (Acetate buffer, pH 4.3). The solution containing the target protein was loaded on the column at a resin capacity of 8 mg/mL. To remove unbound protein, the column was washed with buffer C until the A280 signal approached zero after sample loading. Elution was performed with a linear gradient with a range of 0%–100% buffer D (Acetate and NaCl pH 4.3) to collect the target protein. The experiments were performed at room temperature. Diamond MMC (Bestchrom, Shanghai) and Capto S ImpAct (Cytiva, America) were carried out following the same chromatographic procedure as described above. Purity analysis was performed by SEC-HPLC and AEX-HPLC.

2.8.3. Confirmation of purification process at 7 L scale

Application of the Diamond Mix-A was carried out using the column powered by AKTA Pure following the chromatographic processes described in 2.8.1, with the loading capacity not exceeding 40 mg/mL resin. The column dimensions were 15 mm in diameter (Bestchrom, Shanghai), 18 cm in height, and the retention time was maintained at 3 min during the whole chromatographic process.

Similarly, the application of the Diamond MMC was conducted using a column powered by AKTA Pure following the chromatographic processes described in 2.8.2, with the loading capacity not exceeding 30 mg/mL resin. The column dimensions were 15 mm in diameter (Bestchrom, Shanghai), 17.5 cm in height, and the retention time was 3 min during the whole chromatographic process.

2.9. Scale-up purification at 100L scale

During the purification scale-up from 7 L to 100 L, chromatographic procedures, parameters (such as column height, loading capacity, retention time), and buffer composition remained consistent. However, the column volume underwent a linear amplification process. Specifically, the columns of Diamond Mix-A and Diamond MMC were increased in diameter from 15 mm to 300 mm, while maintaining the same height as in the 7 L scale.

The Diamond Mix-A (multimodal anion exchange resin) was carried out using the C03050 M column (HaiWei) powered by 10 L Process (HaiWei) to capture Nb3341 from the supernatant of the fermentation broth. The Diamond Mix-A loading should not exceed 40 mg/mL resin. To remove unbound proteins, the column was washed with buffer A (Phosphate/Citrate buffer, pH7.0) until the A280 signal approached zero after sample loading. Elution was performed with a linear gradient ranging from 0% to 100% buffer B (Phosphate/Citrate buffer, pH3.0) to collect the target protein. The experiments were performed at room temperature.

The Diamond MMC (multimodal cation exchange resin) was carried out using the C03050 M column (HaiWei) powered by 10 L Process (HaiWei) to further purify Nb3341. The Diamond MMC loading should not exceed 30 mg/mL resin. To remove unbound proteins, the column was washed with buffer C (Acetate buffer, pH 4.3) until the A280 signal approached zero after sample loading. Elution was performed with a linear gradient ranging from 0% to 100% buffer D (Acetate and NaCl pH 4.3) to collect the target protein. The experiments were performed at room temperature. The purity of collection was determined by SEC-HPLC and AEX-HPLC. HCPs was detected using the Picha pastoris 2nd Generation HCP ELISA Kit (Cygnus, USA) according to the manufacturer's instructions. HCD was determined using the Pichia pastoris Residual DNA Quantitative Kit (HZSKBIO, China) with quantitative-PCR assay, following the manufacturer's instructions. The binding activity of Nb3341 to TSLP was detected by ELISA assay. The final drug substances of Nb3341 were tested by non-reducing SDS-PAGE.

3. Results

In order to obtain Nbs against TSLP with superior affinity, specificity, and good diversity, camels immunization and molecules screening were performed (data not shown). After the candidate molecules were identified, the target genes were constructed into expression vectors and electrotransformed into *P. pastoris*. Selection of clones with the highest yield were achieved by antibiotic resistance screening and expression testing. After fermentation, clarification, purification and quality testing, the drug substances of Nbs were obtained. The entire schedule is illustrated in Fig. 1.

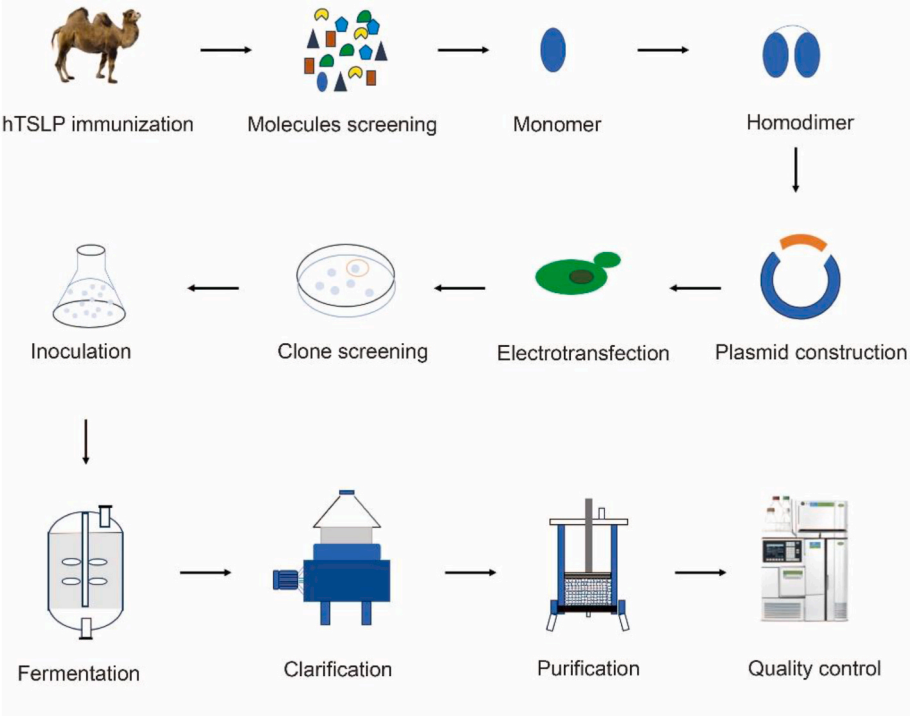

Fig. 1. Schematic depicting the immunization, screening, expression and purification process of Nbs.

3.1. Identification and characterization of Nb3341

The bivalent humanized Nb against TSLP, named Nb3341, demonstrates robust inhibitory properties and excellent drug resistance. It exhibited a high affinity with TSLP and good blocking activity on the interaction between TSLP and TSLPR. Binding kinetics were measured through a BLI-based assay, and the results showed that Nb3341 bound to TSLP with good affinity, as indicated by a Kd value of 1.7 nM (Fig. S1A). Additionally, the blocking assay conducted through ELISA showed that Nb3341 could significantly inhibit the interaction between TSLP and TSLPR, with an IC_{50} value of 0.9501 μg/mL (Fig. S1B).

3.2. Selection of recombinant P. pastoris

The expression level of the cells is critical for the efficient production of the target protein. The schedule of selecting recombinant *P. pastoris* is illustrated in Fig. 2A. To obtain clones with high expression levels, induction were performed in 24 well plates. After a 48 h induction, titers indicated that twenty-nine transformants randomly selected successfully secreted Nb3341 into the medium (Fig. 2B). Among these, T-1 clone exhibited the highest yield which is 0.98 g/L. The clone with the lowest yield is 0.07 g/L, which is less than one-tenth of the highest yield. Clone T-1 was chosen for fermentation process development in the following experiments.

3.3. Relative gene copy number of recombinant P. pastoris

Four transformants (T-1, T-12, T-22, and T-23) were selected as representatives of the recombinants with varying yields. The target gene copy number of clone T-22 was set to 1. The result of the relative gene copy number calculation was rounded to the nearest whole number. As shown in Table 2, the relative gene copy number of T-1, T-23 and T-12 were 15, 7 and 2, respectively. The results indicated a positive correlation between gene copy number and the yield of Nb3341.

3.4. Optimization of expression condition by Plackett-Burman design

The Plackett-Burman design, a widely used statistical experimental approach, efficiently identifies factors with the most significant impact on experimental results using the fewest number of experiments [16]. Twenty-four experiments were carried out with different combinations of individual factors. Among them, factors A, B, C, D, E, and F in shaker culture were comparable with that in 7 L fermentation culture, and factor G served as the inducer of protein expression. Other operation parameters of flask culture not comparable with 7 L fermentation culture were excluded from this experiment. Experimental design and results for the Plackett-Burman experiments are presented in Table 3, with the analysis of variance results provided in Table 4. The model demonstrated extreme significance with a p value of $0.000 < 0.01$. In order of influence, the factors affecting the titer of Nb3341 were G > F > E > D > C > A > B. Methanol feed, Induction pH and Induction temperature (G, F, and E) had a significant impact ($p < 0.05$) and were assessed as key process parameters in the subsequent experiment.

3.5. Fermentation process development of recombinant P. pastoris by 7 L bioreactor

To establish the optimal conditions for the large-scale production of Nb3341, the effect of induction temperature, methanol feed and induction pH on cell growth and Nb3341 expression were systematically investigated at the 7 L scale. The optimization results for different parameters were shown in Fig. 3A/B/C, Fig. 3D/E/F and Fig. 3G/H/I.

The wet cell weight, OD value and titer of induction temperature optimization were shown in Fig. 3A, B and C. Varying induction temperatures minimally affected the wet cell weight, but significantly affected the OD value and titer of Nb3341. The highest Nb3341 production level (23.38 g/L) was achieved at 24 °C after 181 h of induction, which is 15% higher than 28 °C (20.32 g/L) and 60% higher than 20 °C (14.60 g/L) (Fig. 3C). Consequently, the induction temperature of 24 °C was fixed for the subsequent fermentation process development.

During methanol feeding rate optimization, an increasing trend was observed in wet cell weight, OD value and Nb3341 titer with higher methanol feeding rate (Fig. 3D, E and H). When the methanol feeding rate was 9.5 mL/L/h, the highest Nb3341 titer was achieved. However, the increased foam fermentation at this rate was detrimental to the stability of subsequent commercial production. Therefore, a methanol feeding rate of 8.5 mL/L/h was chosen as optimal and applied to the subsequent fermentation process development.

As shown in Fig. 3G and H, the wet cell weight and OD value of the fermentation broth were consistent under the induction conditions of pH 5.5 and pH 6.5. Whereas, an increase in induction pH to 7.5 resulted in an obviously decrease in both wet cell weight and OD value (Fig. 3G and H). The expression of Nb3341 showed different trends with cell growth with pH 5.5 and pH 6.5 induction conditions. The highest Nb3341 production level (23.38 g/L) was achieved at pH 6.5 after 181 h of induction, which bring about a 15% increase over pH 5.5 (20.32 g/L) and a 60% increase over pH 7.5 (14.60 g/L) (Fig. 3I).

In conclusion, the optimal induction temperature, methanol feed and induction pH were determined to be 24 °C, 8.5 mL/L/h and 6.5, respectively. A high protein yield of 23.38 g/L and volumetric

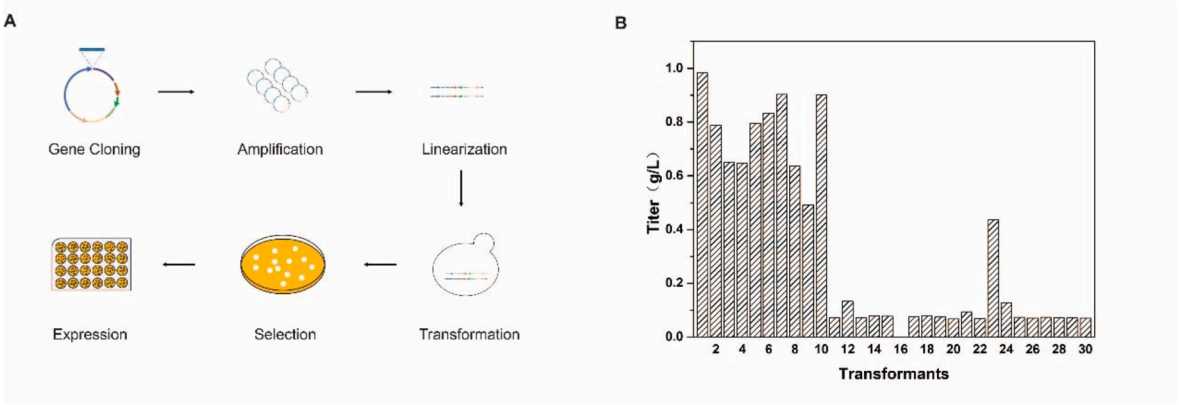

Fig. 2. Selection of recombinant *P. pastoris*. (A) Schematic depicting the construction and selection process of recombinant *P. pastoris*. (B) The titer of different clones. 1~10:10 transformants (T-1 to T-10) selected from YPD plates containing 500 μg/mL zeocin; 11~20:10 transformants (T-11 to T-20) selected from YPD plates containing 200 μg/mL zeocin; 21~30:10 transformants (T-21 to T-30) selected from YPD plates containing 100 μg/mL zeocin.

Table 2
Relative gene copy number of different clones.

Clone	C_t (GAPDH)	C_t (Nb3341)	ΔC_t	$-\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$	Relative gene copy number	Titer (g/L)
T-1	19.44	16.82	−2.62	3.91	15.03	15	0.98
T-23	21.20	19.62	−1.58	2.87	7.31	7	0.44
T-12	21.10	21.50	0.40	0.89	1.85	2	0.13
T-22	20.92	22.21	1.29	0.00	1.00	1	0.07

Table 3
Plackett-Burman experimental design.

Run	A	B	C	D	E	F	G	Response
	Inoculation proportion (%)	Culture temperature (°C)	Culture pH	Culture time (h)	Induction temperature (°C)	Induction pH	Methanol feed (%/day)	Titer (mg/L)
1	10 (+1)	32 (+1)	6.6 (+1)	24 (−1)	28 (+1)	6.6 (+1)	1 (−1)	840.7
2	5 (−1)	32 (+1)	6.0 (−1)	24 (−1)	24 (−1)	6.6 (+1)	2 (+1)	1065.4
3	5 (−1)	32 (+1)	6.0 (−1)	24 (−1)	24 (−1)	6.6 (+1)	2 (+1)	1080.8
4	5 (−1)	32 (+1)	6.6 (+1)	48 (+1)	24 (−1)	6.6 (+1)	2 (+1)	1019.2
5	10 (+1)	28 (−1)	6.6 (+1)	24 (−1)	24 (−1)	6.0 (−1)	2 (+1)	935.9
6	5 (−1)	28 (−1)	6.0 (−1)	24 (−1)	24 (−1)	6.0 (−1)	1 (−1)	636.3
7	10 (+1)	32 (+1)	6.6 (+1)	24 (−1)	28 (+1)	6.6 (+1)	1 (−1)	844.9
8	5 (−1)	28 (−1)	6.0 (−1)	24 (−1)	24 (−1)	6.0 (−1)	1 (−1)	580.3
9	10 (+1)	32 (+1)	6.0 (−1)	48 (+1)	24 (−1)	6.0 (−1)	1 (−1)	639.8
10	10 (+1)	28 (−1)	6.0 (−1)	24 (−1)	28 (+1)	6.6 (+1)	2 (+1)	1248.1
11	5 (−1)	28 (−1)	6.0 (−1)	48 (+1)	28 (+1)	6.6 (+1)	1 (−1)	796.6
12	10 (+1)	28 (−1)	6.6 (+1)	48 (+1)	24 (−1)	6.6 (+1)	1 (−1)	684.6
13	5 (−1)	28 (−1)	6.6 (+1)	48 (+1)	28 (+1)	6.0 (−1)	2 (+1)	952.0
14	5 (−1)	28 (−1)	6.6 (+1)	48 (+1)	28 (+1)	6.0 (−1)	2 (+1)	914.2
15	10 (+1)	32 (+1)	6.0 (−1)	48 (+1)	24 (−1)	6.0 (−1)	1 (−1)	616.7
16	10 (+1)	32 (+1)	6.0 (−1)	48 (+1)	28 (+1)	6.0 (−1)	2 (+1)	1075.2
17	5 (−1)	32 (+1)	6.6 (+1)	24 (−1)	28 (+1)	6.0 (−1)	1 (−1)	777.0
18	5 (−1)	28 (−1)	6.0 (−1)	48 (+1)	28 (+1)	6.6 (+1)	1 (−1)	788.9
19	10 (+1)	28 (−1)	6.0 (−1)	24 (−1)	28 (+1)	6.6 (+1)	2 (+1)	1043.7
20	10 (+1)	28 (−1)	6.6 (+1)	48 (+1)	24 (−1)	6.6 (+1)	1 (−1)	710.5
21	5 (−1)	32 (+1)	6.6 (+1)	48 (+1)	24 (−1)	6.6 (+1)	2 (+1)	961.1
22	10 (+1)	32 (+1)	6.0 (−1)	48 (+1)	28 (+1)	6.0 (−1)	2 (+1)	1014.3
23	5 (−1)	32 (+1)	6.6 (+1)	24 (−1)	28 (+1)	6.0 (−1)	1 (−1)	613.2
24	10 (+1)	28 (−1)	6.6 (+1)	24 (−1)	24 (−1)	6.0 (−1)	2 (+1)	921.9

Table 4
Analysis of Variance in Plackett–Burman experimental.

Factors	Effect	T-Value	P-Value
Inoculation Proportion	32.6	1.55	0.141
Culture temperature	27.9	1.33	0.203
Culture pH	−34.2	−1.63	0.203
Culture time	−34.6	−1.64	0.120
Induction temperature	88.0	4.19	0.001
Induction pH	117.3	5.58	0.000
Methanol feed	308.5	14.67	0.000

productivity of 129 mg/L/h were achieved with optimal culture conditions after about 181 h of induction. These optimal culture conditions were applied in the next scale-up study.

3.6. Scale-up fermentation at 100 L scale

To study the feasibility of commercial production, the fermentation scale of Nb3341 was scaled up to 100 L. As shown in Fig. 4A, the wet cell weight reached 430 g/L at 100 L scale, slightly lower than that at 7 L scale (458 g/L). The OD value (Fig. 4B) and titer (Fig. 4C) of the fermentation broth both at 100 L and 7L scale were comparable, indicating that the 100 L scale achieved a higher output than 7 L scale per unit volume of fermentation broth. SDS-PAGE assay results of supernatant samples at 100 L scale (Fig. 4D) demonstrated a gradual increase in Nb3341 concentration over the fermentation time, which is consistent with the results of the titer (Fig. 4C). At the end of the 100 L fermentation, the total fermentation volume was 72 L, and the supernatant volume was 40.5 L. Ultimately, Nb3341 fermentation production at 100 L scale yielded 22.97 g/L with a volumetric productivity of 126 mg/L/h after approximately 183 h of induction.

3.7. Purification process development

3.7.1. Screening of resin

The comparison between Diamond Mix-A and Capto Adhere as potential resins for capturing Nb3341 from fermentation supernatants, was investigated. As shown in Fig. 5A, chromatograms of Diamond Mix-A and Capto Adhere were almost identical. However, the yield, SEC-HPLC and AEX-HPLC purity of the Diamond Mix-A collection were 73.7%, 96.8% and 85.7%, respectively (Fig. 5B), which were higher than Capto Adhere collection, surpassing by 2.4%, 2.9% and 0.9%, respectively. Consequently, Diamond Mix-A was determined as the capture resin.

In Nb3341 purification process, cation exchange chromatography (Diamond MMC and Capto S ImpAct) served as a polishing step to reduce impurities (Fig. 5C and D). As shown in Fig. 5C, Diamond MMC exhibited superior resolution compared to Capto S ImpAct. Meanwhile, the yield, SEC-HPLC and AEX-HPLC purity of the Diamond MMC collection were 32.7%, 98.1% and 95.7%, respectively (Fig. 5D). Which were higher than the Capto S ImpAct collection, exceeding by 7.8%, 5.6% and 7.2%, respectively. Therefore, Diamond MMC was chosen as the polishing resin for further purification.

3.7.2. Confirmation of purification process at 7 L scale

To verify the stability of the purification process, three batches of purification process were carried out at 7 L scale. The yield and the purity of the collections were presented in Table 5. For Diamond Mix-A

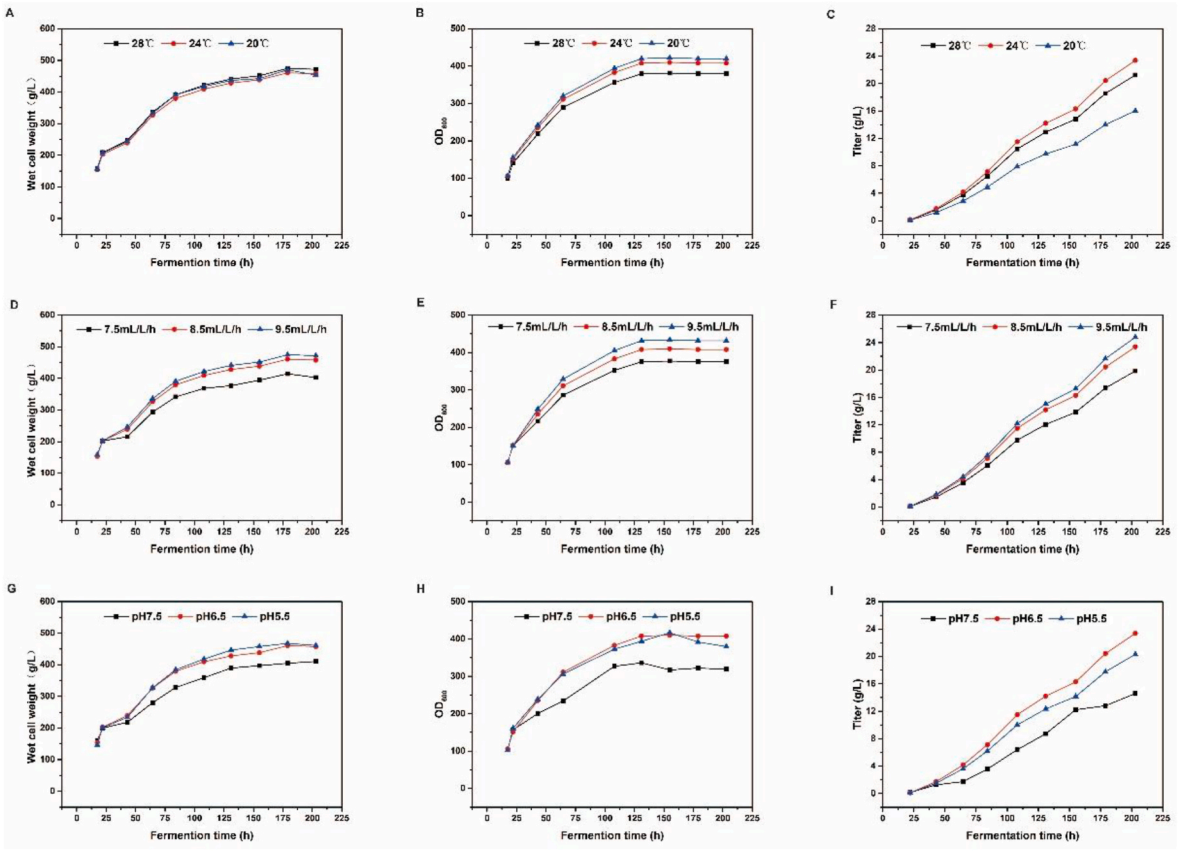

Fig. 3. The time-course of wet cell weight (A, D and G), OD value (B, E and H) and titer of Nb3341 (C, F, and I) in 7 L bioreactors.

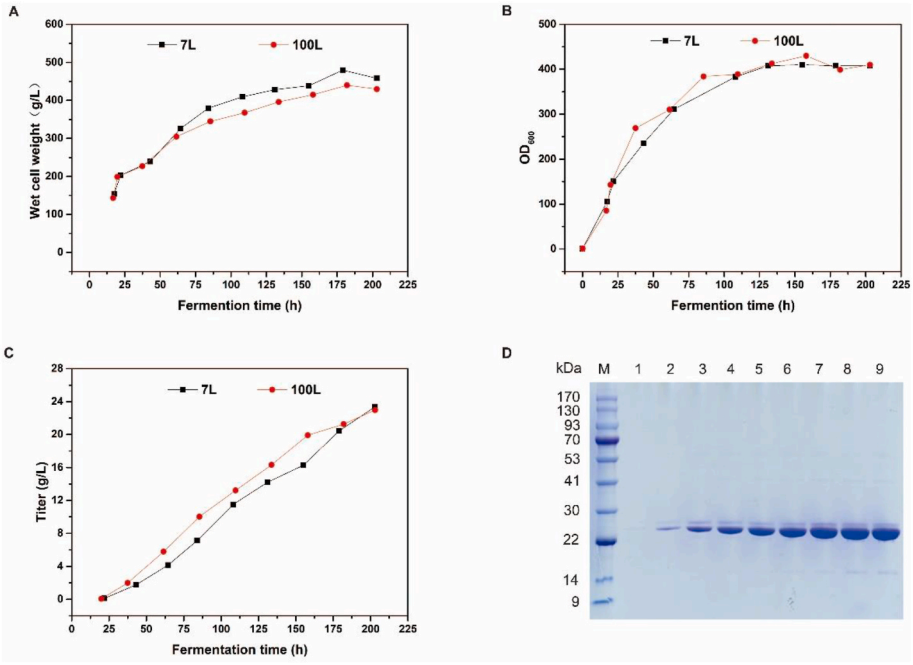

Fig. 4. The time-course of wet cell weight (A), OD value (B), titer (C) and non-reducing SDS-PAGE analysis (D) at 7 L and 100 L scale. M, prestained molecular weight markers; lanes 1–9, 0.5 μL culture supernatants after 0, 18, 42, 66, 90, 114, 138, 162 and 183 h of induction at 100 L scale.

purification, the yield of ranged from 71.8% to 75.1%, with SEC-HPLC and AEX-HPLC purities ranging from 96.9% to 97.3%, and 93.9%–95.2%, respectively. For the Diamond MMC purification process, the yield varied from 69.5% to 74.1%, with SEC-HPLC and AEX-HPLC purities in the range of 98.9%–99.1%, and 96.0%–96.3%, respectively. The purification processes for Diamond Mix-A and Diamond MMC were stable and reproducible at 7 L scale.

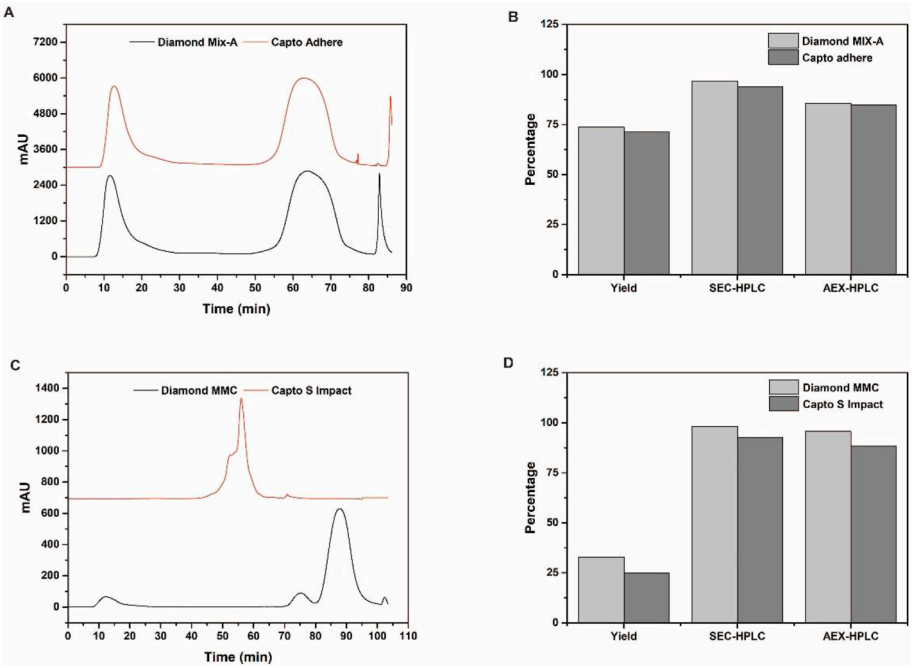

Fig. 5. Purification process development of Nb3341. (A) Chromatograms of Diamond Mix-A and Capto Adhere. (B) Yield, SEC-HPLC and AEX-HPLC of Diamond Mix-A and Capto Adhere. (C) Chromatograms of Diamond MMC and Capto S ImpAct. (D) Yield, SEC-HPLC and AEX-HPLC of Diamond MMC and Capto S ImpAct.

Table 5
Yield and purity of purification process at 7 L scale.

Step	Batch	Loading Volume (mL)	Loaded protein (mg)	Elution Volume (mL)	Eluted protein (mg)	Yield (%)	SEC-HPLC (%)	AEX-HPLC (%)
Diamond Mix-A	1	67.3	1271.6	102.8	919.7	72.3	96.9	93.9
	2	67.3	1271.6	104.9	955.3	75.1	97.2	95.2
	3	67.3	1271.6	105.1	913.3	71.8	97.3	94.8
Diamond MMC	1	96.7	865.2	170.1	601.3	69.5	99.0	96.0
	2	95.1	865.2	176.3	631.6	73.0	99.1	96.0
	3	99.6	865.2	175.9	641.1	74.1	98.9	96.3

Table 6
Yield and purity of purification process at 100L scale.

Step	Batch	Loading Volume (L)	Loaded protein (g)	Elution Volume (L)	Eluted protein (g)	Yield (%)	SEC-HPLC (%)	AEX-HPLC (%)
Diamond Mix-A	1	27.0	510.3	41.2	364.6	71.4	97.1	93.7
	2	27.0	504.9	41.5	368.1	72.9	96.6	95.0
	3	27.0	505.3	42.8	378.5	74.9	97.0	94.2
Diamond MMC	1	41.0	362.3	67.7	253.6	70.0	98.8	95.8
	2	40.0	360.4	69.1	249.7	69.3	98.8	96.0
	3	42.0	369.6	68.8	269.0	72.8	98.7	95.7

3.8. Scale-up purification at 100 L scale

To verify the stability of purification process at a larger scale, three batches of 100 L scale production were carried out, and the results are presented in Table 6 and Fig. 6. As shown in Table 6, For Diamond Mix-A purification, the yield ranged from 71.4% to 74.9%, with SEC-HPLC and AEX-HPLC purities ranging from 96.6% to 97.1%, and 93.7%–95.0%, respectively. For the Diamond MMC purification, the yield varied from 69.3% to 72.8%, with SEC-HPLC and AEX-HPLC purities in the range of 98.7%–98.8% and 95.7%–96.0%, respectively. The chromatograms of Diamond Mix-A (Fig. 6A) and Diamond MMC (Fig. 6B), SEC-HPLC (Fig. 6C) and AEX-HPLC (Fig. 6D), the degree of HCD (Fig. 6E) and HCP (Fig. 6F) removal, binding activity of the final drug substances (Fig. 6G) and non-reducing SDS-PAGE analysis (Fig. 6H) were basically consistent. The content of HCP of Nb3341 purified by Diamond Mix-A has been reduced to below 30 ppm and the residue of HCD has been reduced to 1 pg/mg. Subsequently, the content of HCP of Nb3341 purified by Diamond MMC has been reduced to 4 ppm. Which shows excellent removal ability of host residual impurities. Moreover, relevant chromatograms of purification process (chromatography, SEC-HPLC and AEX-HPLC), binding activity and non-reducing SDS-PAGE analysis also present a high level of consistency.

4. Discussion

P. pastoris is a mature (FDA and EMA approved), safe (GRAS) and highly competitive expression host, known for its potent and effective secretory capacity [8]. To achieve high expression of target protein in the culture supernatant while reducing endogenous protein secretion, multiple strategies have been implemented to enhance the yield and quality of different recombinant proteins of interest. However, the expression and purification of Nbs based on *P. pastoris* expression system

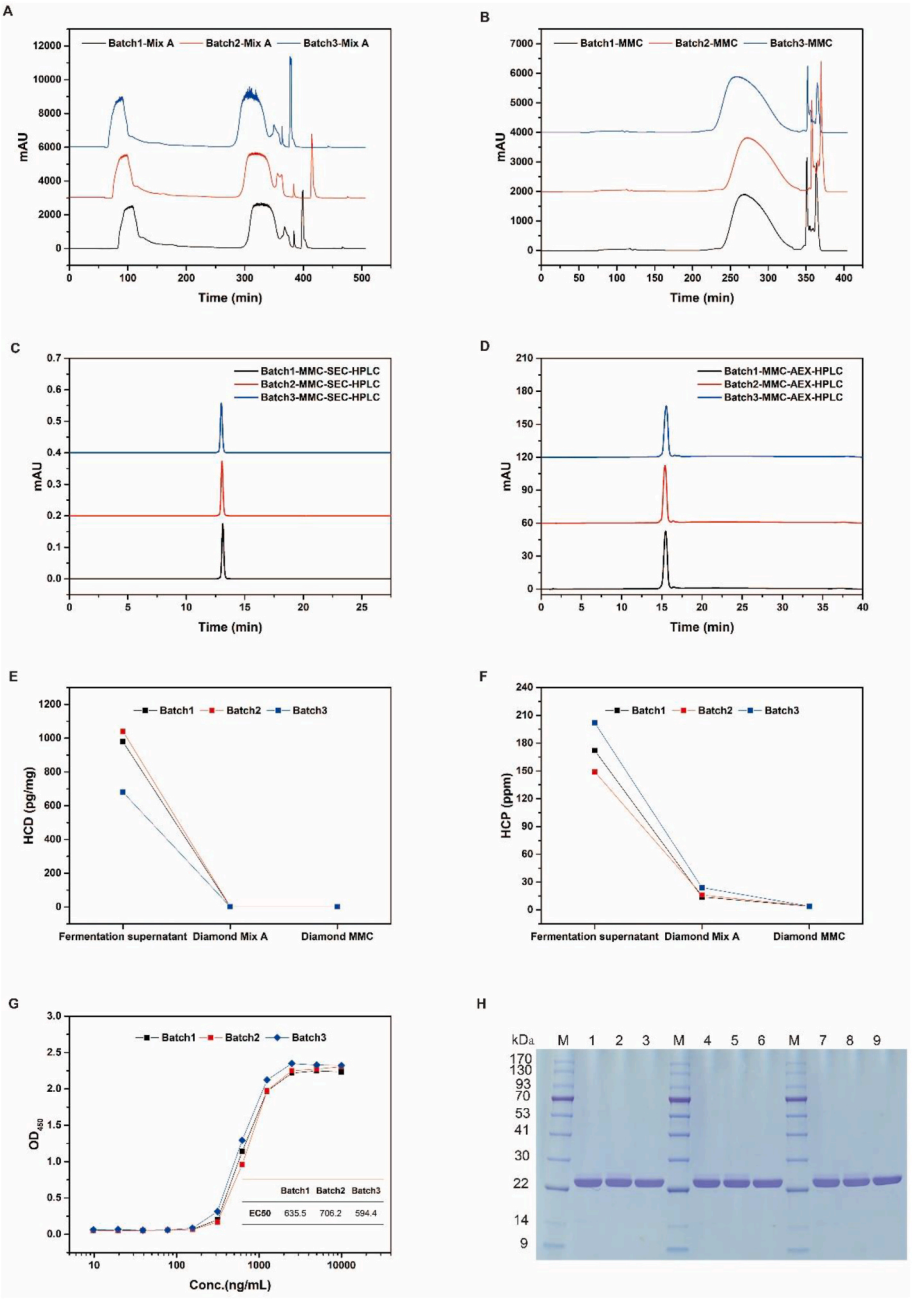

Fig. 6. Purification production of Nb3341 at 100 L scale. (A) Overlay of Diamond Mix-A chromatograms of three batches. (B) Overlay of Diamond MMC chromatograms of three batches. (C) Overlay of SEC-HPLC chromatograms of Diamond MMC. (D) Overlay of AEX-HPLC chromatograms of Diamond MMC; (E) Trend chart of HCD level. (F) Trend chart of HCP level. (G) Binding activity of the final drug substances. (H) Non-reducing SDS-PAGE analysis. M, prestained molecular weight markers; lanes 1, 2 and 3, culture supernatants, harvest of Diamond Mix-A and harvest of Diamond MMC of batch 1; lanes 4, 5 and 6, culture supernatants, harvest of Diamond Mix-A and harvest of Diamond MMC of batch 2; lanes 7,8 and 9, culture supernatants, harvest of Diamond Mix-A and harvest of Diamond MMC of batch 3.

is rarely reported systematically. In this paper, we focus on the development and scale-up of nanobody production and purification processes, and some encouraging and enlightening results were obtained.

Clonal screening procedures for comparison of *P. pastoris* strains rely on the availability of a culture environment that ensures equal growth and production capacity for all transformants evaluated, which is pivotal in the production of recombinant proteins by *P. pastoris*. Since clonal variation in this assay is attributed to differences in target gene copy numbers and possible genomic location of integrated (linearized) expression constructs between transformants, yields from different clones can be very large. Fig. 2 shows a yield difference of more than 10 times among the 30 selected clones. For the recombinant protein with low titer, the productivity assessment of a larger number of strains is an effective way to identify high-yield clones.

Many standard protocols for protein production in *P. pastoris* using bioreactors are available in the reported literature [17,18], optimization is often necessary for specific proteins. The alcohol oxidase (P_{AOX1}) promoter, a prominent promoter system for recombinant protein production in *P. pastoris*, can be induced by methanol but inhibited by several other carbon sources, such as glucose and glycerol. Thus, the typical cultivation strategies of *P. pastoris* strains involve two distinct stages: growth on a carbon source, such as glycerol, to obtain high

biomass concentrations, followed by induction of recombinant protein production by methanol [19]. For the above reasons, the optimal growth conditions of *P. pastoris* was maintained during the growth phase, and the induction pH, which has a great influence on the protein yield and quality, was optimized during the recombinant protein production phase at 7 L scale. Here a high level of 23.38 g/L Nb3341 were obtained by induction at pH 6.5.

The process stability is of utmost importance in nanobody production as it directly influences the quality, yield, and consistency of the antibodies. A stable production process ensures consistent yield and quality characteristics across different batches [20]. Scaling up the fermentation process is essential for commercial production of the recombinant protein. Fed-batch fermentation experiments of Nb3341 was successfully scaled up to 100 L scale in our research, maintaining a consistent yield of 22.97 g/L Nb3341 compared to the 7 L scale.

The multimodal chromatography, encompassing both charge exchanger and hydrophobic functional groups, exhibits higher tolerance to the salt concentration and better resolution compared to single-modal chromatography [15]. The multimodal chromatography proves effective in the purification process of Nbs, addressing challenges associated with capturing and purifying Nbs lacking of labels. Compared with the single-modal chromatography, the multimodal chromatography reduces the chromatography steps [21], and the product of high purity and low host impurities residue can be obtained by two-step multimodal chromatography.

Continuous gradient elution is essential for Nb3341 to achieve high resolution, which is less convenient. Meanwhile, the interaction of ion exchange and hydrophobic involved in the multimodal resin leads to the complexity of process development [22], which requires in-depth research to achieve a more convenient step elution mode. The purities and impurities, such as charge variants, HCPs, HCD, are critical quality attributes in process development of Nbs. In accordance with the 2020 edition of the Chinese Pharmacopoeia (ChP), the HCP and HCD content in drug substrate of Nb3341 should be less than 1000 ppm and 330 pg/mg, respectively. The HCP and HCD content in the drug substrate of Nb3341 were found to be 4 ppm and 1 pg/mg, respectively, representing only 0.4% and 0.3% of the standards. This article presents a comprehensive analytical method for assessing the quality attributes of Nb3341.

5. Conclusions

In conclusion, efficient production and purification process of Nb3341 was established. The T-1 clone demonstrated a yield of 0.98 g/L in 24-well plate. After optimization at the 7 L scale and scale-up to the 100 L scale, the yield of Nb3341 reached 22.97 g/L, reportedly the highest production level of Nbs by *P. pastoris* in literature to date. The SEC-HPLC purity, AEX-HPLC purity, HCP content and HCP content reach 98.7%, 95.7%, 4 ppm and 1 pg/mg, respectively, meeting the requirements of investigational new drug applications [23]. With the development of clinical trials, regulations, and guidelines from regulatory agencies will enable us to further refine our quality standards. The encouraging results obtained in this study not only lay a solid foundation for commercial production of Nb3341, but also provide valuable enlightenment about the industrialization of recombinant proteins using *P. pastoris*.

Declaration of interest

The authors declare that there is no conflict of interests.

CRediT authorship contribution statement

Xiaofei Li: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Peng Qiao:** Writing – original draft, Methodology, Investigation. **Yicai Zhang:** Validation, Methodology. **Guoxin Liu:** Methodology. **Min Zhu:** Writing – review & editing, Visualization, Methodology. **Junwei Gai:** Visualization. **Yakun Wan:** Project administration, Conceptualization.

Data availability

Data will be made available on request.

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2024.106441.

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