

Optimization of Liquid Fermentation Conditions for *N*-Glycosylated *Ganoderma lucidum* Immunomodulatory Protein Production by an Engineered *Pichia pastoris*

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Abstract

The *Ganoderma lucidum* immunomodulatory protein (FIP-glu) holds promise as a potential anti-tumor therapeutic agent. Enhancing the expression and stability of the engineered yeast strain is crucial for its industrial viability. This study focused on optimizing the fermentation parameters to produce an *N*-glycosylated protein, designated T36N, using the engineered *Pichia pastoris* GS115 strain with a mutant gene FIP-glu_T36N, a FIP-glu variant with an N-linked glycan modification. The response surface methodology (RSM) was employed to investigate the fermentation parameters that influence the yield of the *N*-glycosylated protein. Initially, the impact of fermentation duration on the optical density (OD_{600}) and protein yield of *P. pastoris* GS115 were assessed, leading to the determination of a 96-hour fermentation period as optimal. Subsequently, the interplay among fermentation temperature, initial pH, and methanol concentration on protein yield was explored. The RSM analysis revealed the most favorable conditions for fermentation: a temperature of 26 °C, an initial pH of 6.5, and a methanol concentration of 1.00%. Verification tests confirmed that under these optimized conditions, the T36N yield peaked at 346.64 ± 3.47 mg.L⁻¹, marking a 32.9% increase. The results underscored the capability of achieving high yields of T36N through the optimization of *P. pastoris* GS115 fermentation conditions. This research laid the groundwork for the industrial-scale production of *N*-glycosylated immunomodulatory proteins, offering a platform for the development of novel therapeutic agents.

Keywords: *N*-glycosylated, Fungal Immunomodulatory Protein, Fermentation Conditions, Optimization

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INTRODUCTION

Fungal immunomodulatory proteins (FIPs) are a class of small proteins that exhibit immunomodulatory activity like plant hemagglutinins and immunoglobulins. These proteins are primarily derived from macrofungi that are both edible and medicinal.^{1,2} To date, over 30 types of FIPs have been isolated and identified, with the majority originating from the *Ganoderma* genus.³ These proteins have been shown to possess a range of biological activities, including anti-cancer, immunoregulation, and anti-allergy properties.⁴

Glycosylation is a crucial post-translational modification of proteins that plays a significant role in various biological processes, such as cell proliferation, differentiation, immune inflammation, and protein stability.^{5,6} Glycosylated proteins have a stable structure and hold potential for industrial applications.⁷⁻⁹ However, the development and application of FIPs in disease prevention and treatment have been limited, and there is a lack of research on glycoprotein production.

FIP-glu, first isolated from *G. lucidum*,^{10,11} was one fungal immunomodulatory protein. With the advancement of biotechnology, extensive studies have been conducted on the production of recombinant FIPs (r-FIP) using various microorganisms through recombination technology.¹²⁻¹⁵ These studies have laid a solid foundation for the industrial application of FIPs and their potential clinical use.¹⁴ Yeasts, in particular, have been widely used in the industrial production of medicinal proteins due to their numerous advantages.^{16,17}

Fermentation conditions, such as temperature, pH and fermentation time significantly impact the yield of fermentation products. For engineered yeast, the inducer is also a critical factor for protein expression. Response surface methodology (RSM) is an effective technique for identifying the optimal combination of variables to produce the desired outcome in experimental design.^{18,19}

In a previous study, based on bioinformatics analysis, we have successfully constructed an *N*-glycosylated mutant *FIP-glu_T36N* gene by mutating the threonine (Thr,

T, T36) in the FIP-glu sequence to the asparagine (Asn, N), resulting in the production of the fungal immunomodulatory protein FIP-glu_T36N (referred to as T36N), which demonstrated enhanced anti-inflammatory activity *in vitro*.²⁰ In the current study, RSM was employed to optimize the fermentation conditions of the engineered *P. pastoris* GS115/*FIP-glu_T36N* (referred to as *P. pastoris* GS115) to improve the yield of T36N. The goal of this study was to provide a reference for the industrial production of glycosylated proteins.

MATERIALS AND METHODS

Strain preservation and media preparation

The genetically modified *P. pastoris* GS115/*FIP-glu_T36N* strain was stored at -80 °C within the Plant Biotechnology Research Center at Shanghai Jiao Tong University's School of Agriculture and Biology. A Bicinchoninic acid (BCA) protein quantification kit was sourced from Sangon Biotech (C503021, Shanghai, China). The Buffered Glycerol-complex Medium (B540130, BMGY) was formulated with 10 g·L⁻¹ yeast extract, 20 g·L⁻¹ peptone, 10 mL·L⁻¹ glycerin, 13.4 g·L⁻¹ Yeast nitrogen base, potassium phosphate buffer at pH 6.0, and 0.4 mg·L⁻¹ biotin. The Buffered Methanol-complex Medium (BMMY) included 10 g·L⁻¹ yeast extract, 20 g·L⁻¹ peptone, 13.4 g·L⁻¹ yeast nitrogen base, potassium phosphate buffer at pH 6.0, 0.4 mg·L⁻¹ biotin, and 5 mL·L⁻¹ methanol, all procured from Sangon Biotech (B540131, Shanghai, China). Sterilization of all media was conducted at 121°C for 20 min.

Fermentation time impact on *P. pastoris* GS115 growth and protein production

P. pastoris GS115 was cultivated in BMGY until the optical density (OD₆₀₀) value reached between 2.0 and 6.0. Subsequently, the culture was transferred into 250 mL Erlenmeyer flasks containing 60 mL of BMMY, with the OD₆₀₀ adjusted to 1.0, and incubated at 28 °C with agitation at 220 rpm. Methanol at a concentration of 1.00% (v/v) was added daily for a period of 120 hours. To assess the impact of fermentation duration on the growth of *P. pastoris* GS115 and protein yield, the OD₆₀₀ was measured every 24 hours using a microplate reader (BioTek, Winoosik, VT, USA). Post-centrifugation at 4000 × *g* for 20

min, the supernatant was collected for protein quantification using the BCA assay kit, following the manufacturer's protocol.

Fermentation condition optimization via RSM

Recognized for its high protein production capabilities, *P. pastoris* requires fermentation condition optimization to address strain-specific and product-related challenges such as temperature, methanol consumption, and initial pH.²¹ RSM was utilized to identify optimal conditions for protein yield in *P. pastoris* GS115 fermentation, following the Box-Behken central composite design. Based on preliminary findings regarding the influence of methanol concentration, temperature, and pH on protein yield (data not presented), the independent variables and their levels are detailed in Table 1. A total of 17 randomized trials were executed using Design-Expert 13.0 software (Stat-Ease Inc., Minnesota, USA), examining the

interactive effects of temperature (X_1), initial pH (X_2), and methanol concentration (X_3) on the protein yield outcome (Table 2).

Model and peak validation

To ascertain the accuracy of the RSM model, five combinations were selected for validation with peak values confirmed under the optimal fermentation conditions. Protein yield was then determined under these conditions with five replicates.

Statistical analysis

Experiments were conducted in triplicate, with results expressed as mean \pm standard deviation (SD). Analysis of variance (ANOVA) was applied to the independent variables to determine significance levels. Significance was denoted as ns (not significant), * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), and **** ($P \leq 0.0001$).

RESULTS AND DISCUSSION

Influence of fermentation time on *P. pastoris* GS115 growth and protein output

The optical density at 600 nm (OD_{600}) and the protein yield served as indicators to evaluate the impact of fermentation duration on the growth of *P. pastoris* GS115 and its protein production. The findings indicated a positive correlation between fermentation time and protein yield, with a

Table 1. Factors and levels of Box-Behken central design

Independent variables	Coded variables Levels		
	-1	0	1
Temperature ($^{\circ}\text{C}$), X_1	26	28	30
Initial pH, X_2	5.5	6.0	6.5
Methanol content (%), X_3	0.75	1.00	1.25

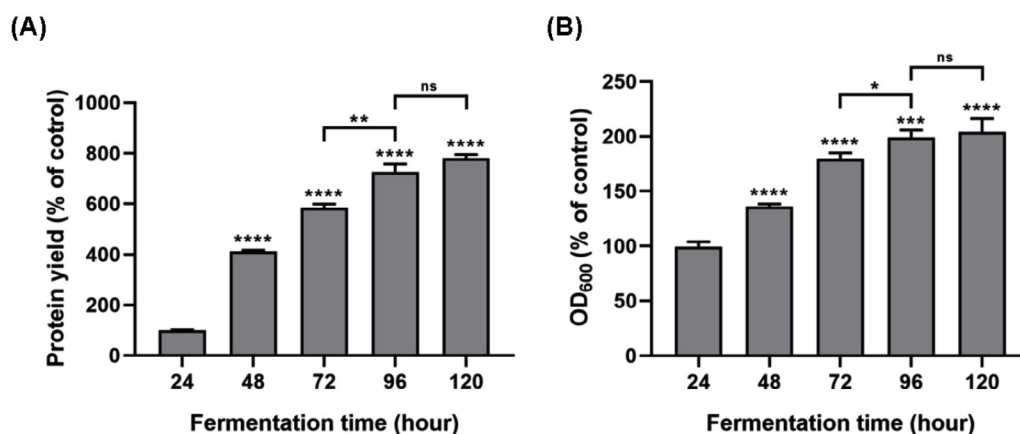


Figure 1. Effects of fermentation time on the protein concentration and the growth of engineered *Pichia pastoris* GS115. Data was expressed as mean \pm SD ($n = 3$). ns (not significant), * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), and **** ($P \leq 0.0001$)

Table 2. Response surface methodology experimental design and results

Run	Independent variables			Protein yield (Y), mg·L ⁻¹	
	Temperature (°C) X_1	Initial pH X_2	Methanol content (%) X_3	Experimental	Predicted
1	-1	0	1	268.16	266.07
2	0	0	-1	250.42	257.43
3	0	0	0	261.84	260.85
4	0	0	0	262.43	260.85
5	0	0	0	259.97	260.85
6	0	-1	1	285.08	285.85
7	-1	-1	0	322.53	323.85
8	0	1	-1	289.16	288.39
9	0	1	1	292.15	300.48
10	0	-1	-1	287.06	278.73
11	0	0	0	263.04	260.85
12	1	-1	0	310.93	317.17
13	-1	1	0	352.37	346.13
14	1	0	1	257.25	250.24
15	1	0	-1	237.57	239.66
16	1	1	0	320.51	319.19
17	0	0	0	256.98	260.85

plateau observed after 96 hours of fermentation. A significant increase was noted when comparing the 96-hour mark to the 72-hour mark (Figure 1; $P \leq 0.0001$). At 96 and 120 hours, the protein yield was 726% and 780% higher than at 24 hours, respectively, with no significant difference between these two time points (Figure 1A). This suggested that the engineered strain achieved a stable protein yield by the 96-hour fermentation mark. Secreted protein is linked to the cell density, as mirrored by the OD_{600} values, which is indicative of *P. pastoris* GS115 growth. Compared to the 24 hours, the OD_{600} values were 176.33% and 195.61% higher after 72 and 96 hours of fermentation, respectively, with a significant difference between these two time points (Figure 1B; $P \leq 0.05$). However, at 120 hours, the OD_{600} value reached 202.15%, with no significant difference observed between the 96 hour and 120 hour marks. Mao *et al.*²² found that the concentration of rFIP-glu expressed by *P. pastoris* GS115 increased from 2-5 days and the OD_{600} value increased from 3-5 days. Externally expressed protein might vary due to different strains and incubation time.²³ Appropriate incubation time was one of the important factors for obtaining high proteins expression.

Optimization of fermentation conditions via RSM

Employing the Box-Behnken design principle, a three-factor, three-level RSM experiment was devised, encompassing 17 test points. These tests were categorized to assess experimental error, with fermentation temperature, initial pH, and methanol concentration as independent variables, and protein yield as the dependent response. The experimental and model-predicted values from the response surface analysis are detailed in Table 2. Design-Expert 13.0 software was utilized to fit the experimental data through a quadratic polynomial regression model. The relationship between the response value Y (protein yield) and the independent variables could be described by the following equation:

$$Y = 260.85 - 8.40 X_1 + 6.07 X_2 + 4.80 X_3 - 5.07 X_1 X_2 - 0.49 X_1 X_3 - 1.24 X_2 X_3 + 15.36 X_1^2 + 50.37 X_2^2 - 22.86 X_3^2$$

In this equation, Y, X_1 , X_2 , and X_3 represented the predicted T36N yield, temperature, initial pH, and methanol content, respectively. The effects of independent

variables and their interaction on the production yield of T36N were analyzed and the results are shown in Figure 2. Further analysis found that the higher the initial pH tending to 6.5 and the lower the temperature tending to 26 °C, accordingly resulted in the 3D surface higher (Figure 2A) and the contoured lines denser (Figure 2B). At this point, the yield of T36N revealed a greater increase. The initial pH played a relatively dominant role in the interaction between temperature and initial

pH, but it was not obvious. On the other hand, at the level of interaction between temperature and methanol content, when the methanol content was inclined to 1.00%, the 3D surface was relatively high (Figure 2C) and the contours were relatively dense (Figure 2D), in the direction of the lower temperature. At this point, the yield of T36N showed a greater increase. The fermentation temperature obviously played a dominant role in the interaction between temperature and

Table 3. ANOVA of regression model

Source	Sum of Squares	Df	Mean Square	F-value	P-value	
Model	14770.86	9	1641.21	32.58	<0.0001	significant
X_1	564.82	1	564.82	11.21	0.0123	significant
X_2	295.12	1	295.12	5.86	0.0461	significant
X_3	184.61	1	184.61	3.66	0.0971	not significant
X_1X_2	102.62	1	102.62	2.04	0.1966	not significant
X_1X_3	0.9409	1	0.9409	0.0187	0.8951	not significant
X_2X_3	6.18	1	6.18	0.1226	0.7365	not significant
X_1^2	993.42	1	993.42	19.72	0.0030	significant
X_2^2	10683.85	1	10683.85	212.08	<0.0001	significant
X_3^2	2200.77	1	2200.77	43.69	0.0003	significant
Residual	352.63	7				
Lack of fit	328.61	3				
Pure Error	24.02	4				
Cor Total	15123.49	16				
R^2	0.9767					
R^2_{Adj}	0.9467					
R^2_{Pred}	0.6499					
C.V.%	2.53					

Table 4. Validation results of the model

Run	Independent variables			Protein yield (Y), mg·L ⁻¹	
	Temperature (°C)	Initial pH	Methanol content (%)	Experimental	Predicted
1	-1	-1	-1	329.74	297.91
2	-1	0	1	268.16	266.07
3	-1	1	0	352.37	346.13
4	0	0	0	261.84	260.85
5	0	1	-1	285.08	288.39
6	0	-1	1	289.16	285.85
7	1	1	1	294.69	302.86
8	1	-1	0	310.93	317.17
9	1	0	-1	247.57	239.66
Correlation coefficient					0.9356

methanol content. In a similar analysis, initial pH played a more dominant role than methanol content, and when methanol content was 1.00%, the higher initial pH was, the greater yield of T36N reached (Figures 2E-F).

In summary, the degree of influence of three factors on protein yield was ranked as B >

A > C, that is, initial pH > temperature > methanol content. As a result, the optimal combination conditions of the model for protein yield were fermentation temperature of 26 °C, initial pH 6.5, methanol content of 1.00%.

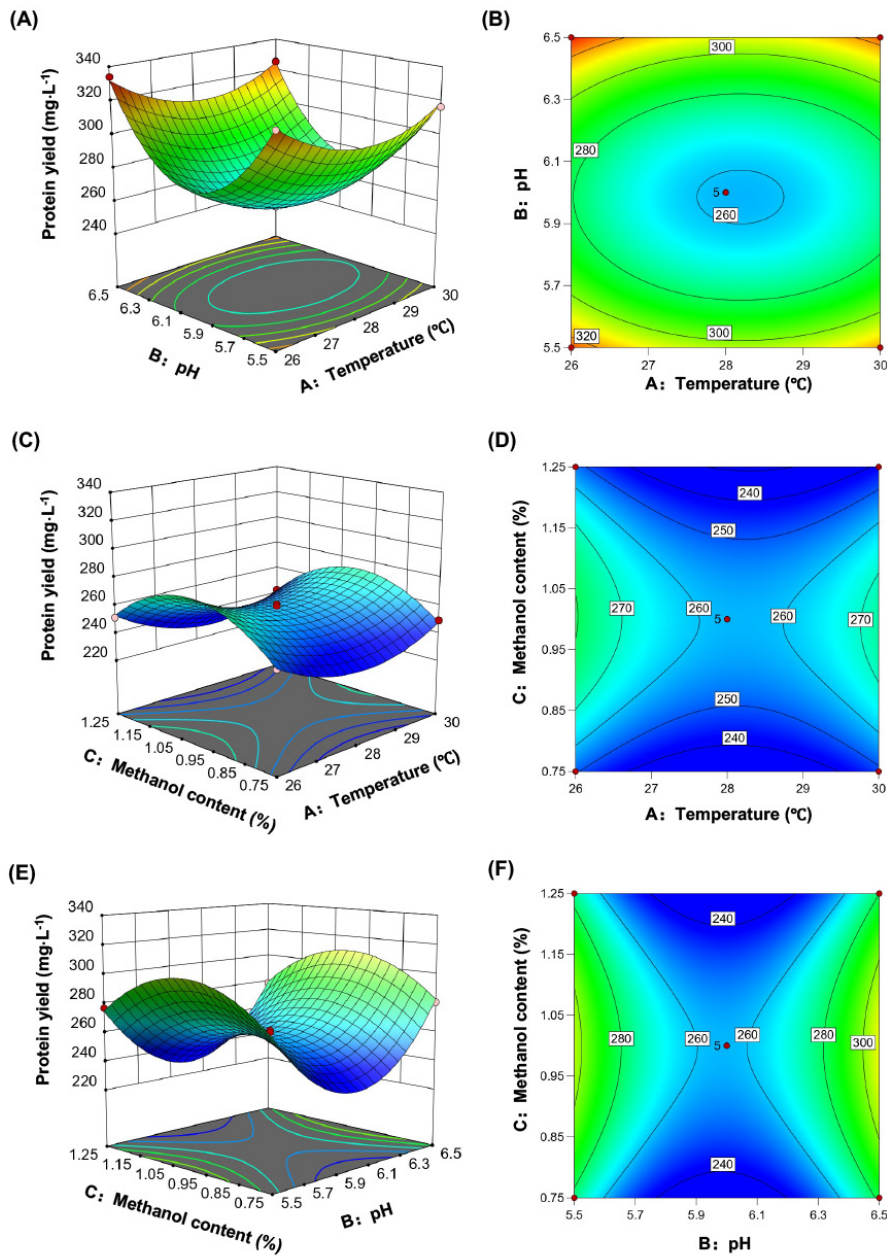


Figure 2. Surface plot and contour plot of the effects of protein concentration by interaction of fermentation temperature, initial pH and methanol content (T36N)

Model variance analysis

The model's variance analysis is presented in Table 3. The regression model was subjected to variance analysis, revealing an overall *P*-value of ≤ 0.0001 , signifying the model's high statistical significance. The determination coefficient R^2 equaled 0.9767, indicating that 97.67% of the response variability was attributed to the model's nine factors, suggesting a robust fit. Additionally, the adjusted determination coefficient R^2_{Adj} equaled 0.9467, further validating the model's predictive accuracy.

To test the reliability of the regression fitting model, 9 sets of validation experiments were conducted (Table 4). The results showed that the correlation coefficient between measured and predicted protein yield was $0.9356 > 0.9$, which demonstrated that the regression fitting model was valid. According to the regression fitting model of response surface optimization, the predicted yield of T36N was $346.13 \text{ mg}\cdot\text{L}^{-1}$ under the optimal combination conditions (temperature 26°C , initial pH 6.5, and methanol content 1.00%) for inducing expression of T36N by *P. pastoris* GS115. At the same time, the *P. pastoris* GS115 fermentation experiment was conducted under the above optimal combination conditions for 5 replicates. The results showed that the actual expression yield of T36N was $346.64 \pm 3.47 \text{ mg}\cdot\text{L}^{-1}$ under the optimal condition, while the original T36N yield was $260.85 \pm 2.45 \text{ mg}\cdot\text{L}^{-1}$, obtaining an increase of 32.89% (Supplementary Table S1). These data indicated that the optimal fermentation combination conditions obtained by RSM were consistent with the actual conditions. Lin *et al.*²⁴ obtained the expression of rFIP-fve produced by *P. pastoris* GS115, with the optimal yield of $258.2 \text{ mg}\cdot\text{L}^{-1}$. The same host, under different fermentation conditions or expressing different heterologous proteins, will lead to different yields.

P. pastoris expression system is one of the most popular and standard tools to produce recombinant protein in the biomedical industry. However, the fermentation conditions of engineered *P. pastoris* are different from those of wild strains. In addition to temperature and pH, it is also necessary to consider the induction time of foreign protein expression and the concentration of inducer.^{23,25} For example, methanol is the inducer of gene expression in

most *P. pastoris* expression systems. However, high concentration of methanol is toxic to the cells, while low concentration of methanol is not conducive to improving the productivity of heterologous proteins.²³ To obtain a lot of FIP-glu, the FIP-glu had successfully expressed in various host cells, and the yield of rFIP-glu in *P. pastoris* ranged from $191.2 \text{ mg}\cdot\text{L}^{-1}$ to $270 \text{ mg}\cdot\text{L}^{-1}$, which was not enough for a pharmaceutical use. After optimization of the fermentation parameters, Mao *et al.*²² improved the yield of rFIP-glu up to $368.71 \text{ mg}\cdot\text{L}^{-1}$ by *P. pastoris* in the shake-flask, under the condition of 1.0% methanol, initial pH 6.5 and 26°C at 280 rpm for 4 days.²² In the present study, the gene *FIP-glu_T36N* was a gene *FIP-glu* variants with a *N*-linked glycan modification. It was still unknown whether the engineered yeast had the potential to produce protein T36N on a large scale after the gene *FIP-glu_T36N* was constructed into *P. pastoris*. This experiment demonstrated that T36N yield under these optimal fermentation conditions reached up to $346.64 \pm 3.47 \text{ mg}\cdot\text{L}^{-1}$, which was increased by 32.9% comparing with not optimized.

In this study, three factors including temperature, initial pH and methanol content were taken into consideration. It is well-known that the suitable fermentation temperature is conducive to the growth of *P. pastoris* and the synthesis or hydrolysis degradation of protein.^{26,27} Some studies have suggested that glycosylation could affect the thermostability of proteins to some extent.^{28,29} In our study, the optimal fermentation temperature of the engineered *P. pastoris* was 26°C that generally consistent with the previous results.²² The glycosylation modification does not seem to have much effect on the yield and thermal stability of the protein, which need further experiments confirmed. Secondly, initial pH of media also played an important role in cell growth, protein formation, and protein stability. In our study, initial pH was the most important of the three variables during *P. pastoris* GS115 fermentation for T36N glycoprotein. Under the condition of initial pH 6.5, the T36N yield reached up to the highest, which was basically consistent with the results of similar studies in previously.²² In Mao *et al.* study, the initial pH had a better effect on the rFIP-glu production of *P. pastoris* GS115 culture than fermentation temperature

and methanol concentration, and under the optimum fermentation parameters (fermentation temperature 26 °C, initial pH 6.5, methanol concentration 1%), rFIP-glu concentration reached $368.71 \pm 10.34 \mu\text{g}\cdot\text{mL}^{-1}$.²² Generally, the optimal initial pH was obtained by running a series of fermentations at different pH. The yield of recombinant porcine lactoferrin expressed in *P. pastoris* increased to $12 \text{ mg}\cdot\text{L}^{-1}$ through increasing initial pH from 6.0 to 7.0.³⁰ Therefore, it is necessary to control initial pH in the media and the best pH is related to protein properties, especially stability. Besides, methanol contributed to cell growth and was an inducer for the expression of foreign proteins in methanotrophic *P. pastoris*. It was reported that in the *P. pastoris* expression system at least 0.5% concentration of methanol was necessary for recombinant protein production, and methanol concentration should not exceed 2-2.5% for cell toxic effect of high level of methanol.²³

CONCLUSION

This research successfully demonstrated the optimization of fermentation conditions for the production of the glycoprotein FIP-glu_T36N using the engineered strain *P. pastoris* GS115, achieved through the application of response surface methodology (RSM). The expression of various bioactive proteins by *P. pastoris* often requires tailored medium compositions and fermentation parameters. The current study revealed that the optimal conditions for T36N production were a fermentation temperature of 26 °C, an initial pH of 6.5, and a methanol concentration of 1.00%. Under these conditions, the T36N yield was maximized, reaching $346.64 \text{ mg}\cdot\text{L}^{-1}$. It is important to note that the experiments were conducted at the shake flask level, highlighting the utility of these methods for fine-tuning fermentation processes. The optimization of such processes in submerged culture offered a viable and efficient alternative to traditional culture techniques, particularly for engineered yeast strains like *P. pastoris* GS115. The findings of this study not only provided a valuable reference for the industrial-scale production of glycoprotein T36N but also underscored its potential for further development and application. The optimized parameters laid a solid foundation

for future research and commercialization efforts in the field of bioactive protein production.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.19.1.38>

Additional file: Additional Table S1.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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