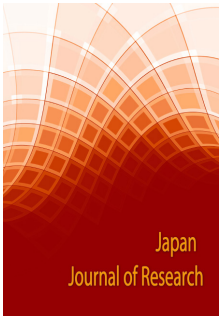
Japan Journal of Research

Original Article



Response Surface Methodological Approach for Optimizing the Expression of Recombinant *Ganoderma leucocontextum* Immunomodulatory Protein in *Pichia pastoris*

Jiayi Yang[1*], Pei Qun[2*], Lida Zhang[1], Juan Lin[3], Xuanwei Zhou[1*]

[1]School of Agriculture and Biology, and Engineering Research Center of Therapeutic Antibody (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China

[2]Xizang Institute for Food and Drug Control, NMPA Key Laboratory for Quality Control of Traditional Chinese Medicine, Tibetan Medicine, Lhasa 850000, People's Republic of China

[3]State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200433, People's Republic of China

*These authors contributed equally to this work.

Correspondence

Xuanwei Zhou

School of Agriculture and Biology, and Engineering Research Center of Therapeutic Antibody (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, P. R. China

Tel: +86-13524360158

Fax: +86-21-34205778

- Received Date: 11 April 2025
- Accepted Date: 25 April 2025
- Publication Date: 28 eApril 2025

Keywords

Ganoderma leucocontextum; fungal immunomodulatory proteins (FIPs); fermentation; response surface method.

Abbreviations

AOX: alcohol oxidase; BBD: box-behnken design; BMGY: buffered glycerol-complex medium; BMMY: buffered methanol-complex medium; BoNT: *botulinum neurotoxin*; FIP-gle 2: FIP from *Ganoderma leucocontextum* 2; FIPs: fungal immunomodulatory proteins; OD: optical density; RBD: *receptor-binding domain*; RSM: response surface methodology; YPD: yeast extract peptone dextrose medium

Abstract

Ganoderma leucocontextum immunomodulatory protein 2 (FIP-gle2) is a recently identified small molecule protein. Based on the successful expression of FIP-gle2 in Pichia pastoris GS115, this study optimized the expression conditions of the engineered P. pastoris using response surface methodology (RSM). Initially, single-factor experiments determined that the optimal parameters for subsequent optimization were a cultivation period of 4 days, a temperature range of 26 to 30 °C, an initial pH of 6.0 to 7.0, and a methanol (MeOH) concentration of 0.75% to 1.25%. Subsequently, a box-behnken design (BBD)-based RSM was employed, with the total protein concentration in the fermentation broth serving as the detection indicator. The optimal fermentation conditions were determined to be a cultivation temperature of 27 °C, an initial pH of 6.7, and a MeOH concentration of 1.11%. Under these conditions, the actual operational parameters were set at a fermentation temperature of 27 °C, an initial pH of 6.5, and a MeOH concentration of 1%, resulting in an average total protein concentration of 225.12 mg·L^{-1}. This study provides preliminary optimization of the cultivation conditions for P. pastoris, offering valuable data support for the industrial production of this protein.

Introduction

Ganoderma leucocontextum, commonly known as "Tibetan white lingzhi" or "white-fleshed lingzhi," is a newly discovered species of the Ganodermataceae family found in the Linzhi region of Tibet in recent years [1]. Research has shown that its fruiting bodies are richer in active components comparing with other *Ganoderma species*. It is currently widely cultivated in the southwestern region of China and is one of the important medicinal materials in the medicinal fungi market [2]. Fungal immunomodulatory proteins (FIPs) are a family of small molecular proteins widely found in fungi [3]. They typically consist of 106 to 133 amino acid residues, with a molecular weight ranging from 12 to 15 kDa. Most FIPs have an isoelectric point between 4~6 and are characterized by a deficiency of histidine, cysteine, and methionine, while being rich in aspartic acid and valine [4]. FIPs have garnered widespread attention due to their diverse biological activities, such as immunomodulation, antitumor effects, and blood coagulation [5,6]. For instance, FIPs may exert their antitumor effects through various molecular mechanisms, including inducing cytokine release [7], promoting cell apoptosis [8], affecting the cell cycle [9], and inhibiting telomerase activity [10].

The whole-genome sequencing and research of *G. leucocontextum* revealed that, compared to *Ganoderma lucidum*, *G. leucocontextum* possesses more genes associated with the synthesis of terpenoid compounds. This may be one of the reasons for their different biological activities. *G. leucocontextum* also contains genes encoding fungal immunomodulatory proteins [11]. However, it is a scientific question worth investigating whether the immunomodulatory proteins encoded by these genes also exhibit the functions characteristic of other genes within this protein family. However, the isolation and purification of the target proteins from natural fruiting bodies involves complex processes and high costs. Therefore, using recombinant protein expression systems to produce the target proteins has become the preferred method.

Citation: Yang J, Qun P, Zhang L, Lin J, Zhou X. Response Surface Methodological Approach for Optimizing the Expression of Recombinant Ganoderma leucocontextum Immunomodulatory Protein in Pichia pastoris. Japan J Res. 2025;6(6):118

The emergence of recombinant protein expression systems has significantly advanced the field of protein production. Currently, the technology for expressing exogenous proteins using *P. pastoris* is highly mature [12,13]. Several recombinant subunit vaccines can be efficiently expressed through this system [14,15]. Additionally, many FIPs can also be expressed using the *P. pastoris* expression system [4]. In prior research, we cloned a fragment of the *FIP-gle* gene (FIP from *G. leucocontextum*) from the genome of *G. leucocontextum* using homologous cloning technology. Because the *FIP-gle* gene sequence is different from the sequences described in the genome sequencing of *G. leucocontextum*, it has been designated as FIP-gle2. We further constructed a *P. pastoris* GS115 strain to produce recombinant FIP-gle2 (rFIP-gle2) through methods such as plasmid construction and electroporation (data not shown). The objective of this study is to optimize the fermentation conditions for the *P. pastoris* GS115 /*FIP-gle2* using response surface methodology (RSM), thereby increasing the expression level of protein and providing a reference for further pharmacological studies and large-scale production of this protein.

Materials and Methods

Materials

The genetically modified *P. pastoris* GS115/*FIP-gle2* strain was constructed and preserved in our laboratory. The yeast extract peptone dextrose medium (YPD), the buffered glycerol-complex medium (BMGY) and the buffered methanol-complex medium (BMMY) were purchased from Qiagen Inc. (Chatsworth, CA, United States), while all analytical-grade reagents were also purchased from Sangon Biotech (Shanghai, China). The Bradford protein concentration assay kit was purchased from Beyotime Biotech (Shanghai, China).

Activation of the engineered P. pastoris GS115

The *P. pastoris* GS115/*FIP-gle2* strain, preserved in the laboratory, was inoculated into a 100 mL Erlenmeyer flask containing 30 mL YPD. The cultivation conditions and methods refer to the previous literature [21].

Single-factor experiments on fermentation conditions for the engineered P. pastoris

To determine the reasonable range of key factors in fermentation, single-factor experiments were designed. After obtaining the optimal values from each single-factor experiment, the ranges for the RSM were set to the values on both sides of the optimal values. In the fermentation experiments, various factors, including fermentation time, temperature, initial pH of the culture medium, and methanol MeOH concentration, significantly influenced the fermentation efficiency of *P. pastoris* GS115/*FIP-gle2*.

Activation of the engineered *P. pastoris* GS115

The fermentation days were set to 1 d, 2 d, 3 d, 4 d, and 5 d, with a fermentation temperature of 30 ℃, a pH value of 6.0 for the potassium phosphate buffer, and a MeOH concentration of 1% (V/V). MeOH was supplemented every 24 h to maintain a final concentration of 1% (V/V). Samples were collected every 24 h to measure the total protein concentration, with each group having three replicates for accuracy.

The effect of fermentation temperatures on the total protein concentration of the culture

The fermentation temperatures were set to 26 ℃, 28 ℃, 30 ℃, 32 ℃, and 34 ℃, with a fermentation day of 4 d, a pH value of the potassium phosphate buffer at 6.0, and a MeOH concentration of 1% (V/V). MeOH was supplemented every 24 h to maintain a final concentration of 1% (V/V). After 96 h of fermentation, samples were taken to measure the total protein concentration, with three replicates for each group.

The effect of initial pH on the total protein concentration of the culture

The pH of the potassium phosphate buffer was set to 5, 5.5, 6, 6.5, and 7, with a fermentation day of 4 d, a temperature of 30 ℃ and a MeOH concentration of 1% (V/V). MeOH was supplemented every 24 h to maintain a final concentration of 1% (V/V). After 96 h of fermentation, samples were taken to measure the total protein concentration, with three replicates for each group.

The effect of MeOH concentration on the total protein concentration of the culture

The MeOH concentrations were set to 0.5%, 0.75%, 1%, 1.25%, and 1.5% (V/V), with a fermentation day of 4 d, a temperature of 30 ℃, a pH value of the potassium phosphate buffer at 6.0. MeOH was supplemented every 24 h to maintain a final concentration of 1% (V/V). After 96 h of fermentation, samples were taken to measure the total protein concentration, with three replicates for each group.

Response Surface Methodology on fermentation conditions for the P. pastoris GS115/FIP-gle2

Based on the results of the single-factor experiments, suitable fermentation temperature, initial pH, and MeOH concentration were selected as independent variables, with the total protein concentration in the fermentation broth as the response value. The corresponding optimization experiments were designed using the software Design Expert v13.0.5.0 (https://www.statease.com/software/ design-expert/), and the levels of each factor are shown in Table 1.

Peak Validation

The outcomes of the RSM study on fermentation conditions for the *P. pastoris* GS115/*FIP-gle2* was analyzed using regression fitting with the Design Expert v13.0.5.0 software, resulting in the derivation of regression equation for rFIP-gle2 induction and the determining of optimal fermentation conditions. Fermentation induction was conducted under these conditions, with six repetitions set, and the measured actual total protein concentration was compared with the predicted total protein concentration.

Total protein concentration detection

The total protein concentration was determined using the Bradford reagent kit, and details can be referenced from the kit's instruction manual.

Table 1. *Optimization of fermentation conditions: Factors and levels setup for RSM*

level	A fermentation temperature (℃)	B initial pH	C MeOH concentration (%)
1	26	6	0.75
2	28	6.5	1
3	30	7	1.25

Statistical analysis

Statistical analysis was performed using SPSS software, with analysis of variance (ANOVA) applied to the independent variables to ascertain significance levels. Significance levels were indicated as ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); and *** ($p \leq 0.001$). The RSM was designed using Design Expert v13.0.5.0, with variance analysis and multiple regression analysis performed.

Results

Results of the Single-Factor Experiment

The effect of fermentation days on the total protein concentration

The effect of different fermentation days on the total protein concentration is demonstrated in Figure 1A. The total protein concentration in the fermentation broth increased with the extension of fermentation days, with the highest concentration observed on the fifth day. When using the total protein concentration measured on the fifth day as a benchmark, statistical differences were observed when compared to days one, two, and three, whereas no significant difference was noted when compared to day four. Therefore, to expedite subsequent experiments, a fermentation duration of four days was chosen.

The effect of fermentation temperatures on the total protein concentration

The effect of different fermentation temperatures on the total protein concentration is demonstrated in Figure 1B. With the increase in fermentation temperature, the total protein concentration in the fermentation broth initially increased and then decreased, reaching the highest concentration at a fermentation temperature of 28 ℃. When using the total protein concentration obtained at 28 ℃ as a reference, a statistically significant difference was observed when compared to 26 ℃, whereas a highly significant difference was noted then compared to temperatures ranging from 30 ℃ to 34 ℃. Therefore, 26 ℃, 28 ℃, and 30 ℃ were chosen as the levels for subsequent RSM.

The effect of initial pH on the total protein concentration

The effect of different initial pH levels on the total protein concentration is demonstrated in Figure 1C. As the initial pH increased, the total protein concentration in the fermentation broth exhibited a trend of rising and then falling, with the highest concentration observed at an initial pH of 6.5. When using the total protein concentration at an initial pH of 6.5 as a benchmark, a highly significant difference was observed when compared to the initial pH level of 5 and 5.5. A significant difference was also noted when compared to an initial pH of 6, and no significant difference was observed when compared to a pH of 7. Consequently, initial pH levels of 6, 6.5, and 7 were chosen for subsequent RSM experiments.

The effect of MeOH concentration on the total protein concentration

The effect of different MeOH concentrations on the total protein concentration is demonstrated in Figure 1D. As the MeOH concentration increases, the total protein concentration in the fermentation broth first rises and then decreases. The total protein concentration is highest when the MeOH concentration is 1%. When using the total protein concentration at 1% methanol as a control, a significant difference was observed when compared to the total protein concentration at 0.5% MeOH, and a noticeable difference was also noted when compared to the total protein concentration at 0.75% MeOH. However, there is no significant difference when comparing the total protein concentrations at 1.25% and 1.5% MeOH. Therefore, 0.75%, 1%, and 1.25% MeOH concentrations were selected as the levels for RSM.

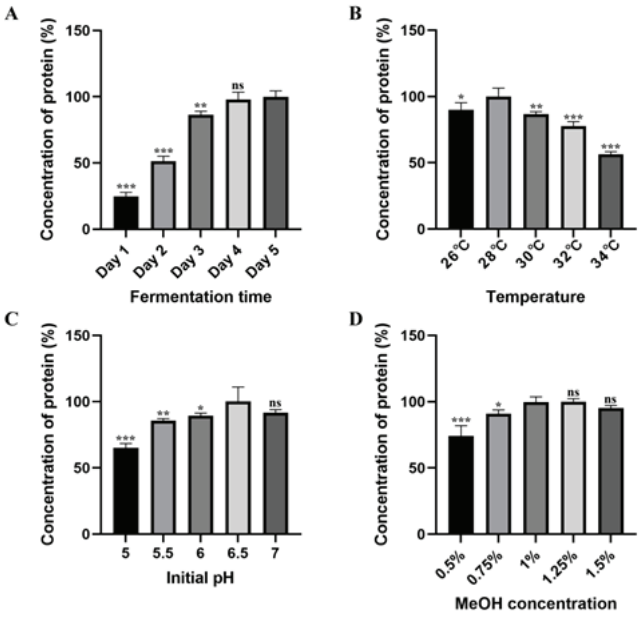


Figure 1. *Effects of different fermentation conditions on the induction of rFIP-gle2.*
(A) Effects of different fermentation durations on the induction of rFIP-gle2; (B) Effects of different fermentation temperatures on the induction of rFIP-gle2; (C) Effects of different initial pH values on the induction of rFIP-gle2; (D) Effects of different methanol concentrations on the induction of rFIP-gle2

The effect of fermentation days on the total protein concentration

Establishment and Analysis of the Response Surface Methodology

The results of the optimization of the single-factor induction conditions indicate that fermentation temperature (A), initial pH (B), and methanol concentration (C) are selected as the three main influencing factors for RSM. The total protein concentration in the fermentation broth (Y) is used as the response value. A three-factor, three-level Box-Behnken response surface experiment is designed using software, comprising a total of 17 experimental groups, with the results summarized in Table 2. A fitting analysis of the experimental results in Table 2 yields the following quadratic polynomial regression equation: $Y=216.08-16.13A+7.59B+4.59C+3.16AB-2.96AC+2.67BC-9.42A^2-8.43B^2-7.34C^2$, where the response value Y represents the total protein concentration, a denotes the fermentation temperature, B indicates the initial pH, and C represents the methanol concentration. The analysis of variance for the regression model is shown in Table 3. The P-value of the regression model is < 0.0001, and the lack of fit is not significant, indicating that the model is highly significant with a small error, making the prediction results reliable. The coefficient R^2 of 0.9820 indicates that the model fits well. The adjusted R^2_{Adj} of 0.9589 and the predicted R^2_{Pred} of 0.9540, with a difference of less than 0.2, suggest that the model can adequately explain the underlying

Table 2. *Design and results of the RSM*

Run	A: temperature (°C)	B: initial pH	C: MeOH (%)	Total protein concentration ($mg \cdot L^{-1}$)
1	30	6.5	1.25	185.08
2	30	6	1	170.65
3	28	6.5	1	216.46
4	26	6.5	1.25	221.93
5	28	6.5	1	214.57
6	28	6.5	1	219.26
7	28	6	0.75	190.45
8	26	7	1	219.51
9	28	6.5	1	210.04
10	30	6.5	0.75	182.63
11	28	7	1.25	215.51
12	28	6	1.25	195.09
13	28	6.5	1	220.08
14	28	7	0.75	200.20
15	26	6	1	210.55
16	26	6.5	0.75	207.65
17	30	7	1	192.25

process. The coefficient of variation C.V. of 1.55%, which is less than 10%, demonstrates the high reliability and accuracy of the experiments. Furthermore, the Adequate Precision value being greater than 4 indicating that the model possesses a good signal-to-noise ratio and can effectively predict the response variable.

In this regression model, the factors A, B, C, A^2, B^2, and C^2 are found to be highly significant, whereas other factors exhibit no significant impact on the results. The effects of factors A, B, and C on the total protein concentration are ranked as A>B>C, suggesting that fermentation temperature has a greater influence than initial pH, which subsequently has a greater influence than methanol concentration.

RSM can visually represent results in three-dimensional (3D) form. A steeper surface indicates a more significant factor, and the contour lines that are closer to an ellipse suggest a stronger interaction between the factors. The three-dimensional response surfaces and contour plots illustrating the interactions among different experimental factors are shown in Figure 2. It is observable that all three response surface curves are convex and exhibit a maximum value. Additionally, the contour lines for the interaction terms AB, AC, and BC are close to elliptical shapes. When combined with the analysis of variance results, it becomes evident that, although these interactions exist, they are not statistically significant.

Peak Validation

The optimal fermentation conditions obtained from the formula are a fermentation temperature of 27 °C, an initial pH of 6.7, and a MeOH concentration of 1.11%. Under these

Table 3. *ANOVA for the regression model*

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	3817.08	9	424.12	42.46	< 0.0001	***
A-temperature	2080.67	1	2080.67	208.31	< 0.0001	***
B-initial pH	461.00	1	461.00	46.15	0.0003	***
C-MeOH	168.20	1	168.20	16.84	0.0046	***
AB	40.04	1	40.04	4.01	0.0854	
AC	35.02	1	35.02	3.51	0.1033	
BC	28.45	1	28.45	2.85	0.1353	
A2	373.36	1	373.36	37.38	0.0005	***
B2	299.09	1	299.09	29.94	0.0009	***
C2	226.96	1	226.96	22.72	0.0020	***
residual	69.92	7	9.99			
Lack of fit	4.81	3	1.60	0.0986	0.9567	not significant
Pure Error	65.11	4	16.28			
Cor Total	3887.00	16				
R^2	0.9820					
Adjusted R^2	0.9589					
Predicted R^2	0.9540					
Adeq Precision	21.3037					
C.V. %	1.55					

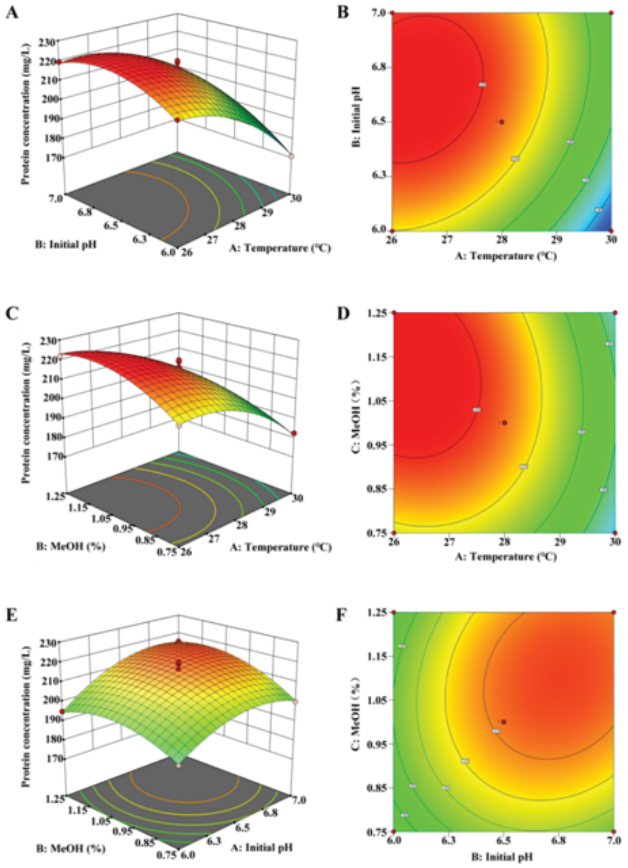


Figure 2. *3D response surfaces and contour plots of the interactions among fermentation temperature, initial pH, and methanol concentration.*
(A) *3D response surface of fermentation temperature and initial pH;* ***(B)*** *Contour plot of fermentation temperature and initial pH;* ***(C)*** *3D response surface of fermentation temperature and methanol concentration;* ***(D)*** *Contour plot of fermentation temperature and methanol concentration;* ***(E)*** *3D response surface of initial pH and methanol concentration;* ***(F)*** *Contour plot of initial pH and methanol concentration*

conditions, the theoretical total protein concentration is 223.49 mg·L^{-1}. To enhance practical feasibility, the fermentation conditions were adjusted to a temperature of 27 °C, an initial pH of 6.5, and a MeOH concentration of 1%. Verification experiments were conducted under these conditions with six repetitions. The results, as shown in Table 4, indicate that the average total protein concentration obtained was 225.12 mg·L^{-1}, with a coefficient of variation of 3.68%. This result closely matches the predicted value, demonstrating the validity of the model.

Discussion

In recent decades, dozens of FIPs have been discovered in various edible and medicinal mushrooms [16]. Among them, FIP-gle2 is a member of this protein family. Modern pharmacological studies have found that FIPs possess antitumor, anti-allergic, anti-rejection, and immunomodulatory effects, highlighting their significant medicinal value and broad application prospects in the fields of medicine, health care, and food supplements [17]. However, the limited availability of these sources and their high costs have constrained their utilization. Therefore, enhancing the expression level of recombinant proteins in *P. pastoris* is

Table 4. *Verification of the optimal fermentation conditions*

	Total protein concentration (mg·L^{-1})
1	229.96
2	217.56
3	220.79
4	215.18
5	234.44
6	232.76
average value	225.12
standard deviation	8.287
c.v.	3.681%

crucial for improving production efficiency, reducing costs, enhancing the quality of recombinant proteins, and meeting diverse application demands.

In previous research, the engineered *P. pastoris* with *FIP-gle2* gene have been constructed in our laboratory. The next task is to increase the expression level of the protein through fermentation to meet the demands of subsequent scientific research or production. Current common methods to increase the expression level of recombinant proteins mainly include the optimization of fermentation conditions, feeding strategies to optimize carbon sources and nutrients, screening of yeast engineering strains through mutation, and modification of the engineered strains using genetic engineering techniques such as CRISPR/Cas9 [18,19]. This study conducted a routine exploration of the optimization of fermentation conditions, including initial pH, MeOH concentration, temperature, and induction time. The pH affects the biomass, protein expression levels, activity, and stability of the cells. In this experiment, at a culture medium pH of 6.5, the protein expression level was 1.53 times that at pH 5.0. Low-temperature induction may enhance the yield of exogenous proteins by reducing the activity of proteases, thus increasing the stability of exogenous proteins. An appropriate MeOH concentration is a key factor for maintaining high cell activity and promoting high protein expression.

In this study, single-factor experiments were conducted with MeOH concentrations ranging from 0.5% to 1.5%. As the concentration of MeOH added to the medium increased, the total protein concentration in the fermentation broth initially increased and then decreased. The total protein concentration in the fermentation broth was highest at a MeOH concentration of 1%, being 1.35 times that at a concentration of 0.5%. Moreover, when MeOH was used as the sole carbon source, cell growth was slow, and the method of supplementing with a mixed carbon source effectively increased the expression level [20]. Therefore, in the subsequent stages of this study, experiments will be attempted to explore the optimization of the culture medium using mixed carbon sources, such as methanol combined with sorbitol or glycerol. Generally, as the cell density increases in the expression system, the protein expression level also correspondingly rises. However, higher cell densities, along with dissolved oxygen levels, nutrient composition, and the metabolic by-products of yeast cells, can affect the yield and stability of the target protein. Mao investigated the optimal fermentation conditions for the rFIP-glu engineered strain and measured its yield in both shake flasks and 5 L fermentation tanks [21]. The results showed that the yield of rFIP-glu was

368.71 μg·mL^{-1} in shake flasks and 613.47 μg·mL^{-1} in the 5 L fermentation tank, indicates that the yield varied with changes in the fermentation environment and scale. Wu induced mutations in the rFIP-glu engineering strain using ultraviolet radiation and successfully increased the yield of rFIP-glu from an initial 242 μg·mL^{-1} to 469 μg·mL^{-1} through high-throughput screening [22]. Therefore, there is still a substantial amount of work needed to further improve production efficiency and reduce costs for FIP-gle2.

Conclusions

In the current study, the conditions of liquid fermentation of an engineered *P. pastoris* strain were successfully optimized using single-factor test and RSM. These optimizations not only enhanced the expression levels of rFIP-gle2 but also provided crucial data support for the transition from laboratory-scale to industrial-scale production. Although challenges remain in the transition from laboratory to industrial production, the findings of this study lay a solid foundation for the large-scale production of rFIP-gle2 and the future development of FIP-gle products. Furthermore, our work offers a reference for the optimization of fermentation processes for other recombinant proteins, particularly within the *P. pastoris* expression system. Future work will focus on addressing these challenges to achieve efficient and stable production of rFIP-gle2 and to further explore its potential applications in immunomodulation and related fields.

Declarations

Conflict of interest

The authors declare no competing interests.

Acknowledgment

This work was supported by the NMPA Key Laboratory for Quality Control of Traditional Chinese Medicine, Tibetan Medicine (XZ202401YD0020).

References

1. Li TH, Hu HP, Deng WQ, Wu SH, Wang DM, Tsering T. *Ganoderma leucocontextum*, a new member of the *G. lucidum* complex from southwestern China. *Mycoscience*. 2015;56:81-85.
2. Liu S, Liu C, Li J, et al. Research progress on artificial cultivation and active components of *Ganoderma leucocontextum*. *Edible Fungi of China*. 2020;39:1-4, 16. (In Chinese)
3. Li Q, Wang X, Zhou X. Recent status and prospects of the fungal immunomodulatory protein family. *Crit Rev Biotechnol*. 2011;31:365-375.
4. Wang XF, Su KQ, Bao TW, et al. Immunomodulatory effects of fungal proteins. *Curr Top Nutraceutical Res*. 2012;10.
5. Li QZ, Zheng YZ, Zhou XW. Fungal immunomodulatory proteins: characteristic, potential antitumor activities and their molecular mechanisms. *Drug Discov Today*. 2019;24:307-314.
6. Dong W, Han J, Zhang, Liu H. Research progress on chemical constituents and pharmacological effects of *Ganoderma leucocontextum*. *J Fungal Res*. 2023;21:170-177. (In Chinese)
7. Hsu HY, Hua KF, Wu WC, et al. Reishi immuno-modulation protein induces interleukin-2 expression via protein kinase-dependent signaling pathways within human T cells. *J Cell Physiol*. 2008;215:15-26.
8. Lin TY, Hua WJ, Yeh H, Tseng AJ. Functional proteomic analysis reveals that fungal immunomodulatory protein reduced expressions of heat shock proteins correlates to apoptosis in lung cancer cells. *Phytomedicine*. 2021;80:153384.
9. Chang YC, Hsiao YM, Wu MF, et al. Interruption of lung cancer cell migration and proliferation by fungal immunomodulatory protein FIP-fve from *Flammulina velutipes*. *J Agric Food Chem*. 2013;61:12044-12052.
10. Liao CH, Hsiao YM, Sheu GT, et al. Nuclear translocation of telomerase reverse transcriptase and calcium signaling in repression of telomerase activity in human lung cancer cells by fungal immunomodulatory protein from *Ganoderma tsugae*. *Biochem Pharmacol*. 2007;74:1541-1554.
11. Liu Y, Huang L, Hu H, et al. Whole-genome assembly of *Ganoderma leucocontextum* (Ganodermataceae, Fungi) discovered from the Tibetan Plateau of China. *G3 (Bethesda)*. 2021;11:jkab337.
12. Cregg JP. *Pichia Protocols*. 2nd ed. Humana Press; 2007.
13. Türkanoğlu Özçelik A, Yılmaz S, Inan M. *Pichia pastoris* Promoters. *Methods Mol Biol*. 2019;1923:97-112.
14. Chen WH, Chag SM, Poongavanam MV, et al. Optimization of the production process and characterization of the yeast-expressed SARS-CoV recombinant receptor-binding domain (RBD219-N1), a SARS vaccine candidate. *J Pharm Sci*. 2017;106:1961-1970.
15. Webb RP, Smith TJ, Smith LA, et al. Recombinant botulinum neurotoxin Hc subunit (BoNT Hc) and catalytically inactive *Clostridium botulinum* holoproteins (ciBoNT HPs) as vaccine candidates for the prevention of botulism. *Toxins (Basel)*. 2017;9:269.
16. Li QZ, Chang YZ, He ZM, Chen L, Zhou XW. Immunomodulatory activity of *Ganoderma lucidum* immunomodulatory protein via PI3K/Akt and MAPK signaling pathways in RAW264.7 cells. *J Cell Physiol*. 2019;234:23337-23348.
17. Ejike UC, Chan CJ, Okechukwu PN, Lim RLH. New advances and potentials of fungal immunomodulatory proteins for therapeutic purposes. *Crit Rev Biotechnol*. 2020;40:1172-1190.
18. Pan Y, Yang J, Wu J, Yang L, Fang H. Current advances of *Pichia pastoris* as cell factories for production of recombinant proteins. *Front Microbiol*. 2022;13:1059777.
19. Karbalaei M, Rezaee SA, Farsiani H. *Pichia pastoris*: A highly successful expression system for optimal synthesis of heterologous proteins. *J Cell Physiol*. 2020;235:5867-5881.
20. Zhang W, Hywood Potter KJ, Plantz BA, et al. *Pichia pastoris* fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement. *J Ind Microbiol Biotechnol*. 2003;30:210-215.
21. Mao PW, Li LD, Wang YL, Bai XH, Zhou XW. Optimization of the fermentation parameters for the production of *Ganoderma lucidum* immunomodulatory protein by *Pichia pastoris*. *Prep Biochem Biotechnol*. 2020;50:357-364.
22. Wu Q, Zhang R, Jin M, et al. Screening of engineered *Pichia pastoris* mutant with enhanced production of a functional rFIP-glu protein and investigating its potential bioactivities. *Lett Appl Microbiol*. 2021;73:770-778.