High-yield production of the human lysozyme by *Pichia pastoris* SMD1168 using response surface methodology and high-cell-density fermentation

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ABSTRACT

Background: Lysozyme plays a crucial role in innate immunity with its well-recognized bacteriolytic activity. In this study, the influence of expression parameters (inoculation volume, culture volume, growth time, induction temperature and time, initial pH and methanol concentration) on human lysozyme (HLZ) production in recombinant *P. pastoris* SMD1168 was investigated through Plackett–Burman (PB) design and response surface methodology (RSM).
Results: It was revealed that induction temperature, induction time and culture volume had significant influence (P < 0.01) on HLZ expression level, which were elected for further optimization with three-dimensional response surface designs for enhanced HLZ production. The highest lysozyme activity reached 3301 U/mL under optimized conditions (at 23.5°C for 90 h with culture volume of 48 mL) in shake flask, which increased 2.2 fold compared with that achieved with the standard protocol (Invitrogen). When high-cell-density fermentation of the recombinant *Pichia pastoris* was performed in a 15 L fermenter under optimized conditions, the extracellular lysozyme activity reached 47,680 U/mL. SDS-PAGE analysis of the product demonstrated that HLZ was produced as a single major protein with a molecular weight of approximately 14.7 kDa, consistent with its expected size.
Conclusions: The results indicated that the optimized culture conditions using PB design and RSM significantly enhanced the expression level of HLZ, and the Pichia expression system for HLZ production was successful and industrially promising.

1. Introduction

Traditional antibiotics are facing escalating challenges as the emergence of multidrug-resistant bacteria. Lysozyme has gained much attention as an antimicrobial agent due to its ability to cleave the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan of bacterial cell wall, thus damaging cell wall and causing bacterial cell lysis [1]. Lysozyme plays an important role in nonspecific immunity and has a direct effect on gram-positive bacteria [2]. The human lysozyme (HLZ) gene is located on chromosome 12 and is widely expressed in a variety of tissues and body fluids [3,4]. In addition to its well-characterized antimicrobial activity, HLZ has exhibited other biological functions, including anti-inflammatory [5], anti-HIV [6], anti-tumor [7], anti-proliferative [8] and immunoregulation [9] activities. As a human-derived protein, HLZ is considered safer than bactericides or antibiotics from other species in clinical applications.

Pichia pastoris has emerged as one of the most common and effective recombinant protein production systems in molecular biology for producing both secreted and intracellular proteins [10,11]. Up to now, more than 500 heterologous proteins have been expressed in *P. pastoris* [12]. With its powerful inducible alcohol oxidase 1 (AOX1) promoter, *P. pastoris* can use methanol as the sole carbon source and inducer for recombinant proteins in high cell-density fermentation [13]. As a eukaryotic single-cell-factory, *P. pastoris* is capable of post-translational modifications such as proteolytic processing, folding, disulfide bridge formation, and glycosylation. Thus, many proteins expressed in prokaryotic system as inactive forms can be produced with biological activity in *P. pastoris*. Other advantages of *P. pastoris* include fast growth in a wide temperature ranging from 15°C to 30°C, and a relatively broad pH tolerance from 3.0 to 7.0 [12,14].

In our previous studies, a synthetic codon-optimized HLZ cDNA was inserted into expression plasmid pPIC9K in frame with the

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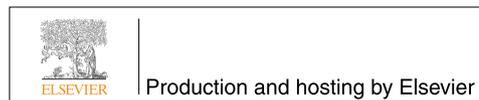
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Saccharomyces cerevisiae pre-pro α-mating factor secretion signal sequence. The HLZ sequence was under the control of AOX1 promoter, and the expression of bioactive HLZ in recombinant *P. pastoris* SMD1168 was successful. In this article, the influences of environmental parameters on HLZ production were evaluated employing PB design and RSM. When high cell-density fed-batch fermentation was performed in a 15 L fermenter under optimized conditions, the production of HLZ was improved to 47,680 U/mL, which was much higher than that achieved under unoptimized conditions.

2. Materials and methods

2.1. Strain and plasmid

P. pastoris SMD1168 (Mut^{S}his4$^-$) was purchased from Invitrogen (Carlsbad, CA, United States). Recombinant plasmid (pPIC9K-pt-LYC6) for secreted expression of HLZ in *P. pastoris* was constructed by our laboratory previously. HLZ cDNA sequence was optimized according to the codon preference of *P. pastoris* to enhance the expression level, and the recombinant plasmid was transformed into SMD1168 and integrated into the genome.

2.2. Culture media

Preserved recombinant *P. pastoris* strain was recovered and cultured in YPD (10.0 g/L yeast extract, 20.0 g/L peptone and 20.0 g/L glucose. For plate, 20.0 g/L agar was added to the medium). The BMGY (10.0 g/L yeast extract, 20.0 g/L peptone, 0.1 M potassium phosphate, pH 6.0, 13.4 g/L YNB, 4×10^{-4} g/L biotin, 1% glycerol) and BMMY (BMMY medium with 0.5% methanol instead of glycerol) media were used for cultivation and expression of HLZ, respectively, in shake flask. The basal salt medium (0.93 g/L CaSO$_4$, 18.2 g/L K$_2$SO$_4$, 14.9 g/L MgSO$_4 \times$ 7H$_2$O, 4.13 g/L KOH, 26.7 mL/L H$_3$PO$_4$, 40.0 g/L glycerol, and 4.0 mL/L PTM1 (6 g/L CuSO$_4 \times$ 5H$_2$O, 0.08 g/L NaI, 2 g/L MnSO$_4 \times$ H$_2$O, 20.2 g/L Na$_2$MoO$_4$, 0.2 g/L H$_3$BO$_3$, 20 g/L CoCl$_2$, 65 g/L FeSO$_4 \times$ 7H$_2$O, 0.2 g/L biotin, and 0.5 g/L H$_2$SO$_4$)) was used for fermentation.

2.3. Shake flask culture

Preserved recombinant strains stored at -80°C were streaked onto YPD plates for recovery and isolation of single colonies. A single colony was inoculated in 5 mL YPD and incubated overnight at 30°C, 220 rpm. The seed culture was transferred to 50 mL BMGY and was grown for 24 h under the same condition as above. The expression of HLZ was induced by resuspending the cells in the same volume of BMMY media after centrifugation, and 100% methanol was added to a final concentration of 1% (v/v) every 24 h to maintain induction.

2.4. High cell-density fed-batch fermentation

The fermentation was carried out in a 15 L bioreactor (NC-Bio, Shanghai, China) under the following conditions: medium volume, 10 L; inoculation volume, 10% (v/v); aeration rate, 1.0–3.0 vvm; agitation speed, 200–600 rpm; dissolved oxygen (DO), 20%. Growth and induction temperature were maintained at 30°C and 23.5°C, respectively. Growth pH was controlled at 5.5, and induction pH values were set at 4.5, 5.0, and 5.5 for three parallel experiments to determine the optimal induction pH. Samples were taken periodically at 8 h intervals, and the induction period lasted for 96 h. The fermenter was equipped with oxygen and pH electrodes to monitor and control DO and pH by adjusting aeration/agitation and addition of NH$_4$OH (25%, w/v), respectively. Residual ethanol concentration was determined by gas chromatography.

2.5. Analytical methods

Lysozyme activity was determined by measuring the decrease of absorbance at 450 nm described by Olmo et al. [15]. 50 μL sample solution was added into 2.95 mL of *Micrococcus lysodeikticus* cell suspension (OD 450 approximately 0.7) in 0.05 M potassium phosphate buffer (pH = 7.0). One unit of lysozyme activity corresponds to an absorbance decrease of 0.001 at 450 nm/min. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% gel according to Laemmli [16].

2.6. Experimental design and statistical analysis

2.6.1. Plackett–Burman (PB) design

The experiments were designed by Design-Expert.8.05b software (Stat Ease Inc., Minneapolis, Minn., USA). The effects of seven factors (inoculation volume, culture volume, growth time, induction temperature, induction time, initial pH and methanol concentration) on HLZ production were investigated using PB design [17]. PB is a kind of two-level experimental design method, applicable for quickly and efficiently screening for the significant factors from many using the least experiments [18]. The high and low levels of the investigated variables are given in Table 1.

2.6.2. Box–Behnken design (BBD) and response surface methodology

Significant factors were chosen for further optimization by RSM based on the results of PB design. BBD and RSM are the combination of mathematical and statistical methods exploring the relationships between several explanatory variables and one or more response variables, normally using a second-degree polynomial model to do this. The ultimate goal is to optimize the response values through modeling and analysis of multiple independent variables affecting the response [19]. The mathematical model is as follows [Equation 1]:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2. \qquad \text{[Equation 1]}$$

Y is the predicted response value, β_0 is the constant term, β_i is the factor influence coefficient, β_{ij} is the interactive response coefficient between factors, and β_{ii} is the factors of the second order influence coefficient. X_i and X_j are the levels of the independent variables.

3. Results

3.1. Screening the major factors for HLZ expression with PB design

A 7-factor and a 2-level method were used in PB design, and the high levels of the variables were 25% higher than the low levels. Table 2 showed the experimental design using Design Expert software and the results. All the experiments were carried out in 500 mL shake flasks, triplicates from three independent experiments were measured and means were calculated. The significant factors were determined and the analysis of variance was performed (Table 3).

Table 1
Factors and levels of PB design.

Coded	Factors	Levels	
		-1	+1
X1	Inoculation volume (%)	5	6.25
X2	Culture volume (mL)	40	50
X3	Growth time (h)	24	30
X4	Induction temperature (°C)	24	30
X5	Induction time (h)	72	90
X6	Initial pH	5	6.25
X7	Methanol concentration (%)	1	1.25

Table 2
Experimental design and results of PB.

Run	Factors							Lysozyme activity Y (U/mL)
	X1	X2	X3	X4	X5	X6	X7	
1	-1	1	1	-1	1	-1	1	2631
2	1	1	-1	1	1	1	-1	2039
3	-1	-1	-1	-1	-1	-1	-1	3066
4	1	1	1	-1	-1	1	1	2095
5	-1	-1	-1	1	-1	1	1	1808
6	1	-1	-1	-1	1	-1	1	2979
7	1	-1	1	1	1	-1	-1	2279
8	1	-1	1	1	-1	1	1	1519
9	-1	1	-1	1	1	1	1	2172
10	-1	-1	1	-1	1	1	-1	3229
11	-1	1	1	1	-1	-1	-1	1630
12	1	1	-1	-1	-1	-1	-1	2431

The values of "Prob > F" (P-value) indicated the significant levels of the model and the factors. The influences of seven investigated factors on HLZ production were ranked as: X4 > X5 > X2 > X7 > X1 > X3 > X6. X2, X4 and X5 (induction temperature, induction time and culture volume) had significant effects ($P < 0.01$) on HLZ production in tested range and were chosen for further optimization.

3.2. Optimization of the significant factors using RSM

As revealed by the PB experiments, induction temperature, induction time and culture volume, which were encoded as A, B, and C, had significant influence on the response values. Subsequently, BBD was used to investigate the interactions and the optimal levels of the three factors. The mathematical equation was shown below in [Equation 2]:

$$Y = 3194 + 228.56A + 158.56 B + 175.75C$$
$$+ 368.13AB - 309.25 AC \qquad \text{[Equation 2]}$$
$$+ 420.25BC - 677.19A^2 - 555.19B^2 - 216.06C^2.$$

Y was the predicted lysozyme activity, A, B and C were coded values for induction temperature, induction time and culture volume, respectively. Each factor had three coded levels (1, 0, -1, see Table 4) and a total of 17 runs were performed.

Analysis of variance (ANOVA) was carried out to verify the significant level of the parameters and the regression model, and the results were shown in Table 5. The Model F-value of 80.58 implied that the model was significant. There was only a 0.01% chance that a "Model F-value" this large could occur due to noise. The value of "Prob > F" less than 0.05 indicates that the model term is significant. In this case, A, B, C, AB, AC, BC, A^2, B^2, and C^2 were significant terms. Simulation loss curve (lack of Fit) indicates the probability that model prediction does not fit the actual value. The "lack of Fit F-value" of 0.51 implied that the lack of Fit was not significant compared to the pure error. There was a 69.90% chance that a "lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. Coefficient of variation (CV) reflects the confidence of the model, and the lower the CV value is, the higher the confidence of the model will be [20]. The CV value of 3.69% indicated that the model equation could reflect the real experimental

Table 3
Analysis of the results of PB design.

Factor	F value	P value (Prob > F)	Significant
X1	9.93	0.0345	5
X2	24.66	0.0077	3
X3	8.61	0.0426	6
X4	151.26	0.0003	1
X5	53.81	0.0018	2
X6	6.37E-05	0.9940	7
X7	13.14	0.0222	4

Table 4
BBD and RSM model predicted values of lysozyme activity.

Run	Factors			Lysozyme activity Y (U/mL)	
	A: Induction temperature (°C)	B: Induction time (h)	C: Culture volume (mL)	Actual value	Predicted value
1	23 (0)	84 (0)	40 (0)	3021	3194
2	26 (1)	72 (-1)	40 (0)	1665	1664
3	23 (0)	84 (0)	40 (0)	3194	3194
4	23 (0)	84 (0)	40 (0)	3227	3194
5	26 (1)	96 (1)	40 (0)	2716	2716
6	26 (1)	84 (0)	30 (-1)	2708	2663
7	20 (-1)	72 (-1)	40 (0)	1944	1943
8	23 (0)	84 (0)	40 (0)	2540	2457
9	23 (0)	72 (-1)	50 (1)	3302	3194
10	20 (-1)	84 (0)	50 (1)	2512	2557
11	23 (0)	72 (-1)	30 (-1)	2462	2509
12	23 (0)	84 (0)	40 (0)	3227	3194
13	26 (1)	84 (0)	50 (1)	2350	2396
14	23 (0)	96 (1)	30 (-1)	1941	1985
15	20 (-1)	96 (1)	40 (0)	1522	1524
16	20 (-1)	84 (0)	30 (-1)	1633	1587
17	23 (0)	96 (1)	50 (1)	3224	3177

value well. The correlation coefficient R^2 of the model was 98.09% ($R^2 > 90\%$), indicating that the predicted and actual values fitted well. Thus, this model can be applied to predict HLZ production effectively.

In order to gain a better understanding of the effects of the variables on HLZ production, the 2D contour plots and the 3D response surface were constructed (Fig. 1, Fig. 2 and Fig. 3). The contour map reflects the cross-interaction between two variables by keeping the other variable at constant and middle level. An elliptical contour plot (as in Fig. 1a, Fig. 2a and Fig. 3a) implies a significant interaction between variables. All of the three contour plots exhibit similar relationships with respect to the influence of each variable. The statistically optimal values of variables were achieved by moving along the major and minor axes of the contour. The highest predicted value is obtained in the smallest ellipse in the contour diagram. The optimal solution was found to be induction temperature 23.36°C, induction time 89.76 h and culture volume 47.89 mL for a predicted response of 3315 U/mL. Taking into account the actual situation, induction temperature 23.5°C, induction time 90 h and culture volume 48 mL were employed. Under the optimized expression parameters, the highest lysozyme activity production reached 3301 U/mL.

3.3. Cultivation in a 15 L bioreactor under the optimal conditions

To confirm the model feasibility for predicting maximum HLZ production, fermentation was carried out in a 15 L bioreactor under the

Table 5
ANOVA for response surface quadratic model.

Source	Sum of squares	df	Mean square	F value	P value (Prob > F)
Model	6.25E+06	9	6.94E+05	80.58	<0.0001
A	4.18E+05	1	4.18E+05	48.53	0.0002
B	2.01E+05	1	2.01E+05	23.36	0.0019
C	2.47E+05	1	2.47E+05	28.70	0.0011
AB	5.42E+05	1	5.42E+05	62.95	<0.0001
AC	3.83E+05	1	3.83E+05	44.42	0.0003
BC	7.06E+05	1	7.06E+05	82.04	<0.0001
A2	1.93E+06	1	1.93E+06	224.22	<0.0001
B2	1.30E+06	1	1.30E+06	150.71	<0.0001
C3	1.97E+05	1	1.97E+05	22.83	0.0020
Residual	60,279.56	7	8611.37	0.51	0.6990
Lack of fit	16,574.06	3	5524.69		
Pure error	43,705.5	4	10,926.38		
Cor total	6.31E+06	16			

$R = 0.9904$, $R^2 = 0.9809$, R^2 (adjust) $= 0.9567$, R^2 (predicted) $= 0.8970$, coefficient of variation (CV) $= 3.69\%$.
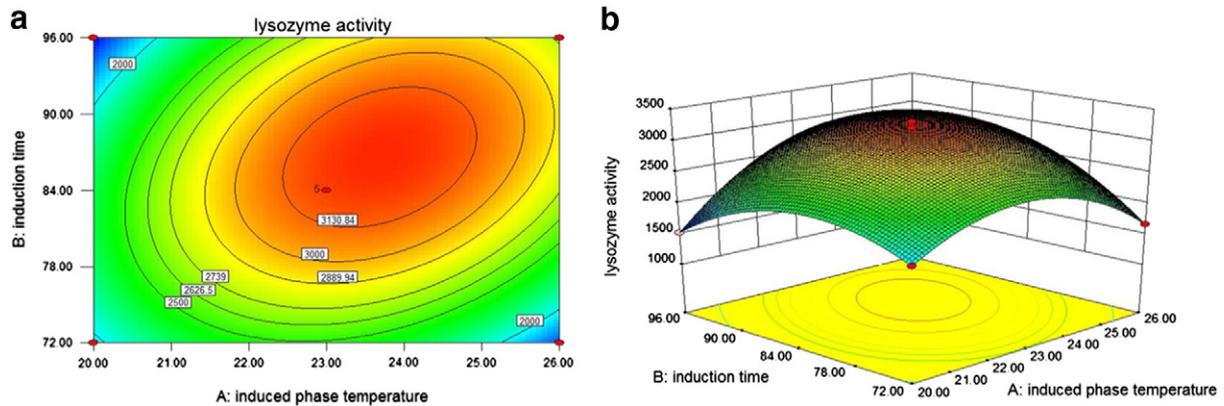
Fig. 1. 2D contour plot and 3D response surface for the effect of cross-interaction between A (induction phase temperature) and B (induction time) on the lysozyme activity.
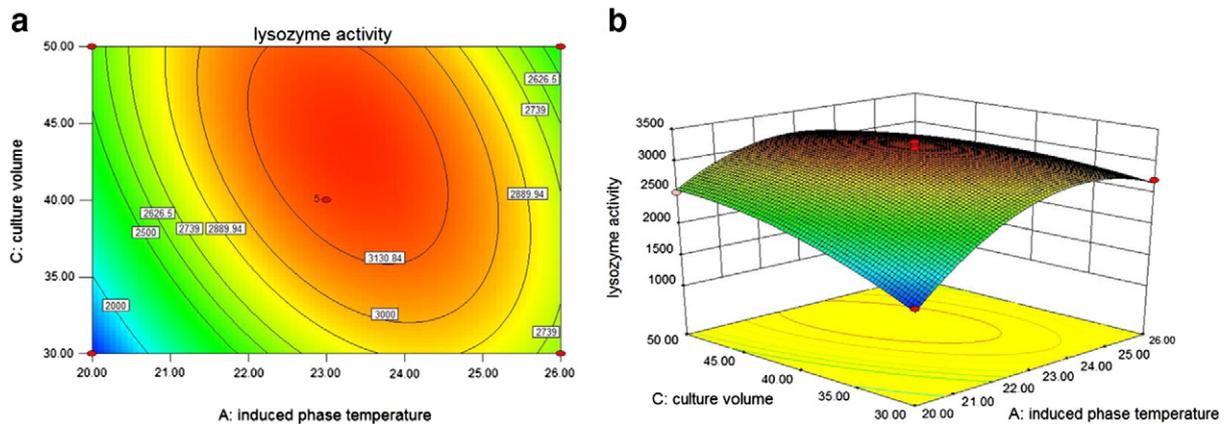

Fig. 2. 2D contour plot and 3D response surface for the effect of cross-interaction between A (induction phase temperature) and C (culture volume) on the lysozyme activity.

optimal conditions determined by RSM. In addition, three parallel experiments were carried out to investigate the influence of pH on HLZ production, with pH controlled at 4.5, 5.0, and 5.5, respectively. The time-course profiles of HLZ production in fermentation were shown in Fig. 4, resulting in the maximum HLZ production of 47,680 U/mL at 88 h of induction under pH 4.5. SDS-PAGE analysis for the samples of the fermentation supernatants revealed the expression of HLZ, shown as a single predominant protein band of approximately 14.7 kDa (Fig. 5, lanes 3–6), consistent with its expected size and the control (Fig. 5, lane 2).

4. Discussion

A notable problem in high-cell-density fermentation is the degradation of recombinant proteins due to release of proteases from host cells. *P. pastoris* SMD1168 is a protease-deficient strain, which could reduce the degree of potential proteolysis [21].

Wei et al. [22] performed a 'one-factor-at-a-time' method study in which the expressed HLZ activity was up to 533 U/mL by *P. pastoris* GS115. However, the 'one-factor-at-a-time' strategy is unable to reveal the interaction between multiple factors. Ercan et al. [23] used a three-factor BBD of response surface method in terms of temperature, pH and aeration levels to find the optimum combination of growth parameters of *Kluyveromyces lactis* K7 for human lysozyme production in biofilm reactor and the highest lysozyme production was 141 U/mL.

By evaluating the influence of fermentation parameters on HLZ production using PB design, we found that induction temperature, induction time and culture volume were highly significant factors ($P < 0.01$). Then a series of experiments and RSM analysis concerning
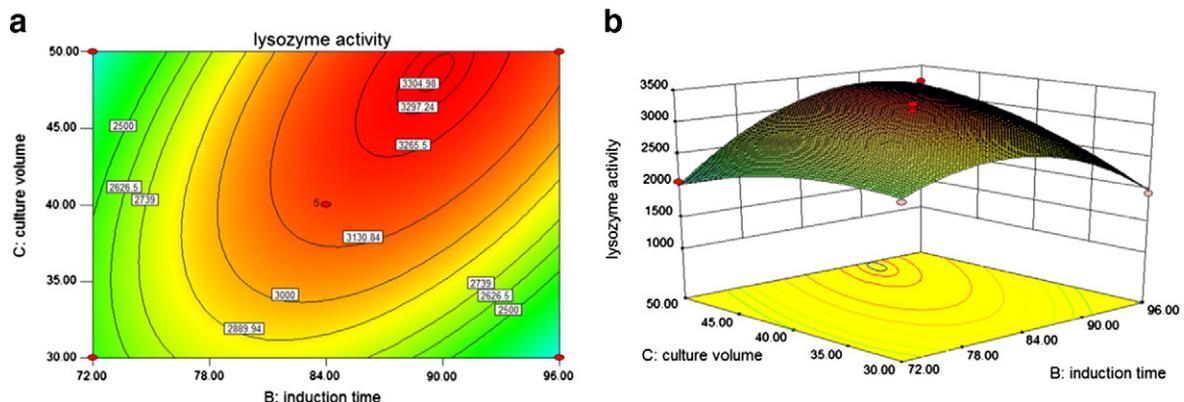

Fig. 3. 2D contour plot and 3D response surface for the effect of cross-interaction between B (induction time) and C (culture volume) on the lysozyme activity.
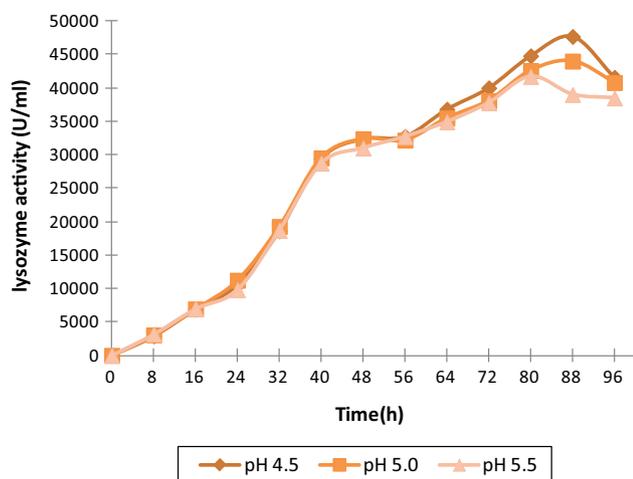
Fig. 4. Time-course profile of lysozyme production with different pH during fed batch fermentation.

these factors were performed. The highest lysozyme activity obtained under optimized conditions (at 23.5°C for 90 h with culture volume of 48 mL in 500 mL shake flask) was 3301 U/mL, which was 2.2 fold higher than that under standard conditions (1515 U/mL) and was in good accordance with the predicted value (3315 U/mL) by RSM.

Higher temperature is generally beneficial to the growth and metabolism of *Pichia* and protein expression. However, over temperature can lead to premature aging of the host strain and shorten the fermentation period thus reducing heterologous protein production. The optimal temperature for *Pichia* growth is 30°C and employed induction temperature range from 20°C to 28°C. It has been reported that lower temperature was beneficial to extracellular expression, as it is advantageous to the correct processing and secretion of recombinant proteins [24,25]. The induction period also plays a vital role in the production of recombinant proteins for expression level increase over time during induction, while the products may suffer severe degradation under some circumstances if the induction period is overdue. Therefore, it is necessary to determine the appropriate temperature and induction time during expression, and a temperature-changing fermentation strategy was adopted in this study. Huang et al. [26] used *K. lactis* K7 as the host strain to produce recombinant human lysozyme, and it was demonstrated that 25°C with no pH control (initial pH 6.0) resulted in better production (64.1 U/mL) than with pH controlled near neutrality.

Growth of *Pichia* and expression of heterologous proteins are oxygen-consuming processes, so the DO is an important factor during the production of HLZ. In shake flask, oxygen supply is enhanced by
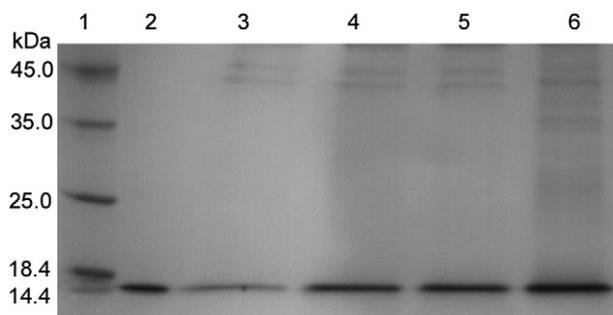
increasing shaking speed or reducing culture volume. However, in this study, lysozyme activity was not increased as the reduction of culture volume. It could be that the methanol evaporated quickly as the culture volume went to low, leaving the culture lack of carbon source and inducer. In our study, the optimal culture volume was approximately 10% of the shake flask volume, which is in good accordance with a previous report [27].

The results of batch-fermentation experiments in a 15 L fermenter demonstrated that the optimized expression conditions could also improve HLZ production during high-cell-density fermentation, and the induction pH value of 4.5 was more preferable for HLZ expression than a pH of 5.0 or 5.5 (Fig. 4). In addition, the relatively low temperature (23.5°C) and acidic condition (pH 4.5) employed in this study could help to limit the protease activity, according to previous studies [28,29].

To the best of our knowledge, this is the first study concerning the optimization of process parameters for HLZ production in *Pichia* SMD1168 combining PB design and RSM, and the extracellular lysozyme activity (47,680 U/mL) was the highest by far. The high-yield production of HLZ using fed-batch fermentation under optimized conditions was successful and industrially promising, which could shed some light on the scale-up production of HLZ with higher activity in a cost-effective manner and provide enough quantity of protein for future studies and applications.

Author contributions

Proposed the theoretical frame: YY, XZ, LY; Conceived and designed the experiments: YY, XZ; Contributed reagents/materials/analysis tools: SW; Wrote the paper: YY, XZ; Performed the experiments: YY, XZ, TW; Analyzed the data: XZ.

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Fig. 5. SDS-PAGE analysis of the culture supernatants of the recombinant *P. pastoris*. Lane 1, protein marker; lane 2, positive control: HLZ standard (Sigma-Aldrich Trading Co., Ltd., Shanghai, China); lane 3, HLZ expressed under optimized conditions in flask; lines 4–6, HLZ production in 15 L fermenter under induction pH of 5.5, 5.0 and 4.5, respectively.

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