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Expression of *HpaI* in *Pichia pastoris* and optimization of conditions for the heparinase I production

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ABSTRACT

Heparinase I has important applications in the fields of biomedicine and pharmaceuticals. The heparinase I gene (*HpaI*) from *Flavobacterium heparinum* was cloned and overexpressed in *Pichia pastoris* GS115, and the conditions for the heparinase I production were optimized by RSM. PCR analysis indicated that *HpaI* was integrated into the *P. pastoris* GS115 genome. The concentrations of key factors that affected the heparinase I activity were optimized, and were as follows: oleic acid, 0.07%, liquid volume in flask, 34.3 ml/L, and methanol, 0.96%. Under the optimal conditions, the activity of heparinase I was up to 323 U/L in shake flask. A maximal heparinase I activity of 398.5 U/L from the transformant 2 was achieved in a 5 L fermentor. This study demonstrates the overproduction of heparinase I by recombinant *P. pastoris*.

1. Introduction

Heparinase I is a polysaccharide lyase that can degrade heparin and heparin sulfate (Björk & Lindahl, 1982; Lindahl, 2000; Linker & Hovingh, 1972; Michelsen et al., 1996). A large number of studies have been made since heparinase I was discovered from *Flavobacterium heparinum* (Sasisekharan, Bulmer, Moremen, Cooney, & Langer, 1993; Sasisekharan, Moses, Nugent, Cooney, & Langer, 1994; Yang, Linhardt, Bernstein, Cooney, & Langer, 1985). Many reports have shown that heparinase I has important biofunctions, including the detection and removal of heparin (Ammar & Fisher, 1997; Langer et al., 1982), the regional heparinization of blood (Blaufox, Hampers, & Merrill, 1966; Desjardins & Duling, 1990; Kaplan & Petrillo, 1987), and the inhibition of the invasion and metastasis of tumor cells (Nakajima, Irimura, & Nicolson, 1988; Okada et al., 2002). Heparinase I is involved in the inhibition of the tumor angiogenesis (Ferrara, 2004; Sasisekharan, Moses, Nugent, Cooney, & Langer, 1996; Toi, Inada, Suzuki, & Tominaga, 1995), and so becomes a promising target of the anti-tumor therapy (Ernst et al., 1997; Zhang, Tang, Gao, Fang, & Yang, 2011). Moreover, it can cleave the heparin chain to produce low-molecular-weight heparins (LMWHs) that can retain the anticoagulant property of the unfractionated heparin, and that have not side effect on the human body (Laurent et al., 2002). Currently, heparinase I has been prepared from a few microorganisms, such as *F. heparinum*, *Bacteroides stercoris* and *Bacillus circulans* (Gesner & Jenkin, 1961; Sasisekharan, Moses, et al., 1996; Sasisekharan, Vetetraman, et al., 1996; Yoshida et al., 2002). *F. heparinum* is a major commercial producer of heparinase I (Galliher, Cooney, Langer, & Linhardt, 1981; Zimmerman & Cooney, 1992). To date, however, heparinase I has been expensive due to its low yield.

An efficient method for improving the enzymatic production is the use of the recombinant DNA technology. In the past twenty years, a large number of enzyme-encoding genes have been cloned and expressed by this technology (Chen, Shen, & Wu, 2009; Yamabhai et al., 2008; Yu & Li, 2008). *Pichia pastoris* has been widely used for the industrial production of various heterologous proteins (Cereghino & Cregg, 2000; Macauley-Patrick, Fazenda, McNeil, & Harvey, 2005; Yu & Tang, 2012). This strain has an enormously potential for the high production of heterologous proteins, the efficient secretion of expressed products and the easy culture to a high cell density (Cereghino & Cregg, 2000; Yu & Tang, 2012). Thus, it is interesting to investigate if the heparinase I from *F. heparinum* can be overproduced by *P. pastoris* GS115.

In the present study, the heparinase I gene (*HpaI*) from *F. heparinum* was cloned and overexpressed in *P. pastoris* GS115. The transformant 2 that could effectively express *HpaI* was screened and identified. The conditions for the heparinase I production were optimized by response surface methodology at the shake flask level. Production profiles of heparinase I by recombinant *P. pastoris* in a

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5 L fermentor were also investigated in detail. To our knowledge, this is the first report regarding the overproduction of heparinase I by recombinant *P. pastoris*.

2. Materials and methods

2.1. Plasmids, strains and reagents

Escherichia coli DH5α was used as the cloning host, and was grown in a Luria-Broth medium supplied with 50 μg/mL kanamycin. *F. heparinum* DSM 2366 was purchased from the Germany Collection of Microorganisms and Cell Cultures. *P. pastoris* GS115 and the plasmid pPIC9K were purchased from the Invitrogen Co. Ltd., USA. PCR reagents and restriction endonucleases were purchased from the TaKaRa Biotech Co. Ltd., Japan. G418, sodium heparin and azure A were purchased from the Sigma, Co. Ltd, USA. The High Pure Plasmid Isolation Kit, BMMY and BMGY media were purchased from the Invitrogen Life Technology Corporation, USA. Biotin, Tween-80, oleic acid, yeast nitrogen base (YNB) and PTM1 were purchased from the Sangon Bioengineering Co. Ltd, Shanghai, China. All other reagents used in the experiments were of analytical grade, and were used as the routine method.

2.2. Construction of the expression plasmid and P. pastoris transformation

The degenerated primer set, F_1: 5′-GCTGAATTCAAAAAAC-AAATTCTATATC-3′ and F_2: 5′-A TTCGCGGCCGCCTATCTGGC-AGTTTCGC-3′, was designed to amplify *HpaI* from *F. heparinum* DSM 2366 based on the reported sequence of *HpaI* from EMBL (accession number: L12534.1). Restriction sites *Eco*RI and *Not*I were respectively added to the upstream of the primer F_1 and the downstream of the primer R_1 for the cloning purpose. PCR conditions consisted of an initial denaturation at 94 °C for 5 min, and 35 cycles of the amplification consisted of the denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min, and extension at 72 °C for 1 min. Then the further extension at 72 °C was performed for 10 min. PCR products were purified and digested with *Eco*RI and *Not*I, and were ligated into the *Eco*RI–*Not*I digested plasmid pPIC9K to construct the recombinant plasmid pPIC9K-HpaI. This plasmid was then transformed into *E. coli* DH5α by $CaCl_2$-heat shock (Sambrook, Fritsch, & Maniatis, 1989). The recombinant plasmid was isolated from the positive transformant using the High Pure Plasmid Isolation Kit. The presence and correct orientation of the insert sequence was confirmed by PCR and DNA sequencing.

The plasmid pPIC9K-HpaI was linearized with *Bpu*1102I for the transformation into *P. pastoris* GS115. This process resulted in a stable integration of one or multiple copies of the linearized plasmid at 5′ AOX1 chromosomal locus of *P. pastoris* GS115 by homologous recombination. Competent *P. pastoris* GS115 cells were prepared by combining chemical transformation with electroporation. 1 μg of the linearized plasmid was mixed with competent GS115 cells. The mixture was immediately transferred to a pre-chilled electroporation cuvette, and was incubated on ice for 5 min. Electroporation was carried out on the following conditions: charging voltage 1.5 kV, capacitance 25 F and resistance 200 X. 1 mL of 1M ice-cold sorbitol was immediately added to the cuvette after electroporation. The transformed mixture was patched onto YPD plates containing different concentrations of G418 (0.50, 1.00, 2.00 and 4.00 mg/mL). YPD plates were incubated at 30 °C until the single colony appeared.

2.3. Screening and identification of P. pastoris transformant

Sixteen transformants with a faster growth rate on YPD plates containing the highest concentration of G418 were screened. Each transformant was inoculated into a 50 mL buffered minimal glycerol medium (BMGY, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 1% glycerol), and was grown at 30 °C overnight with vigorous agitation at 250 rpm. When the optical density (OD_{600}) reached 5.0, cells were harvested by centrifugation at $3000 \times g$ for 10 min. The cell pellet was resuspended in a BMMY medium (BMGY with 0.5% methanol instead of 1% glycerol) with a starting OD_{600} of 30. The culture was grown at 30 °C for 48 h. Methanol was added to a final concentration of 0.5% every 24 h. After the induction for 48 h, the sample was taken for the analysis of the heparinase I activity. The activity of heparinase I was determined as described by Galliher et al. (1981) with minor modification. The sample was centrifuged at $3000 \times g$ for 10 min to remove the cell pellet. 200 μL of the supernatant was mixed with 100 μL of the heparin solution (25 mg/mL in 0.25 M sodium acetate–0.0025 M calcium acetate at pH 7.0). The mixture was incubated at 36 °C in a water bath. At different time intervals, 10 μL of the mixture was taken and added to 10 mL of 0.02 mg/mL Azure A dye solution. The dye showed a metachromatic shift from blue to red in the presence of heparin. The change in the optical density at 620 nm was measured within 1 h. One unit of the heparinase I activity is defined as the amount of enzyme that can degrade 1 mg heparin per minute at 36 °C and pH 7.0. The transformant with the highest activity of heparinase I was further analyzed by PCR amplification and SDS-PAGE. Its genomic DNA was isolated according to the specification of the Multicopy *Pichia* Expression Kit. PCR amplification was carried out to confirm if *HpaI* was integrated into the genome of *P. pastoris* GS115 using the primer set F_1 and R_1. SDS-PAGE analysis was performed on a 15% running gel as described by Laemmli (1970). Resolved proteins were visualized by staining with Coomassie Brilliant Blue R250.

2.4. Plackett–Burman design

Seven factors, including Tween-80 (*A*, ml/L), methanol (*B*, ml/L), liquid volume in flask (*C*, ml/250 mL), PTM1 (*D*, ml/L), oleic acid (*E*, ml/L), inoculation (*F*, %) and YNB (*G*, %), are supposed to have an impact on the activity of heparinase I. It is known that the Plackett–Burman design can evaluate the main effect of factors. Factors having a significant effect on the activity of heparinase I, were identified by this design. The activity of heparinase I was given as a response value. Each factor was investigated at a high (+1) and a low (−1) level. Factors that were significant at 95% of the confidence level ($p < 0.05$) from the regression analysis were considered to have a significant effect on the activity of heparinase I. These factors were further optimized by response surface methodology with a Box–Behnken design. The first-order model that was used to fit the results of the Plackett–Burman design was represented as follows:

$$Y = \beta_0 + \sum \beta_i \chi_i \quad (1)$$

where Y is the predicted response; β_0 is the intercept; β_i is the linear coefficient and χ_i is the coded independent factor.

2.5. Box–Behnken design

The Box–Behnken design was used to optimize three most significant factors for further improving the activity of heparinase I. Each factor was studied at three different levels, and a set of 17

experiments was carried out. For statistical calculations, the coding of factors was done according to the following equation:

$$\chi_i = \frac{X_i - X_0}{\Delta X}, \quad i = 1, 2, 3, \ldots k \tag{2}$$

where χ_i is the coded value of an independent factor, X_i is the actual value of an independent factor, X_0 is the actual value of an independent factor at the center point, and ΔX is the step change. For predicting the optimal point, a second-order polynomial equation was fitted to correlate the relationship between factors and the response. The model equation used for the analysis was given below:

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ij} \chi_i \chi_j + \sum \beta_{ii} \chi_i^2, \quad i = 1, 2, 3, \ldots k \tag{3}$$

where Y is the predicted response, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the squared coefficient, and β_{ij} is the interaction coefficient. χ_i, and χ_j represent independent factors in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by determination coefficient R^2. Each design was performed in duplicate, and the mean value was given.

2.6. Validation of optimal conditions

To verify the optimal conditions obtained by response surface methodology, three experiments were performed to compare the actual value of the heparinase I activity with its predicted value obtained by model equation.

2.7. Fermentation of the recombinant 2 in a 5 L fermentor

A 5 L bioreactor pre-equipped with a dissolved oxygen electrode, a pH electrode and three peristaltic pumps was used for the fermentation of the recombinant 2 to produce heparinase I. The fermentative period included three stages: incubation, carbon starvation and induction. The recombinant 2 was inoculated into a BMGY medium, and was cultured at 30 °C for 24 h. 175 mL of the culture was transferred into a 5 L fermentor pre-equipped with a 2.5 L BMGY medium. The starting conditions were as follows: DO > 20% (controlled by the automatic control of agitation) and 30 °C. After the culture was incubated for 28 h, DO sharply increased to 90%, indicating that glycerol was used up. After the carbon starvation for 1 h, methanol feeding was initiated at a rate of 12 mL/h, and gradually increased to a final rate of 21 mL/h. The sample was taken once every 12 h for analyzing the activity of heparinase I (Galliher et al., 1981), the wet cell weight (Yu & Shen, 2012) and the total protein content.

2.8. Statistical analysis

Design expert, version 8.0 (Statease Inc., Minneapolis, USA) was used for experimental designs and the regression analysis of experimental data. The statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was statistically judged by determination coefficient R^2, and its statistical significance was determined by F-test.

3. Results and discussion

3.1. Screening and identification of P. pastoris transformant

Sixteen transformants with a faster growth rate on YPD plates containing the highest concentration of G418 (4.0 mg/mL) were screened. Their activities of heparinase I were determined, and the result is shown in Fig. 1a. Obviously, the transformant 2 had the highest activity of heparinase I (210.26 U/L). Based on the manual of the "multi-copy *P. pastoris* expression kit" from the Invitrogen, multiple integrated copies can result in an increase in the G418 resistance level from 0.5 mg/mL (1–2 copies) to 4 mg/mL (7–12 copies). Therefore, the copy number of *HpaI* in the transformant 2 was predicted to be 7–12. PCR amplification and SDS-PAGE were carried out to confirm the integration and expression of *HpaI* in *P. pastoris* GS115, and results are shown in Fig. 1b. PCR amplification showed that the transformant 2 had an expected band of 1080 bp in 1% agarose gel (Fig. 1b), but no band was found with the genome from the control strain GS115/pPIC9K as the template. This indicates that the linear construct pPIC9K-HpaI has been integrated into the genome of *P. pastoris* GS115.

3.2. Screening of the important factors by the Plackett–Burman design

The Plackett–Burman design offers an effective screening procedure, and evaluates the significance of a large number of factors in one experiment, which is time-saving and maintains the convincing information on each factor. Table 1 shows seven independent factors and their concentrations at different coded and uncoded levels, and the experimental responses for 16 runs. The activity of heparinase I varied widely as the concentrations of these factors varied in the medium. By applying the regression analysis on the experimental data, the corresponding first-order model equation fitted to the data obtained from the Plackett–Burman design was given as follows:

$$Y = 177.55 + 5.31A + 18.09B - 34.15C + 4.55D - 29.67E - 0.23F - 2.74G \tag{4}$$

Regression results are shown in Table 2. The model was significant ($p < 0.05$). The statistical analysis of the data showed that methanol, oleic acid and liquid volume in flask had a significant effect on the activity of heparinase I, with the confidence level above 95% ($p < 0.05$). The other factors had the confidence level below 95%, and were therefore considered insignificant.

3.3. Box–Behnken design and response surface methodology

Following screening, response surface methodology with a Box–Behnken design was employed to determine the optimal levels of three factors that significantly affected the activity of heparinase I. Their respective low, zero and high levels are defined in Table 3. The concentrations of the other factors with negative and positive effects were chosen as a low and a high level in Table 1, respectively. Results of the Box–Behnken design are shown in Table 3. Based on the regression analysis of data from Table 3, the effects of three factors on the activity of heparinase I were predicted by a second-order polynomial equation:

$$Y = 308.82 + 39.46X_1 + 16.84X_2 + 5.72X_3 + 25.98X_1X_2 - 36.76X_1X_3 + 31.90X_2X_3 - 61.18X_1^2 - 35.05X_2^2 - 20.67X_3^2 \tag{5}$$

The statistical significance of Eq. (5) was checked by F-test, and the analysis of variance (ANOVA) is shown in Table 4. It was obvious that the model was significant, as suggested by the model F value and a low probability value ($p = 0.0018$). The analysis of factor (F-test) showed that the second-order polynomial model was well matched with experimental data. The coefficient of variation (CV) indicates the degree of precision to which treatments is compared. Usually, the higher the value of CV, the lower the reliability of experiment. Here, a lower value of CV (8.72) indicated better

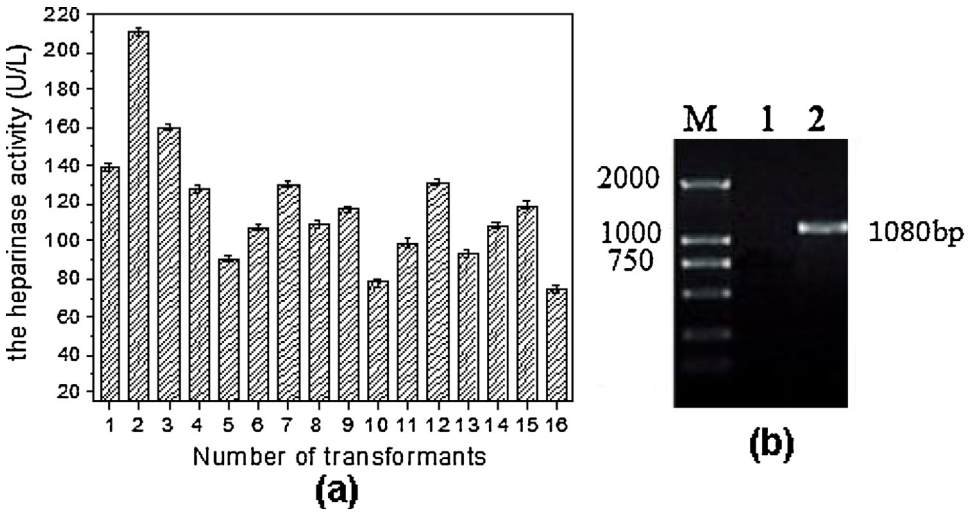

Fig. 1. (a) The activity of heparinase I in sixteen screened transformants. Experiments were carried out three times, and data are represented as the mean ± SD. (b) PCR identification. Lane M: Marker DL2000; lane 1: the control strain transformed with the plasmid pPIC9K; lane 2: the screened transformant 2.

Table 1
Experimental results of the Plackett–Burman design.

Runs	Coded levels							Uncoded levels							The activity of heparinase I (U/L)
	A	*B*	*C*	*D*	*E*	*F*	*G*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	
1	1	1	1	1	1	1	1	0.5	2	45	0.05	0.08	7	2.0	125.65
2	−1	1	1	−1	−1	−1	1	0.1	0.5	15	0.01	0.02	1	0.6	167.76
3	1	−1	1	−1	−1	1	−1	0.5	2	45	0.05	0.08	7	2.0	136.05
4	−1	1	−1	−1	1	1	−1	0.1	0.5	15	0.01	0.02	1	0.6	197.21
5	1	−1	−1	−1	1	−1	1	0.5	2	45	0.05	0.08	7	2.0	195.39
6	−1	1	−1	1	1	−1	1	0.1	0.5	15	0.01	0.02	1	0.6	160.86
7	−1	1	1	1	−1	1	−1	0.1	0.5	15	0.01	0.02	1	0.6	212.32
8	1	−1	1	1	−1	−1	1	0.5	2	45	0.05	0.08	7	2.0	152.97
9	1	1	−1	1	−1	−1	−1	0.5	2	45	0.05	0.08	7	2.0	318.71
10	−1	−1	1	−1	1	1	1	0.1	0.5	15	0.01	0.02	1	0.6	115.47
11	1	−1	−1	1	1	1	−1	0.5	2	45	0.05	0.08	7	2.0	151.47
12	1	1	1	−1	1	−1	−1	0.5	2	45	0.05	0.08	7	2.0	120.73
13	1	1	−1	−1	−1	1	1	0.5	2	45	0.05	0.08	7	2.0	261.89
14	−1	−1	−1	1	−1	1	1	0.1	0.5	15	0.01	0.02	1	0.6	218.5
15	−1	−1	−1	−1	−1	−1	−1	0.1	0.5	15	0.01	0.02	1	0.6	189.53
16	−1	−1	1	1	1	−1	−1	0.1	0.5	15	0.01	0.02	1	0.6	116.29

Note: *A* (ml/L): Tween-80, *B* (ml/250 mL): methanol, *C* (ml/250 mL): liquid volume in flask, *D* (ml/L): PTM1, *E* (ml/L): oleic acid, *F* (%): inoculation, and *G* (%): YNB.

Table 2
Regression results of the Plackett–Burman design.

Model term	Sum of squares	Degree of freedom	Mean square	*F*	*p* (*Prob* > *F*)
Model	38,873.82	7	5553.40	5.85	0.0051[a]
A	453.48	1	453.48	0.48	0.5039
B	5227.29	1	5227.29	5.50	0.0388[a]
C	18,671.86	1	18,671.86	19.66	0.0010[a]
D	333.06	1	333.06	0.35	0.5657
E	14,065.96	1	14,065.96	14.81	0.0027[a]
F	0.73	1	0.73	7.695E−004	0.9784
G	121.44	1	121.44	0.13	0.7275

Note: *A* (ml/L): Tween-80, *B* (ml/250 mL): methanol, *C* (ml/250 mL): liquid volume in flask, *D* (ml/L): PTM1, *E* (ml/L): oleic acid, *F* (%): inoculation, and *G* (%): YNB.
[a] Statistically significant at 95% of the confidence level ($p < 0.05$).

precision and reliability of experiments (Box, Hunter, & Hunter, 1978). The precision of a model can be checked by determination coefficient (R^2) and correlation coefficient (R). The determination coefficient (R^2) was calculated to be 0.9319, indicating that 93.19% of the variability in the response could be explained by this model. A regression model having a R value higher than 0.9 is considered to have a very high correlation. The closer the R value to 1, the better the correlation between experimental values and predicted ones. Here, the R value (0.9674) for Eq. (5) indicated a good agreement between experimental results and theoretical ones predicted by model equation.

Model coefficients and probability values are shown in Table 5. The model was proved to be suitable for the adequate representation of the real relationship among selected factors. Among these factors, oleic acid was found to have a more significant effect on the activity of heparinase I. The positive coefficient showed a linear effect on increasing the activity of heparinase I. Quadratic terms of three factors, and interactions between X_1 and X_2 and between X_1

Table 3
Experimental results of the response surface methodology.

Runs	Coded levels			Uncoded levels			The activity of heparinase I (U/L)
	X_1	X_2	X_3	X_1	X_2	X_3	
1	−1	−1	0	0.03	20	9	175.49
2	1	−1	0	0.09	20	0.9	205.67
3	−1	1	0	0.03	40	0.9	167.53
4	0	0	0	0.06	30	0.9	289.90
5	−1	0	−1	0.03	30	0.3	160.74
6	1	0	−1	0.09	30	0.3	315.95
7	0	0	0	0.06	30	0.9	327.45
8	1	0	1	0.09	30	1.5	213.67
9	0	−1	−1	0.06	20	0.3	250.50
10	0	0	0	0.06	30	0.9	305.39
11	0	−1	1	0.06	20	0.9	232.36
12	0	1	1	0.06	40	0.9	319.49
13	0	0	0	0.06	30	0.3	318.81
14	−1	0	1	0.03	30	0.9	217.5
15	0	1	−1	0.06	40	0.3	210.05
16	0	0	0	0.06	30	0.6	302.53
17	1	1	0	0.09	40	0.6	301.64

Note: X_1 (%): oleic acid, X_2 (ml/250 mL): liquid volume in flask, and X_3 (%): methanol.

Table 4
Analysis of variance (ANOVA) for the second-order polynomial model.

Source	Sum of squares	Degree of freedom	Mean square	F	p ($Prob > F$)
Model	52,890.31	9	5876.70	11.99	0.0018[a]
X_1	12,455.94	1	12,455.94	25.42	0.0015[a]
X_2	2267.67	1	2267.67	4.63	0.0685
X_3	261.98	1	261.98	0.53	0.4884
X_1X_2	2700.36	1	2700.36	5.51	0.0513
X_1X_3	6323.43	1	6323.43	12.9	0.0088[a]
X_2X_3	4069.16	1	4069.16	8.30	0.0236[a]
X_1^2	15,762.16	1	15,762.16	32.16	0.0008[a]
X_2^2	5172.42	1	5172.42	10.55	0.0141[a]
X_3^2	1798.38	1	1798.38	3.67	0.0970
Residual error	3430.49	7	490.07		
Lack of fit	2574.32	3	858.11	4.01	0.1066
Pure error	856.17	4	214.04		
Total	56,320.80	16			
R^2					0.9319

Correlation coefficient (R) = 0.9674. Coefficient of variation (CV) = 8.72.
Note: X_1 (%): oleic acid, X_2 (ml/250 mL): liquid volume in flask, and X_3 (%): methanol.
[a] Statistically significant at 95% of the confidence level ($p < 0.05$).

and X_3, had also a significant effect on the activity of heparinase I. Negative coefficients of X_1X_3, X_1^2, X_1^2 and X_1^2 showed a significant effect on decreasing the activity of heparinase I. In this case, X_1, X_1X_3, X_2X_3, X_1^2, X_1^2 and X_1^2 were more significant model terms.

3D response surface plots and 2D contour plots described by regression model were drawn to illustrate the effects of independent factors, the interactive effects of each independent factor on the response factor, and the optimal concentration of each component required for the activity of heparinase I (Fig. 2a–f). Each figure presents the effect of two factors while the other factor is held at zero level. These plots provide a visual interpretation of the interaction of two factors, and facilitate the location of optimal conditions. The optimal values of tested factors predicted by the model were as follows: oleic acid: 0.07%, liquid volume in flask: 34.3 ml/L, and methanol: 0.96%. The maximal activity of heparinase I predicted by the model was 320.2 U/mL. Three experiments were performed to verify the predicted activity under the optimal conditions. The average activity of heparinase I was 323 U/mL, which was in a good agreement with the predicted activity. The final medium components optimized were: yeast extract 10 g/L, tryptone 20 g/L, YNB 1.34 g/L, potassium phosphate 100 mM, methanol 9.6 ml/L, oleic acid 0.7 ml/L, Tween-80 5.0 ml/L, PTM_1 4.0 ml/L and

Table 5
Regression coefficient and significance of the second-order polynomial model.

Factor	Coefficient estimate	Degree of freedom	Standard error	95% CI low	95% CI high	VIF
Intercept	308.82	1	9.90	285.41	332.23	1
X_1	39.46	1	7.83	20.95	57.97	1
X_2	16.84	1	7.83	−1.67	35.34	1
X_3	5.72	1	7.83	−12.78	24.23	1
X_1X_2	25.98	1	11.07	−0.19	52.16	1
X_1X_3	−39.76	1	11.07	−65.93	−13.59	1
X_2X_3	31.89	1	11.07	5.72	58.07	1
X_1^2	−61.18	1	10.79	−86.69	−35.67	1.01
X_2^2	−35.05	1	10.79	−60.56	−9.54	1.01
X_3^2	−20.67	1	10.79	−46.18	4.84	1.01

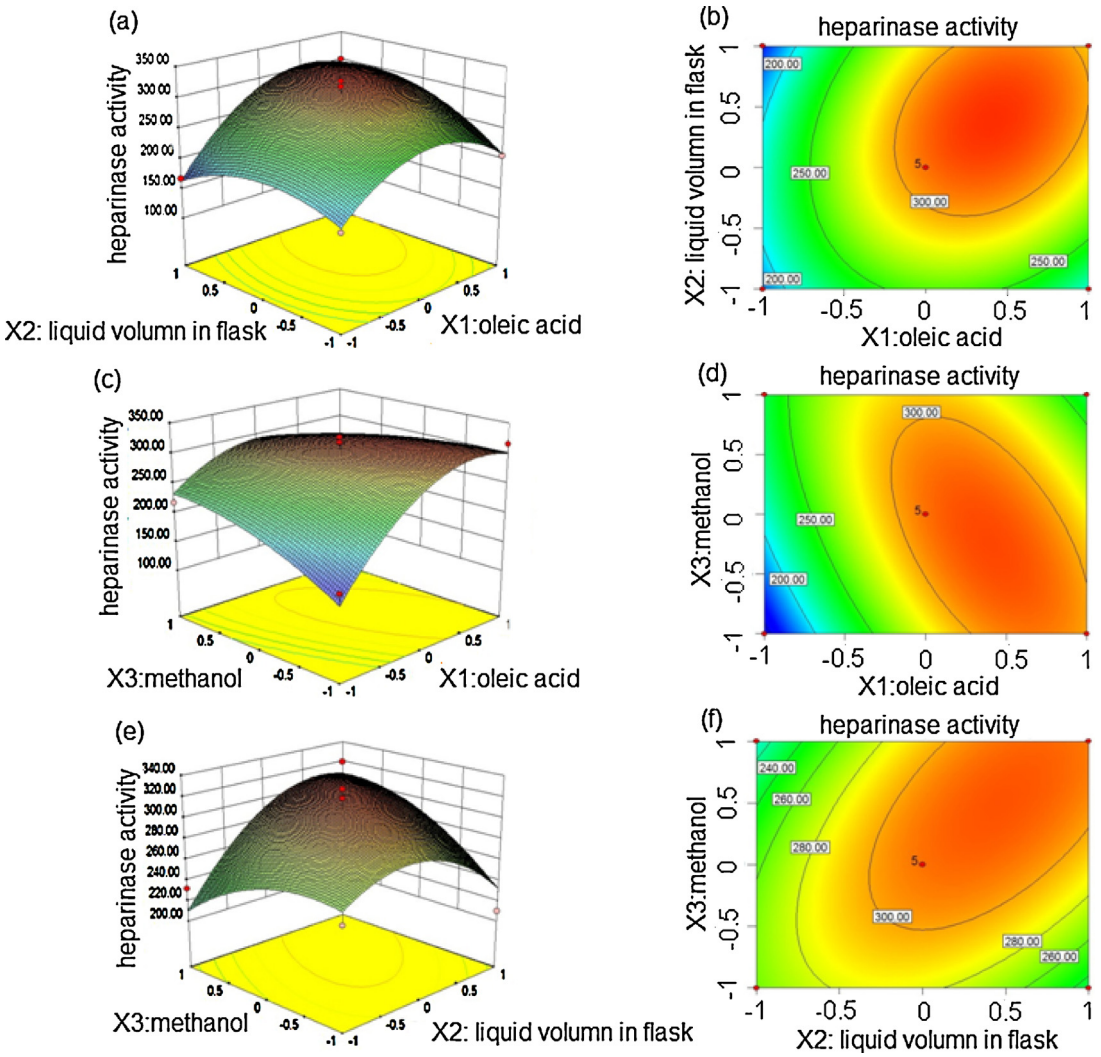

Fig. 2. 3D response surface plots and 2D contour plots showing the effect of oleic acid, liquid volume in flask and methanol on the activity of heparinase I. (A) $Y=f(X_1, X_2)$, (C) $Y=f(X_1, X_3)$, (E) $Y=f(X_2, X_3)$. Y: the activity of heparinase I, X_1: oleic acid, X_2: liquid volume in flask, X_3: methanol.

biotin 4.00×10^{-4} g/L. The activity of heparinase I was improved by 1.54-fold after optimization.

3.4. Fermentation of the transformant 2 in a 5 L fermentor

Fermentative profiles of the transformant 2 in a 5 L fermentor are shown in Fig. 3. At the initial stage, glycerol was used as the only carbon source to stimulate the cell growth. When glycerol was used up at 28 h, the wet cell weight reached 175.5 g/L. After carbon starvation for 1 h, methanol was fed to induce the expression of *HpaI*. By changing the feeding rate of methanol, its concentration in the fermentor was maintained at the optimal level to minimize the toxic effect on yeast cells. The maximal biomass and the highest activity of heparinase I were 202 g/L and 398.5 U/L after the methanol induction for 100 h, respectively. Furthermore, it was found that there was a good correlation between the total protein concentration and the heparinase I activity (Fig. 3). The maximal protein concentration was 1.29 g/L after the methanol induction for 100 h.

In the previous studies, *HpaI* from *F. heparinum* was cloned and successfully expressed in *E. coli* (Chen, Xing, & Lou, 2005; Chen, Xing, Ye, Kuang, & Luo, 2007; Yin, Xinhui, Fengchun, & Ying, 2007). Recently, Chen et al. (2013) and Yin et al. (2007) have combined site-directed mutagenesis and calcium ion addition to improve the production of thermostable MBP-fused heparinase I in engineered *E. coli*. Although an enormous success has been achieved in expressing the heparinase I in *E. coli* in the above-mentioned studies, a part of the expressed heparinase I was found in the form of insoluble inclusion body. Restoring the heparinase I activity by refolding involves complicated and time-consuming procedures. Our study is the first time to report the secreted expression of *HpaI* in *P.*

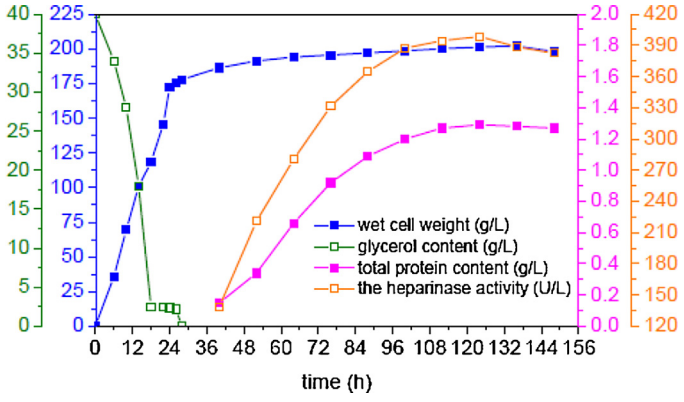

Fig. 3. Changes in the glycerol content, the wet cell weight, the total protein content and the activity of heparinase I during fermentation by transformant 2.

pastoris, and to optimize the conditions for the heparinase I production by response surface methodology at the shake flask level. In comparison to *E. coli*, the α-factor secretory signal sequence from *Saccharomyces cerevisiae* in the *P. pastoris* expression plasmid allows the expressed heparinase I to be directly secreted into the culture medium, thereby avoiding the renaturation process in addition to other benefits. Finally, a maximal heparinase I activity of 398.5 U/L was reached in a 5 L fermentor. This study lays a good foundation for the industrial production of heparinase I by recombinant *P. pastoris* in future.

4. Conclusions

This study demonstrates the overproduction of heparinase I by constructing recombinant *P. pastoris* bearing *HpaI* from *F. heparinum*. The transformant 2 that could effectively express *HpaI* was screened and identified. The components of the optimized medium were as follows: yeast extract 10 g/L, tryptone 20 g/L, YNB 1.34 g/L, potassium phosphate 100 mM, methanol 9.6 ml/L, oleic acid 7.1 ml/L, Tween-80 5.0 ml/L, PTM_1 4.0 ml/L and biotin 4.00×10^{-4} g/L. The activity of heparinase I was 323 U/L in the optimized medium, a 1.54-fold increase compared to the original activity. The highest activity of heparinase I obtained in a 5 L fermentor was 398.5 U/L. Continuous efforts should be given to further improve the heparinase I production by directed evolution, or codon optimization based on the codon bias of *P. pastoris* in future.

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