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Response surface optimization for lactose inducible expression of recombinant fructosyl peptide oxidase enzyme in *Escherichia coli*

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Abstract

Background: Fructosyl peptide oxidase (FPOX), a flavoenzyme classified as an oxidoreductase, serves as a diagnostic enzyme in HbA1c measurement tests. This research focuses on statistically optimizing lactose-induced expression to produce soluble recombinant FPOX.

Methods: A Plackett–Burman design was used to identify key factors influencing enzyme expression, which were further optimized using a central composite design.

Results: The results indicated that glycerol, yeast extract, tryptone, and lactose significantly affected FPOX activity. The maximum enzyme activity and biomass concentration were achieved under the optimum conditions of yeast extract (10.12 g/L), tryptone (13.44 g/L), K_2HPO_4 (2.62 g/L), and lactose (12.79 g/L). When the lactose-inducible induction strategy was examined at the shake flask scale, FPOX activity (28.77 U/mL) was 18.5-fold higher than with the IPTG induction protocol. Additionally, the increased biomass yield (49.0 g/L compared to 22.0 g/L) further supported the appropriateness of utilizing lactose-inducible expression.

Conclusion: Together, our findings indicated that the design of experiment methodology can be utilized effectively to enhance the production of the FPOX enzyme with lactose as the inducer.

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Highlights

What is current knowledge?

Lactose-inducible fermentation of the FPOX enzyme was optimized using statistical design. The enzyme activity under the optimized expression conditions was found to be 28.77 U/mL.

What is new here?

The correlation between predicted and experimental values confirmed the practicability of the optimization strategy.

Introduction

Fructosyl peptide oxidase (FPOX) belongs to the oxidoreductase family and is responsible for catalyzing the oxidative deglycation process of fructosyl peptide into unglycated peptide, glucosone, and hydrogen peroxide (1,2). The FPOX enzyme, obtained from various microorganisms, has been utilized to enzymatically test glycated proteins such as hemoglobin A1c (HbA1c) and glycated albumin (3). HbA1c is a crucial test for detecting and controlling diabetes in patients (4). Several studies have been performed on optimizing the production and cost-effective bioprocessing of FPOX, a diagnostic enzyme with industrial significance (5,6). However, industrial uses of this biocatalyst require large quantities of the enzyme, along with efficient production and cost-effectiveness (7). The culture medium components and type of expression inducer are important factors influencing microbial growth and enzyme production cost (8).

Statistical designs such as Plackett-Burman design (PBD) and response surface methodology (RSM) are effective methods for optimizing experiment conditions and obtaining desired results (9,10). RSM involves a variety of statistical techniques to assess the relationship between the response of an experiment and a group of variables. Identifying effective factors, optimal levels, and potential interactions helps improve the accuracy of predicting optimal conditions (11,12). RSM has proven effective in improving the production of different recombinant enzymes, e.g., α-amylase (13), laccase (14), and L-glutaminase (15). In this communication, recombinant FPOX was produced in *Escherichia coli* BL21 (DE3) with lactose-inducible expression, and production yield was optimized using statistical methods.

Methods

Expression of recombinant enzyme

The nucleotide sequence of *Eupenicillium terrenum* FPOX was synthesized and inserted into the pET-28a (+) expression vector through cloning. The recombinant plasmid pET28FPOX was expressed in *E. coli* BL21 (DE3) cells grown in Luria-Bertani (LB) medium with 100 µg/mL ampicillin at 37ºC and 200 rpm until reaching OD600 = 0.6–0.8. Induction was done with lactose or IPTG at 37°C and 250 rpm for 5 h. After induction, the bacteria were collected, rinsed with 0.9% NaCl solution, and frozen at −20 °C. The cells were then dissolved in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 1% glycerol, pH 8.0), disrupted by sonication, and clarified through centrifugation at 9000 rpm for 30 min at 4 °C. The supernatant of the cell lysate was used for the subsequent experiments (16).

Optimization of FPOX expression by statistical designs

The optimization plan involved identifying the most important elements through PBD and then assessing the best concentrations using central composite design (CCD). The variables, including glycerol, tryptone, yeast extract, K_2HPO_4, MgSO4, induction time, temperature, and lactose, were examined at two levels (Table 1). The experiments were performed three times each, and the mean enzyme activity was determined. Student's t-test was used to calculate the p-value for each variable. The variables that had a significant impact based on the PBD test were studied using a three-level CCD with 44 runs. ANOVA was used to test the importance of variables. The significance of the model was supported using Fisher's statistical test (F-test) and the low p-value. Three-dimensional graphs were employed to assess the apparent connections among the important variables (14,16). The statistical designs were conducted using Design Expert 11.0 software from State-Ease, Inc. in the USA.

Table 1. Variables used for finding factors affecting FPOX expression

Variable code	Variables	Value	
		-1	+1
A	Tryptone (g/L)	5.0	15.0
B	Glycerol (g/L)	0.2	3.0
C	Yeast extract (g/L)	5.0	15.0
D	K2HPO4 (g/L)	0.4	2.0
E	Induction time (h)	20.0	37.0
F	Temperature (°C)	20.0	37.0
G	Lactose (g/L)	10.0	20.0

Enzyme expression analysis

Enzyme expression was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and an enzyme activity assay. The electrophoresis was conducted for 5 hours at 50 V and 15 mA. The gel was stained with Coomassie Brilliant Blue R-250 and subsequently destained through diffusion in a mixture containing 40% (v/v) methanol and 10% (v/v) acetic acid (17). The activity of FPOX was assessed using the HRP-linked reaction system. The components mixed in a 1 mL reaction mixture at 25°C included 0.1 M potassium phosphate buffer (pH 8.0), 3.0 mM substrate (Fru-ValHis), 0.45 mM 4-aminoantipyrine, 0.5 mM TOOS, 2.0 U/mL HRP, and enzyme solution. Quinoneimine dye formation was then assessed at 555 nm using

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spectrophotometry. One unit of FPOX activity is defined as the amount of enzyme that can oxidize 1 µmol of substrate per minute (16). Protein concentration was determined using the Bradford method, with bovine serum albumin as a reference standard.

Results

Optimization of FPOX expression

PBD was used to identify key variables with a significant impact on the production of the FPOX enzyme. Table 2 illustrates the statistical analysis of PBD for seven variables. Examination of p-values indicated that yeast extract, tryptone, K2HPO4, and lactose had significant impacts on FPOX production ($p < 0.05$) (Table 2). Therefore, yeast extract, tryptone, K2HPO4, and lactose were chosen to be further optimized through CCD. A total of 44 experiments were conducted to investigate how the combination of these variables affects FPOX expression. The variables and experimental outcomes are presented in Table 3. The R^2 coefficient of 0.9885 for FPOX production showed a good agreement between the experimental and predicted data. Table 4 contains statistical analysis of CCD. To examine how the test variables interacted, p-values were analyzed to determine the importance of each coefficient (Table 4). Model terms are considered significant if they have p-values below 0.05. The linear model showed that X_1, X_2, X_3, and X_4 were statistically significant terms according to the findings. ANOVA (Table 4) confirmed the appropriateness of the model. The F-value and the value of "Prob > F" were 4.36 and 0.0017, respectively, indicating that the model was statistically significant. The R^2 coefficient, expected R^2, and adjusted R^2 values were 0.9960, 0.9875, and 0.9733, respectively. The CV value of 1266.64 indicated a high level of reliability in the experiment. As illustrated in Figure 1, a linear pattern is observed, showing good consistency between the actual and predicted response values for enzyme activity and biomass concentration. In Figures 2A, 2D, and 2F, it can be observed that FPOX activity increases at moderate levels of yeast extract, tryptone, and lactose. On the other hand, the activity of FPOX rises as the concentrations of glycerol and K2HPO4 increase (Figures 2B and 2C).

Experimental validation

The calculated enzymatic activity of FPOX under optimal conditions was 28.77 U/mL. Table 5 illustrates the comparison between IPTG-inducible and lactose-inducible methods for FPOX expression. Testing the same induction strategy in shake flasks resulted in an FPOX activity of 28.77 U/mL, which was 18.56-fold greater than with the IPTG induction protocol. Figure 3 shows a higher level of FPOX activity in the lactose-induced protocol compared to the IPTG-induced method. Figure 4 depicts the SDS-PAGE gel analysis of recombinant FPOX produced using both IPTG and lactose induction techniques. The electrophoresis results display the presence of a protein weighing 50.0 kDa, suggesting that recombinant FPOX is expressed in response to lactose induction.

Table 2. Statistical analysis of experimental design for each variable affecting FPOX expression

Variables	Coefficients	df	Standard error	F-value	P-value
Model	1.07	10	0.0211	22.77	0.0372
A- Tryptone	- 0.1652	1	0.0219	65.25	0.0272
B-Glycerol	- 0.1410	1	0.0219	53.42	0.2111
C-Yeast extract	- 0.1658	1	0.0219	82.34	0.0270
D- K2HPO4	0.0602	1	0.0219	11.44	0.0353
E-Induction time	0.0633	1	0.0219	10.34	0.2019
F-Temperature	- 0.0535	1	0.0219	8.75	0.2546
G-Lactose	0.0388	1	0.0219	6.34	0.0254

Table 3. Experimental design results of FPOX activity and biomass concentration

Run	X_1 (Tryptone)	X_2 (Yeast extract)	X_3 (K2HPO4)	X_4 (Lactose)	Enzyme activity (U/mL)		Biomass concentration (g/L)	
					Experimental	Predicted	Experimental	Predicted
1	12	10	2	20	9.84	50.63	21.40	26.07
2	2	15	2	20	7.70	187.74	15.10	20.68
3	12	10	2	20	8.01	- 55.88	18.10	29.37
4	12	15	0.2	8	6.47	6.53	14.20	26.07
5	7	10	1.1	14	9.38	42.59	19.50	21.81
6	12	10	2	20	13.36	-53.23	28.30	23.88
7	7	15	1.1	14	7.80	42.59	13.80	16.20
8	2	15	0.2	8	13.87	- 55.98	31.20	26.38
9	7	10	1.1	14	17.39	42.59	38.50	30.15
10	7	5	1.1	14	16.37	42.59	35.60	25.28
11	12	15	0.2	20	8.62	263.81	19.20	19.62
12	12	15	0.2	8	12.34	27.54	29.60	21.74
13	7	15	1.1	14	11.73	42.59	25.20	28.37
14	12	5	0.2	20	17.75	28.55	35.50	35.76
15	2	5	0.2	8	12.09	9.42	31.20	26.07
16	2	15	2	20	6.83	- 59.68	15.20	24.23
17	2	5	0.2	20	6.07	25.49	16.30	18.82
18	12	5	2	20	14.40	- 8.79	25.10	28.63
19	12	10	0.2	8	16.68	- 54.77	35.10	34.45
20	2	5	2	8	6.93	190.17	16.60	19.08
21	12	15	0.2	20	11.70	- 57.89	25.30	26.31
22	2	5	2	20	9.94	- 14.40	22.30	30.71
23	7	5	1.1	14	13.10	42.59	26.90	21.90
24	2	5	0.2	20	9.95	10.64	19.50	22.57
25	12	5	0.2	8	8.50	- 62.12	20.60	23.23
26	2	10	0.2	20	12.30	- 57.97	31.10	24.14
27	12	15	2	8	13.50	188.02	32.80	29.75
28	2	15	2	8	1150.00	539.35	27.70	22.66
29	2	10	0.2	8	15.30	13.51	31.50	31.22
30	7	5	1.1	14	14.50	42.59	33.20	36.31
31	12	15	2	8	15.20	- 53.25	37.70	28.12
32	2	10	0.2	20	16.50	- 56.85	36.50	31.97
33	7	15	1.1	14	13.20	42.59	31.20	31.53
34	2	10	0.2	8	102.00	229.87	24.90	23.37
35	12	15	2	20	10.50	26.41	26.50	25.96
36	2	5	2	8	9.50	189.46	20.50	27.94
37	2	15	2	20	13.20	- 8.49	29.10	30.09
38	12	5	2	8	13.50	- 6.81	35.50	29.09
39	2	15	2	8	15.40	- 8.77	32.20	26.99
40	12	15	1.1	8	12.20	- 9.37	36.60	28.10
41	7	10	1.1	14	12.10	79.59	33.60	29.98
42	7	10	- 1.04057	14	9.80	2.71	21.70	28.21
43	7	10	1.1	14	10.50	- 44.56	24.50	30.24
44	7	21.8921	1.1	14	15.00	- 55.04	30.20	33.41

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Table 4. ANOVA of the CCD results affecting FPOX expression

Term	Sum of squares	df	Mean of square	F-Value	P-Value
Model	12843.10	1	856.21	4.36	0.0017
A-Tryptone	1059.10	15	1059.10	4.18	0.0483
B-Yeast extract	1068.88	1	1068.88	4.22	0.0474
C-K2HPO4	3.60	1	3.60	0.0142	0.0458
D-Lactose	1084.11	1	1084.11	4.28	0.0459
AB	1420.91	1	1420.91	5.61	0.0234
AC	1455.94	1	1455.94	5.74	0.0219
AD	3.51	1	3.51	0.0138	0.9070
BC	1411.97	1	1411.97	5.57	0.0238
BD	3.92	1	3.92	0.0154	0.9018
CD	7.06	1	7.06	0.0278	0.8684
Residual	9125.92	1	253.50	-	-
Lack of fit	9124.91	36	337.96	3027.37	0.7018
Pure error	1.00	27	0.1116	-	-
Corrected total	22242.89	9	Mean of square	F-Value	P-Value

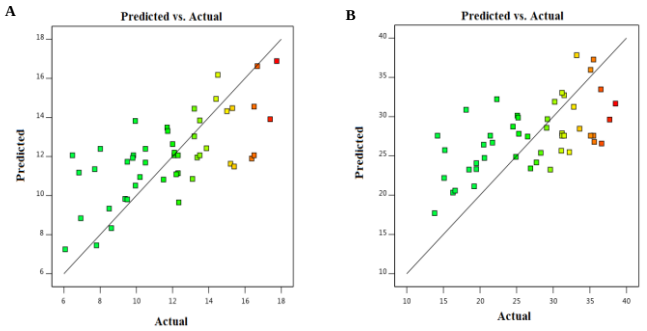

Figure 1. Correlation between the actual and predicted values. (A) Modeling of enzyme activity of FPOX. (B) Modeling of biomass concentration

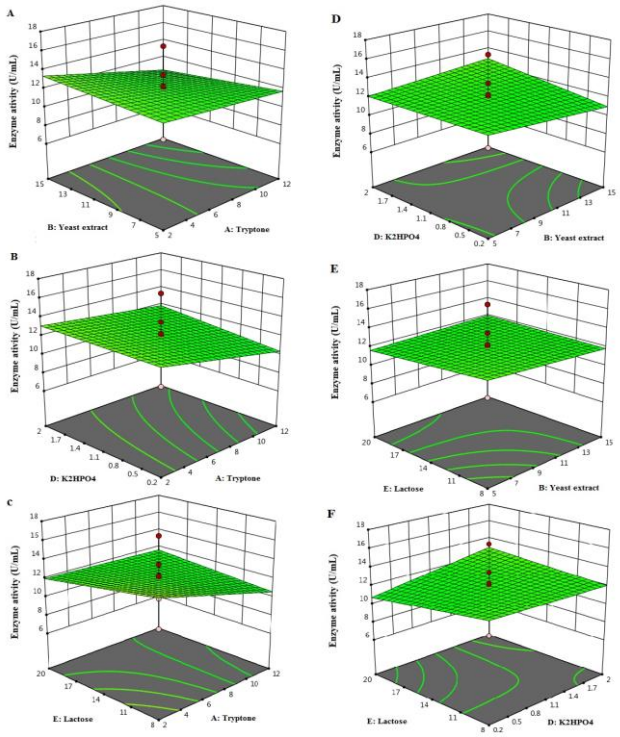

Figure 2. The interaction effects of variables on FPOX activity. (A) Effect of tryptone and yeast extract. (B) Effect of tryptone and K2HPO4. (C) Effect of tryptone and lactose. (D) Effect of K2HPO4 and yeast extract. (E) Effect of lactose and tryptone. (F) Effect of K2HPO4 and lactose

Table 5. Comparison of IPTG and lactose induction protocol for expression of recombinant FPOX

Parameter	Induction with 0.5 mM IPTG	Induction with 14.25 g/L lactose
Type of cultivation	Batch	Batch
Medium volume	1.2 L	1.2 L
Temperature	25 °C	25 °C
Agitation	300 rpm	300 rpm
Total fermentation time	24 hours	24 hours
Enzyme activity (U/mL)	1.55	28.77
Biomass yield (g/L)	22.0	49.0

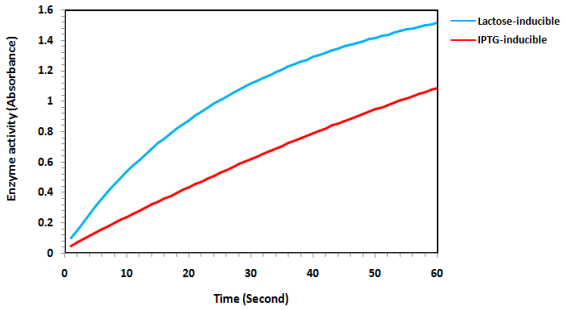

Figure 3. Comparison of FPOX activity in IPTG-inducible and lactose-inducible methods

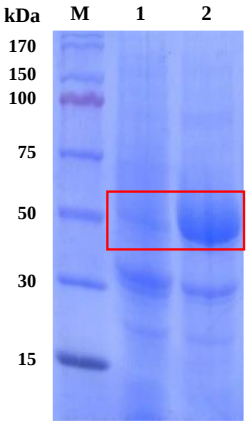

Figure 4. SDS-PAGE analysis of lactose-inducible expression. Lane M: Protein marker. Lane 1: Whole cell lysate of *E. coli* producing recombinant FPOX before optimization. Lane 2: Whole cell lysate of *E. coli* producing recombinant FPOX after optimization of lactose-inducible expression

Discussion

Techniques for producing enzymes in the lab are seldom suitable for producing enzymes on a large industrial scale. Although costs are not a significant factor in small-scale enzyme production, they become a major obstacle when scaling up for large-scale enzyme production (18).

In this paper, we discuss a method that enables the affordable manufacturing of recombinant FPOX through a lactose-based expression system. This expression system is frequently utilized because of its numerous benefits (18). Our batch strategy enabled us to express enzymes by utilizing lactose as an inducer. In addition, through a precise choice of the host organism, affordable mineral media could be utilized. Moreover, the enzyme titers achieved fall within the same range as those of commonly employed IPTG-inducible expression systems. Under ideal conditions, the biomass concentration reached 49.0 g/L, which is higher than the 22.0 g/L achieved through the IPTG induction protocol. The target enzyme's expression was higher during the lactose induction process than with the IPTG method. Therefore, it can be inferred that using lactose for induction improves enzyme expression better than using IPTG.

Conclusion

In summary, PBD and RSM methods were used to screen and optimize seven variables to identify the key factors influencing FPOX activity. Findings showed that yeast extract (10.12 g/L), tryptone (13.44 g/L), K2HPO4 (2.62 g/L), and lactose (12.79 g/L) resulted in the highest FPOX activity (28.77 U/mL), indicating increased production of recombinant FPOX. Together, the design of experiment methodology is an effective technique for enhancing the lactose-inducible fermentation of FPOX in E. *coli.*

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Ethical statement

This study was approved by the Ethics Committee of Qazvin University of Medical Sciences with an approval code of (IR.QUMS.REC.1401.286).

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Conflicts of interest

The authors declared no conflicts of interest.

Author contributions

This article is the result of a research project (No. 401000249) at Qazvin University of Medical Sciences. Dr. Hamid Shahbazmohammadi supervised and was responsible for conducting the research.

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