Lactose Inducible Fermentation in Escherichia coli for Improved Production of Recombinant Urate Oxidase: Optimization by Statistical Experimental Designs

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Research

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Abstract

The enzyme urate oxidase (UOX) is used as a drug for preventing and treatment of chemotherapy-induced hyperuricemia. This study deals with the statistical optimization of lactose inducible fermentation for production of soluble recombinant *Aspergillus flavus* UOX. 10 variables were investigated by Plackett–Burman design (PBD), and the most significant factors were further optimized by central composite design (CCD). PBD results indicated that glycerol, yeast extract, tryptone, and lactose affected UOX activity significantly. The CCD results showed that the maximum enzyme activity (19.34 U/ml) could be achieved under the optimum conditions of glycerol 0.87 g/L, yeast extract 9.11 g/L, tryptone 10.29 g/L, K_2HPO_4 1.81 g/L, and lactose 12.79 g/L. When the same induction strategy was tested at shake flask, 19.34 U/mL of UOX activity was obtained, which was 12.5 folds higher than IPTG induction protocol. Furthermore, the lower total cost (0.7 vs. 13.5 €) was additionally feature that confirmed the suitability of the lactose induction method. Collectively, our results showed that design of experiment methodology can be applied as a suitable tool for improved production of UOX using lactose as the inducer.

Introduction

Uric acid is the final product of purine metabolism in hominoids. Because of low solubility of uric acid, various species have specific enzymes to change it into more soluble substances such as allantoin (Hayashi et al., 2000). Urate oxidase (UOX, EC 1.7.3.3) is the main enzyme in this reaction, and catalyzes uric acid into 5-hydroxyisourate. In the second step, uric acid converts to allantoin and CO_2, which can be enzymatic or non-enzymatic (Collings et al. 2020; Cendron et al. 2007). UOX has a homotetrameric structure without any glycosylated group or disulfide bonds, and is expressed in bacteria, fungi and most mammals. The increment in the amount of uric acid is associated with some disorders like gout, renal nephropathy, cardiovascular diseases, stroke, and tumor lysis syndrome (Hummel et al., 2005; Kang et al., 2003; Garay et al., 2012). UOX enzyme is used to determine uric acid in biological fluids, and can be a good candidate for diagnostic goals.

Higher species like hominoid primates have no enzyme for uric acid degradation due to a nonsense mutation in *uox* gene occurred in evolutionary process (Keilin, 1959). This is mainly related to uric acid antioxidant properties to prevent cancer (Gabison et al., 2014). Production of UOX has been reported from different microorganisms such as *Aspergillus flavus*, *Nocardia farcinica*, *Candida tropicalis*, *Sphingobacterium thalpophilum, Bacillus subtilis* and *B. fastidious* (Zhang et al., 2012; Ishikawa et al., 2004; Nishiya et al., 2002). The recombinant *A. flavus* UOX is accepted under the brand name Rasburicase for treatment purposes by Food and Drug Administration (FDA). Because of the industrial importance of UOX as a drug enzyme, various studies have focused on the production optimization, and cost effective bioprocessing. One of the most effective parameters in this area is the culture medium components and the type of expression inducer, which affects microbial growth, and enzyme production (Sorensen et al., 2005; Shahbazmohammadi and Omidinia, 2017; Huang et al., 2010). Since the interactions among different constituents in culture media are complex, it is beneficial to screen those

using statistical models. Statistical experimental designs including Plackett–Burman design (PBD) and response surface methodology (RSM) are useful strategies to optimize conditions, and achieve favorable response in an experiment (Shahbazmohammadi et al., 2019; Papaneophytou et al., 2013; Ghoshoon et al., 2015). RSM is a complex of statistical methods for evaluating relation between experiment response, and a set of variables. It helps to identify the effective factors, optimum levels, and possible interactions which lead to a more credible prediction of optimum conditions (Ghoshoon et al., 2015). RSM has been applied to enhance production of various recombinant enzymes e.g., cutinase (Kumari et al., 2016), streptokinase (Aghaeepoor et al. 2017), and protease (Chidambaram and Lakshmanaperumalsamy, 2009). In this communication, *uox* gene from *A. flavus* was expressed in *Escherichia coli* BL21 (DE3) using lactose inducible expression system, and statistical methods were applied to increase the production yield. First,

Materials And Methods

Strains, Media and reagents

E. coli BL21 (DE3) and pET-42a (+) were from Novagen (Philadelphia, PA). *Nde*I and *Xho*I restriction enzymes were procured from Thermo Fisher Scientific (Wilmington, DE, USA). N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-toluidine (TOOS) was purchased from Santa Cruz (Santa Cruz, CA, USA). 4-Aminoantipyrine (4-AA), horseradish peroxidase (HRP) and uric acid were from Sigma-Aldrich (St. Louis, MO, USA). *A. flavus* UOX gene was synthesized by Biomatik Company (Ontario, Canada).

Cloning and expression of recombinant enzyme

The synthetic coding DNA sequence of *A. flavus* UOX (Accession number; X61766) was cloned into pET-42a (+) using the forward (CATATGTCCGCAGTAAAAGCAGCCCGC) and reverse (CTCGAGTTACAATTTAGACTTCAGAGAGGACCGG) primers which had the endonuclease restriction sites for *Nde*I and *Xho*I, respectively (Fig. 1). The construct (pET42-UOX) was introduced into *E. coli* BL21 (DE3) competent cells. Then, transformed cells were cultured in Luria-Bertani (LB) containing kanamycin (40 µg/ml) at 37 ºC, and 180 rpm to reach OD_{600} = 0.6–0.8, and induced by adding lactose at 37 °C and 250 rpm for 5 h. The cells were harvested by centrifugation at 4 °C and 8000 rpm for 20 min, and stored at -80 °C for the further use. Harvested cells were re-suspended in lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 0.1 mM EDTA, 1% glycerol, pH 8.0), and sonicated for 10 min (15 s on and 10 s off). The supernatant was obtained by centrifugation (10000 rpm for 30 min at 4 °C) and, used for enzyme activity assay experiments (Shahbazmohammadi and Omidinia, 2008).

Optimization of soluble expression of UOX by statistical designs

Optimization strategy included the determination of the most significant medium components by PBD and, evaluation of the optimal concentrations using CCD. Design Expert 11.0 software (State-Ease, Inc., USA) was used for the statistical designs. 10 independent variables including glycerol, tryptone, yeast

extract, K_2HPO_4, NaCl, $MgSO_4$, $CaCl_2$, induction time, temperature and lactose (as the inducer) were examined in two levels, high and low represented +, - respectively (Table 1). According to the PBD, a total of 12 runs were performed, and the effect of each variable was calculated by the Eq. (1):

$$E(X_i) = 2(\sum M_i^+ - M_i^-)/N \quad \text{(Eq. 1)}$$

Where, E is the effect of the tested variable (X_i), M_i^+ and M_i^- are responses of experiments (enzyme activities) at which the factor is at its high or low levels respectively, and N is the number of trails. All the experiments were carried out in triplicates, and the average activity was reported as the final response. The *p*-value of each variable was determined by using Student's t-test. Since PBD cannot describe interaction effects among the variables, and their optimal levels, it was followed by CCD. Among the five variables which showed significant effect from Plackett-Burman test, three-level CCD with 53 runs was conducted. The significance of variables was checked by analysis of variance (ANOVA). Fisher's statistical test (F-test), and the low *p*-value was further used to support the significance of the model. Three-dimensional plots were used to evaluate the visible relationships between the significant independent factors (Shahbazmohammadi and Omidinia, 2017).

Enzyme expression analysis

The analysis of enzyme expression was done through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme activity assay. Electrophoresis was run at 50 V and 15 mA for 5 h. The gel was stained with Coomassie Brilliant Blue R-250, and then destained by diffusion in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid (Sambrook et al., 1994).

Enzyme assay and protein determination

UOX activity was determined using a colorimetric method combined with HRP, TOOS and 4-AA at 555 nm. This protocol is based on the production of colored product from the reaction between H_2O_2 and 4-AA in the presence of HRP. The reaction mixture was composed of 0.1 M sodium tetraborate (pH = 8.0), 5.0 mM uric acid, 0.7 mM 4-AA, 0.5 mM TOOS, HRP (0.83 U/ml), and enzyme solution. The unit of UOX activity is defined as the amount of enzyme that catalyzes the reaction of 1 µmol uric acid to allantoin and H_2O_2 per minute (Xianyu et al., 2013). The total protein concentration was measured using Bradford method (Bradford, 1976).

Results And Discussion

Selection of significant factors by PBD

PBD was applied to screen the important variables that significantly affect the production of UOX enzyme. As shown in Fig. 2, Pareto chart indicates higher effects showed in the upper portion and then progress down to the lower effects. Pareto chart is a convenient way to view the results of PBD and

represents the magnitude of each variable. Large positive or negative coefficients indicate that these factors including A, B, C, K and D play an important role in UOX production, while J, H, E and G indicates negative effects. The $MgSO_4$ concentration (F) exhibits positive effect, but its t-value is lower than t-value limit and negligible. Statistical analysis of PBD for 10 variables is described in Table 2. The *p*-value is the probability used to check the significance of each of the coefficients. Analysis of *p*-values shows that glycerol, yeast extract, tryptone, and lactose play significant roles in UOX production ($p < 0.05$) (Table 2). Although the K_2HPO_4 concentration (D) is a non-significant parameter (*p*-value = 0.1111), but it has positive effect on enzyme production. As a result, glycerol, yeast extract, tryptone, K_2HPO_4, and lactose were selected for further optimization by CCD. The coefficient of R^2 was 0.9998 for UOX production, indicating a reasonable agreement between the experimental data and the predicted values. Different observations have been made by others. For example, in the study of Abdel-Fattah et al. for the production of recombinant *Pseudomonas aeruginosa* UOX, culture volume, and $FeSO_4$ had positive effect, and glucose and $MgSO_4$ showed negative effect (Abdel-Fattah et al. 2005).

Optimization of UOX production by RSM

According to the results of PBD, glycerol, yeast extract, tryptone, K_2HPO_4, and lactose were selected for optimization by CCD. A total of 53 experiments were performed to examine the combinatorial effects of these variables on UOX production. The design matrix of variables and the experimental results are listed in Table 3. Statistical analysis of CCD is also presented in Table 4. To study the interaction pattern between the test variables, *p*-values were checked to understand the significance of each coefficient (Table 4). Model terms are significant for *p*-values less than 0.05. Based on the results, the liner model terms including X_1, X_2, X_3, X_4, and X_5 were significant. The suitability of model was supported using ANOVA (Table 4). F-value and the value of “Prob > F” were 3.38, and 0.0014, respectively, implying the model was significant. The determination coefficient R^2, predicted R^2, and adjusted R^2 were 0.9890, 0.9695, and 0.9821, respectively. The R^2values emphasizes that the model was significant, and suitable for sufficient representation of the real relationship between variables. The lower value of CV (1266.64) demonstrated that the performed experiment was highly reliable. As shown in Fig. 3, a linear distribution is observed which is indicative of a well agreement between the actual response values, and the predicted response values. The graphical representations of a regression equation or three dimensional (3D) response surface plots provide a significant contribution for understanding the interactions between two variables and finding their optimum levels (Abdel-Fattah et al., 2005). Two variables were kept at optimum levels, and the remaining variables were held at zero level. The resulted 3D plots can be used to predict the enzyme activity at different concentration of variables. The plots are not perfectly elliptical and are rather horizontal, which explains that there may be less interaction occurring among the independent variables corresponding to the response surfaces (Jhample et al., 2015). As shown in Fig. 4A, Fig. 4D and Fig. 4F, UOX activity increases in the intermediate concentrations of yeast extract, tryptone, and lactose. In contrast, UOX activity increases with the increment of concentrations of glycerol and K_2HPO_4 (Fig. 4B and Fig. 4C). Similar results have been obtained by other studies where the effects of different medium components on recombinant UOX production were investigated by RSM (Abdel-

Fattah et al., 2005, El-Ahmady El-Naggar, 2015, Nanda et al., 2012). For example, in production optimization of *Gliocladium viride* UOX, enzyme activity was drastically affected by glucose, yeast extract and K_2HPO_4 (Nanda et al., 2012).

Expression of soluble recombinant enzyme under optimized condition

Enzymatic activity of UOX under optimized condition was calculated to be 19.34 U/ml. The enzyme activity is higher than earlier reported values from *A. flavus* (0.43 U/mL) (Fazel et al., 2014), *Pseudomonas aeruginosa* (7.1 U/mL) (Abdel-Fattah et al., 2005), *Candida utilis* (2.6 U/mL) (Chen et al., 2008), and *B. subtilis* strain SP6 (15.87) (Pustake et al., 2019). However, it is lower than the reported activity of recombinant UOX from *B. cereus* (29.7 U/mL) (Khade et al., 2018). Table 5 shows the comparison of IPTG and lactose induction protocols for UOX production. When the same induction strategy was tested at shake flask, 19.34 U/mL of UOX activity was obtained, which was 12.5 folds higher than IPTG induction protocol. It can be concluded that lactose-based induction enhances protein expression more efficient compared to IPTG based process. Furthermore, the lower total cost (0.7 vs. 13.5 €) was additionally feature that confirmed the suitability of the lactose induction method. The expression of target enzyme in lactose induction process was also comparable to that obtained from the IPTG protocol. Figure 5 shows the SDS-PAGE analysis of recombinant UOX obtained by IPTG and lactose induction methods. SDS-PAGE gel shows the expression of a soluble protein with molecular weight of 34.0 kDa, indicating the lactose inducible expression of recombinant UOX in the optimized medium. Therefore, lactose induction for recombinant UOX production was cost-effective, and important from the commercial viewpoint.

Conclusions

Collectively, 10 variables were screened through PBD to select the most significant variables affected on UOX activity. Subsequently, further optimization using RSM was applied. Based on the results, design of experiment methodology can be used successfully as an efficient technique to optimize the lactose inducible fermentation of UOX in *E. coli*. Results indicated that presence of glycerol (0.87 g/L), yeast extract (9.11 g/L), tryptone (10.29 g/L), K_2HPO_4 (1.81 g/L), and lactose (12.79 g/L) lead to the maximum UOX activity (19.34 U/ml) which referred to high yield production of soluble recombinant UOX.

Declarations

Ethics approval and consent to participate

Not applicable, the work does not include any studies with human or animals.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included either in this research article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Abbas Najari carried out the design of experiments with RSM and purification and characterization of the target enzyme. Hamid Shahbazmohmmadi did the general molecular biology methods in this research. Eskandar Omidinia participated in the coordination of the manuscript as well as doing the statistical analysis. All authors read and approved the final manuscript.

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Tables

Table 1
Variables and their levels employed in PBD for screening of conditions affecting on UOX production.

Variable code	Variable	Value	
		-1	+1
A	Tryptone (g/L)	2.0	12.0
B	Glycerol (g/L)	0.1	2.0
C	Yeast extract (g/L)	5.0	15.0
D	K_2HPO_4 (g/L)	0.2	2.0
E	NaCl (g/L)	1.0	3.0
F	$MgSO_4$ (g/L)	0.2	2.0
G	$CaCl_2$ (g/L)	0.1	0.5
H	Induction time (h)	5.0	20.0
J	Temperature (° C)	20.0	37.0
K	Lactose (g/L)	8.0	20.0

Table 2
Statistical analysis of Plackett-Burman experimental design showing coefficient values, F- and p- values for each variable affecting on UOX production

Variables	Coefficients	df	Standard error	F-value	P-value
Model	1.07	10	0.0211	20.60	0.0472
A- Tryptone	-0.1652	1	0.0219	56.72	0.0172
B-Glycerol	-0.1410	1	0.0219	41.34	0.0233
C-Yeast extract	-0.1658	1	0.0219	57.18	0.0170
D- K_2HPO_4	0.0602	1	0.0219	7.53	0.1111
E- NaCl	0.0778	1	0.0219	12.60	0.0710
G-$CaCl_2$	-0.0418	1	0.0219	3.64	0.1967
H-Induction time	0.0633	1	0.0219	8.34	0.1019
J-Temperature	-0.0535	1	0.0219	5.95	0.1349
L-Lactose	0.0388	1	0.0219	3.14	0.0218

Table 3
Experimental design matrix and results of CCD.

Run	X_1 (Tryptone)	X_2 (Yeast extract)	X_3 (Glycerol)	X_4 (K_2HPO_4)	X_5 (Lactose)	Experimental value	Predicted value
1	12	10	0.1	2	20	9.84	50.63
2	2	15	2	2	20	7.70	187.74
3	12	10	0.1	2	20	8.01	-55.88
4	12	15	2	0.2	8	6.47	6.53
5	7	10	1.05	1.1	14	9.38	42.59
6	12	10	2	2	20	13.36	-53.23
7	7	15	1.05	1.1	14	7.80	42.59
8	2	15	0.1	0.2	8	13.87	-55.98
9	7	10	1.05	1.1	14	17.39	42.59
10	7	5	1.05	1.1	14	16.37	42.59
11	12	15	0.1	0.2	20	8.62	263.81
12	12	15	0.1	0.2	8	12.34	27.54
13	7	15	1.05	1.1	14	11.73	42.59
14	12	5	0.1	0.2	20	17.75	28.55
15	2	5	2	0.2	8	12.09	9.42
16	2	15	0.1	2	20	6.83	-59.68
17	2	5	0.1	0.2	20	6.07	25.49
18	12	5	2	2	20	14.40	-8.79
19	12	10	0.1	0.2	8	16.68	-54.77
20	2	5	2	2	8	6.93	190.17
21	12	15	2	0.2	20	11.70	-57.89
22	2	5	0.1	2	20	9.94	-14.40
23	7	5	1.05	1.1	14	13.10	42.59
24	2	5	2	0.2	20	9.95	10.64
25	12	5	2	0.2	8	8.50	-62.12
26	2	10	0.1	0.2	20	12.30	-57.97

27	12	15	2	2	8	13.50	188.02
28	2	15	2	2	8	1150.00	539.35
29	2	10	0.1	0.2	8	15.30	13.51
30	7	5	1.05	1.1	14	14.50	42.59
31	12	15	0.1	2	8	15.20	-53.25
32	2	10	2	0.2	20	16.50	-56.85
33	7	15	1.05	1.1	14	13.20	42.59
34	2	10	0.1	0.2	8	102.00	229.87
35	12	15	2	2	20	10.50	26.41
36	2	5	0.1	2	8	9.50	189.46
37	2	15	0.1	2	20	13.20	-8.49
38	12	5	2	2	8	13.50	-6.81
39	2	15	3.30949	2	8	15.40	-8.77
40	12	15	1.05	1.1	8	12.20	-9.37
41	7	10	1.05	1.1	14	12.10	79.59
42	7	10	1.05	-1.04057	14	9.80	12.71
43	7	10	1.05	1.1	14	10.50	-44.56
44	7	21.8921	1.05	1.1	14	15.00	-55.04
45	7	10	-1.20949	1.1	14	16.50	12.71
46	18.8921	10	1.05	1.1	14	9.50	-54.49
47	7	10	1.05	1.1	14	12.00	-54.18
48	7	10	1.05	1.1	28.2705	12.30	-55.87
49	7	-1.89207	1.05	1.1	14	13.50	80.45
50	7	10	1.05	1.1	14	15.40	12.71
51	-4.89207	10	1.05	3.24057	14	13.20	79.91
52	7	10	0.1	1.1	14	12.20	69.98
53	7	10	2	2	-0.270485	13.20	81.29

Table 4
ANOVA of the experimental results of CCD affecting on UOX production.

Term	Sum of squares	df	Mean of square	F-Value	*P*-Value
Model	12843.10	1	856.21	3.38	0.0014
A-Tryptone	1059.10	15	1059.10	4.18	0.0483
B-Yeast extract	1068.88	1	1068.88	4.22	0.0474
C-Glycerol	1053.45	1	1053.45	4.16	0.0489
D-K_2HPO_4	3.60	1	3.60	0.0142	0.0458
E-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
AE	1453.13	1	1453.13	5.73	0.0220
BC	1411.97	1	1411.97	5.57	0.0238
BD	3.92	1	3.92	0.0154	0.9018
BE	1433.25	1	1433.25	5.65	0.0228
CD	7.06	1	7.06	0.0278	0.8684
CE	1378.06	1	1378.06	5.44	0.0254
DE	6.22	1	6.22	0.0245	0.8764
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			

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

Parameter	Induction with 0.4 mM IPTG	Induction with 12.79 g/L lactose
Type of cultivation	Batch	Batch
Cultivation condition	Shake flask	Shake flask
Medium volume	1 L	1 L
Temperature	37 °C	37 °C
Agitation	250 rpm	250 rpm
Total fermentation time	5 hours	5 hours
Enzyme activity (U/mL)	1.55	19.34
Biomass production (g/L)	18.0	44.0
Cost of inducer	9.5 €	0.7 €

The cost for IPTG and lactose was estimated by considering that the medium volume was 1 L.

Figures

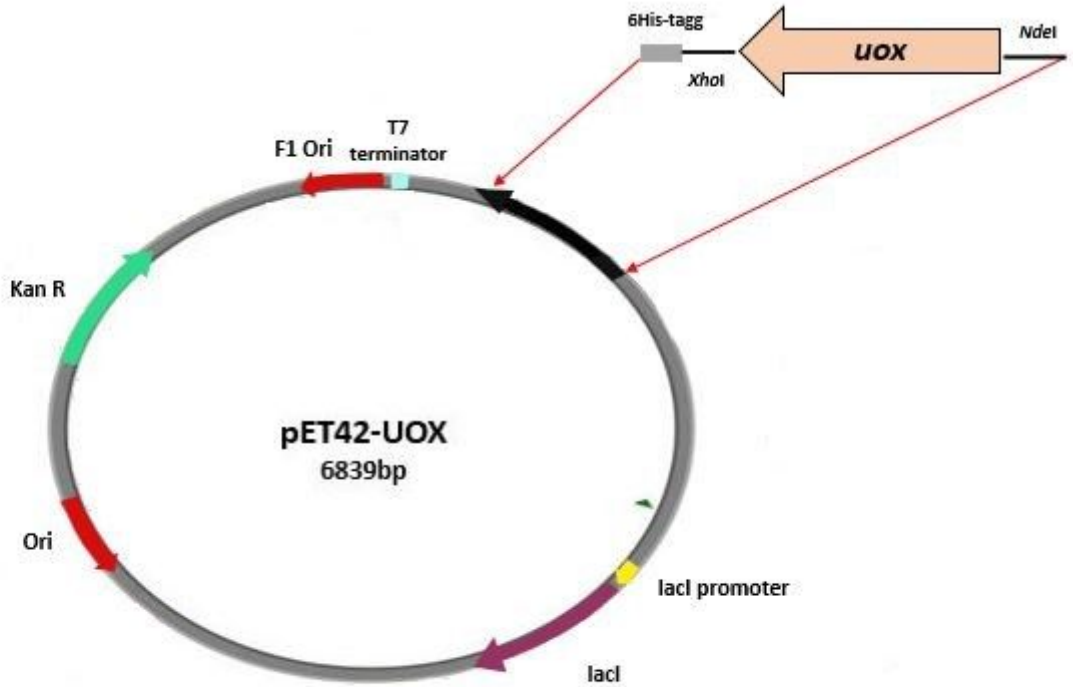

Figure 1

Physical map of recombinant plasmid pET42-UOX.

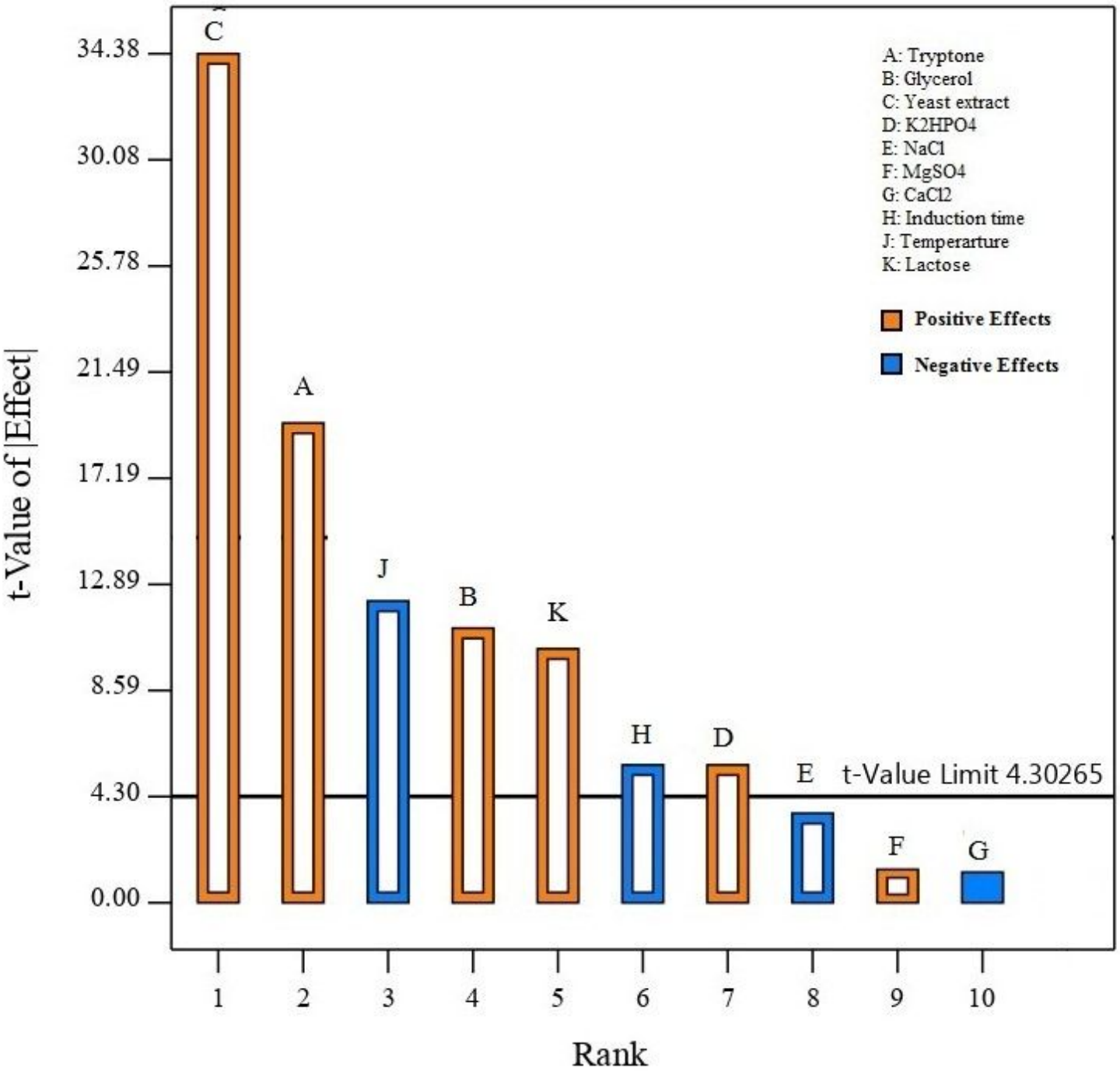

Figure 2

Effect of different conditions on recombinant UOX production in Plackett-Burman experimental design. (Y: main effects, X: variables)

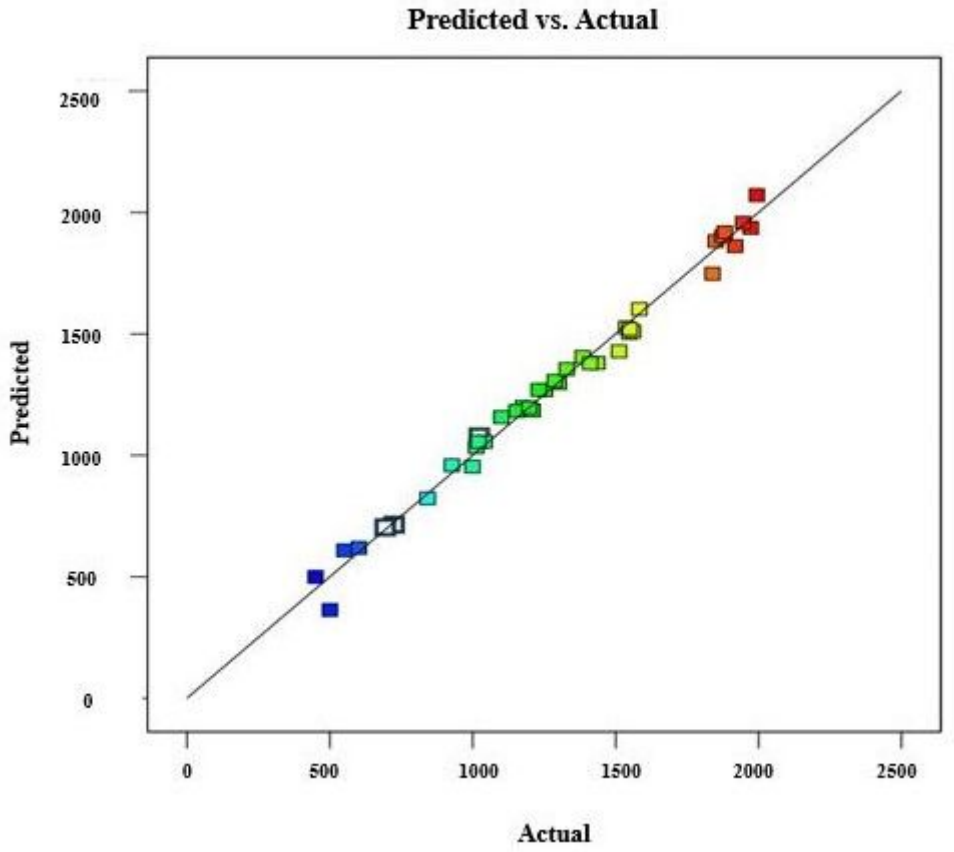

Figure 3

Plots showing correlation of actual conversions and values predicted by the model of UOX production.

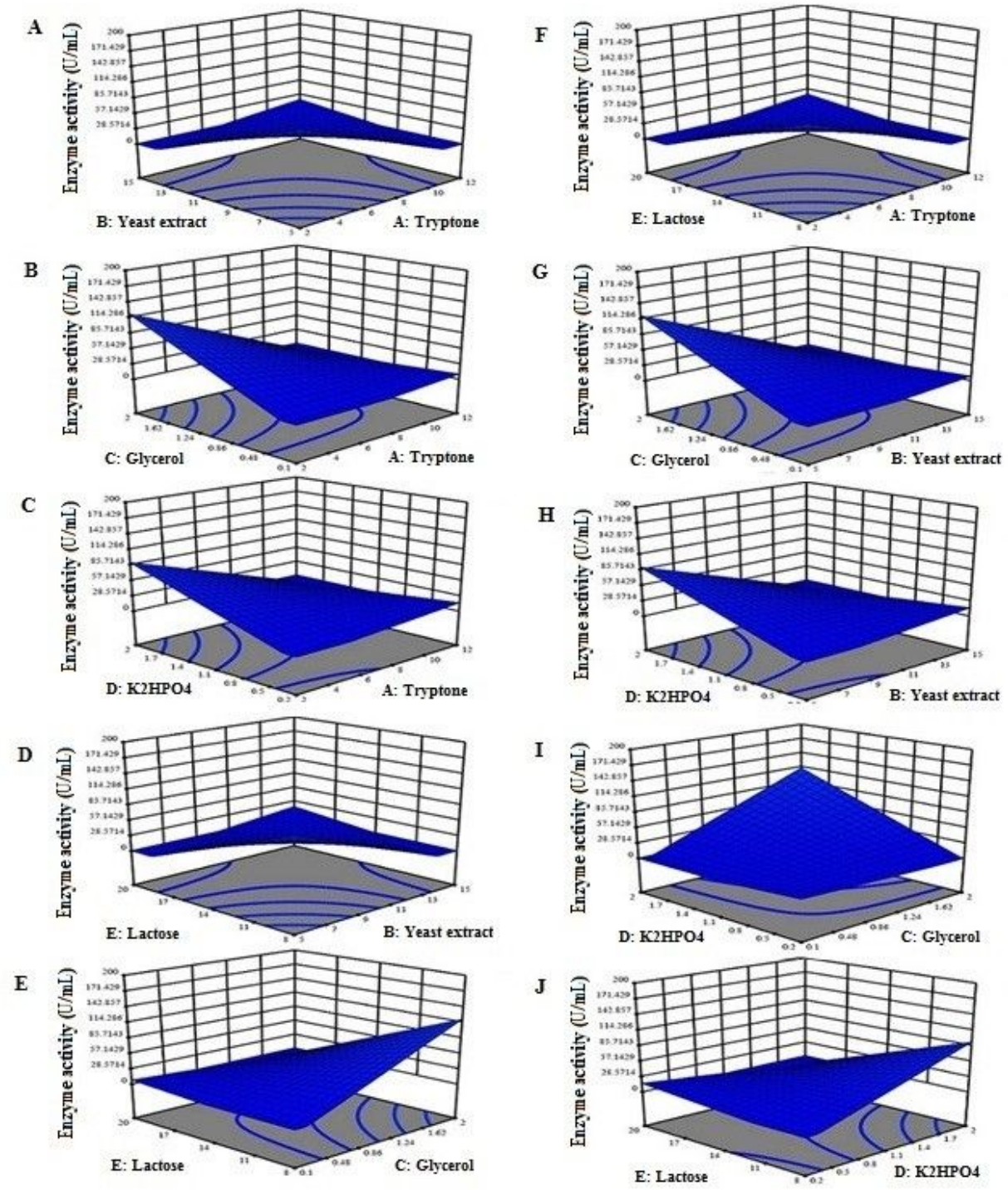

Figure 4

Three-dimensional graphs showing the interaction effects of variables on UOX activity. (A) Effect of tryptone and yeast extract. (B) Effect of glycerol and tryptone. (C) Effect of K2HPO4 and tryptone. (D) Effect of yeast extract and lactose. (E) Effect of glycerol and lactose. (F) Effect of lactose and tryptone. (G) Effect of yeast extract and glycerol. (H) Effect of K2HPO4 and yeast extract. (I) Effect of K2HPO4 and glycerol. (J) Effect of K2HPO4 and lactose. In all plots non-mentioned variables were fixed at zero level.

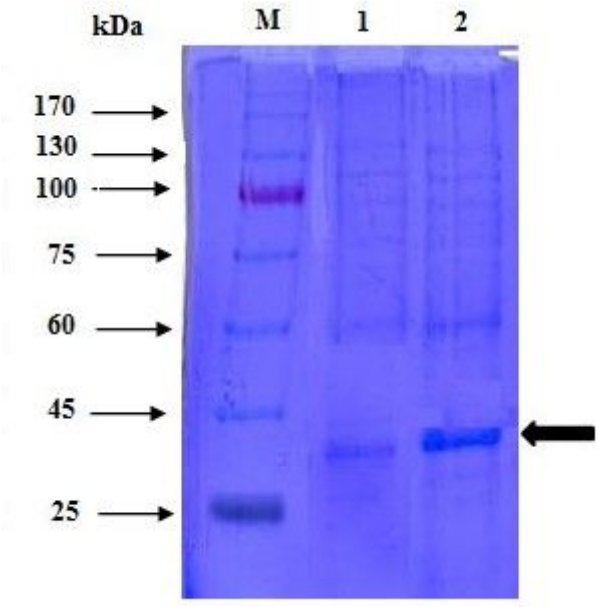

Figure 5

SDS-PAGE profile of recombinant UOX expression analysis. Lane M: Protein molecular weight marker. Lane 1: Whole cell lysate of E. coli producing recombinant UOX before optimization of lactose-inducible expression. Lane 2: Whole cell lysate of E. coli producing recombinant UOX after optimization of lactose-inducible expression. Arrow indicates the UOX protein band.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalabstractUOX.jpg