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Optimization of Glycosyl Aesculin Synthesis by *Thermotoga neapolitana* β-Glucosidase Using Response-surface Methodology

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Glycosyl aesculin, a potent anti-inflammatory agent, was synthesized by transglycosylation reaction, catalyzed by *Thermotoga neapolitana* β-glucosidase, with aesculin as an acceptor. The key reaction parameters were optimized using response-surface methodology (RSM) and 2 μg of the enzyme. As shown by a statistical analysis, a second-order polynomial model fitted well to the data (p<0.05). The response surface curve for the interaction between aesculin and other parameters revealed that the aesculin concentration and reaction time were the primary factors that affected the yield of glycosyl aesculin. Among the tested factors, the optimum values for glycosyl aesculin production were as follows: aesculin concentration of 9.5 g/l, temperature of 84℃, reaction time of 81 min, and pH of 8.2. Under these conditions, 61.7% of glycosyl aesculin was obtained, with a predicted yield of 5.86 g/l. The maximum amount of glycosyl aesculin was 6.02 g/l. Good agreement between the predicted and experimental results confirmed the validity of the RSM. The optimization of reaction conditions by RSM resulted in a 1.6-fold increase in the production of glycosyl aesculin as compared to the yield before optimization. These results indicate that RSM can be effectively used for process optimization in the synthesis of a variety of biologically active glycosides using bacterial glycosidases.

Key words : Aesculin, β-glucosidase, response surface methodology, *Thermotoga neapolitana,* transglycosylation

Introduction

Aesculin, a 6, 7-dihydroxycoumarin-6-*O*-D-β-glucopyranoside, is a coumarin glucoside naturally found in stem barks of *Fraxinus rhynchophyllycosidea* and widely used for the treatment of diarrhea, leukorrhea, and inflammatory diseases such as arthritis and gout [14, 18, 19]. The anti-inflammatory activity of aesculin is attributed to activating nuclear factor-E2-related factor 2 (Nrf2) as well as inhibiting NF-κB [5, 15]. Although aesculin has been reported to show an anti-inflammatory effect, it marginally activates Nrf2 [5]. Glycosylation is one of important tailoring reactions in the synthesis or modification of biologically active compounds. Addition of a sugar moiety to the biologically active compounds by glycosylation can improve their biological activities as well as the low water solubility and low absorption rate in small intestine [6, 7, 9, 20]. The β-glucosidase from hyperthermophilic bacterium *Thermotoga neapolitana* has been successfully used to attach the sugar moiety to the various biologically active compounds and the resultant glycosylated compounds enhanced solubility and biological half-life [4, 8, 16]. The glycosylated aesculin was also synthesized using aesculin as an acceptor by this enzyme and its chemical structure was identified as 3-*O*-β-D-glycosyl aesculin by mass spectrometry and 2-dimensional NMR analysis [5]. Although the glycosylation did not give a benefit to aesculin in terms of solubility and stability, the aesculin conferred the ability to activate Nrf2 after glycosylation, enhancing the anti-inflammatory function [5].

Process optimization has been used to increase the yield and productivities of many bioprocesses. Optimization of the process parameters by classical methods, which requires the change of one variable at a time, is extremely time-consuming because a large number of variables are usually considered. It is also difficult to obtain the valuable information about the combined effect of various factors. In order to solve these difficulties and analyze the interactions between the variables, response surface methodology (RSM) based on factorial central composite design has been em-

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ployed for optimization of the process parameters [3, 12, 13]. Hence in this study, optimization of critical factors for enzymatic synthesis of glycosyl aesculin by the β-glucosidase mutant from *T. neapolitana* was carried out by using RSM. The yield of the glycosylated product was continuously monitored by TLC during the optimization process of reaction conditions.

Materials and Methods

Chemicals, bacterial strain, and growth conditions

Aesculin, cellobiose, maltose, and maltotriose were purchased from Sigma-Aldrich (St. Louis, MO, USA). *E. coli* MC1061 cells, which were used as host for enzyme expression study, were grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml).

Purification of mutant enzyme

The β-glucosidase mutant (N291T) from *T. neapolitana* (BglA) was obtained as previously [2]. *E. coli* cells harboring the p6xHis119 vector containing a mutated *bglA* gene from *Thermotoga neapolitana* was cultured at 37°C in a shaking incubator for 18 h and then harvested by centrifugation at 10,000× g for 30 min at 4°C. The cell pellet was resuspended in 100 mM Tris-HCl buffer (pH 7.5) and disrupted by sonication (Sonifier 450, Branson, Danbury, CT, USA; output 4, 6 times for 30 s, constant duty) on an ice bath. The cell lysate was centrifuged at 16,000× g for 30 min at 4°C to remove the cellular debris. The supernatant was incubated at 80°C for 30 min, and heat-labile proteins of *E. coli* were removed by centrifugation at 16,000× g for 30 min at 4°C. The supernatant was filtered through a 0.4 μm pore-sized filter and then passed through a nickel-NTA resin (Qiagen). The column was washed twice with two volumes of washing buffer [100 mM Tris-HCl buffer (pH 7.5), 10 mM imidazole], and the mutant BglA was eluted with the same buffer containing 250 mM imidazole. Proteins from the eluted fractions were pooled and dialyzed against Tris-HCl buffer (pH 7.5) to remove the excess imidazole, and then concentrated using a Vivaspin 2 concentrator (Sigma-Aldrich).

Transglycosylation reaction

In all experiments, the reaction mixture contained 2 μg of BglA (N291T) enzyme in a 100 mM each buffer, unless otherwise indicated. The aesculin concentration (1.7-15.3 g/l), reaction temperature (70-90°C), reaction pH (5-9), and reaction time (0-120 min) were varied in accordance to the experimental plan. The reaction was quenched by boiling for 10 min. The glycosyl aesculin produced in the reaction mixture was analyzed by TLC. TLC was performed on Whatman K5F silica gel plates using *n*-butanol/ethanol/ water (5:3:2, v/v/v) as the solvent, and the spots were visualized by immersion in a solution containing 0.3% (w/v) *N*-(1-naphtyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol followed by heating at 110°C for 10 min. The quantification of the aesculin and the glycosyl aesculin was evaluated by the density of each spot using ImageJ program (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

Experimental design and data analysis

To optimize the conditions for transglycosylation reaction of aesculin by BglA (N291T) enzyme, Design-Expert 9.0 software (Stat-Ease, Minneapolis, MN, USA) was used in the experimental design and data analysis. Experiments were conducted using a central composite design (CCD) with 30 experimental points, that helps in investigating linear, quadratic, and cross product effects of four factors, each varied at five levels [10, 17]. The assay conditions for reaction parameters were taken at zero level (center point), level one (+1 and -1) and level two (+2 and -2). The design of the experiments employed is presented in Table 1, with both, actual and coded values of factors. For the data evaluation RSM was used, and the experimental data was fitted via the following second-order polynomial equation:

$$Y_i = \beta_o + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_i j X_i X_j$$

where Y_i is the predictive response, X_iX_j the independent variables, β_0, β_i, β_{ii}, β_{ij} are regression coefficient of the applied equation. The independent variables were coded as X_1, X_2, X_3, and X_4. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The significance of the model equation and model terms was evaluated by Fisher's test [1]. The fitted polynomial equation was expressed as three-dimensional response surface curves showing the effect of interactions between the responses and the experimental levels of each variable used in this study.

Results and Discussion

The β-glucosidase mutant (N291T) from *T. neapolitana* has been previously reported as an efficient catalyst in trans-

Table 1. Experimental design with experimental yields of glycosyl aesculin production

Standard order	Aesculin (g/l) (X_1)	Time (min) (X_2)	Temperature (°C) (X_3)	pH (X_4)	Experimental yield (%)
1	-1 (5.1)	-1 (30)	-1 (70)	-1 (5)	12.27
2	1 (11.9)	-1 (30)	-1 (70)	-1 (5)	9.57
3	-1 (5.1)	1 (90)	-1 (70)	-1 (5)	14.08
4	1 (11.9)	1 (90)	-1 (70)	-1 (5)	20.68
5	-1 (5.1)	-1 (30)	1 (90)	-1 (5)	13.38
6	1 (11.9)	-1 (30)	1 (90)	-1 (5)	20.56
7	-1 (5.1)	1 (90)	1 (90)	-1 (5)	27.70
8	1 (11.9)	1 (90)	1 (90)	-1 (5)	47.95
9	-1 (5.1)	-1 (30)	-1 (70)	1 (9)	13.97
10	1 (11.9)	-1 (30)	-1 (70)	1 (9)	23.65
11	-1 (5.1)	1 (90)	-1 (70)	1 (9)	20.33
12	1 (11.9)	1 (90)	-1 (70)	1 (9)	32.74
13	-1 (5.1)	-1 (30)	1 (90)	1 (9)	22.92
14	1 (11.9)	-1 (30)	1 (90)	1 (9)	33.52
15	-1 (5.1)	1 (90)	1 (90)	1 (9)	30.67
16	1 (11.9)	1 (90)	1 (90)	1 (9)	56.72
17	-2 (1.7)	0 (60)	0 (80)	0 (7)	3.68
18	2 (15.3)	0 (60)	0 (80)	0 (7)	35.79
19	0 (8.5)	-2 (0)	0 (80)	0 (7)	0
20	0 (8.5)	2 (120)	0 (80)	0 (7)	78.12
21	0 (8.5)	0 (60)	-2 (60)	0 (7)	47.90
22	0 (8.5)	0 (60)	2 (100)	0 (7)	44.57
23	0 (8.5)	0 (60)	0 (80)	-2 (3)	43.86
24	0 (8.5)	0 (60)	0 (80)	2 (11)	66.63
25	0 (8.5)	0 (60)	0 (80)	0 (7)	66.23
26	0 (8.5)	0 (60)	0 (80)	0 (7)	62.42
27	0 (8.5)	0 (60)	0 (80)	0 (7)	63.63
28	0 (8.5)	0 (60)	0 (80)	0 (7)	65.28
29	0 (8.5)	0 (60)	0 (80)	0 (7)	57.86
30	0 (8.5)	0 (60)	0 (80)	0 (7)	54.54

glycosylation reaction [2]. Previously, the glycosyl aesculin was enzymatically synthesized using aesculin and cellobiose as an acceptor and a donor, respectively [5]. The reaction was carried out at the condition of 20 g/l aesculin, 10 g/l cellobiose and 1.8 μg of enzyme in the presence of 100 mM sodium phosphate buffer (pH 7.0) at 80°C. After 1 hr reaction, the reaction product was examined using TLC. Only one major product, glycosyl aesculin, was identified and purified from the reaction mixture by recycling prep HPLC, whose yield was approximately 15% according to the densitometric analysis.

For optimization process, cellobiose supposed to be a donor for transglycosylation reaction was not added in the reaction because glycosyl aesculin was still synthesized without cellobiose. It has been known that cellobiose has an inhibitory effect on most microbial β-glucosidase [11, 21]. In this respect, the use of β-glucosidase without adding cellobiose will have a significant advantage for the enzymatic

Table 2. ANOVA for the fitted quadratic polynomial model of glycosyl aesculin production

Source	Sum of squares	Degree of freedom	Mean square	F value	*p*-value
Model	11230.49	14	802.18	4.60	0.0029[a]
Residual	2616.84	15	174.46		
Lack of fit	2513.26	10	251.33	12.13	0.0065[a]
Pure error	103.58	5	20.72		
Cor total[b]	13847.33	29			

[a]$p<0.05$ Significant
[b]Cor total: corrected total

conversion of aesculin to glycosyl aesculin. It was confirmed that the glucose moiety released from the aesculin by hydrolytic reaction of BglA was transferred to the aesculin to produce the glycosyl aesculin.

Therefore, in order to maximize the yield of the product, the reaction conditions including aesculin concentration (X_1), reaction time (X_2), temperature (X_3), and pH (X_4) were investigated. CCD was used to assess the interactions between factors within a range of -2 to +2 for synthesis of glucosyl aesculin. A total of 30 experiments was conducted utilizing different combinations of four factors (Table 1). RSM was adopted because it determines the optimum values of key experimental factors, as well as explains the relationships between factors. Statistical analysis showed that the proposed second-order polynomial model was adequate for fitting experimental results at the applied significance value (p<0.05). The final empirical model in terms of four factors in coded units was as follows:

$$Y = 61.66 + 6.43X_1 + 10.72X_2 + 4.14X_3 + 4.74X_4 + 2.53X_1X_2 + 2.38X_1X_3 + 1.71X_1X_4 + 2.77X_2X_3 - 0.51X_2X_4 + 0.009X_3X_4 - 12.99X_1^2 - 8.15X_2^2 - 6.36X_3^2 - 4.11X_4^2$$

where X_1 is aesculin, X_2 is reaction time, X_3 is temperature, and X_4 is pH. From ANOVA, p-value (Prob > F) less than 0.05 indicated that the model terms were significant. In this case, X_1, X_2, X_1^2, X_2^2, and X_3^2 were significant model terms. The model F-value was 4.60 and the F-value for lack of fit was 12.13(Table 2). The coefficient of determination (R^2) of 0.8110 (a value > 0.75 indicates fitness of the model) showed reasonably a good agreement with the adjusted R^2 of 0.6346. The "adequate precision" measures the single to noise ratio. In this model, a ratio was found to be 7.294(a ratio greater than 4 is desirable), which indicated a satisfactory outcome.

The response surface curve for the interaction between aesculin and other parameters are shown in Fig. 1. The experimental yield of glycosyl aesculin varied with changes in X_2, X_3, and X_4. The yield of glycosyl aesculin was decreased at longer reaction time and lower aesculin concen-

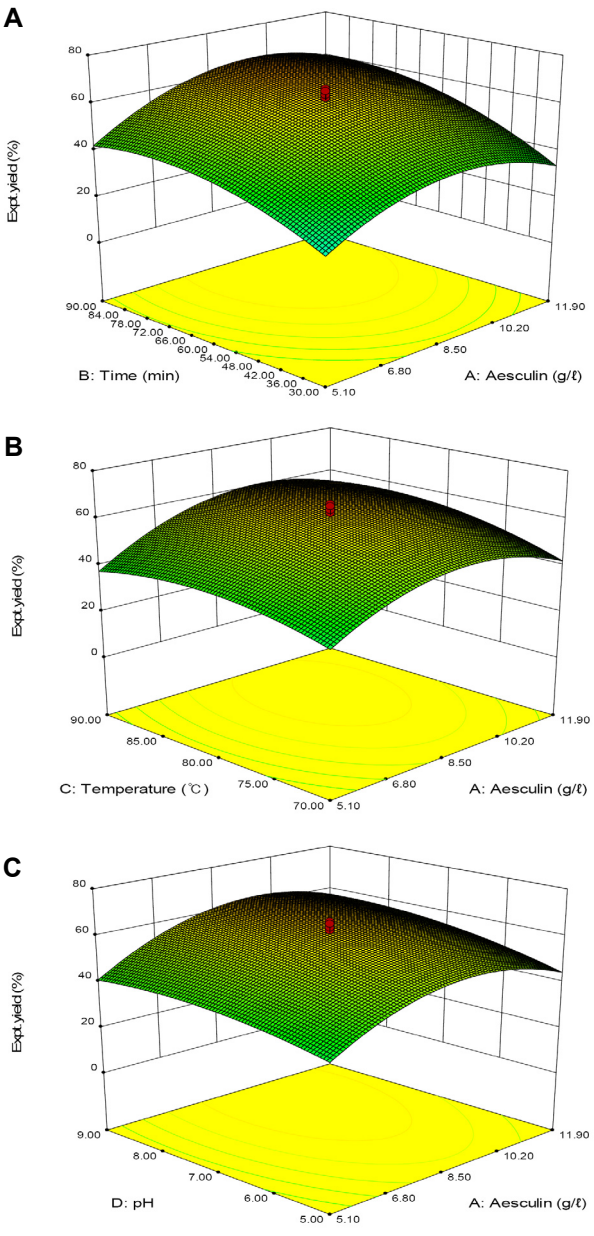

Fig. 1. Three-dimensional response surface curves showing the effect of interactions of (A) aesculin and time, (B) aesculin and temperature, and (C) aesculin and pH for glycosyl aesculin production.

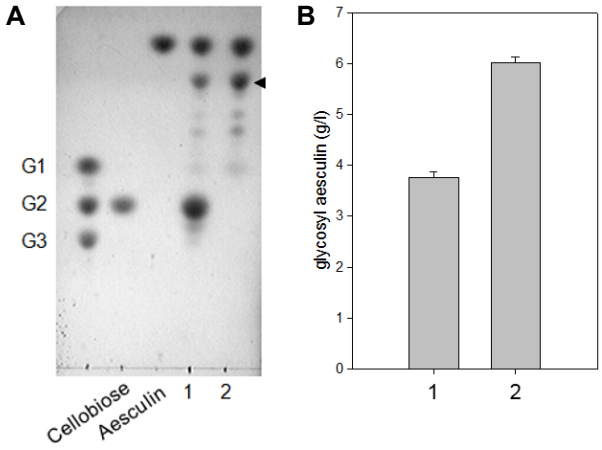

Fig. 2. TLC chromatogram (A) and yield (B) of the glycosyl aesculin by the transglycosylation reaction using the β-glucosidase mutant. Arrow indicates a glycosyl aesculin. 1, before optimization, 2, after optimization with RSM. G1, glucose; G2, maltose; G3, maltotriose.

tration (Fig. 1A). The synthesis of glycosyl aesculin was drastically affected by slight changes in the values of these two factors. The coefficient value from second-order polynomial model also indicated that the aesculin concentration and the reaction time seem to be the influential factors, since the corresponding linear and quadratic coefficients have the high values. Other factors exhibited moderate effects in the product yield. The optimum values were X_1 = 9.5 g/l, X_2 = 81.2 min, X_3 = 84°C, and X_4 = pH 8.16. At these conditions, the yield of maximum glycosyl aesculin was 61.7% with a predicted yield of 5.86 g/l. The actual yield obtained was 6.02 g/l. The 1.6% deviation from the predicted value was observed between the experimental and predicted values, which validate the model. The glycosyl aesculin production before optimization was 3.76 g/l at 1 hr. Thus the optimization of reaction conditions resulted in 1.6-fold increase in the production of glycosyl aesculin over the yield before optimization (Fig. 2). In conclusion, RSM might be effectively applied to the process optimization for the synthesis of a variety of biologically active glycosides using bacterial glycosidases.

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초록 : 반응표면분석법을 이용한 *Thermotoga neapolitana* β-glucosidase의 당전이 활성을 통한 glycosyl aesculin 합성 최적화

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강한 항 염증 활성을 갖는 glycosyl aesculin이 *Thermotoga neapolitana* β-glucosidase의 당전이 활성을 통하여 aesculin을 수용체로 이용하여 합성되었다. 약 2 μg의 효소를 이용하여 반응표면분석법을 통한 주요 반응 매개변수들의 최적화가 시도되었다. 각 반응 변수들의 통계분석 결과 2차 다항식모델이 적용된 유의값(p<0.05)에 잘 맞았다. Aesculin과 다른 매개변수사이의 상호관계를 의미하는 반응표면곡선 그래프는 glycosyl aesculin의 수율이 주로 aesculin의 농도와 반응시간에 영향을 받음을 나타내었다. Glycosyl aesculin의 생산을 위한 반응최적조건은 aesculin의 농도 9.5 g/l, 온도 84°C, 반응시간 81분, 그리고 pH 8.2로 나타났으며, 이러한 조건하에서 효소반응 시 61.7%의 전환율로 5.86 g/l 의 수율이 예상되었다. 실험을 통한 실질적인 수율은 6.02 g/l으로 나타났다. 실질적인 수율과 가까운 값을 예측 가능하다는 것을 통하여 반응표면분석법이 효소반응의 전환율을 최적화하는데 타당하다는 것이 입증되었다. 본 연구에서는 반응표면분석법을 활용하여 최적화 이전에 비하여 약 1.6배의 glycosyl aesculin을 얻을 수 있었다. 이러한 결과들은 반응표면분석법이 미생물유래 당화효소를 이용한 생물학적 활성을 갖는 배당체 합성의 생산 최적화에 효과적으로 활용할 수 있다는 것을 보여준다.