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Optimization of theaflavin biosynthesis from tea polyphenols using an immobilized enzyme system and response surface methodology

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Abstract

Theaflavins were synthesized from tea polyphenols extracted from green tea using an immobilized polyphenol oxidase system. To optimize the production of theaflavins, response surface methodology was applied to determine the effects of five critical variables and their mutual interactions on theaflavin biosynthesis at five levels. A total of 52 individual experiments were performed and a statistical model predicted that the highest theaflavin concentration was 0.766 mg ml^{-1} at optimized conditions. Using these optimal parameters under experimental conditions in three independent replicates, the average value of the biosynthesized theaflavin concentration reached 0.75 $\pm$ 0.017 mg ml^{-1} and matched the value predicted by the model.

Introduction

Tea is a popular beverage all over the world. The growing evidence of the potential health benefits of tea has prompted a large number of investigations on tea, its major components and their biological activities. The overall results indicate that both green and black tea have significant anti-mutagenic and anti-clastogenic effects (Gupta *et al.* 2002, Ganguly 2003). Theaflavins are a major group of polyphenol compounds in black tea and have strong antioxidant, anticancer and other bioactive properties (Ganguly 2003, Huang & Xu 2004, Tu *et al.* 2004). They are formed from the oxidation of tea polyphenols by polyphenol oxidase during black tea processing.

There were some reports on the synthesis of theaflavins from tea polyphenols. Robertson (1983a,b) reported using an *in vitro* model fermentation system containing purified catechins and partially purified polyphenol oxidase from green tea shoots to form theaflavin and thearubigin and using the model system to assess the reaction conditions and to improve black tea quality. Sang *et al.* (2004) reported using enzymatic coupling (horseradish peroxidase/H_2O_2) of selected pairs of compounds to prepare theaflavin derivatives based on a benzotropolone skeleton. Li & Xiao (2002) described a bi-liquid phase system to prepare tea pigments from the oxidation of tea polyphenols. These reports provided useful information on theaflavin biosynthesis despite of their different experimental purposes. Although some progress has been achieved, so far there are few papers on theaflavin biosynthesis from tea polyphenols, such as the use of immobilized enzyme systems and response surface methodology for optimization of production, that would provide useful information for scale-up or commercial scale production of theaflavins.

Immobilized enzyme system has the advantages for multiple and effective uses of enzyme, even distribution in substrate solution and increasing reaction speed, easy handling and separation from the substrates, which is suitable for commercial scale applications. Stability of an immobilized enzyme is an important factor for effective utilization of the enzyme. There will be much more commercial benefits only when high stable immobilized enzyme system has been achieved.

Response surface methodology has long been used to solve process optimization problems in the fields of chemical engineering and agro-biotechnology. It can be defined as a statistical method that uses quantitative data from appropriate experimental designs to determine and simultaneously solve multivariate equations and to determine the optimum combination of factors that yield a desired response near the optimum. It also shows how a specific response is affected by changes in the level of the factors over the specified levels of interest.

The objective of this study was to use an immobilized polyphenol oxidase system in combination of response surface methodology to optimize the conditions for theaflavin biosynthesis, which may provide useful information on a low-cost but efficient way for theaflavin biosynthesis using inexpensive source of polyphenol extracts from green tea and a stable immobilized polyphenol oxidase system.

Materials and methods

Tea polyphenols

Samples of tea polyphenols (TP95) as spray-dried powder were obtained from Hainan Groupforce Pharmaceutical Co., Ltd. (Hainan, P.R. China). They were extracted from green tea and purified, with 95% purity and containing more than 40% epigallocatechin gallate (EGCG) and less than 1% caffeine. Authentic theaflavin standards were purchased from Sigma.

Preparation of polyphenol oxidase and immobilized enzyme plate

Fresh new shoots of tea plant were plucked from tea trees and stored overnight at −20 °C. The frozen leaves were placed in cold 80% (v/v) acetone and then homogenized with a blender at high speed for 5 min. The suspension was vacuum filtered through a filter paper. The enzyme powder was washed repeatedly with cold 80% (v/v) acetone until colourless and then washed a few times with 100% acetone. The dried enzyme powder was stored at −20 °C until using. The enzyme powder (10 g) was mixed with 10 g polyvinyl polypyrolidone, 2 g sea sand and 100 ml cold 0.1 M citrate buffer (pH 5.6) containing 0.2 M potassium phosphate buffer, 0.35 M KCl and 0.5% Triton-X 100 using a mortar. The mixture was transferred to a container and agitated for 10 h at 10 °C and then centrifuged at 10 000 *g* for 10 min to remove the residues. The resulting supernatant was the crude extract of polyphenol oxidase for immobilization.

Polyphenol oxidase was immobilized according to a patented method (Tu & Xia 2004). In brief, the crude extract of polyphenol oxidase (75 ml) was mixed with 100 ml 2% (v/v) sodium alginate solution and stirred for 5 min. A microporous metal plate was placed into this liquid polymer–enzyme mixture for 10 min and then put into 0.1 M $CaCl_2$ for 30 min to form a membrane at 4 °C. Finally, the plate was cross-linked in a 0.025% glutaraldehyde aqueous solution for 1 h. The immobilized polyphenol oxidase plate was used straightaway or stored in a 0.1 M citrate buffer (pH 5.6) and at 4 °C for future use.

Assay of the immobilized enzyme activity

The immobilized polyphenol oxidase plate was incubated at 37 °C in a reaction mixture containing 0.1 M citrate/Na_2HPO_4 buffer (pH 5.6), 0.1% praline and 1% catechol at a ratio of 10:2:3. The catechol oxidation was monitored by measuring the absorbance of the mixture at 415 nm. One unit of enzyme activity was defined as the change of 0.1 unit absorbancy unit per min.

Stability of the immobilized enzyme

The stability of the immobilized polyphenol oxidase was tested by measuring the enzyme activity and the formed yield of theaflavin at various times of usage (Table 1). Its activity and the formed yield of theaflavin were also measured on different storage days of the immobilized polyphenol

Table 1. The enzyme activity and the formed yield of theaflavin at different used times of the immobilized polyphenol oxidase.

Time of usage	Yield of theaflavin (mg ml^{-1})	Activity (Unit g^{-1})
1	0.784	48
5	0.781	47.8
10	0.777	47.6
15	0.761	46.6
20	0.754	46.2
25	0.733	44.9
30	0.753	46.1
35	0.663	40.6
45	0.681	41.7
50	0.696	42.6
55	0.666	40.8
60	0.677	41.5
65	0.653	40
70	0.642	39.3
75	0.637	39
80	0.628	38.5

Table 2. The enzyme activity and the yield of theaflavin formed on different storage days of the immobilized polyphenol oxidase.

Storage days	Activity (unit g^{-1})	Yield of theaflavin (mg ml^{-1})
0	48	0.783
15	45	0.741
30	42	0.686
45	38	0.657
60	37	0.593
75	35	0.563

oxidase when it was stored in 0.1 M citrate buffer (pH 5.6) and at 4 °C (Table 2).

Theaflavin biosynthesis

The immobilized polyphenol oxidase plate was placed into aqueous tea polyphenol solution and incubated at 37 °C for biosynthesis of theaflavins. The tea polyphenol concentrations tested were from 1 to 9 mg ml^{-1}. The pH range was between 2.8 and 6.0. Aeration volumes used were from 0 to 40 l min^{-1} by an air pump with a volume controller. The reaction time was between 11 and 59 min. The ratio of immobilized enzyme to the substrate (E/S) was from 4.9 to 196. The reacted polyphenol solution was extracted with an equal volume of ethyl acetate. Theaflavin was concentrated by evaporation of ethyl acetate from the extract. The concentrated theaflavins were dissolved in distilled water and analysed by HPLC.

Theaflavin analysis

The synthesized theaflavin mixture was analysed on a Diamonsil C18 column (4.6 × 250 mm, 5 μm particle size, Japan) using a Shimadzu LC-2010 HPLC (Shimadzu, Japan). The eluate was monitored at 280 nm. Mobile phase A and B were made of acetic acid/acetonitrile/water (A: 0.5:3:96.5 and B: 0.5:30:69.5, by vol.). The flow rate was set at 1 ml min^{-1} and 10 μl sample was injected into the column. The column was equilibrated initially with 100% A, and then eluted with a gradient up to 100% B in 45 min. The column temperature was set to 35 °C. Peaks were identified by comparison with the retention time of authentic standards of theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3-3′-digallate. Concentrations of the individual theaflavin were calculated using corresponding standard curves.

Experimental design and statistical analysis

Response surface methodology was applied to determine the optimum conditions for the production of theaflavins using an immobilized polyphenol oxidase plate. Literature data and results of our preliminary trials were taken into account for selection of the number and range of process variables in the experimental design. A five-level, five-variable, and central composite, uniform precision design was chosen for this experimental design using the SAS System for Windows software (SAS Institute Inc., Cary, NC). The five independent variables were pH, aeration volume (l min^{-1}), tea polyphenol concentration (mg ml^{-1}), reaction time (min), and the ratio of immobilized enzyme to the substrate. Coded levels for independent variables are presented in Table 3. The experimental design for a five-level and five-factor scheme with 52 treatments in total is shown in Table 4. The response surface values are the concentrations of resulting theaflavin production which are also shown in Table 4. SAS software was used for the experimental design and data analysis.

Table 3. Experimental factors and coded levels of the independent variables.

Level	Factor				
	Reaction time (min)	E/S (AU ml^{-1})	Aeration volume ($l\ min^{-1}$)	Tea polyphenol concentration ($mg\ ml^{-1}$)	pH
−2.38	11.2	4.86	0	1	2.8
−1	25	60	10	3.32	3.73
0	35	100	20	5	4.4
1	45	140	30	6.68	5.07
2.38	58.8	195.14	40	9	6

Table 4. RSA structural matrix and experimental results for theaflavin production.

Treatment	Factor					Theaflavin Y ($mg\ ml^{-1}$)
	X_t	X_e	X_a	X_c	X_p	
1	−1	−1	−1	−1	−1	0.2189
2	−1	−1	−1	−1	1	0.2123
3	−1	−1	−1	1	−1	0.43
4	−1	−1	−1	1	1	0.2458
5	−1	−1	1	−1	−1	0.4038
6	−1	−1	1	−1	1	0.329
7	−1	−1	1	1	−1	0.3222
8	−1	−1	1	1	1	0.2941
9	−1	1	−1	−1	−1	0.3644
10	−1	1	−1	−1	1	0.4733
11	−1	1	−1	1	−1	0.5046
12	−1	1	−1	1	1	0.259
13	−1	1	1	−1	−1	0.5204
14	−1	1	1	−1	1	0.4962
15	−1	1	1	1	−1	0.4478
16	−1	1	1	1	1	0.3647
17	1	−1	−1	−1	−1	0.4044
18	1	−1	−1	−1	1	0.3766
19	1	−1	−1	1	−1	0.5194
20	1	−1	−1	1	1	0.4526
21	1	−1	1	−1	−1	0.4785
22	1	−1	1	−1	1	0.5166
23	1	−1	1	1	−1	0.5242
24	1	−1	1	1	1	0.6121
25	1	1	−1	−1	−1	0.5083
26	1	1	−1	−1	1	0.4786
27	1	1	−1	1	−1	0.6587
28	1	1	−1	1	1	0.4878
29	1	1	1	−1	−1	0.5238
30	1	1	1	−1	1	0.7019
31	1	1	1	1	−1	0.7311
32	1	1	1	1	1	0.6587
33	−2.38	0	0	0	0	0.2654
34	2.38	0	0	0	0	0.6201
35	0	−2.38	0	0	0	0.3229
36	0	2.38	0	0	0	0.5957
37	0	0	−2.38	0	0	0.0833
38	0	0	2.38	0	0	0.5269
39	0	0	0	−2.38	0	0.2237
40	0	0	0	2.38	0	0.5419
41	0	0	0	0	−2.38	0.5163
42	0	0	0	0	2.38	0.4608
43	0	0	0	0	0	0.6041
44	0	0	0	0	0	0.7206
45	0	0	0	0	0	0.6348
46	0	0	0	0	0	0.615
47	0	0	0	0	0	0.7207
48	0	0	0	0	0	0.6324
49	0	0	0	0	0	0.659
50	0	0	0	0	0	0.7267
51	0	0	0	0	0	0.6545
52	0	0	0	0	0	0.6956

Results and discussion

The stability of the immobilized enzyme is an important parameter for commercial production of theaflavin. The results listed in Tables 1 and 2 show that the immobilized polyphenol oxidase was stable over a long period of storage and after many times of use for theaflavin biosynthesis. After 80 times of usage, the enzyme still had 80% of its original activity. After 75 d storage in 0.1 M citrate buffer (pH 5.6) and at 4 °C, the enzyme had 73% of its original activity. These results indicate that the patented method (Tu & Xia 2004) is effective for achieving high stability of the immobilized polyphenol oxidase system.

Response Surface Analysis (RSM) uses multiple regression and correlation analysis as tools to examine the influence of two or more independent variables on the dependent variable. All the 52 designed experiments were performed and the resulting data were multiregression analysed. Coefficients of a full model were evaluated by regression analysis and tested for their significance. The insignificant coefficients were eliminated on

Table 5. Analysis of variables in the RSM model.

Factor	Degree of freedom	Sum of squares	S^2	F value	$p > F$
X_t	6	0.403774	0.067296	14.82	< 0.0001
X_a	6	0.371943	0.061991	13.66	< 0.0001
X_c	6	0.222831	0.037138	8.18	< 0.0001
X_e	6	0.211114	0.035186	7.75	< 0.0001
X_p	6	0.103249	0.017208	3.79	0.006

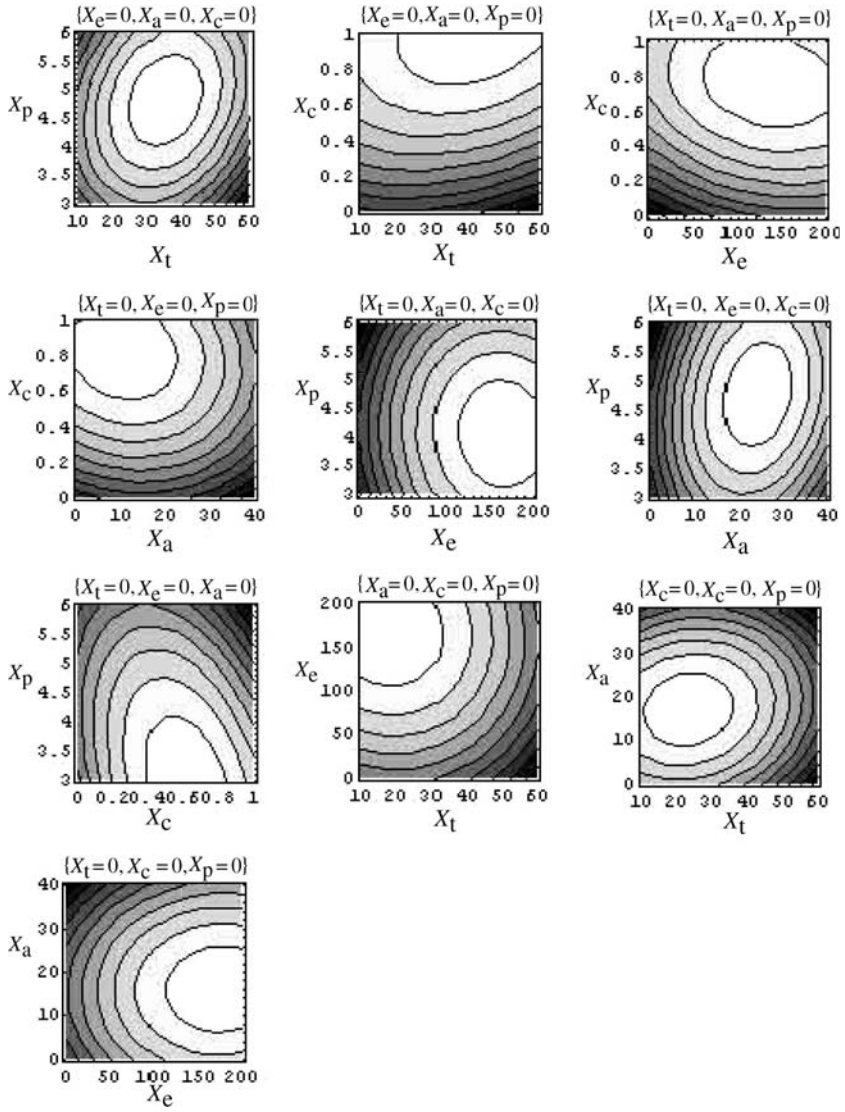

Fig. 1. The contour maps of RSM show the interaction of two variables in the enzyme-catalyzed reaction of theaflavin biosynthesis.

the basis of p values after examining the coefficients, and the model was finally refined. It was observed that four linear coefficients (X_e, X_a, X_c, and X_p), all the quadratic coefficients and two cross-product coefficients (X_cX_t and X_pX_c) were significant. The final response model to predict the synthetic theaflavin concentration after eliminating the insignificant terms was as follow:

$$Y = -2.2482 + 0.00701X_e + 0.025166X_a + 2.792401X_c + 505665X_p - 0.000349X_t^2 - 0.000020298X_e^2 - 0.000819X_a^2 + 0.014974X_cX_t - 1.626765X_c^2 - 0.255562X_pX_c - 0.060351X_p^2,$$

where Y is the response variable, theaflavin concentration (mg ml^{-1}). X_t, X_e, X_a, X_c, and X_p, are the values of the independent variables, reaction time (min), the ratio of E/S, aeration volume (l min^{-1}), tea polyphenol concentration (mg ml^{-1}), and pH, respectively.

The coefficient of determination in a multiple regression equation measures the strength of the relationship between the independent variables and the dependent variables. The value of the determination coefficient for the equation is $r^2 = 0.9038$, suggests that only less than 10% of the total variations are not explained by the model. The value of r ($= 0.9507$) for the above equation being closed to 1 indicates that a good correlation between the independent and dependent variables. From this model, the synthetic theaflavin concentration was a function of reaction time, aeration volume, the substrate concentration, the ratio of E/S, and pH in the range of variables tested ($p < 0.0001$).

Table 5 shows the effect of a single variable on the formation of theaflavin. Variables X_t and X_a had a much greater effect on F values, indicating that the reaction time and aeration volume are more important parameters, while pH has less effect on the biosynthesis of theaflavins.

The contour maps of RSM are shown in Figure 1, in which the levels of two factors were changed while the other factors were kept constant. There were strong interactions between the variables. Each contour plot of the 11 maps had a central point, indicating that the experimental parameters were in the optimal range and the optimum experimental parameters for biosynthesis of theaflavin from tea polyphenols using an immobilized enzyme system were identified. The optimal conditions for theaflavin production predicted by the equation were: $X_t = 49$ min, $X_e = 12.81$, $X_a = 23.81$ l min^{-1}, $X_c = 5.95$ mg ml^{-1} and $X_p = 4.3$. The theoretical theaflavin concentration predicted under these conditions was $Y = 0.766$ mg ml^{-1}. To confirm the prediction by the model, the optimal conditions were applied to three independent replicates for theaflavin production. The average value of the synthesized theaflavin concentration reached 0.75 ± 0.017 mg ml^{-1} and matched well with the value predicted by the model, demonstrating that response surface methodology with appropriate experimental design can be effectively used to optimize the process parameters in complex biotechnological processes.

This study benefited from the use of response surface methodology to optimize the conditions for theaflavin production and an immobilized polyphenol oxidase system. It may provide useful information on a low-cost but efficient way of theaflavins biosynthesis using inexpensive source of tea polyphenols and a highly stable immobilized enzyme in commercial scale.

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