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Research Article

Optimisation of Immobilisation Conditions of β-galactosidase onto Chitosan Beads Using Response Surface Methodology

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Abstract: Response Surface Methodology (RSM) was advantageously used to optimize production conditions of immobilization of β-galactosidase onto chitosan beads. The influence of immobilization conditions on the immobilized enzyme activity was studied by using Box–Behnken experimental design, resulting in the appropriate average values of OD_{420} and the maximum value of immobilized enzyme activity. This illustrated that the experimental model was reliable and the experimental results were of good stability. Analysis of variance was performed to determine the adequacy and significance of the quadratic model. Various model parameters also could be seen from F value that the interaction between three factors was not particularly obvious, but effects of X_1, X_1^2, X_2^2 and X_3^2 on the activity of immobilized enzyme were very obvious. The optimisation parameters studied in accordance with the results were: the appropriate average values of OD_{420} loaded in the range from 0.713 to 0.721. Furthermore the immobilized enzyme activity reached about 162U/g when the adsorption time, adsorption pH and cross-linking pH were 4h, 7 and 7, respectively. Thus, the relative activity of β-galactosidase immobilized increased more than 37% compared with the previous single factor research.

Keywords: β-galactosidase, chitosan beads, immobilization, response surface methodology

INTRODUCTION

Lactose intolerance is a clinical syndrome that can cause lactose maldigestion, the symptoms of the disease are diarrhea, pain, nausea and flatulence, this will lead to lots of people to avoid milk or milk products (Devesh and Arvind, 2012; Kocin, 1988) due to the inability to digest lactose into its constituents, glucose and galactose, because of low levels of lactase (Valentina *et al*., 2011). Thus, it is necessary to remove the lactose through the use of enzymes. One of the more common ways to accomplish this is through the use of enzymes.

β-Galactosidase (EC 3.2.1.23) was selected for immobilization (Emese *et al*., 2008) and it is one of the most frequently used enzymes in the dairy industry that it hydrolyses lactose (Qiuyun *et al*., 2011; Pedro and Francisco, 2012), catalyses the conversion of lactose into glucose and galactose (Yoon *et al*., 2012; Kim *et al*., 2001; Puri *et al*., 2010). This enzyme is broadly used to prepare lactose-hydrolysed products for lactose-intolerant or lactase-deficient people (Carpio *et al*., 2000).

Response surface methodology (RSM) is one of the popular statistical methods (Neelesh *et al*., 2014) commonly applied for optimization of immobilization of enzymes and cells (Piyushkumar *et al*., 2007; Park and Chang, 2000; Ariga *et al*., 1997). This method involves various statistical and mathematical techniques based on the multivariate non-linear model that has been widely used method of modeling and analyzing the relationships between several independent variables and response variable(s) (Neelesh *et al*., 2014; Alessandro *et al*., 2013; Piyushkumar *et al*., 2007).

Chitosan and its derivatives are known as a swollen bead support for preparation of immobilized enzyme (Juang *et al*., 2002; Muzzarelli, 1980). Chitosan has very good biocompatibility, low toxicity, chemically inert and high hydrophilicity (Alka and Arvind, 2009).

As far as can be ascertained, the present literatures mainly contain studies relating to the optimisation of immobilization of β-galactosidase onto epoxy-activated (Pedro and Francisco, 2012), alkylamine glass (Devesh and Arvind, 2012), Sephadex (Alka and Arvind, 2009), even cell immobilization (Piyushkumar *et al*., 2007) and so on. Unfortunately, the main disadvantages do not like particularly high immobilization efficiency and heavy leakage of enzyme have not been desirably improved (Qiuyun *et al*., 2011).

The present work reported that various factors implicated in immobilization of β-galactosidase onto chitosan beads were optimized using the response

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surface methodology. Furthermore, a comparison study involving the values of OD_{420} and immobilized enzyme activity in the previous single factor research (He *et al*., 2013) were carried out. Finally, optimum production conditions were determined when higher OD_{420} value and immobilized enzyme activity.

MATERIALS AND METHODS

Materials: Glutaraldehyde was purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. Chitesan, O-Nitrophenyl-β-d-Galactopyranoside (ONPG) and O-Nitrophenol (ONP) were obtained from X`A Luosenbo Technology. Co., Ltd. The β-galactosidase was purchased from Harbin Meihua Biological Technology Co., Ltd.

Preparation of chitosan beads: With 20 g/L acetic acid solution dissolved 30 g/L chitosan. Using 1mL medical needle tube added chitosan into 1mol/L NaOH solution, the chitosan beads were rinsed several times until neutral with distilled water after coagulating. Finally, the chitosan beads were filtered and air-dried (Adriano *et al*., 2008).

Preparation of immobilized lactase: The β-galactosidase was immobilized on chitosan beads by using glutaraldehyde. Added 1 g chitosan beads into 10 mL 0.5% glutaraldehyde and cross-linked at 25°C for 1 h, then washed chitosan beads with distilled water until there was no residual glutaraldehyde solution. Chitosan beads were placed into 10 mL 1g/mL lactase solution and soaked at 25°C for 2 h and then washed the immobilized enzyme with distilled water and filtered by suction until enzyme activity could not be detected in the distilled water, finally the immobilized lactase activity was measured.

Enzyme activity assays: Using ONPG as substrate, the activity of β-galactosidase could be assayed by colorimetric test. The β-galactosidase can catalyze ONPG to ONP and galactose, The ONP in alkaline medium was yellow, which has the absorbance value at the wavelength of 420 nm in the solution. A standard curve was constructed by using ONP at various concentrations (Cavaille and Combes, 1995).

Dissolved 100 mg β-galactosidase into phosphate buffer (pH 6.5) and diluted to 100mLwith distilled water to prepare enzyme solution. Then 1mL the enzyme solution was diluted by using 100mLphosphate buffer (pH 6.5). The 3 mL ONPG solution prepared with phosphate buffer (4 mg/mL) was added to test tube and set at 38°C for 7 min, the ONPG solution was well mixed with the 1mLdiluted enzyme solution and kept at 38°C for 10 min. Added 2 mL1 mol/ L Na_2CO_3 solution to terminate the reaction, then the absorbance value was measured at 420 nm.

Under the measurement conditions (38°C, pH6.5, for 10 min), an enzyme activity Unit (U) was the amount of enzyme that catalyses to generate 1μmol ONP per min under standard assay conditions. The immobilized enzyme activity was measured by the same method, However, we had to consider the immobilized protein content (He *et al*., 2013).

Determination of immobilization parameters: "Immobilized lactase activity" was defined as the unit of the amount of immobilized lactase was required when 1 μg ONP was generated per hour under the conditions (38°C, pH 6.5, reaction time of 20 min). This was calculated as:

$$Y(U/g) = \frac{139.11E_{(OD)}}{1.8436 \times T \times M}$$

where,

Y = The immobilized lactase activity unit
$E_{(OD)}$ = The absorbance value, 1.8436 is the conversion ratio
T(h) = The reaction time
M(g) = The amount of immobilized lactase

RESULTS AND DISCUSSION

According to the results of P-B test and the test results to determine the adsorption time (X_1), the adsorption of pH (X_2) and cross linked pH (X_3), the response surface analysis method regarded the three factors as variables. And the response value was the value of OD_{420} for optimization of component of lactase immobilization; the Y value was the immobilized enzyme activity. Table 1 the factor level coding table of Box-Behnken experiment design. Table 2 the experimental design and results of Box-Behnken test.

Table 1: The factor level coding table of Box-Behnken experiment design

	Adsorption time /h	Adsorption pH	Cross linking pH
	X_1	X_2	X_3
-1	3.7	6.7	6.7
0	4.0	7.0	7.0
1	4.3	7.3	7.3

Table 2: The experimental design and results of Box-Behnken test

RUN	X_1	X_2	X_3	$E_{(OD)}$	Y
1	-1	-1	0	0.257	57.970
2	1	-1	0	0.286	64.740
3	-1	1	0	0.468	105.94
4	1	1	0	0.522	118.16
5	-1	0	-1	0.406	91.900
6	1	0	-1	0.548	124.05
7	-1	0	1	0.508	114.99
8	1	0	1	0.481	108.88
9	0	-1	-1	0.414	93.720
10	0	1	-1	0.517	117.03
11	0	-1	1	0.467	105.71
12	0	1	1	0.488	110.47
13	0	0	0	0.714	161.63
14	0	0	0	0.712	161.17
15	0	0	0	0.711	160.95

Table 3: Analysis of variance for response surface model pertaining to percent immobilization

Source	DF	S.S	Mean square	F-value	P-value
Model	9	0.247728	0.027525	9.578083	0.011405*
X_1	1	0.040755	0.040755	14.1817	0.013078*
X_2	1	0.001740	0.001740	0.605648	0.471635
X_3	1	0.002415	0.002415	0.840399	0.401337
X_1^2	1	0.108810	0.108810	37.86307	0.001649**
X_2^2	1	0.091495	0.091495	31.83796	0.002426**
X_3^3	1	0.017664	0.017664	6.146637	0.055893
X_1*X_2	1	0.000156	0.000156	0.054371	0.824870
X_1*X_3	1	0.001681	0.001681	0.584943	0.478904
X_2*X_3	1	0.007140	0.007140	2.484617	0.175787
X^2	3	0.193840	0.064613	22.48369	0.002485**
Lack of fit	3	0.014364	0.004788	2052.036	0.000487**
Residual	5	0.014369	0.002874	—	—
Pure error	2	4.667E-6	2.333E-6	—	—
Total (corr.)	14	0.262097	—	—	—

R: 0.9722; R^2: 0.9452; R^2_{Adj}: 0.8465; Df: degree of freedom; **p<0.0: *p<0.05

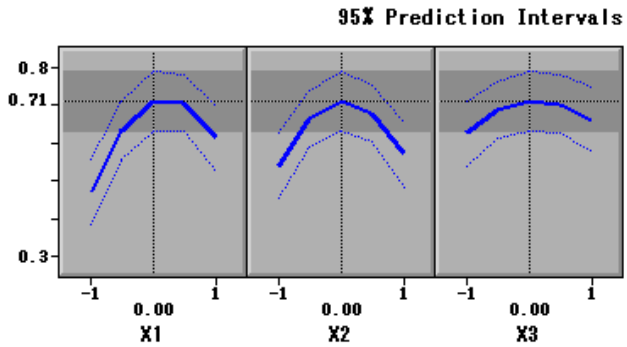

Fig. 1: The variation tendency of activity of immobilized lactase with each factors

As shown in Table 1, three factors were set to three gradients, respectively. And there were more reasonable range between each factor gradient. Analysis of Table 2 experimental data using SAS software was also done to determine the adequacy and significance of quadratic model and then could find the optimal response factor. The response surface model was provided in Table 3. The final equation in terms of actual factors, which governed the response, was as follows:

$$Y = 0.71+0.071X_1+0.015X_2+0.017X_3-0.17X_1^2+0.00625X_1X_2-0.021X_1X_3-0.157X_2^2-0.042X_2X_3-0.069X_3^2$$

As shown in Table 3 regression analysis revealed a coefficient of determination (R-squared) value of 0.9452, indicating that the model was able to explain total variations. Various model parameters also can be seen that the interaction of the F value was very small and the interaction between these 3 factors indicated very little effect. Effects of X_1X_2, X_1X_3, X_2X_3 on the activity of immobilized enzyme were not significant, the interaction between the effects of each other included no statistical significance, but effects of X_1, X_1^2, X_2^2 and X_3^2 on the activity of immobilized enzyme were more obvious.

As Fig. 1 showed variation tendency of each factor and response value of each factor level affect the activity of immobilized lactase. Figure 2 to 4 directly reflected the influence of each factor on the response value. When one factor was under optimal conditions, the relationship between other 2 factors and response value was performed with three-dimensional coordinate graph.

As Fig. 2 showed when the value of cross linking pH was in the center level, the activity of immobilized enzyme would firstly increase and then decrease with prolonging adsorption time and increasing adsorption pH. And as the contour plot displayed that it approximated to roundness, the interaction between each other was not obvious.

As Fig. 3 showed when the value of adsorption pH was in the center level, the activity of immobilized enzyme would firstly increase and then decrease with prolonging adsorption time and increasing cross linked pH. And as the contour plot displayed that it approximated to an oval, the interaction existed between each other, but the effect is not significant.

As Fig. 4 showed when the value of adsorption time was in the center level, the activity of immobilized enzyme would firstly increase and then decrease with prolonging adsorption pH and increasing cross linked

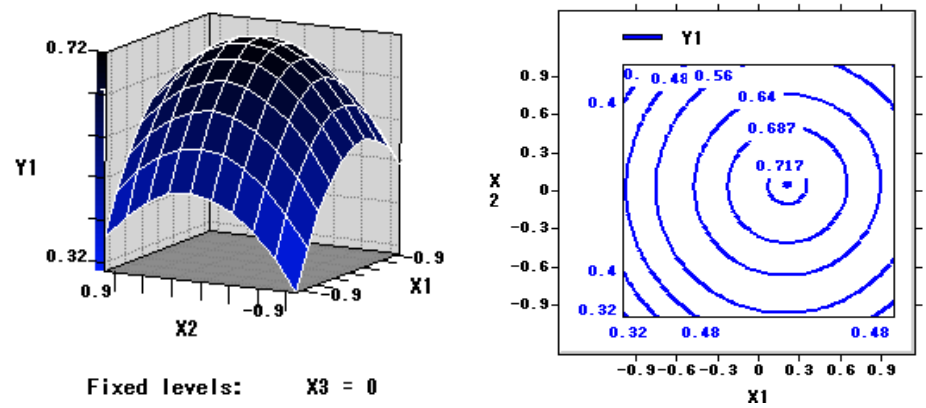

Fig. 2: Response surface plot and contour plot of influence of X_1, X_2 on the response of Y1

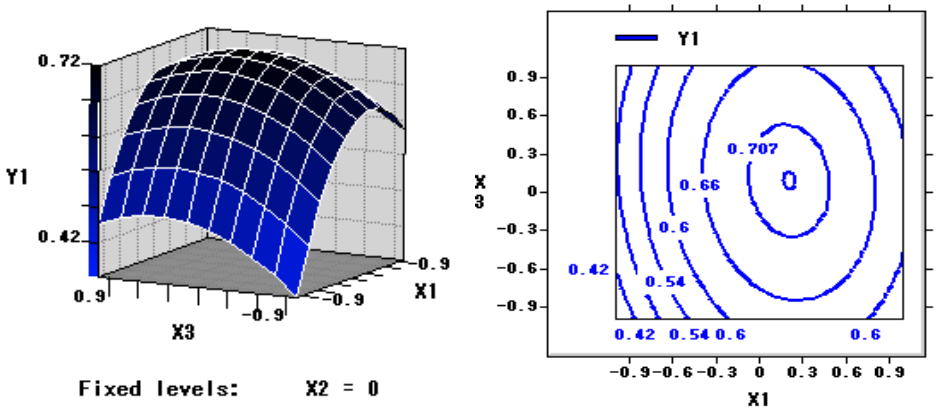

Fig. 3: Response surface plot and contour plot of influence of X_1, X_3 on the response of Y1

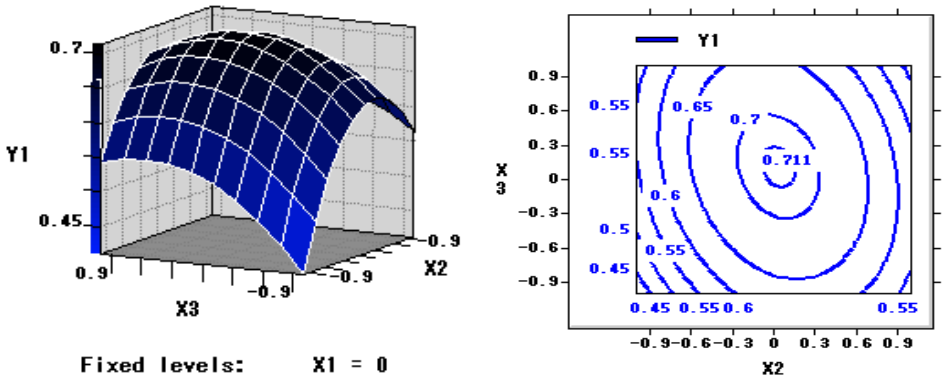

Fig. 4: Response surface plot and contour plot of influence of X_2, X_3 on the response of Y1

pH. And as the contour plot displayed that it approximated to an oval, the interaction existed between each other, but the effect is not significant.

Analysis of regression by SAS software was done to obtain the partial derivatives of X1, X2, X3 respectively and the required partial derivatives were easily worked out. Then the maximum point (0, 0, 0) was worked out and achieved. Under the above conditions prediction of activity of the immobilized lactase would reach 0.712. On second thoughts the repeated experiments were conducted. To be specific, added accurately 1 g chitosan beads into 10 mL 0.3% glutaraldehyde and cross-linked at 25°C and pH7.0 for 2.5 h, then washed chitosan beads with distilled water until there was no residual glutaraldehyde solution. Chitosan beads were placed into 10 mL 7.5 g/mL lactase solution and soaked at 30°C and pH7.0 for 4 h and then washed the immobilized enzyme with distilled

water and filtered by suction until enzyme activity could not be detected in the distilled water, finally the immobilized lactase activity was measured. The above experiment was performed 3 times in parallel. Ultimately, the test results of OD_{420} values were 0.721, 0.718 and 0.713 and the average is 0.717±0.004. The results increased about 0.7% than the predictive value (predictive value was 0.712). At this point, the immobilized enzyme activity reached the maximum value of 162 U/g. This illustrated that the experimental model was reliable and the experimental results were of good stability.

CONCLUSION

Statistically designed experimentation for optimum immobilization of β-galactosidase onto chitosan beads exhibited best production conditions due to the optimal activity of the immobilized enzyme achieved. The optimisation parameters studied in accordance with the results were: the immobilized enzyme activity reached about 162 U/g when the adsorption time, adsorption pH and cross-linking pH were 4h, 7 and 7, respectively. Thus, the relative activity of β-galactosidase immobilized increased more than 37% compared with the previous single factor research.

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