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Research Article

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Optimization for immobilization of β-galactosidase using plackett-burman design and steepest ascent method

He Chen, Jianhua Zhang, Yichao Dang and Guowei Shu*

College of Life Science & Engineering, Shaanxi University of Science & Technology, Xi'an, China

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ABSTRACT

The Plackett-Burman experimental design was applied for screening of the influence of various factors on the immobilization of β-galactosidase on chitosan beads. The steepest ascent method was required the optimum conditions effectively and efficiently to approach the maximum of immobilized enzyme activity. Experimental results indicated that the Plackett-Burman design showed enzyme concentration, adsorption pH, adsorption temperature, glutaraldehyde concentration and cross-linking pH were the positive effect, but adsorption time and cross-linking temperature were the negative effect. Analysis of the results showed that the adsorption time, adsorption pH and cross-linking pH were the main effective parameters on the immobilization of β-galactosidase. Further, the steepest ascent experiment indicated that the immobilized enzyme activity reached 161.85U/g when the adsorption time, adsorption pH and cross-linking pH was 4h, 7 and 7, respectively. And the results increased about 37.60% than the previous single factor experiment.

Keywords: β-galactosidase; Chitosan beads; Immobilization; Plackett–Burman design; steepest ascent method

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INTRODUCTION

Lactose intolerance is a very common disorder 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 [1] due to the inability to digest lactose into its constituents, glucose and galactose, because of low levels of lactase[2]. Thus, it is necessary to remove the lactose through the use of enzymes. But the use of free enzymes has been limited, because they easily denatured and have a short lifetime and be unstable [3]. Moreover, the use of enzymes in their soluble form in large scale industrial processes is limited by their high cost of production and stability.

Therefore, β -Galactosidase was selected for immobilization[4] and the immobilization of β-galactosidase could be an alternative to minimize those drawbacks [5-6], to be specific, the enzyme stability can be improved [7], its activity and stability characteristics can be easily determined therefore it is especially suitable as a model enzyme [4]. Moreover, the immobilization of enzymes on a solid support is an important tool to attract the use of enzymes, because provides many important advantages over the use of soluble enzyme such as reusability, improvement of its thermalstability, avoids enzyme aggregation and autolysis, increases flexibility of reactor design and facilitates the removal from the reactionmedium [8-9], using it as a requirement of industrial utilization [10-11].

Chitosan and its derivatives are known as a swollen bead support for preparation of immobilized enzyme [12-13]. In addition, using glutaraldehyde as cross-linking reagent, the adsorption of β -galactosidase on chitosan beads avoid a direct contact of the enzyme with the surrounding medium, it also enable the reagents to reach the catalytic site[2,14].

The previous research attempted to the analyses of single factor test on the immobilization of β-galactosidase on chitosan beads. And the results were that the immobilized enzyme had optimal cross-linking time of 3h, optimal

cross-linking pH of 6.5, optimal cross-linking temperature of 25℃, under these conditions, the immobilized enzyme activity reached 101U/g, 96U/g, 90U/g, respectively[15]. Due to the numerous factors and far from particularly high of immobilized enzyme activity, the present work was designed to elucidate an effective approach that applied the Plackett-Burman design and steepest ascent method to achieve main effective parameters and find the optimum point.

EXPERIMENTAL SECTION

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 20g/L acetic acid solution dissolved 30g/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 [16].

Preparation of immobilized lactase: The β -galactosidase was immobilized on chitosan beads by using glutaraldehyde. Added 1g chitosan beads into 10 mL 0.5% glutaraldehyde and cross-linked at 25℃for 1h, 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℃for 2h, 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[17].

Dissolved 100mg 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 3mL ONPG solution prepared with phosphate buffer (4mg/mL) was added to test tube and set at 38℃ for 7 min, the ONPG solution was well mixed with the 1mLdiluted enzyme solution and kept at 38℃ 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℃, pH6.5, for 10min), 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 [15].

Plackett–Burman design: Twelve experiments representing the Plackett–Burman design have been designed to conduct the randomization step as in Table 1 and to a correctly enable the regression analysis. Each experiment contained only either the +1 or -1 value of each variables. However, it contained an entirely different variable represented by +1 or -1. None of the twelve experiments was similar to the other [18]. Using Plackett–Burman design was to screen out the main factors of the effect on the activity of immobilized β-galactosidase on Chitosan beads.

Steepest ascent method: The steepest ascent method [19] is an effective experimental procedure for moving sequentially along the direction of the maximum increase in the response, and thus, can approach the optimum neighborhood rapidly and efficiently [20].

RESULTS AND DISCUSSION

Screening of the main factors using Plackett–Burman design

Although the influence of various factors on the immobilized lactase had a general understanding through the single factor experiment, the relationship between primary and secondary could not be determined. If immobilized lactase was conducted in accordance with the single factor test results and used in industrial production, it might greatly increase the workload, the production cycle and operation complexity, especiallly not conducive to the sustainable production. Therefore, Plackett-Burman (PB) designs were screening designs that each factor at two levels was carried on the analysis. Through comparing the differences between each factor at two levels with the overall in order to determine the significant factor, several main influencing factors could be screened out

and avoid to waste test resources in the later period of the optimization test due to the factor number too much or partial factor not significant enough.

The design used was Plackett–Burman design that comprised 11 factors spanning over 12 runs with each factor fixed at two levels (namely a low level and a high level). And the effect analysis of single factor was carried on so as to find the larger influence factors. The factor level coding table and the experimental results of Plackett-Burman were shown in Table 1 and Table 2. Table 2 showed that the X2 and X6 were virtual items, the response value of Y1 was the OD_{420} value, the Y2 value was the immobilized enzyme activity.

Table 1 the factor level coding table of Plackett-Burman

Factor Level (g/100ml)	X1 (adsorption time) (h)	X3 (Enzyme concentration) (mg/ml)	X5 (adsorption pH)	X7 (adsorption temperature) (℃)	X8 (glutaraldehyde solution concentration) (%)	X9 (cross-linking time) (h)	X10 (cross-linking pH)	X11 (cross-linking temperature) (℃)
- 1	4.8	6	5.2	25	0.24	2.5	5.2	25
1	6	7.5	6.5	30	0.3	3	6.5	30

Table 2 the experimental design and results of Plackett-Burman

RUN	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Y1	Y2
1	1	- 1	1	- 1	- 1	-1	1	1	1	- 1	1	0.056	12.68
2	1	1	- 1	1	- 1	-1	- 1	1	1	1	- 1	0.034	7.70
3	- 1	1	1	- 1	1	-1	- 1	- 1	1	1	1	0.132	29.88
4	1	- 1	1	1	- 1	1	- 1	- 1	- 1	1	1	0.037	8.38
5	1	1	- 1	1	1	-1	1	- 1	- 1	- 1	1	0.052	11.77
6	1	1	1	- 1	1	1	- 1	1	- 1	- 1	- 1	0.056	12.68
7	- 1	1	1	1	- 1	1	1	- 1	1	- 1	- 1	0.031	7.02
8	- 1	- 1	1	1	1	-1	1	1	- 1	1	- 1	0.238	53.88
9	- 1	- 1	- 1	1	1	1	- 1	1	1	- 1	1	0.128	28.97
10	1	- 1	- 1	- 1	1	1	1	- 1	1	1	- 1	0.096	21.73
11	- 1	1	- 1	- 1	- 1	1	1	1	- 1	1	1	0.035	7.92
12	- 1	- 1	- 1	- 1	- 1	-1	- 1	- 1	- 1	- 1	- 1	0.040	9.05

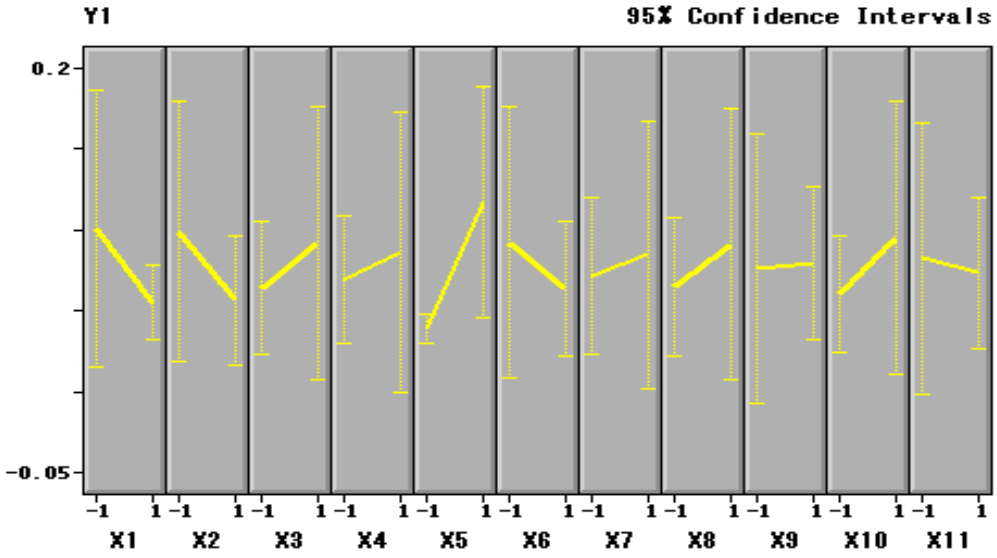

Fig.1 95% CI interval graph of factor

As illustrated in Fig.1 effects of various factors on the activity of immobilized enzyme can be displayed, where X3, X4, X5, X7, X8 and X10 were positive effect, but X1, X2, X6 and X11 were negative effect, and the effect of X9 was not obvious. As shown in Fig.2 the relationship between primary and secondary of the influence of the effect on the activity of immobilized enzyme can be found as follows: X5 > X1 > X10. The effect of X5 was the most obvious and its proportion reached more than 40%. Except the virtual items of X2 and X6, the ratio of X3 and X8 were about 5% and their impacts were very small. And even the ratio of X4, X7, X9 and X11 were less than 3%, so their effects were too trifling. Analysis of the results showed that the X1, X5 and X10 were the main effective

parameters on the immobilization of β-galactosidase.

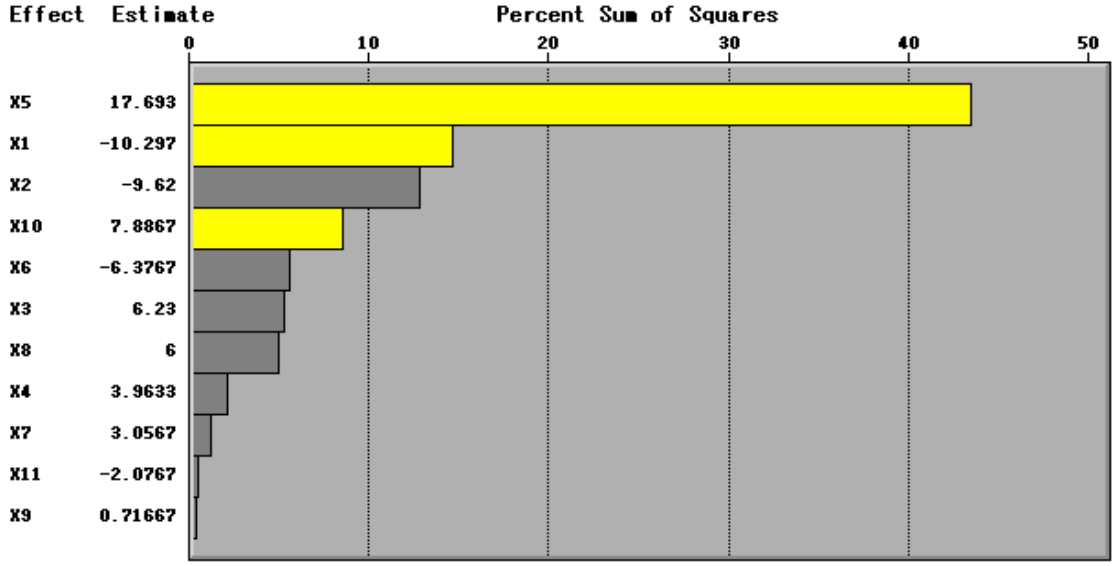

Fig.2 Screening results of the main factor of Plackett-Burman

Steepest ascent experiment

The response surface fitting equation fully approaches to real situation only considered in the study of immediate regions, and in other regions do not exist any similarities between the fitting equation and approximate functional equation, moreover the fitting is also almost meaningless. Therefore, approximation to the maximum activity of immobilized enzyme region is very significant. The path of steepest ascent is the direction in which Y1 and Y2 increases most quickly. According to the effect value of every factor to determine the step-length, the optimal value area can be approximate rapidly. As illustrated in Fig. 1 X5, X1 and X10 were the main factors according to the influence effect, direction and step-length. Results were shown in Table 3.

Table 3 the experimental design and results of steepest ascent experiment

RUN	X1	X3	X5	X7	X8	X9	X10	X11	Y1	Y2(U/g)
1	5	7.5	6	30	0.3	2.5	6	25	0.073	16.53
2	4.5	7.5	6.5	30	0.3	2.5	6.5	25	0.221	50.023
3	4	7.5	7	30	0.3	2.5	7	25	0.715	161.85
4	3.5	7.5	7.5	30	0.3	2.5	7.5	25	0.517	117.03
5	3	7.5	8	30	0.3	2.5	8	25	0.181	40.97

Table 3 showed the new experiment of the path of steepest ascent. The Y1 and Y2 value increased along the path to step 3 and then decreased gradually, indicating that the optimal conditions were close to the operating conditions in step 3. When the experiments were performed to step 3, the Y1 value increased from 0.073 to 0.715, and also the Y2 value increased from 16.53U/g to 161.53U/g. The results increased about 37.60% than the previous single factor experiment [15]. Therefore, as shown in Table 3 the immobilized enzyme activity reached 161.85U/g when X1, X5 and X10 was 4h, 7 and 7, respectively.

CONCLUSION

In conclusion, the influence of each factor on immobilized enzyme activity had certain effect. The Plackett-Burman designs showed the adsorption time, adsorption pH and cross-linking pH are the main factors. The steepest ascent experiment further indicated that the immobilized enzyme activity reached 161.85U/g when the adsorption time, adsorption pH and cross-linking pH was 4h, 7 and 7, respectively.

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