RESEARCH ARTICLE

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Application of Factorial Design to Study the Effects of Temperature, Initial pH and Agitation on the Production of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. G1

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ABSTRACT Production of cyclodextrin glucanotransferase (CGTase) from *Bacillus* sp. G1 was investigated. Two level Full Factorial Design has been employed to study the effect of different experimental variables on the production of CGTase. Three variables of pH (8, 9, and 10), temperature (32 °C, 34.5 °C, and 37 °C) and agitation (100 rpm, 200 rpm, and 300 rpm) were used to identify the significant effects and interactions in the batch studies. A polynomial regression model has been developed using the experimental data. The results show that production of CGTase was strongly affected by the variations in pH. The maximum CGTase activity was achieved when the production was carried out at 32°C, pH 10 with 100 rpm of agitation. The predicted value produced 27.85 U/ml is in close agreement with CGTase activity produce from experiment, which is 27.82 U/ml.

KEYWORDS: cyclodextrin glucanotransferase, factorial design.

INTRODUCTION

Cyclodextrins (CDs) are cyclic molecules, which can be produced by the reaction of an enzyme called cyclodextrin glucanotransferase (CGTase) on starch or starch derivatives. CDs exist mainly as three different types: α-CD, β-CD and γ-CD containing 6, 7 or 8 glucose residues respectively, and linked by α (1-4) glycosides bond.[1] The interior of CD is relatively nonpolar compared to water, and thus CD can easily form inclusion complex with other organic substances. As a result, CDs can change physical and chemical properties of the encapsulated guest molecule.[2] The primary advantage of CD complexation is to stabilize and protect sensitive host molecules such as flavors, odors or drugs. The volatility, chemical, thermal and photo reactivity of molecules can be reduced sharply by CD. Therefore, CD is widely used in food, pharmaceutical, dairy and cosmetic industry.[3] β-CD is the most widely used form of CDs and represents at least 95% of all produced and consumed CDs. CGTase, which involved in the conversion of starch into CD, is one of the most unusual members of the amylolytic glucosylase family. A variety of CGTase that produce CDs have been described. CGTase is an extra cellular enzyme that can be derived from several different bacterial sources, and varies in properties such as optimum pH, optimum temperature, stability and the relative proportion of α-, β- and γ-CD produced. Bacterial are the only known sources of CGTase. CGTases from several bacterial species have been isolated, however the predominant genus is *Bacillus*. Microorganisms such as *B. macerans, B. megaterium, B. circulans, B. stearothermophilus, B. ohbensis, Klebsiella pneumoniae* and *Thermoanaerobacter* species are known to produce CGTase.[4-8] CGTase produced by bacteria is a multifunctional enzyme. The enzyme exhibited three different reactions: cyclization, coupling reaction as well as a weak hydrolyzing activity.[9] Culture conditions were developed for supporting cell growth and CGTase production. Various types of medium compositions, pH, temperature and agitation for fermentation of CGTase had been investigated in several published paper [3,7,10] but none was focused on the effect of pH, temperature and agitation for CGTase

production using statistic experimental design approach. In this study, we have carried out preliminary experiment to observe effect of physical environmental and the parameter (initial pH, temperature and agitation) on the production of CGTase from isolated alkalophilic *Bacillus* sp. G1.

Materials and Methods

Source of Microorganism

CGTase producing bacteria was isolated from soil samples. This isolate was taxonomically identified as *Bacillus* sp. G1 according to Bergey's manual of Systematic Bacteriology.[11] The microorganism was maintained on a medium containing 2% (w/v) agar, 1% (w/v) soluble starch, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.1% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4.7H_2O$ and 1% (w/v) Na_2CO_3 (autoclaved separately). It was stored at 4 °C for further use.

Preparation of Bacteria Inoculums

A small loop of fresh bacteria culture was inoculated in a 250 ml conical flask containing 20 ml medium [0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.1% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4.7H_2O$ and 1% (w/v) Na_2CO_3 (autoclaved separately)] for 18 hours at 37 °C with shaking at 200 rpm. The cells were then washed once with normal saline. 10% (v/v) of the cells with optical density of 0.5 were then inoculated in a 500 ml conical flask containing 100 ml medium [1% (w/v) tapioca starch, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.02% (w/v) $MgSO_4.7H_2O$, 0.02% (w/v) $FeCl_2$ and 0.1% (w/v) KH_2PO_4]. The pH of the medium was adjusted using 1 M Na_2CO_3 (autoclaved separately).

Activity of CGTase

Enzyme assay was carried out according to the method of Kaneko *et al.*[12] After incubation for 24 hours, the culture was centrifuged at 5000 rpm for 2 minutes. 0.5 ml of crude enzyme solution was added to 1.0 ml of 0.04 g soluble starches in 1.0 ml of phosphate buffer, pH 6.0. After incubation at 70°C for 10 minutes in water bath, 0.03 M NaOH was immediately added to the solution to stop the CGTase reaction. Then 0.5 ml of 0.02% phenolphthalein in 0.005 M Na_2CO_3 was added. The reduction in the color intensity of the reaction mixture was measured at 550 nm using Hitachi spectrophotometer. One unit enzyme activity was defined as 1 μmol of β-CD formed per minute. Standard graph was plotted with β-CD replacing crude enzyme solution.

Statistical Analysis for Experimental Design

In order to maximize the enzyme CGTase production, Full Factorial Design for three independent variables was adopted. The Experimental Design was based on Statistica 5.0 (Statsoft Inc.). Full Factorial Design was used to obtain the combination of values that can optimize the response within the region of the three dimensional observation spaces, which allows one to design a minimal number of experimental runs.[13-15] The variables were temperature, pH and agitation, were submitted for the analysis in the design.

The variable of each constituent at levels –1, 0 and +1 is given in Table 1. The selection of low, middle and high levels for all these variables were based on a prior screening done in our laboratory (unpublished data). A 2^3 full factorial design with 2 replicates at the center point, leading the total number of 10 experiments. The behavior of the present system described by the following equation (1), which includes all interaction terms regardless of their significance:

$$\hat{Y}_u = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

where $\hat{Y}_u$ is predicted response, *ie* yield of enzyme CGTase; x_1, x_2 and x_3 are independent variables; b_0 is coefficient constant for offset term; b_1, b_2 and b_3 are coefficient constant for linear effects and b_{12}, b_{13}, b_{23} are coefficient constant for interactions effects. The model evaluates the effect of each independent variable to a response.

The variables studied were temperature (32-37 °C), pH (8-10) and agitation (100-300 rpm). The experiments were carried out based on the analysis using Statistical 5.0 (Statsoft Inc.) to estimate the responses of the dependent variable.

Table 1. Optimization of physical condition for the production of CGTase by bacteria *Bacillus* sp. G1: Independent variables in a 2^3 full factorial experiment design. The parameters used in this experiment are temperature, pH and agitation.

Variable	Parameter	Coded Level		
		-1	0	1
X_1	Temperature, °C	32	34 .5	37
X_2	pH	8	9	10
X_3	Agitation (rpm)	100	200	300

Results and Discussion

Factorial Designs and Analysis of Results

The quantitative description of the physical condition effects on CGTase production was performed. Response Surface Methodology is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed results.[14-16] The results were analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used. The regression equation obtained after analysis of variance gives the level production of CGTase as a function of different variables: pH, temperature and agitation. All terms regardless of their significance are included in the following equation:

$$\hat{Y}_u = 24.87447 + 0.39444x_1 + 2.14481x_2 + 0.52399x_3 - 0.50421x_1x_2 + 0.24616x_1x_3 - 0.99956x_2x_3 \quad (2)$$

where $\hat{Y}_u$ is the response, that is, the enzyme activity (U/ml) and x_1, x_2 and x_3 are the coded values of the test variables temperature, pH and agitation, respectively. Regression model containing 3 linear, 3 interaction terms and 1 block term was employed by using the Statistica 5.0 (Statsoft Inc.). The production of CGTase from the model at each experimental point is summarized and listed in Table 2 along with experimental and predicted values. According to equation (2), all the factors have positive effects, except the interaction between pH-temperature (x_1x_2) and pH-rpm (x_2x_3). These results can be further interpreted in the Pareto Chart (Fig 1), which graphically displays the magnitudes of the effects from the results obtained. The effects are sorted from largest to smallest.

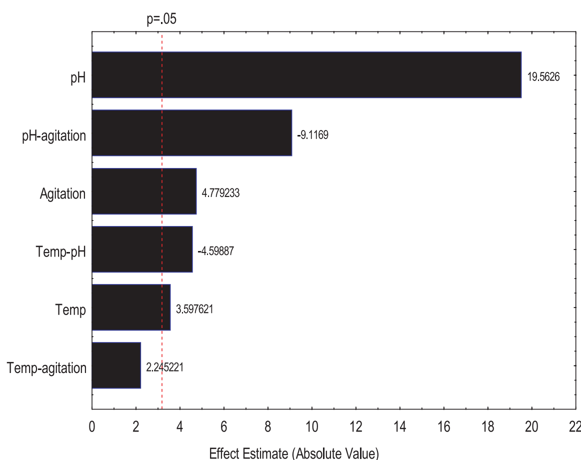

Fig 1. Pareto chart shows that pH was found to be the most sufficient parameter that effects the production of CGTase from *Bacillus* sp G1.

The Pareto chart clearly shows that pH is by far the most important factor affecting the production of CGTase followed by pH-agitation interaction, agitation, temperature-pH interaction, temperature and temperature-agitation interaction. To improve process performance, it is obvious that we should begin by adjusting the factor, which had the biggest effect. The advantages of the model can be checked by several criteria. The fit of the model was expressed by the coefficient of determination. R^2, which was found to be 0.994, indicating that 99.4% of the variability in the response can be explained by the model. The value also indicates that only 0.6% of the total variation is not explained by the model. This shows that equation (2) is a suitable model to describe the response of the experiment pertaining to CGTase production. The value of adjusted determination coefficient (Adj = 0.983) is also very high to advocate for a high significance of the model. A higher value of the correlation coefficient (R = 0.997), justifies an excellent correlation between the independent variables. This indicates a good agreement between the experimental and predicted values of CGTase production as shown in Table 2. The corresponding analysis of variance (ANOVA) is presented in Table 3. Statistical testing of the model can also be done by the Fisher's statistical test for analysis of variance. The F value is the ratio of the mean square due to regression to the mean square due to the real error. Generally, the calculated F-value should be several times the tabulated value if the model is a good predictor of the experimental results.

The response taken from Table 3 reveals that the linear term of temperature (x_1), pH (x_2) and rpm (x_3) have remarkable effects on the CGTase yield. The significance of each coefficient was determined using p-value ($p < 0.05$) and the smallest p-value indicates high significance of the corresponding coefficient. It can be seen that the variables with largest effect was pH (x_2). All the linear term and interaction term are significant except for interaction between temperature-rpm (1 by 3). The three-dimensional contour plot helps in assessing the effect of any two factors in combination on the production of CGTase. Thus, the effects of temperature-pH, temperature-agitation and pH-agitation on the response can be obtained. The darker the color means higher CGTase activity. Fig 2 corroborates the fact that the maximization of CGTase production is possible in the presence of higher pH (10 or above). Stefanova *et al*[7] has reported that the best initial pH of medium for the production of CGTase enzyme from *Bacillus stearothermophilus* 2/2 was at pH 9.0. As we can see in Fig 2 at lower temperature of 32 °C, the pH value needed for maximum CGTase production was about 10. Whereas at a higher temperature of 37 °C, the pH value needed for maximum

Table 2. Experimental design with experimental and predicted values of CGTase activity. The best CGTase activity was observed when temperature of 32 °C, pH 10 and agitation of 100 rpm were used in the production.Results are the average of triplicate analysis.

Run	Temperature (°C),X_1	pH, X_2	rpm, X_3	Activity 1 (U/ml)	Activity 2 (U/ml)	Activity 3 (U/ml)	Average Activity (U/ml)	Standard Deviation
1	32.00	8	100	19.83	19.77	21.22	20.55	0.74
				21.22	20.04	21.22		
2	32.00	8	300	22.67	23.36	22.94	23.08	0.34
				22.67	23.36	23.36		
3	32.00	10	100	27.68	27.90	27.83	27.83	0.09
				27.90	27.90	27.83		
4	32.00	10	300	25.46	26.94	27.57	26.40	0.92
				25.65	26.94	27.57		
5	37.00	8	100	22.21	20.09	23.03	21.83	1.06
				21.97	21.15	22.56		
6	37.00	8	300	26.16	25.57	24.86	25.39	0.46
				25.45	24.98	25.33		
7	37.00	10	100	27.53	27.07	27.13	27.13	0.30
				27.40	26.67	27.00		
8	37.00	10	300	26.51	26.22	26.37	26.65	0.33
				26.87	27.02	26.94		
9	34.50	9	200	24.09	25.49	23.58	24.56	0.88
				24.34	25.79	24.09		
10	34.50	9	200	25.71	25.96	23.75	25.31	0.86
				25.79	25.79	24.85		

Table 3. Regression analysis (ANOVA) for the production of CGTase by Bacillus sp. G1: R = Coefficient of correlation = 0.997; R^2=Coefficient of determination = 0.994; Adjusted R^2 = 0.983. SS: Sum of square; DF: Degree of freedom; MS: Square means; test F with 95% of confidence interval.

Factor	SS	DF	MS	F	P-value
(1) Temp	1.24465	1	1.24465	12.9429	0.036824
(2) pH	36.80177	1	36.80177	382.6953	0.000292
(3) rpm	2.19650	1	2.19650	22.8411	0.017412
1 by 2	2.03384	1	2.03384	21.1496	0.019325
1 by 3	0.48477	1	0.48477	5.0410	0.110426
2 by 3	7.99300	1	7.99300	83.1179	0.002789
Error	0.28849	3	0.9616		
Total SS	51.04302	9			

CGTase production was at least higher than 10.1. The difference in CGTase activity at pH 10, 32°C (A = 27.12 U/ml) and at pH 10, 37°C (A = 26.90 U/ml) was about 0.22 U/ml. However, at lower temperature and lower pH value, the production of CGTase declines. This may be due to the nature of the alkalophilic bacteria itself. At lower pH value, higher temperature was needed for higher CGTase production. The difference in CGTase activity at pH 8, 32°C (A = 21.82 U/ml) and at pH 8, 37°C (A = 23.62) was about 1.79 U/ml. Fig 3 shows the effects of temperature and agitation on the production of CGTase from *Bacillus* sp. G1. when pH value was fixed at 9. It indicates that the maximum CGTase production was obtained at higher temperature and agitation. The difference in CGTase activity at 32°C, 300 rpm (A = 24.91 U/ml) and at 37°C, 300 rpm (A = 26.21) was about 1.30 U/ml. However, at lower temperature and lower agitation speed, the production of CGTase was not significant. At lower rpm speed, higher temperature was needed for higher CGTase production. The difference between CGTase activity at 32°C, 100 rpm (A = 24.25 U/ml) and at 37°C, 100 rpm (A = 24.55) was about 0.30 U/ml. Fig 4 shows the effect of pH and agitation on the production of CGTase when temperature was fixed at 34.5 °C. CGTase production was high when high pH and low agitation was used. The difference in CGTase activity at pH 10, 100 rpm (A = 27.49 U/ml) and at pH 8, 100 rpm (A = 21.20) was about 6.28 U/ml. However, at lower pH value and higher rpm speed, the production of CGTase does not seem so good. At higher rpm speed, higher pH value was needed for higher CGTase production. The difference in CGTase

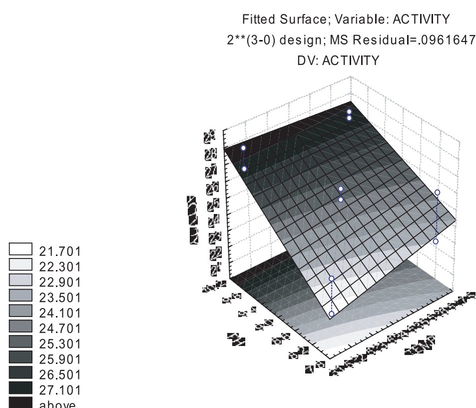

Fig 2. Fitted surface of temperature-pH-enzyme activity. Three dimensional surface plot showing predicted CGTase activity (U/ml) as a function of pH and temperature (ºC).

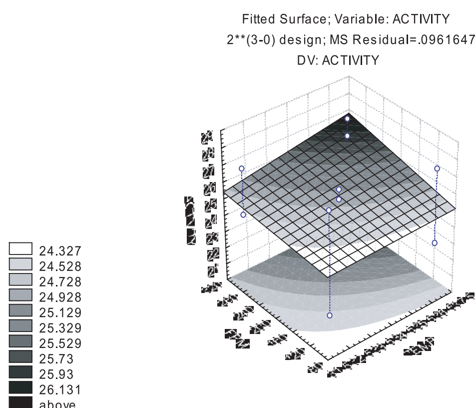

Fig 3. Fitted surface of temperature-agitation-enzyme activity. CGTase activity (U/ml) on three-dimensional graphics for response surface plot versus agitation (rpm) and temperature (ºC).

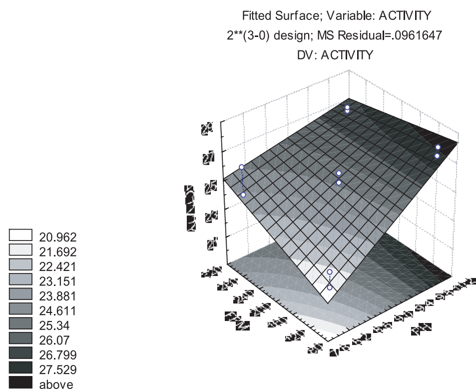

Fig 4. Fitted surface of pH-agitation-enzyme activity. Three dimensional graphics plot showing effects of pH and agitation (rpm) on the activity of CGTase (U/ml)

activity at pH 8, 300 rpm (A = 24.24 U/ml) and at pH 10, 300 rpm (A = 26.53) was about 2.28 U/ml. The bacteria *Bacillus* sp. G1 is an alkalophilic bacteria. Preliminary physiological studies of this bacteria shows that it cannot grow well at pH lower than 8 and higher than 11. This could be one of the reasons for low CGTase yield at low pH values.

Conclusion

The analysis using full factorial design reveals that the maximum CGTase production can be achieved only at high pH of about 10. As shown previously, pH has the most significant effect on CGTase production. Therefore, at pH 10 (final pH 8.54), 32 °C and 100 rpm agitation, the highest CGTase production was observed. However, there was no significant difference in the CGTase when the process was carried out at 37 °C. In our experiment, to achieve optimal results and to lower energy consumption, CGTase production was conducted at 32 °C, pH 10 (final pH 8.54) and 100 rpm.

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