Production of Cyclodextrin Glucanotransferase (CGTase) from *Bacillus stearothermophilus* HR1 in Shake Flasks

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Abstract

The objective of this paper is to increase CGTase production from Bacillus stearothermophilus HR1 by optimization of medium formulation and fermentation variables. Central composite factorial design was used to achieve this purpose. Interpretation of results was carried out using the analysis of variance (ANOVA) and analyzed using the 3-D response surface plot. The parameters studied in the medium formulation were concentration of sago starch, peptone casein, K_2HPO_4, $CaCl_2$ and initial pH of the medium. Increase in the enzyme activity up to 14.20 U/ml was observed from the model of experimental design compared to the initial activity of 4.20 U/ml, with the regression constant (R^2) of 0.9486. For optimization of pH and agitation speed, culture was incubated in shake flasks at 55°C. CGTase produced was 16.15 U/ml, with a regression of 92.34%. An increase of 3.8 fold in CGTase activity was obtained after optimization. We have successfully optimized the production of CGTase from Bacillus stearothermophilus HR1 using experimental design approach.

Keywords:

CGTase. *Bacillus stearothermophilus*. Optimization. Response Surface Methodology.

Introduction

Cyclodextrin (CD) is a closed-ring structure containing six, seven or eight glucose units and known as α-, β- and γ-CD, respectively [1]. The interior of CDs is relatively apolar compared to water, and thereby CDs can easily form inclusion complex with many organic substances which can change the physicochemical properties of the guest molecule; thus increasing their water solubility and stability [2, 3, 4, 5]. These properties made CDs became increasingly important for industrial application particularly in food, pharmaceutical, dairy and cosmetics industry [4].

CDs are products of starch degradation by cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19), a type of extracellular enzyme excreted by bacteria. CGTase is produced by various bacterial species and mostly reported are from *Bacillus* species. Microorganisms such as *Bacillus stearothermophilus* [6], *Bacillus autolyticus* 11149 [7] and *Bacillus macerans* [8] are known to produce CGTase. Production of CGTase depends much on the reaction of producer organisms towards substrate and fermentation conditions.

The objective of this study is to optimise production of the CGTase enzyme from *Bacillus stearothermophilus* HR1 by using design of experiment method. Mathematical models were developed to correlate all the factors analysed and to obtain the optimal conditions for CGTase production.

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Materials and Methods

Preparation of Bacterial Inoculum

Bacillus stearothermophilus HR1 were grown in 20 ml medium containing 2% (w/v) sago starch, 1.75% (w/v) peptone from meat, 0.1% (w/v) K_2HPO_4 and 0.02% (w/v) $MgSO_4.7H_2O$ with initial pH 7.2 in a 250 ml conical flask. The culture was incubated at 50°C with shaking at 200 rpm for 18 hours. The cells were then harvested by centrifugation at 5000 rpm for 5 minutes and washed once with normal saline solution to give an optical density (OD) reading of 0.5 at 550 nm.

Preparation of Crude Enzyme

Ten percent of *Bacillus stearothermophilus* HR1 inoculum was cultivated at 50°C for 24 hours with continuous shaking in 500 ml conical flask containing 100 ml of medium. After cultivation, the cells were removed by centrifugation at 5000 rpm for 2 minutes. The supernatant was used as crude enzyme solution for assaying enzyme activity.

Assay of CGTase

Enzyme activity was determined using phenolphthalein method by Kaneko [9].

Experimental Design and Optimization

Design of experiment [10] was used to show the statistical significance of the variables such as concentration of carbon, nitrogen, phosphorus and mineral salt sources including initial pH in the medium formulation of CGTase production. Response surface methodology was used to indicate the optimum condition of the variables and to understand the interaction between two variables [11].

The low, middle and high levels of each variable were given in Table 1. A 2^{5-1} fractional factorial central composite design for five independent variables with six star points ($\alpha = 2$) and six replication of the central points, leading to a total 32 sets of experiments were carried out in this study.

Table 1 - Level of variables for medium formulation

Factor	High	Low
Sago starch (g/l), X_1	35	15
Peptone casein (g/l), X_2	20	10
K_2HPO_4 (g/l), X_3	1.4	0.6
$CaCl_2$ (g/l), X_4	0.8	0.2
Initial pH, X_5	7.6	6.2

Initial screening discovered that 55°C yields the highest CGTase compared to other temperatures [12]. Thus, cultures were incubated at this temperature for the optimization of fermentation variables (pH and agitation speed). This process was performed using a 2^3 full factorial central composite design, leading to a total 15 sets of experiments. The design also takes into account 2 replicates, 2 star points and 3 centre points. The coded values for different variables are shown in Table 2 and the design matrix chosen for the initial study are shown in the Table 4. Response surface plots were generated by Design Expert (StatEase, USA, Ver. 6.0.4) software.

Table 2 - Coded values for fermentation variables optimization

Factor	High	Low
Initial pH, A	10.0	5.0
Agitation speed, B	210	100

From the experimental results, an approximate polynomial's relationship for dependent variables of CGTase activity was obtained. The design experiments were carried out for analysis by using Design Expert software (State-Ease Inc., Statistics made easy, Minneapolis, MN, US. Version 6.0.4).

Results and Discussions

Experiments with different combination of sago starch, peptone from casein, K_2HPO_4, $CaCl_2$ and initial pH were performed. CGTase activity was assayed and calculated after *Bacillus stearothermophilus* HR1 inoculum was cultivated at 50°C for 24 hours. This result was analysed using the analysis of variance (ANOVA) as appropriate to the experimental design used. The coefficient of determination, R^2 obtained was 0.9486 which implies that the sample variation of 94.86% for CGTase production is attributed to the independent variables tested. The R^2 value also indicates that only 5.14% of the total variation was not explained by the model.

The regression equation (Eq. 1) obtained after analysis of variance gives the production of CGTase from *Bacillus stearothermophilus* HR1 as a function of the different variables sago starch (X_1, g/l), peptone from casein (X_2, g/l), K_2HPO_4 (X_3, g/l), $CaCl_2$ (X_4, g/l) and initial pH (X_5). All terms regardless of their significance was included in the following second order polynomial equation:

$$Y = 11.84 - 0.81X_1 + 1.02X_2 + 0.43X_3 + 0.28X_4 + 0.70X_5 - 1.58X_1^2 - 0.85X_2^2 - 0.13X_3^2 - 0.46X_4^2 - 1.62X_5^2 - 0.56X_1X_2 - 0.37X_1X_3 + 0.17X_1X_4 - 1.01X_1X_5 + 0.050X_2X_3 - 0.33X_2X_4 + 0.40X_2X_5 - 0.44X_3X_4 + 0.018X_3X_5 - 0.96X_4X_5 \quad (1)$$

Regression model with 1 offset, 5 linear, 5 quadratic and 10 interaction terms was employed by using the Design Expert software.

When an optimisation programme was run within the tested range, the optimum levels of the variables obtained were 16.02 g/l sago starch, 20 g/l peptone from casein, 1.4 g/l K_2HPO_4, 0.2 g/l $CaCl_2$ and pH 7.54. The concentration of nitrogen source (pharmamedia in their study) was at 21.0 g/l for production of CGTase from *Bacillus firmus* by using experimental design [4]. Besides, Gawande and Patkar in their study found that the concentration of mineral salts

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used (magnesium sulphate in their case) was 0.5 g/l to increase the CGTase production from *Klebsiella pneumoniae* AS-22 by using experimental design [13].

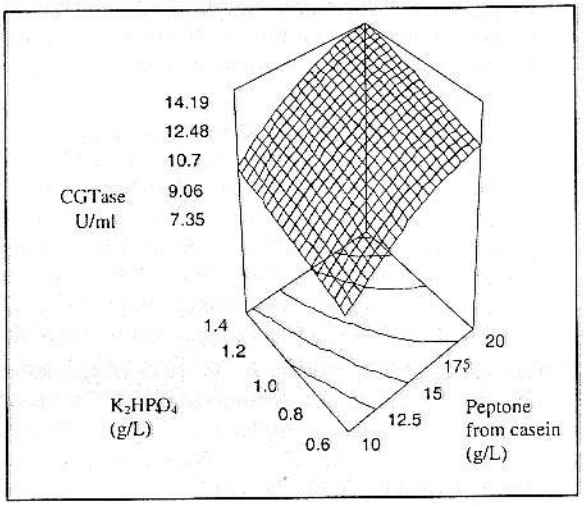

Figure1- Effect of peptone (from casein) concentration and K_2HPO_4 concentration on CGTase production by Bacillus stearothermophilus HR1.

The maximum response predicted from the model was 14.20 U/ml of CGTase. Repeated experiments were performed for the production of CGTase by *Bacillus stearothermophilus* HR1 cultivated in the optimised medium. The experimental results from three replications gave CGTase activity of 14.80 U/ml and the model was proven to be adequate.

For the fermentation variables optimization, the observed enzyme activity was correlated with experimental variables as follows:

$$\text{CGTase} = 15.25 + 0.85A - 2.61B - 5.46A^2 - 2.13B^2 - 0.74AB \quad (2)$$

where A=pH and B= rpm. For this equation, the R^2 value of 0.9234 confirmed that the variability in the enzyme activity could be associated to the experimental factors to the extent of 92.34%, with only 7.66% not explained by the model.

The optimum levels for pH and agitation speed were 7.54 and 120 rpm respectively. Lee and Chen discovered that *Aspergilus niger* produced CGTase in the pH range of 5.0 to 8.0 [14]. However, CGTase production is best at the neutral zone for *B. stearothermophilus* HR1.

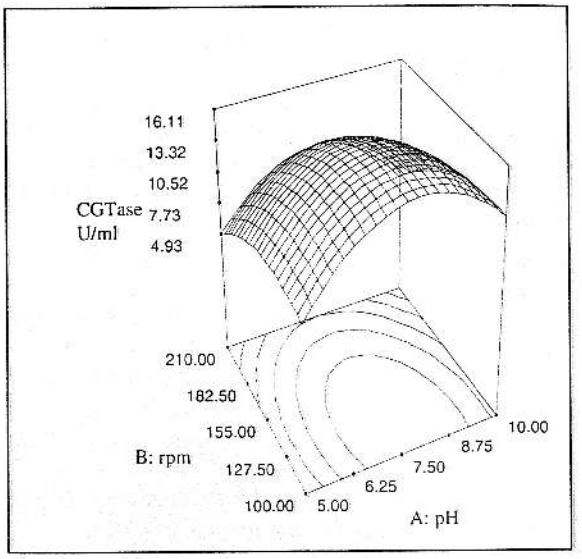

Figure 2- Effect of pH and agitation speed on CGTase production by Bacillus stearothermophilus HR1.

Experimental results of three replications gave CGTase activity of 16.15 U/ml, which is close to the predicted model value of 16.08 U/ml. This is a 3.8 fold increase from the initial CGTase activity of 4.20U/ml. Thus, CGTase production has successfully been optimized.

Conclusion

CGTase from *Bacillus stearothermophilus HR1* had been successfully optimized with overall 3.8 fold increase in the activity. Optimization of the medium had increased CGTase from 4.20 U/ml to 14.80 U/ml. Further optimization of fermentation variables thus increased CGTase to 16.15 U/ml. The coefficient of determination for both regression models were above 90% and this proved that the equations generated by experimental design were adequate.

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