Production of Cyclodextrin Glucanotransferase (CGTase) from *Bacillus* sp. TS1-1: Process Optimisation

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Abstract

The effect of pH and temperature were investigated for an optimised condition of cyclodextrin glucanotransferase (CGTase) production from Bacillus sp. TS1-1. The optimisation process was analysed using Central Composite Experimental Design in Response Surface Methodology (RSM) by Design Expert Version 6.0.4 (StatEase, USA). This design was employed to derive a statistical model for the effects of pH and temperature on the production of CGTase from Bacillus sp. TS1-1. The coefficient of determination, R^2 was found to be 97.85%. The temperature of 29.59°C and the pH of 10.32 had been found to be the optimum conditions for the maximum production of CGTase after 24 hours of incubation. In these conditions, the CGTase activity was attained to be 75.48 U/ml, which was close to the model predictions.

Keywords:

Cyclodextrin Glucanotransferase, Optimisation Condition, CGTase Activity, Production, Response Surface Methodology

Introduction

Cyclodextrin glucanotransferase, CGTase (EC 2.4.1.19) has been found in several bacterial species and catalyses in both intermolecular and intramolecular transglycosylation of α-1,4-glucan. The reaction between CGTase with starch produced cyclodextrins (CDs) with variety of maltooligosaccharides [1]. CGTase is a multifunctional enzyme, whereas besides cyclization (the conversion of starch and related α-1, 4-glucans into CDs through an intramolecular transglycosylation reaction) this enzyme manages to catalyse a coupling reaction (opening of CD rings and transferring of linear maltooligosaccharides to acceptors) through intermolecular transglycosylation reactions. Nevertheless, CGTase had a week contribution towards hydrolysing activity with water molecules [2].

Cyclodextrins (CDs) have a structure of oligosaccharide rings comprising □-1, 4-linked glucopyranose residues. Cyclodextrins are also known as cycloamyloses or Schardinger Dextrins. The are mainly three common types of CDs, which are made up of 6, 7 or 8 molecules, identified as □-, □- and □-cyclodextrins respectively. CD molecules have a torus-shaped structures, where the hydrophilic hydroxyl groups at the exterior site and the hydrophobic C-H groups and glycosidic oxygen at the interior cavity. In the recent years, the requirements of CDs have received great attentions, especially in food, pharmaceutical, chemical, cosmetic as well as the agricultural industries [3]. The reason being is the capable of CDs molecules to form inclusion complexes with a wide variety of guest molecules (organic or inorganic) without changing the properties of the guest molecule it selves. CDs are also capable to stabilize labile materials, mask off

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odours, emulsify oils and change viscous or oily compounds into powder.

Among the three main types of CDs produced, □-CD is more widely used and developed for various applications. Owing to its low solubility in water, □-CD is readily separated from the reaction mixture without any treatment with organic solvents and its inclusion complexes can be easily prepared. The capability of predominantly □-CD producing can reduce subsequent purification costs, leading to commercially desirability. Therefore, efforts are focused on finding the suitable CGTase producers for an efficient of -CD production.

Approach and methods

Culture Conditions for CGTase Production

Bacillus sp. TS1-1 was grown in optimised medium with the composition of 2% (w/v) sago starch, 1% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4 and 0.02% (w/v) Mg $SO_4.7H_2O$ and added with 10% (w/v) of Na_2CO_3 separately, depending on the pH of the medium. The cells were cultured in 250 ml conical flasks and incubated at the temperature of 37°C with the agitation of 200 rpm for 18 hours. The cells were then centrifuged at 5000 rpm for 4 to 5 minutes, washed once with normal saline solution (0.85% NaCl), giving a turbidity (optical density) of 0.5 at 600 nm.

About 10% of bacterial inoculum was inoculated into the medium, giving the total volume of 100 ml (including the medium) in each 500 ml conical flasks. The cultures were then incubated at the rotation speed of 100 rpm with temperature shown in Table 1 for 48 hours in an orbital shaker. Samples were harvested every 2 hours for the first 24 hours and every 4 hours for the next 24 hours. In every sampling, the sample was centrifuged at 5000 rpm for 2 minutes to separate the bacterial cell and the clear supernatant.

CGTase Assay

The CGTase activity was measured using the method established by [4]. The reaction mixtures containing 40 mg of soluble starch in 1.0 ml of 0.1 M sodium phosphate buffer (pH 6.0) and 0.5 ml supernatant was incubated at 60°C for 10 minutes. The reaction was stopped by adding 3.5 ml of 30 mM NaOH, followed 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 solution. The colour intensity of the samples was measured at 550 nm after standing up for 15 minutes at room temperature. The blank solution, which lack of enzyme (medium) was prepared for each batch of assays. Standard curve using 0.1% w/v of β-CD (Fluka) to replace soluble starch and crude enzyme respectively was plotted. A unit of enzyme activity was defined as the amount of enzyme that forms 1 µmol of β-CD per minutes.

Dry Cell Weight Determination

There were two approaches in the cell mass determination, depending to the starch presence in the culture. If the culture contains starch, 0.1 ml enzyme □-amylase (Novo Nordisk) was added to 1 ml of culture to hydrolyse the residue starch to soluble sugars [5]. The mixture was incubated at 100°C for 20 minutes and centrifuged at 3000 rpm for 30 minutes. The supernatant was then decanted and the cells were filtered onto pre-weighted 0.2 □m cellulose nitrate filter (Whatman), washed twice with distilled water and dried in an oven at 95°C for 24 hours. Fresh medium was treated similarly and used as blank for reduction of starch impurities in the sample.

If starch was depleted from the sample, the cell mass was measured by taking 1 ml of culture and centrifuged at 5000 rpm for 3 minutes in a microcentrifuge. The cell pellet was washed twice with saline solution and dried in vacuum drier, followed by drying in the oven at 80 °C to constant the cell weight. The presence of starch in the culture can be determined by using iodine solution.

Starch Concentration

Starch concentration in the sample was carried out according to the method of [6]. 1.0 ml of supernatant was mixed with 4 ml of 0.01 M iodine in 0.25 M potassium iodide (KI) and diluted with 15 ml of distilled water. The colour intensity was measured at 465 nm against blank of distilled water treated in the same manner above. The starch in culture filtrate was quantified according to the standard curve of starch between 0 to 3.5 mg/ml.

Protein Content

Protein content was determined according to Modified Lowry Protein Assay [7].

Experimental Design

Experimental design was determined based on the established method [8][9]. The effect of temperature and pH were studied using Central Composite Design in Response Surface Methodology (RSM). The optimisation was performed to the total sets of 17 experiments, consisting four factorial points, eight axial (star) points and five centre points. The ranges chosen for both process variables were 9 to 11 for pH and 25°C to 35°C for temperature. The CGTase activity was chosen at the duration of 24 hour in every run of experiments.

gives the CGTase production from *Bacillus* sp. TS1-1 as a function of temperature and pH. The final equation (actual value) was expressed in second order polynomial equation (1).

$$X=-2252.35464+51.59356A+301.62010B-0.90418A^2-15.05414B^2+0.27550AB$$

(1) where X=CGTase activity, U/ml, A=Temperature, °C and B=pH.

Results

From the experimental design, experiments with different combination of temperature and pH were performed. The CGTase activities of each sets of experiment were determined after the culture was cultivated for 24 hours. The equation obtained after analysis of variance (ANOVA)

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The three dimensional presentation (correlation between temperature and pH on the CGTase production) was shown in Figure 1. The optimum temperature and pH for the maximum CGTase production were 29.59°C and 10.32 respectively, giving a predicted CGTase production of 76.17 U/ml.

The optimum conditions for both temperature and pH were applied in the shake flasks to compare the CGTase production with the predicted ones. Besides, the growth kinetics of the microbe was also studied. The CGTase production at 24 hours incubation was obtained to be 75.48 U/ml, which was closed to the model had predicted. The CGTase production found to be constant after 18 hours of incubation.

Discussion

The CGTase production as a function of temperature and pH is shown in equation (1). The fitness of the model was expressed by the coefficient of determination, R^2. The R^2 value, 0.9785 or 97.85% indicate the variability in the response of the model, where 2.15% of the total variation was not explained by the model (equation 1). The value of adjusted determination coefficient (Adj R^2 = 0.9687) was found to be high and advocated for a high significance of the model. The correlation coefficient, R was mainly used to show the correlation measurement of estimation in the regression model. The closer the R value to the unity, the better correlation between the observed and the predicted values was gain. From ANOVA, the R value was found to be 0.9842.

As mentioned previously, the optimum conditions for maximum CGTase production were pH 10.32 and temperature of 29.6°C, giving a predicted value of 76.17 U/ml. From the two-dimensional presentation of the response surface, it was observed that the optimum CGTase production response was more sensitive to changes in one pH unit rather than one temperature unit (°C). Therefore, the response surface analysis of the experimental results clearly reveals the interrelationship between temperature and pH as affecting the CGTase production.

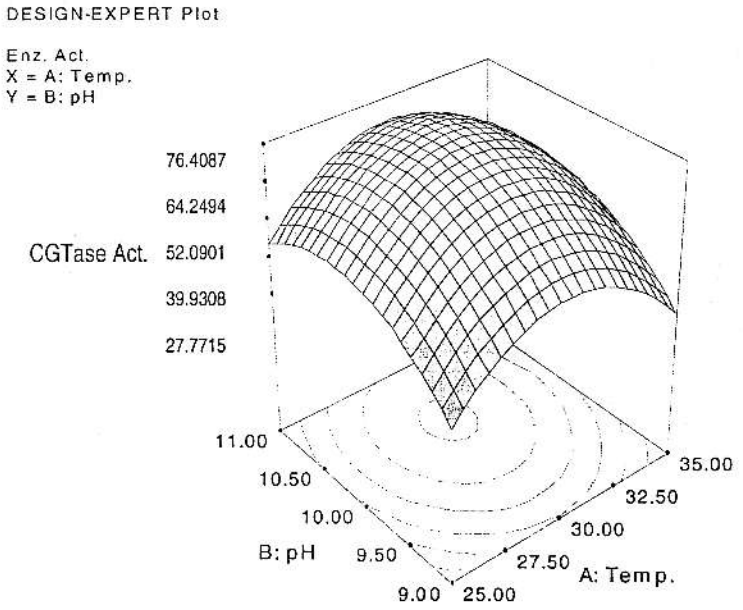

Figure 1 : The three-dimensional presentation of the response surface for the CGTase activity of Bacillus sp. TS1-1. Temperature units are in degrees Celcius while the CGTase activity units were in U/ml.

The temperature above or below the optimum temperature (29.6°C) was observed to give low CGTase production. At lower temperature limit, it seem that insufficient activation energy was produced that allow the critical process (decrease the effectiveness of CGTase production) to occur [10] While, at the upper limit, the increment of the production rate as proportional to the temperature increment was offset by the changes in protein structures resulting inactivation or denaturation of critical cell proteins, with an accompanying cessation of growth.

The alkalinity of the growth environment of the microbes also response to the specific changes in protein structures. In one study, the response of an alkali-tolerant *bacillus* sp. to growth at pH 7.5 and 10.2, it was shown that there were quantitative differences in the cell membrane protein compositions [11] Studies on an alkalophilic *bacillus* sp.

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that grow at pH 10.0 and 8.2 also show that there were specific difference in the protein compositions, which were reflected in the greater negative charge of the proteins at pH 10.0 [12][13].

Similarly, analysis of the cell envelope of a large number of alkalophilic *Bacillus* sp. show that in some strains there were large amounts of negatively charged compounds [14]. This may be one of the reasons the cell membrane required alkaline pH for the transport charged substances, while intracellular enzymes function optimally at neutral pH [15].

Conclusion

The optimisation of CGTase production by *Bacillus* sp. TS1-1 was conducted in batch culture. From the Response Surface Methodology (RSM), the optimum conditions for both temperature and pH observed to be 29.59°C and 10.32, where the expectation of CGTase production was 76.17 U/ml. While from the experiment applied, the CGTase production obtained to be 75.48 U/ml, which was close to the model prediction.

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References

[1] Szetli J. 1998. Introduction and general overview of cyclodextrin chemistry. *Chem. Rev.* 98:1743-1753.

[2] Tonkova A. 1998. Bacterial Cyclodextrin Glucanotransferase. *Enzyme and Microbial Technology.* 22:678-686.

[3] A. Biwer, G. Antranikian, E. Heinzle 2002. Enzymatic production of cyclodextrins. *Appl Microbiol Biotechnol.* 59:609-617.

[4] Kaneko T., Kato T., Nakamura N. and Horikoshi K. 1987. Spectrophotometric Determination of Cyclization Activity of □-Cyclodextrin-Forming Cyclodextrin Glucanotransferase. *J. Jpn.SocStarch Sci.* 29:45-48.

[5] Ariff A.B., Karim M.I., Madihah M.S., Sahaid K.M. and Suriani A. 2001. Direct Fermentation of Gelatinized Sago Starch ta Acetone-Butanol-Ethanol by Clostridium acertobutylicum. *World Journal of Microbiology and Biotechnology.* 17:1-10.

[6] Kitahata S., Tsuyuma N. and Okada S. 1973. Purification and some properties of cyclodextrin glycosyltransferase from the strain of *Bacillus* sp. Ournal of Agricultural Biological Chemistry. 38(2):387-393.

[7] Pierce Chemical Company. (1996). "Modified Lowry Protein Assay". Rockford, U.S.A. Instructions.

[8] Haaland P.D. (1989). "Experimental Design in Biotechnology". New York. Marcel Dekker.

[9] Montgomery D.C. Second Edition. 1992. *Design and Analysis Experiments.* New York. John Wiley & Sons Inc. 540.

[10] Caldwell D.R. 1995. *Microbial Physiology and Metabolism.* IA Wm C Brown Communications, Inc.

[11] Koyama N., Takinishi H. and Nosoh Y. 1983. A possible relation of membrane proteins of the alkalostabilityof a facultatively alkalophilic *Bacillus. FEMS Microbiology Letters.* 16:213-216.

[12] Koyama N. and Nosoh Y. 1976. The effect of the pH of culture medium on the alkalophilicity of a species of *Bacillus. Achieves of Microbiology.* 109:105-108.

[13] Koyama N, Kiyomiya A. and Nosoh Y. 1976. Na^+-dependant uptake of amino acids by an alkalophilic *Bacillus. FEBS Letters.* 72:77-78.

[14] Horikoshi K. and Akiba T. 1982. *Alkalophilic Microorganisms.* Springer, Berlin, Heidelberg and New York.

[15] Ohta K., Kiyomiya A., Koyama N. and Nosch Y. 1975. The basis of the alkalophilic property of a species of *Bacillus. Journal of General Microbiology.* 86:259-266.

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