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ORIGINAL ARTICLE

Cyclodextrin glucosyltransferase production by *Bacillus megaterium* NCR: evaluation and optimization of culture conditions using factorial design

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Abstract Statistically-based experimental designs were used to optimize the production of cyclodextrin glucosyltransferase (CGTase) from a local isolate of *Bacillus megaterium* using shack culture fermentation. Seven cultural conditions were examined for enzyme production and specific activity using Plackett-Burman factorial design. Fermentation time and K_2HPO_4 level were the crucial for factors improving enzyme production process. The steepest ascent design was adopted-based on the results recorded with Plackett-Burman design. Maximal enzyme estimates (activity 56.1 U/ml, and specific activity 62.7 U/mg protein) were achieved. A verification experiment was carried out to examine model validation of this optimization.

Keywords *Bacillus megaterium* · Cyclodextrin glucosyltransferase · Optimization · Factorial design

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Introduction

Cyclodextrin glucosyltransferase (CGTase) [α-1.4-glucan-4-glucosyl-transferase, cycling EC 2.4.119] catalyzes the formation of cyclodextrin (CD) from starch and related carbohydrates such as amylase, amylopectin and malto-oligosaccharide [1]. The CD which is a ring structure molecule built-up of 6, 7 or 8 glucopyranose units is referred to as α, β or γ CDs, respectively. Depending on the major product of the cyclization reaction, CGTase are characterized as α-, β- or γ-CGTase. The cyclic products can form versatile inclusion complexes with many organic and inorganic compounds. This property is of particular importance giving a widespread application in the pharmaceutical, food and chemical industries [2, 3].

In this work different microorganisms were tested for their abilities to produce CGTase and a local isolate of *Bacillus megaterium* National Research Center (NRC) was considered to be the most active. The CGTase productivities of *B. megaterium* were physiologically optimized. Screening and evaluation of some nutritional (medium components) and environmental requirements (fermentation time and initial pH) of the microorganism considered to be as an important step for process development. The optimization studies involved a one factor at a time is tedious and tends to overlook the effect of interactivity factors and might lead to misinterpretation of the obtained results. In contrast, statistically planned experimental studies effectively tackle the problem which involves the specific design of experiment which minimizes the error in determining the effect of parameters and the results are achieved in an economical manner [4].

Therefore, the present study aims to determine on, statistical basis, the best conditions for production of extracellular CGTase by *B. megaterium* NRC adopting submerged culture technique. Optimization of the most significant components through the statistical experimental design not only creating the optimum conditions for production of CGTase but also evaluating the importance of certain significant effective factors.

Materials and methods

Microorganism

The bacterium used throughout this study namely; *B. megaterium* NRC was obtained from the Center of Culture of National Research Center, Giza, Egypt.

Chemicals

The chemicals used in this work were analytical grade reagents purchased from Merck Company.

Cultivation conditions, crude enzyme extraction and dry weight estimation

Stock cultures of *B. megaterium* NRC were maintained on potato dextrose agar (PDA) slants, stored at 4°C, with periodic monthly transfer. Aliquots of 50 ml of the production medium composed of (g/l): soluble starch 12.5, peptone 5, K_2HPO_4 1, KH_2PO_4 2; $MgSO_4$ 0.2 were dispensed in 250 ml Erlenmeyer flasks. The flasks were autoclaved for 15 min at 121°C and inoculated with 2% of the bacterial suspension and then shacked at 200 rpm at 30°C for the requested time. At the end of fermentation period each flask was centrifuged at 5,000 rpm for 20 min and the cell clear culture filtrate was used as crude enzyme preparation, while the obtained bacterial biomass was repeatedly washed and dried at 60°C to constant dry weight values.

Statistical design

The Plackett-Burman statistical design [5] was used to refer to the relative importance of various nutritional and fermentation factors for the production of CGTase enzyme. This design comprises one type of two levels screening design which is practical specially when the researchers faces large number of factors and doubts which setting are likely to produce optimal or near optimal responses [6]. Table 1 illustrated the factors under investigation as well as levels of each factor used in the experimental design. The main effect of each independent variable was determined according to the following equation:

$$Ex_i = (\sum M_i^+ - \sum M_i^-)$$

where, EX_i is the variable main effect, M_i^+ and M_i^- are the sum of the observation recorded by trials containing high and low concentrations of the independent variables (x_i), respectively. The main effect with positive sign indicates that the high concentration of this variable increases to optimum, while negative sign indicates that the low concentration of this variables is nearer to optimum.

Table 1 Factors examined as variable affecting CGTase activity produced by *B. megaterium*

Factors	Level		
	–1	0	+1
pH	6	7	8
Time	24	48	72
Starch	10	12.5	75
Peptone	2.5	5	7.5
K_2HPO_4	0.5	1	1.5
KH_2PO_4	1	2	3
$MgSO_4$	0.1	0.2	0.3

The steepest ascent method

The steepest ascent method was applied based on the results of Plackett-Burman experiment design. The design calculations were performed as described by [7]. The signs attributed to the effect (in factorial design) were used to initiate this phase of the optimization procedure. The figure of the effect total is divided by the number of trials examined which is equal to each of the generated figure was called slope. The slope was then multiplied by the unit of variation used in the factorial experiment (i. the extent of +ve and –ve values used). The figure generated for each folder was transferred relative to one of the factors was chosen arbitrarily. These final figures for each factor were then progressively added to (if possesses a +ve sign) or subtracted from (if possessed a –ve sign) the base level concentrated of each factor until a reasonable series had been completed or until one of the factors reached zero.

Analysis

Enzyme assay

CGTase activity was measured by phenolphethaline CD complex formation by incubating 0.1 ml enzyme solution with 1% soluble starch in 1 ml 50 mM phosphate buffer

(pH 6.5) at 60°C at for 20 min the reaction was stopped by the addition of 3.5 ml of 30 mM NaOH solution, followed by 0.5 ml 0.02% (w/v) phenolphethaline solution prepared in 5 mM sodium carbonate. The absorbance was measured spectrophotometrically at 550 nm. The activity of the enzyme was estimated as µg of CD produced/ml culture/min under the assay conditions [8].

Protein determination

The protein content was determined according to Lowry's method [9].

Statistical analysis

All data in this work were statistically analyzed by ANOVA test.

Results and discussion

Role of incubation period

In a preliminary experiment the time course production of CGTase in relation to the growth phases of the experimental organism was investigated as shown in Fig. 1. The enzyme synthesis begins at the early exponential phase and maximum CGTase activity and specific activities were obtained after 72 h of cultivation, with spontaneous increase in cell biomass yield. Thereafter, the enzyme estimates were gradually decreased with the prolongation of the fermentation periods to 100 h.

Evaluation of the factors affecting CGTase activities

Ten trials were conducted using the Plackett-Burman design (Tables 2 and 3). The results of both CGTase activity and specific activity of this screening exhibited a wide variation of the enzyme estimates. This variation reflected the importance of medium optimization to attain higher enzyme productivities. The pH value of the culture medium was initially adjusted using phosphate buffer (0.1 M) to cover range of 5–8.

The main effects of the examined factors on CGTase activity and specific activity were illustrated in Fig. 2. On analysis of the main effect and *t*-value of the seven tested ingredients (Table 4), those which showed a positive effect for CGTase activity were the fermentation time and potassium dihydrogen phosphate level (KH_2PO_4). The *t*-test for any individual factor allows an evaluation of the probability of finding the observed effect. Some investigators found that confidence levels >76% are acceptable [10]. In these experiments, variable with confidence levels >90% were considered as significant. The positive effect of KH_2PO_4 on the fermentation process and enzyme activity may be attributed to its positive role on the pH value of the fermentation medium, in addition to its nutritional value for the growth of the experimental organism. The negative terms were considered insignificant. The negative insignificant terms can be neglected [11].

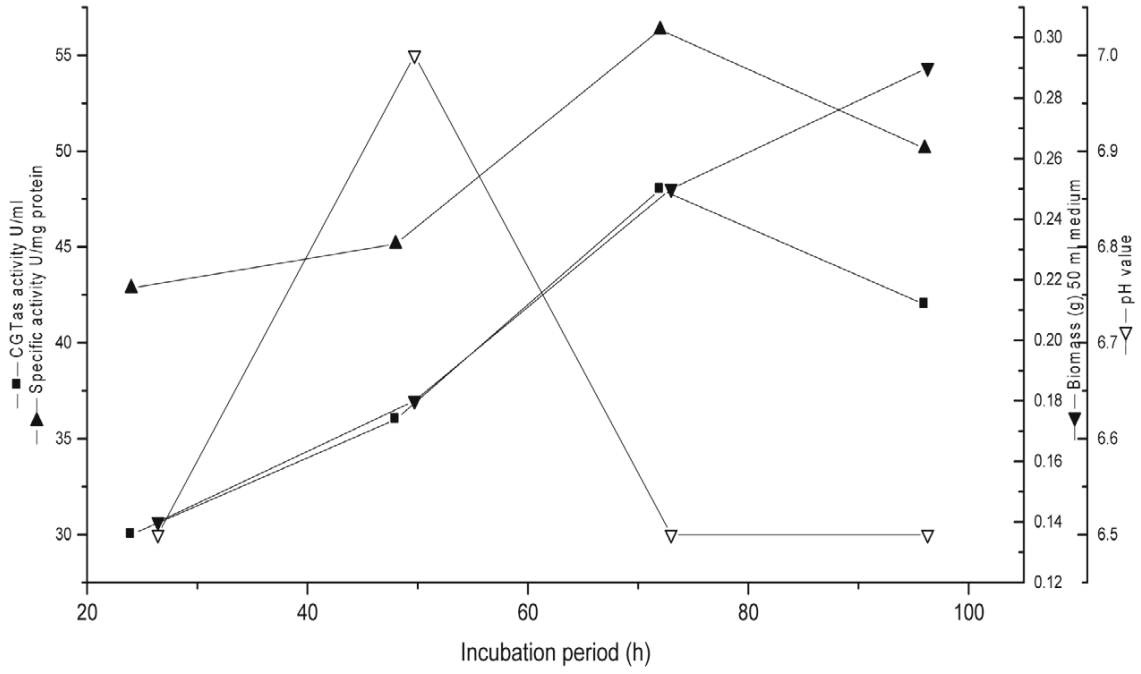


Fig. 1 Growth, pH, CGTase activity and CGTase specific activity of *B. megaterium* NRC as affected by the periods of incubation.

Table 2 The Plackett-Burman experimental design for evaluation of factors affecting GCTase production and activity produced by *B. megaterium* NRC

Trials No.	pH value	Total time	Starch	Peptone	K_2HPO_4	KH_2PO_4	$MgSO_4$
1	–	–	–	+	+	+	–
2	+	–	–	–	–	+	+
3	–	+	–	–	+	–	+
4	+	+	–	+	–	–	–
5	–	–	+	+	–	–	+
6	+	–	+	–	+	–	–
7	–	+	+	–	–	+	–
8	+	+	+	+	+	+	+
9	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0

Table 3 CGTase activity and specific activity of *B. megaterium* according to Plackett-Burman design

Trial	CGTase activity U/ml	Growth dry weight (g)	Protein mg/ml	Enzyme sp. activity
1	33.3 ± 0.2	0.14 ± 0.01	0.87 ± 0.01	38.27
2	39.4 ± 0.2	0.11 ± 0.01	0.69 ± 0.01	57.10
3	30.1 ± 0.58	0.07 ± 0.006	0.81 ± 0.005	37.16
4	36.5 ± 0.65	0.08 ± 0.35	0.90 ± 0.01	40.55
5	18.2 ± 0.15	0.28 ± 0.01	0.88 ± 0.01	20.68
6	21.9 ± 0.60	0.17 ± 0.1	0.72 ± 0.01	30.41
7	51.8 ± 0.64	0.09 ± 0.005	1.14 ± 0.01	59.1
8	18.2 ± 0.64	0.47 ± 0.01	0.85 ± 0.01	21.41
9	40.1 ± 0.62	0.15 ± 0.15	0.85 ± 0.03	47.05
10	40.3 ± 0.65	0.18 ± 0.015	0.81 ± 0.03	47.41

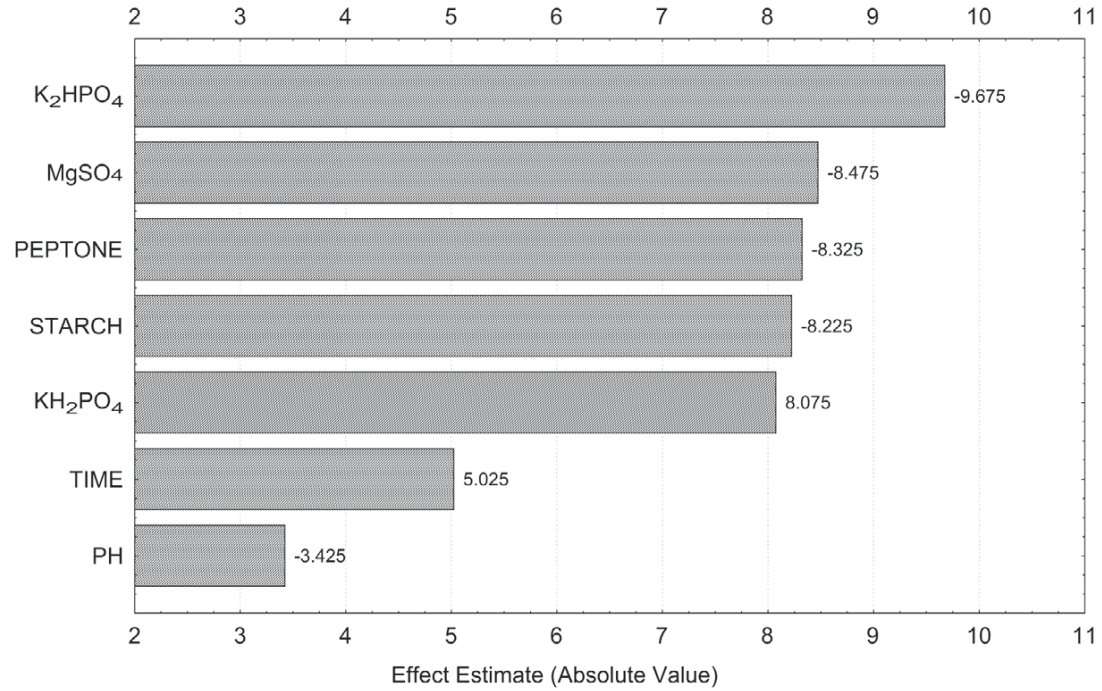
t-test at $p \geq 0.01$ highly significant; $p \geq 0.05$ significant.

Table 4 Statistical analysis of Plackett-Burman experimental results

Variable	CGTase activity U/ml		Enzyme specific activity	
	Main effect	*t*-value	Main effect	*t*-value
pH	–1.9	–1.48	–4.9	–1.15
Time	5	2.12	6.1	12.01
Starch	–8.22	–2.59	–7.2	–2.70
Peptone	–8.37	–3.88	–18.9	–6.96
K_2HPO_4	–9.7	–4.38	15.7	–5.77
KH_2PO_4	8.1	3.58	14.95	5.83
$MgSO_4$	–8.5	–3.78	–11.2	–3.79

Optimization of the most effective cultural factors

In order to attain maximum enzyme yields, two important variables namely; the fermentation time and KH_2PO_4 concentration were simultaneously optimized by applying the steepest ascent statistical design (Tables 5 and 6). The other medium components were omitted from the model as variables and were fixed at their suggested optimum levels. The change units of both fermentation time and KH_2PO_4 were calculated depending on their effect on total parameters (Table 5). The enzyme estimates recorded in Table 6 showed that the increase in concentration of KH_2PO_4 together with the expansion of the fermentation time caused a considerable increase in both the enzyme and specific activities up to 56.1 U/ml and 62.7 U/ml proteins, respectively at fermentation time (72 h) which aimed at trial number 7.



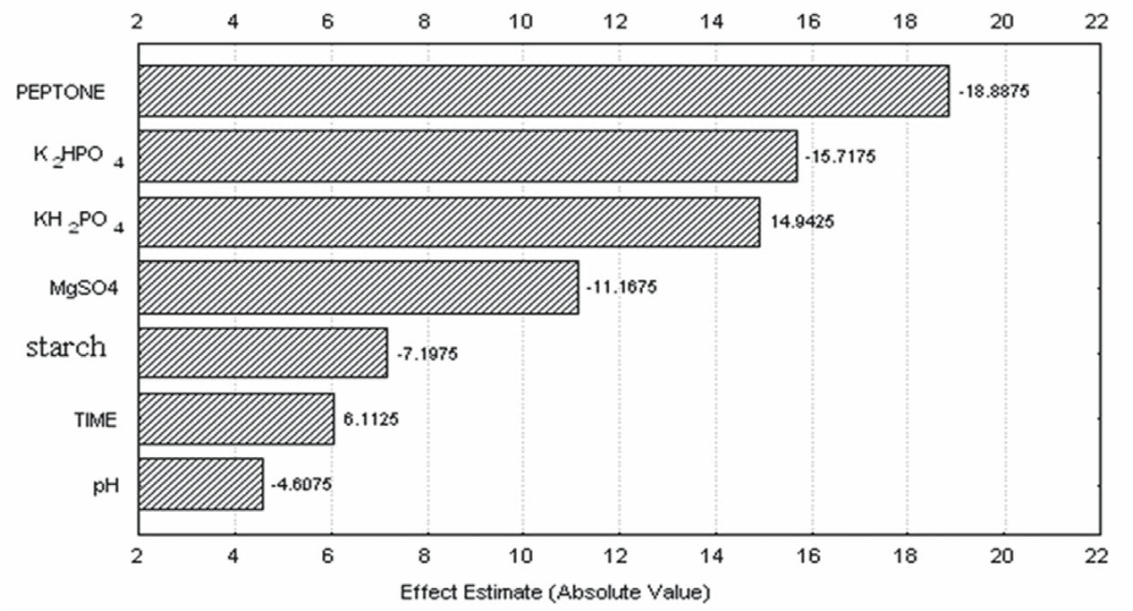


Fig. 2 Effect of physiological factors CGTase activity and CGTase specific activity produced by *B. megaterium* based on results of Plackett-Burman design.

Table 5 The statistical analysis of the second phase factorial experiment results (steepest ascent method)

Factors	Effect total	Slope	Relative change unit
Time	24.4	195.2	4.71
KH_2PO_4	52.32	418.56	0.212

Table 6 The steepest ascent method for two effective factors

Trials	Dry weight (g)	CGTase (U/ml)	Protein (mg/ml)	Specific activity
1	0.15 ± 0.01	25.6 ± 0.35	0.83 ± 0.07	30.8
2	0.15 ± 0.01	24.2 ± 1.01	0.52 ± 0.01	29.5
3	0.15 ± 0.01	32.4 ± 1.30	0.80 ± 0.07	40.5
4	0.14 ± 0.005	36.5 ± 0.32	0.75 ± 0.04	47.4
5	0.13 ± 0.01	44.4 ± 0.50	0.76 ± 0.04	58.4
6	0.23 ± 0.01	50.3 ± 0.70	0.82 ± 0.06	61.3
7	0.23 ± 0.01	56.1 ± 0.32	0.9 ± 0.007	62.7
8	0.13 ± 0.01	52.6 ± 0.80	0.9 ± 0.007	58.4
9	0.13 ± 0.01	48.3 ± 0.20	0.9 ± 0.007	53.6
10	0.13 ± 0.01	47.20 ± 0.06	0.9 ± 0.003	52.4

t-test at $P \geq 0.01$ highly significant; $P \geq 0.05$ significant

The result of this experiment supported the stimulation effect of KH_2PO_4 as a nutritional requirement and as buffering agent on the enzyme activity and production yield. Beside, the fermentation time play and important role in the potential growth (biomass yield), enzyme production and its activity.

Verification experiment

The optimal conditions realized from the previous study were verified experimentally by application of the obtained optimized medium and growth conditions. At the end of optimization approximately 1.25-fold increase in the enzyme activity was achieved if it compared with the basal production medium. These results indicated the validity of applied factorial design.

Conclusion

The application of the Plackett-Burman screening design identified the factors that can enhance the production and activity of CGTase by *B. megaterium* NRC. These included the fermentation time and KH_2PO_4 levels. The results also underwent further estimation by application of the steepest ascent method which paved the way to maximize the enzyme activity and specific activity. As a result this investigation both the enzyme activity and specific activity were elevated to 56.1 U/ml and 62.3 U/mg protein. It is hoped that such experimental design will be useful to maintain high efficiency in one of the economically important microbial enzymes, namely CGTase enzyme.

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