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RESEARCH PAPER

Statistical optimization of cyclodextrin glycosyltransferase (CGTase) production from *Bacillus macerans* in batch cultivation and its purification

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ABSTRACT

Cyclodextrin glycosyltransferase (CGTase) catalyzes the formation of Cyclodextrins from starch by an intramolecular transglycosylation reaction. In the present study, the CGTase enzyme was produced from *Bacillus macerans* NCIM 2131, using shake flask fermentation. Soluble starch and yeast extract were screened as the best carbon and nitrogen source at an individual factor level for enhancing CGTase activity. Similarly, ferrous ion and arginine were found to activate CGTase production in comparison to various other trace metals and amino acids tested. The synergistic effect of individual parameters of CGTase activity and process optimization of cultural condition was performed by response surface methodology (RSM). The optimized media comprised 24 g/L soluble starch; 32.5 g/L ferrous ion, and 15.0 g/L arginine with CGTase activity of 24.23 IU/mL. The final CGTase activity was very close to the predicted value of 24.82 IU/mL with 97.0% validation. Shake flask optimized conditions were further scaled up to 7.5 L fermenter (working volume: 3.0 L) which gave 2.6 folds (64.3 IU/mL) increase in enzyme activity. The enzyme was partially purified by ammonium sulfate precipitation and scanning electronic microscopy (SEM) was used to study the qualitative properties of produced enzyme.

Keywords: *Bacillus macerans*, CGTase activity, optimization, scale up in bioreactor, scanning electronic microscopy (SEM)

Cyclodextrin glucanotransferase (CGTase) is a member of the α-amylase superfamily, which catalyzes the cleavage of the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. CGTase, catalyzes four different reactions viz., cyclization, coupling, disproportionation and hydrolysis (Van der Veen *et al.*, 2000). The enzyme is mainly produced by members of the genus *Bacillus* (Tonkova, 2006) and is able to convert starch and related α-1, 4 glucans into Cyclodextrins (CDs), cyclic non-reducing oligosaccharides, composed of six or more glucose units joined by means of α-1, 4-glucosidic bonds (Terada *et al.*, 1997). The ability of

CDs to form inclusion complexes with many organic and inorganic molecules, changing their physical and chemical properties makes CGTase as an important enzyme for the medicine, food, cosmetic, pharmaceutical industries and in environmental protection (Szejtli 1997; Singh *et al.,* 2002). Bacteria are still regarded as an important source of CGTases. *Bacillus macerans* is the first reported source of CGTases but a wide variety of bacteria have been determined as CGTase producers, namely aerobic mesophilic bacteria, anaerobic thermophilic and aerobic alkalophilic bacteria (Tonkova 1998). The bacterial CGTase is a multifunctional enzyme and is produced by a variety of bacteria such as *B. macerans, B. megaterium, Klebsiella oxytoca, Micrococcus spp*. and *B. circulans* (Iyer *et al.,* 2003).

Cyclodextrins (CDs) are categorized into three different types: α-CD, β-CD and γ-CD, containing six, seven and eight glucose residues, respectively. The torous-shaped cyclodextrin have a unique structure with hydrophobic CH groups on the inside of the ring structure and hydrophobic hydroxyl groups on the outside of the ring. As separation of different CDs is costly and time consuming, CGTase that synthesizes predominantly one type of cyclodextrin is of interest. Generally, the majority of the *Bacillus* CGTases converts starch into β-CD as the main product (Sabioni and Park 1992a; Marechal *et al.,* 1996; Chung *et al.,* 1998; Gawande *et al.,* 1999; Park *et al.,* 1999; Martins and Hatti-Kaul 2002; Sian *et al.,* 2005). The β-form of cyclodextrin is reported to be more desirable for industrial use since inclusion complexes can be prepared easily which are very stable due to the low solubility of β-CDs in water. In addition, the yield of β-CD from starch is usually higher than that of other CDs (Thatai *et al.,* 1999). CDs and especially the β-CDs are applied in environmental protection by the solubilization of organic contaminants and removal of highly toxic substances from land, water and air.

CDs are also used to lower the volatility of odor molecules in perfumes and room freshener for controlled release of the odor. In the chemical industry, CDs are used in the separation of enantiomers to extract the toxic chemicals from the waste streams (Martin Del Valle 2009). β-CDs play a major role in the bioremediation of chronically contaminated soil by increasing the water solubility and reducing the toxicity of fungicides making them more susceptible to biodegradation. CDs are also used for the synthesis of organic nano polymers (pore size 0.7-1.2 nm), possessing the ability to absorb organic contaminants and by this way reduce their quantity to parts-per-trillion levels (Li and Ma 2000). In addition to production of CDs through the cyclization reaction, CGTase can also be used for its coupling and disproportional reactions for the synthesis of modified oligosaccharides by alternative acceptor substrates and limit dextrin's (Van derVeen *et al.,* 2000). A CGTase gene from more than 30 bacteria has been isolated and several of the enzymes have been identified and biochemically characterized (Qi and Zimmermann 2005). The enzyme can be utilized for the production of CDs and oligosaccharide with novel properties.

In the present study, individual factors were used as an initial screening process to identify the significant nutrients. A second level study was conducted with the central composite design (CCD) experiment to develop a mathematical model between the significant nutrients for the optimum production of CGTase from *Bacillus macerans*. SEM was performed to observe the structural degradation of starch granules during CGTase production. Finally the partial purification of the enzyme was also carried out by using ammonium sulfate precipitation.

MATERIALS AND METHODS

Materials

All the microbiological media were obtained from HI-Media (M/s Himedia, Mumbai, India), while other chemicals, buffer and reagents were of analytical grade and were procured from Sigma Laboratories Pvt. Ltd. (India) and Sisco Research Laboratories (Mumbai, India). Soluble starch was obtained from Merck Ltd. (India). ELICO SL-164 double beam UV–vis spectrophotometer was used for analysis purpose.

Source of the Bacterial Strain

The bacterial strain, *Bacillus macerans* NCIM 2131 was obtained from National Chemical Laboratory, Pune, India, and was maintained using a medium that contained (in g/L of distilled water); soluble starch, 1; peptone, 0.5; yeast extract, 0.4; K_2HPO_4, 0.1; $MgSO_4$, 0.02; Na_2CO_3, 0.06 at pH 7.0 ± 0.2 at 30°C.

EXPERIMENTAL METHODS

Production of Cyclodextrin glycosyltransferase

The enzyme was produced by cultivating the bacterium in 250 mL Erlenmeyer flasks containing 5.0% (v/v) inoculum of *Bacillus macerans* with 100 mL of the enzyme production medium (gL^{-1} of distilled water: soluble starch, 1.0; peptone, 0.5; yeast extract, 0.4; K_2HPO_4, 0.1; $MgSO_4$, 0.02; Na_2CO_3, 0.06 and *p*H 7.0.) in an incubator shaker (Lab Tech, Germany) at 37°C for 24 h at 150 rev min^{-1}. The culture fluid was centrifuged at 8000 rpm for 10 min at 4°C. The cell-free supernatant was used as the source of crude extracellular cyclodextrin glycosyltransferase enzyme.

Enzyme Assay

The enzyme was assayed by the method based on the reduction in color intensity of phenolphthalein after complexing with CDs, according to the method of Goel and Nene (1995). The cell free supernatant containing cyclodextrin glycosyltransferase was used for the determination of enzyme activity.

Total protein content

The total protein content was determined by the method of Lowry *et al.,* (1951) with bovine serum albumin as the standard. The protein content was calculated by checking the absorbance at 620nm.

Enzyme purification

The cell-free supernatant was partially purified by ammonium sulfate precipitation method (Kaulpiboon and Pongsawasdi, 2004). All steps of purification were performed at 4°C using 10 mM phosphate buffer (pH 7.5). The crude CGTase was precipitated overnight with 40-60% ammonium sulfate. The precipitates formed were collected by centrifugation and dissolved in phosphate buffer (pH 7.5).

Parametric Optimization

Preliminary trials for the optimization process was done by using various carbon sources (soluble starch, lactose, corn starch, maltose, glucose and sucrose), complex nitrogenous sources (yeast extract, beef extract, and corn steep liquor), trace metal ions (Ca^{+2}, Mg^{+2}, Fe^{+2} and Zn^{+2}), amino acids (arginine, phenylalanine, methionine, cysteine, tyrosine and tryptophan) and by varying the agitation speed (50 -200 rpm) in an incubator shaker (Lab Tech, Germany). Table 1 each parameter was examined after taking into account the previous optimized condition, i.e., 'one variable at a time' approach. All the experiments were performed in triplicate and the average values are presented.

Experiment design

The preliminary data were further expanded to a central composite design (CCD) with CGTase activity as the dependent variable and the significant term from preliminary trials as the independent variables. A total of 20 sets of experiments with different combinations of three variables viz., carbon source, amino acid and metal ions were employed in this study to determine the significant factors affecting the CGTase activity. The statistical software package MINTAB 14 (Minneapolis, USA) was used to analyze the experimental design. The design matrix with different variables was set at five levels ($-\alpha$, -1, 0, +1, $+\alpha$) (Table 2). All the variables were taken on a central coded value considered as zero. The minimum and maximum ranges of variables were investigated.

Scale up using bioreactor

Seed culture was prepared in 500 ml conical flask containing 100 mL of media production. Batch cultivation was carried out at 37°C in a 7.5 L bioreactor (BioFlo/Celli-gen 115, New Brunswick, USA) containing 3.0 L of media. The reactor was sterilized in an autoclave (Tomy Digital,Tokyo, Japan)

of capacity 50.0 L at the 121°C for 20 min. 5 % (v/v) seed inoculum was added in 3.0 L sterilized media. After inoculating with 5% (v/v) of seed culture, pH of the culture broth was maintained at optimum pH by automatic addition of 0.1 N HCl/NaOH by pH-mV program controller (Mettler Toledo, USA). DO (Dissolved Oxygen) concentration was analyzed by DO probe (Mettler Toledo, USA) and was maintained at 40% saturation value by cascading the speed of the agitator and air flow rate. Aeration rate was 1.5 L/min and the fermentation was carried out for 24.0 h to obtain maximum CGTase production under optimized condition.

Kinetics of CGTase production

The enzyme activity was observed at at its optimum temperature and pH with different substrate concentrations (1.0, 1.8, 2.7, 4.0 and 5.0 g/L, respectively). A Lineweaver Burk (LB) graph was plotted between reciprocal of enzyme activity (1/V) and the reciprocal of substrate concentration (1/S) for determination of kinetic constants K_m and V_{max}.

Scanning electron microscopy

Qualitative analysis of CGTase activity was performed by observing starch granules degradation after CGTase reaction using SEM (Scanning electron microscope, FEI Company, QUANTA 200, The Netherlands) at 20 kV, according to the method of (Yamamoto *et al.*, 2000).

RESULTS AND DISCUSSION

Effect of various carbon sources on CGTase activity

Effect of different carbon sources on CGTase activity was monitored in submerged batch cultivation. Significantly high CGTase activity was observed when soluble starch was supplemented as a carbon source, while with glucose, significantly less activity was obtained (Fig. 1*A*). Previously, Sivakumar and Bhanu, (2011) reported similar findings, where soluble starch gave the maximum CGTase production in *B. macerans* in similar condition (Sivakumar and Bhanu 2011). Kulpreecha *et al.*, (2009) used various carbon sources such as rice, corn and soluble starch for the synthesis of CGTase. Varavinit *et al.* (1998) reported that the production of CGTase by *Bacillus* sp. MP 523 was repressed significantly by glucose. However, studies carried out by Stefanova *et al.*, (1999) showed that 0.5% (w/v) glucose was found to be the most suitable substrate for CGTase production.

Effect of various nitrogen sources on CGTase activity

Fig 1b represents the effect of different nitrogen source on CGTase production. Among the three different nitrogen sources studied the productivity of CGTase was most significantly influenced by yeast extract (Fig. 1*B*). CGTase production was found to be higher when an organic nitrogen source was present in the medium. This is in conformity with previous findings where sago starch and yeast extract combination showed a pronounced effect on CGTase production (Mahat *et al.*, 2004).

Effect of various trace metal solutions on CGTase activity

The effect of trace metal ions on the activity of CGTase was studied by substituting different metal ions in the basal media. All metal ions inhibited the activity of CGTase. However Fe^{+2} supplemented media gave enhanced CGTase activity, suggesting the enzyme to be a metalloprotein needing a cofactor for its maximum activity while Zn^{+2} was the strong inhibitor of CGTase activity as shown in Fig. 1*C*. Sian *et al.*, (2005) reported that Fe^{+2} (in the form of $FeCl_2$) promoted CGTase activity when incubated with an enzyme without substrate for 10 min at 25°C (Sian *et al.*, 2005).

Previous studies showed that CGTase from *Brevibacterium* sp. no. 9605 (Mori *et al.*, 1994), *Bacillus halophilus* INMIA-3849 (Abelian *et al.*, 1995), *Bacillus firmus*, *Bacillus* sp. AL-6 (Fujita *et al.*, 1990) and *Bacillus macerans* ATCC 8244 (Arya and Srivastava 2006) was strongly inhibited by Zn^{+2}. The inhibitory effect could be due to the oxidation of amino acid residues essential for the cyclization reaction. But, it was also reported that Mg^{+2} and Ca^{+2} showed less activation on CGTase activity (Freitas *et al.*, 2004). The effect of

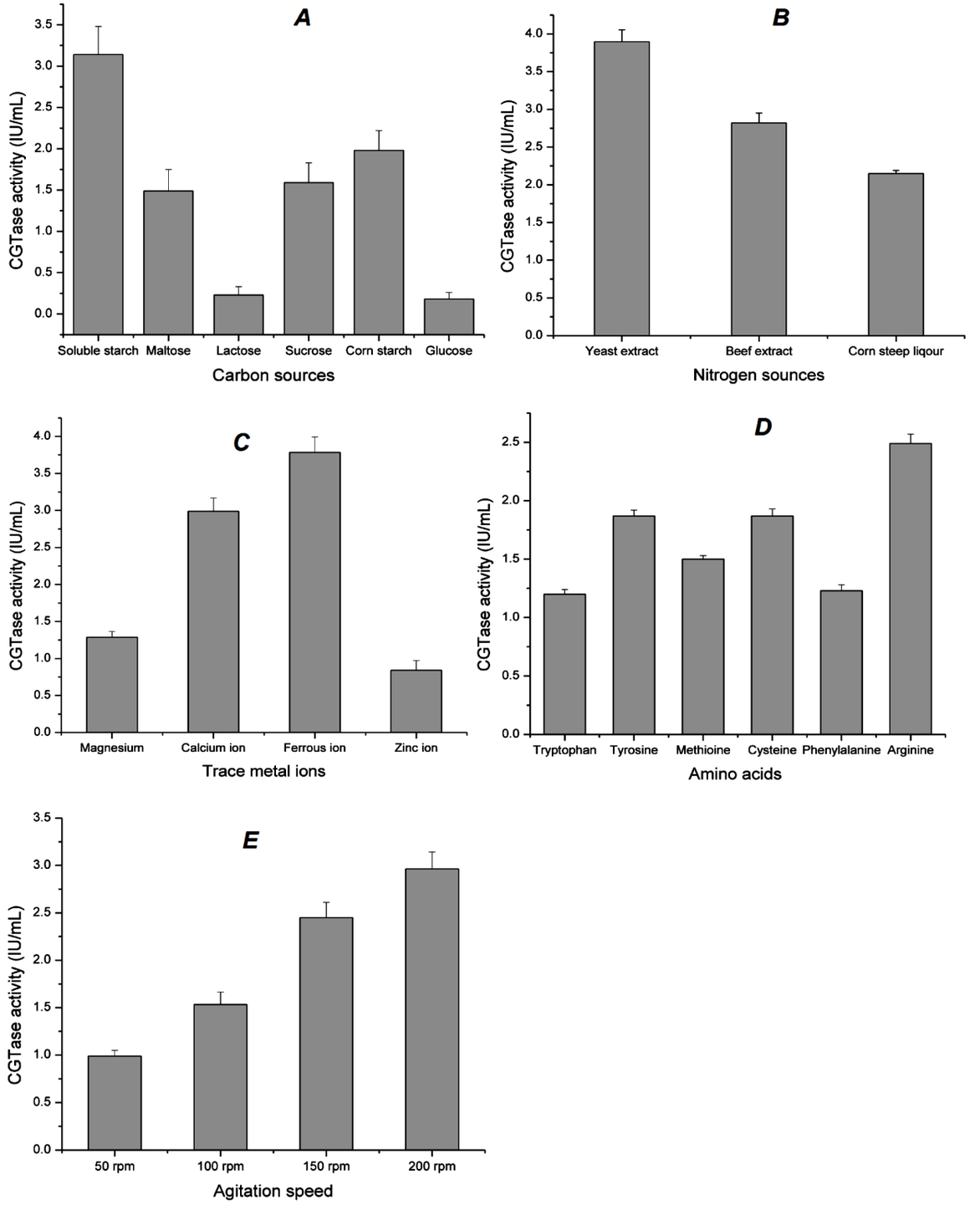

Fig. 1: Effect on CGTase activity in the production media due to (a) carbon sources (b) complex nitrogen sources (c) trace metal ions (d) amino acids and (e) agitation speed

metal ions on enzyme activity seems to depend on the enzyme source (Higuti *et al.,* 2003).

Effect of various amino acids on enzyme activity

The effect of amino acid supplementation on CGTase activity was studied in comparison to other aromatic and aliphatic amino acid. Arginine showed maximum enzyme activity among the present aromatic and aliphatic amino acids (Fig. 1*D*). As arginine is basic and aliphatic amino acid and is not produced by *Bacillus macerans* therefore when supplemented, it led to increase in the cell density and enzyme activity. However, tyrosine and tryptophan supplementation inhibited the CGTase activity.

Effect of different agitation speed on enzyme activity

Figure 1E represents the effect of varying agitation speed on CGTase activity. The highest enzyme activity was obtained at an agitation speed of 200 rpm (Fig. 1d). However, enzyme activity was the least at an agitation speed of 50 rpm due to inadequate mixing of media components. Similarly, it decreased as the agitation speed was increased to more than 200 rpm due to shear loss.

Statistical optimization of cultural conditions by the CCD

The individual factor study revealed that soluble starch, trace metal solution (Fe^{2+}) and arginine showed a pronounced effect on CGTase activity. Taking these three factors into account, a total of 20 sets of experiments with different combinations of soluble starch, trace metal solution and amino acid were performed (Table 1).

Table 1: CCD design for the optimization of the cultural conditions, including the soluble starch, ferrous ion and arginine concentration

Expt No.	Soluble starch (g/L)	Ferrous ion (g/L)	Arginine (g/L)	Enzyme activity (IU/mL)
1	20.0	50.0	20.0	16.6
2	14.0	32.5	6.5	20.0
3	14.0	32.5	15.0	19.3
4	8.0	15.0	20.0	15.3
5	3.9	32.5	15.0	3.0
6	24.0	32.5	15.0	22.3
7	8.0	50.0	20.0	19.3
8	20.0	50.0	10.0	22.0
9	14.0	32.5	15.0	22.3
10	8.0	50.0	10.0	18.9
11	14.0	61.9	15.0	12.6
12	8.0	15.0	10.0	3.5
13	14.0	32.5	15.0	20.3
14	20.0	15.0	20.0	19.3
15	14.0	32.5	15.0	20.0
16	14.0	3.0	15.0	3.5
17	14.0	32.5	15.0	20.3
18	14.0	32.5	23.4	22.2
19	14.0	32.5	15.0	20.3
20	20.0	15.0	10.0	20.3

The ranges and levels of independent variables used in CCD were obtained from experimental design (Table 2).

Table 2: Experimental range and levels of independent variables used in CCD

Independent variables	Range and levels				
	-α	**-1**	**0**	**1**	**+α**
Soluble starch (g/L)	3.90	8	14	20	24.0
Ferrous ion (g/L)	3.0	15	32.5	50	61.9
Arginine (g/L)	6.5	10	15	20	23.4

*Significant terms in the model; DF: degree of freedom; Seq SS: sequential sum of squares; Adj MS: adjusted mean square.

The results obtained were analyzed using analysis of variance (ANOVA) as appropriate to the experimental design used. The regression equation obtained after analysis of variance gives the production of CGTase from *Bacillus macerans* as a function of different variables of soluble starch concentration (A, g/L),

trace metal ion, i.e. ferrous ion concentration (B, g/L), amino acid i.e. arginine concentration (C, g/L). All the terms regardless of their significance were included in the second order polynomial equation represented by Eq. 1.

$$\text{CGTase activity} = 20.3082 + 3.9291A + 2.4679B + 0.6956C - 2.0369A^2 - 3.6632B^2 + 0.9506C^2 - 2.5500AB - 2.3250AC - 1.9750BC \quad (1)$$

The determination coefficient (R^2) of the model was found to be 0.897 (a value of > 0.75 indicates fitness of the model). An R^2 value can be between 0 to 1, and the closer value to 1.0 is better fits the experimental data. Thus the study indicates that 89.7% of the variation in the response (i.e. Enzyme activity) was attributed to the independent variables, whereas 10.3% of the total variance could not be explained by the model. An adjusted R^2 was 0.804, which accounted for the number of predictors in the model. Both the obtained R^2 values suggested that the model fitted the data well. The F statistic was 9.70 and corresponded to a value of P = 0.001 (the confidence interval was 0.05), which indicated that the model was both adequate and significant (Table 3).

Table 3: ANOVA for the full quadratic model for optimization of soluble starch, arginine and ferrous ion

Source	DF	Seq SS	Adj SS	Adj MS	F value	P value
Regression	9	692.739	692.739	76.971	9.70	0.001*
Linear	3	300.616	7320.0	100.205	12.63	0.001*
Square	3	265.652	265.652	88.551	11.16	0.002*
Interaction	3	126.470	126.470	42.157	5.31	0.019*
Residual error	10	79.347	79.347	7.935		
Lack of fit	5	74.338	74.338	14.868	14.84	0.005*
Pure error	5	5.008	5.008	1.002		
Total	19	772.085				

*Significant terms in the model; DF: degree of freedom; Seq SS: sequential sum of squares; Adj MS: adjusted mean square.

The values A, B, A^2, B^2, AB and AC were significant (Table 4). The probability (P) values were used as a tool to check the significance of each of the coefficients. The larger the magnitude of the t-value and smaller the P-value, the more significant the corresponding factor the significant the coefficient (Dobbins D C 1994). For the first order effects, judging from the regression coefficient and t-values, it could be concluded that the soluble starch (A) concentration had the most significant effect on the CGTase activity, followed by trace metal ion (B).

Table 4: Estimated regression coefficients for soluble starch, ferrous ion and arginine

Term	Coefficient	Standard coefficient	t-value	P-value
Constant	20.3082	1.1488	17.677	0.000*
A	3.9291	0.7622	5.155	0.000*
B	2.4679	0.7622	3.238	0.009*
C	0.6956	0.7622	0.913	0.383
A^2	-2.0369	0.7420	-2.745	0.021*
B^2	-3.6632	0.7420	-4.937	0.001*
C^2	0.956	0.7420	1.281	0.229
AB	-2.5500	0.9959	-2.560	0.028*
AC	-2.3250	0.9959	-2.335	0.042*
BC	-1.9750	0.9959	-1.983	0.075

*Significant terms in the model; A: Soluble starch concentration in g/L.; B: Ferrous ion concentration in g/L.; C: Arginine concentration in g/L.

The quadratic main effect of soluble starch (P < 0.021) and trace metal ion (P < 0.001) were the significant factors. The arginine concentration was not significant at the quadratic level (P > 0.05). Therefore, soluble starch and ferrous ions act as limiting factors and a little variance in their concentration may alter either growth of an organism or the rate of the enzyme activity or both to a considerable extent. It was shown that increasing the concentration of soluble starch and arginine from 6-24 g/L and 3.0-10 g/L, respectively increased enzyme activity (Fig. 2a). The P-value is 0.042 which is indicating that it was significant to some extent (P < 0.05) in this model of enzyme activity (Table 4). However, the soluble starch concentration was the significant variables.

Interaction between the concentration of soluble starch and ferrous ion were significant (P = 0.001) (Table 4). Thus increasing the concentration of soluble starch will increase the CGTase activity (Fig. 2b), where each increase in the concentration of soluble starch and ferrous ion was followed by a significant increase in CGTase activity. Maximum CGTase activity was obtained at 22.45 g/L concentrations of soluble starch with ferrous ion at concentrations of 47.0 g/L.

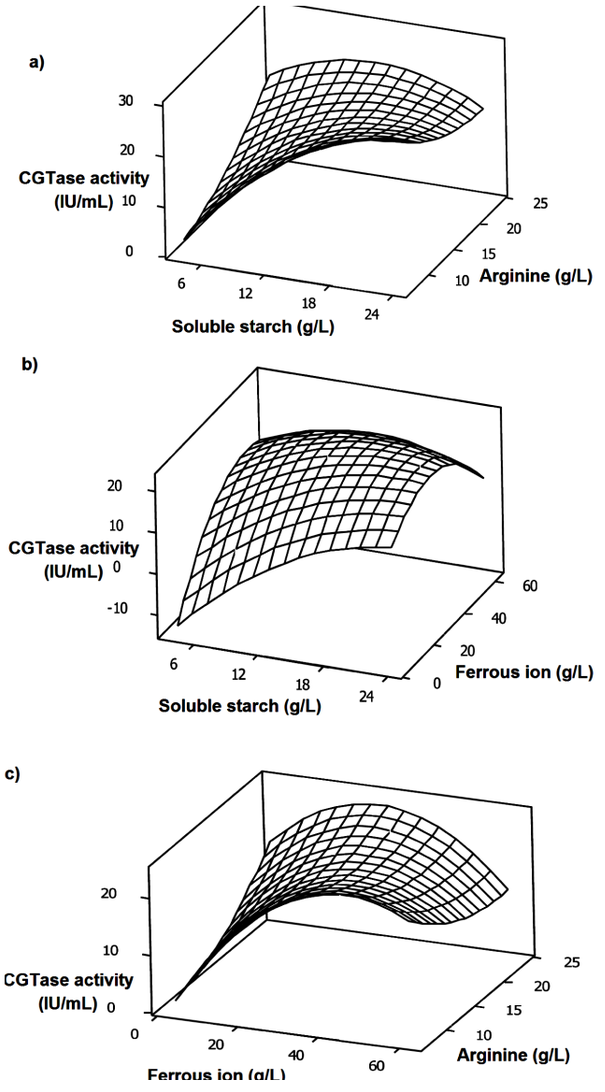

Fig. 2: Response surface plots showing the effect of a) soluble starch and arginine b) soluble starch and ferrous ion and c) ferrous ion and arginine on CGTase activity.

The nature and concentration of carbon source are highly important in enzyme production, especially when carbon source also plays an important role in enzyme induction (Gawande and Patkar 2001). Results shown by Mahat *et al.,* (2004) demonstrated that CGTase production could be increased due to increase in the concentration of sago starch.

The last interaction in the CCD is the interaction of ferrous ion and arginine. This interaction was however, insignificant to enhance the CGTase production (Fig. 2c). The P-value is 0.075, indicating that it was not significant, i.e. P > 0.05 (Table 4). The peaks for ferrous ion and arginine were obtained at a concentration of 47.0 g/L and 6.59 g/L, respectively. It was found that the optimum concentrations of soluble starch, ferrous ion and arginine were 24.0 g/L, 32.5 g/L and 15.0 g/L, respectively. Under these optimized conditions, the model predicted a CGTase activity of 24.23 IU/mL and through experimental re-checking the model, an activity of 24.82 IU/mL was attained.

Scaleup in bioreactor

Shake flask cultivation was then, scaled up in 7.5 L bioreactor with working volume 3.0 L in batch cultivation. After scaling up at the optimized conditions in the biorector, the enzyme activity increased up to 64.3 IU/mL. The reason for increase in changes in the levels of enzyme activity may be due to the differences in hydrodynamic conditions, oxygen limitation and shear rate created in the shake-flask. Ibrahim *et al.,* (2010) reported the enzyme activity of 62.63 IU/mL in batch mode bioreactor using tapioca starch as the carbon source for *Bacillus* G1. The maximum CGTase activity at 37°C was observed to be 3.1 IU/mL, when *Bacillus licheniformis* was used as the producing organism (Sian *et al.,* 2005).

Partial purification of CGTase

The cell free supernatant of the *Bacillus macerans* culture was concentrated by using 40-60% saturation of ammonium sulfate. The purified enzyme activity was 12.3 IU/mL and the total protein was purified by 50% ammonium sulfate. The specific activity of the purified CGTase has been increased tremendously,

from 10.84 IU/mg to 18.49 IU/mg in crude culture broth (Table 5). The partial purification of protein by 50% ammonium sulphate is in agreement with the study previously conducted by Arya and Srivastava (2006). The purified CGTase was obtained due to the hydrophobic interaction between the enzyme and β-CD.

Table 5: Summary of purification of the CGTase from *Bacillus macerans*

Fraction	Volume (mL)	Total protein (mg)	Total activity (IU/mL)	Specific activity (IU/mL)
Culture broth	3000	6724	72900	10.84
Ammonium sulphate precipitation	800	532	9840	18.49

Kinetics of CGTase production

Fig. 3 represent Lineweaver Burk (LB) plot between reciprocal of enzyme activity (1/V) and the reciprocal of substrate concentration (1/S) for determination of kinetic constants K_m and V_{max}. K_m and V_{max} values for CGTase enzyme were found to be 1.6 g/L and 34.2 μg/mL/min, respectively.

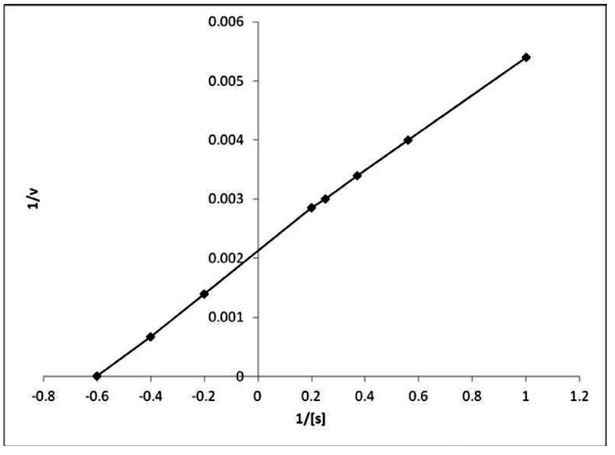

Fig. 3: Lineweaver Burk (LB) plot between 1/V and 1/S for determination of K_m and V_{max}

Scanning electron microscopy

Scanning electron microscopy revealed distinct morphological changes resulting due to the enzymatic treatment. Raw starch granules are resistant to enzymatic hydrolysis because of the larger granule size. The potential application of raw starch digesting CGTase preparation from *Bacillus macerans* was evaluated by studying the extent of hydrolysis of starch granules. Fig. 4a shows that untreated soluble starch had a smooth surface, but of irregular shapes without any enhanced artifacts. The numerous depression or pores on the surface and ruptured cell wall of starch granules were found due to the action of CGTase as shown in Fig. 4b.

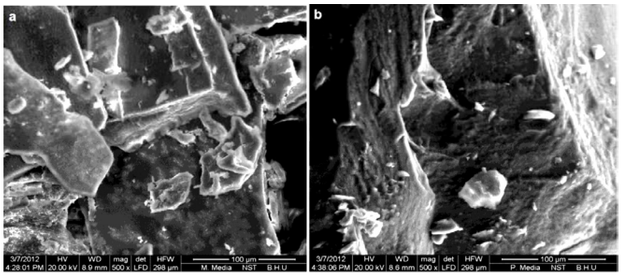

Fig. 4: Scanning electron micrographs showing a) untreated soluble starch granule and b) starch granule after treatment with CGTase from *Bacillus macerans*

CGTase from *Bacillus macerans*, degraded the intact granules of soluble starch and converted them into CDs. Digestion of starch granules by α- amylase alone was slightly less than digestion by the combination of α- and β-amylases (Maeda *et al.*, 1978). CGTase catalyzes multiple reactions (hydrolysis, cyclization, coupling, and disproportionation), whereas other amylases such as α-amylase, β-amylase, and glucoamylase only catalyze *endo*- or *exo*-hydrolysis. Therefore, it is suggested that the unique morphology of the degraded soluble starch granules might be associated with the unique action mechanism of CGTase.

Soluble starch as a carbon source gives the highest production of CGTase. Presence of ferrous ions showed a relative increase in the enzyme activity. Completely randomized design and response surface analysis were useful to determine the optimum levels of medium concentration and other factors that significantly influenced the production of CGTase.

The final composition of CGTase production medium contains 24.0 g/L of soluble starch, 32.5 g/L of ferrous ion and 15.0 g/L of arginine. This optimized media were expected to produce 24.23 IU/mL of CGTase while in practice; the production of CGTase was 24.82 IU/mL. Therefore, this model adequately predicted the optimization of CGTase production from *Bacillus macerans.* Further, enzyme activity of 64.3 IU/mL was obtained which clearly shows the 2.6 fold enhancement in the enzyme activity when the optimized conditions as scaled up in the bioreactor.

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