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OPTIMISATION OF INOCULUM AND MOISTENING MEDIA FOR ENHANCED CGTase PRODUCTION IN SOLID SUBSTRATE FERMENTATION

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ABSTRACT

β-Cyclodextrins (β-CD) are produced by degradation of starch by cyclodextrin glucanotransferase (CGTase). Due to its widespread applications in agricultural, pharmaceutical, textile, food, and cosmetic industries, cost effective CGTase production strategies are essential for the commercial β-cyclodextrin manufacturing. In the present study, CGTase production was optimised using agro-waste wheat bran as a substrate under solid state fermentation (SSF), which is an attractive alternative to conventional submerged fermentation (SmF) methods. CGTase was produced using *Bacillus* sp. NCIM 5799, a strain that exclusively synthesises β-CD. The effects of different inoculum compositions, pH, carbon sources, and nitrogen sources, on CGTase activity were evaluated. Inoculum at pH 10, soluble starch as the carbon source, and peptone as the nitrogen source supported the highest enzyme activity. Various moistening media were also assessed, and Murashige and Skoog (MS) micronutrients-only solution supported enhanced CGTase production. Further statistical optimisation of the MS (micronutrients only) medium was performed using Plackett-Burman Design (PBD) and Central Composite Design (CCD), which identified $CuSO_4.5H_2O$ as a significant linear factor, while the interactions between H_3BO_3-$MnSO_4.4H_2O$, as well as between $MnSO_4.4H_2O$-$CoCl_2.6H_2O$, were found to be the most influential factor interaction. Under optimised condition, using 100% (v/w) inoculum and 100% (v/w) optimised moistening medium, CGTase activity reached 13,653.6 U/gds at 48 hour, representing a 5.2-fold increase compared to the unoptimized conditions.

Keywords: CGTase, β-cyclodextrin, production optimisation, solid state fermentation, Plackett-Burman design, central composite design

INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) is a bacterial enzyme of the α-amylase family (family 13) that hydrolyses starch or starch-related substrates to produce cyclodextrins (CDs). CDs are cyclic oligosaccharides, which are biodegradable and non-toxic. The most notable feature of CDs is their ability to form inclusion complexes (host–guest complexes) with a wide range of solid, liquid and gaseous compounds by molecular complexation. CD cavities feature hydrophobic interiors and hydrophilic exteriors, facilitating the inclusion of hydrophobic molecules within the inner cavity to form inclusion complexes. Inclusion in CDs significantly influences the physicochemical properties of guest molecules, including solubility, volatility, sublimation, and stabilisation **(Sarabia-Vallejo *et al.*, 2023)**.

CGTase converts starch into different ratios of alpha (α), beta (β), and gamma (γ) CDs having six, seven, and eight glucose moieties, respectively, bonded together by α-1,4 glycosidic bonds. Among these types, β-CD holds significant industrial value due to its cavity size, low aqueous solubility, and low toxicity **(Tarannum *et al.*, 2020)**. The rapid formation of inclusion complexes with β-CD makes it highly desirable in various applications, including pharmaceuticals, analytical chemistry, agriculture, cosmetics, food, and biotechnology **(Bezerra *et al.*, 2020; Kolarič and Šimko, 2022; Liu *et al.*, 2022; Puskás et al., 2025)**.

CGTase is predominantly an extracellular enzyme produced by various bacteria, primarily by alkaliphilic *Bacillus* species **(Mora *et al.*, 2012)** which catalyzes the intra- and intermolecular transglycosylation of α-1,4-glucan. CGTase produce CDs by intramolecular transglycosylation (cyclisation) reaction. Beside cyclisation, the enzyme also catalyzes coupling reaction and disproportionation reaction. The coupling activity is the reverse of their cyclisation function, where CGTase can hydrolyse the ring structure of CDs and attach the resulting linear oligosaccharide to another sugar chain. In disproportionation reaction, enzyme transfers a portion of a linear maltooligosaccharide (donor) onto another linear maltooligosaccharide (acceptor) via double displacement mechanism. CGTase also possesses a weak hydrolyzing activity **(Lim *et al.*, 2021)**.

CGTases are typically produced by submerged fermentation (SmF) utilising starch as a carbon source. In this system, the chemical composition of culture medium is nearly homogeneous, which can inhibit enzyme synthesis and activity through catabolite repression (CR) **(Viniegra-González and Favela-Torres, 2006)**. Solid-state fermentation (SSF) is an alternative for enzyme production, which significantly reduces the effects of CR. As SSF uses solid substrate (often industrial or agricultural waste) with only enough moisture for microbial growth, the diffusion of sugars from the matrix into the microbial cells is slow and is controlled by microscopic concentration gradients (**Viniegra-González and Favela-Torres, 2006**). This means that while the overall substrate concentration might be high, the local, immediate concentration around the individual microbial cells remains low or growth-rate-limiting, preventing the trigger for CR.

Only a handful of studies have reported CGTase production under SSF, and even those have not focused exclusively on SSF. In most cases, SSF was evaluated only relative to SmF. Pinto et al. (2007) reported a maximum activity of 1,155 U mL^{-1} under SmF, whereas the activity achieved under SSF was substantially higher (Table 1), highlighting the potential of low-cost agro-industrial substrates for CGTase production. Similarly, Ramakrishna et al. (1994) reported a 1.48-fold increase in CGTase activity under SSF compared with SmF. Alves Prado et al. (2007), however, observed comparable CGTase activities under SSF and SmF. Overall, these findings support SSF as a potential alternative to conventional SmF for CGTase production.

In the present study, we investigated CGTase production by *Bacillus* sp. NCIM 5799 under SSF conditions. Thus far, the majority of reported CGTases produces α-, β- and γ-CDs from starch in varying ratios depending on the reaction conditions and the nature of the CGTase (Mahat et al., 2004). A notable feature of this strain is its ability to exclusively synthesise β-CD, which is advantageous because it eliminates the need for additional purification steps to separate other CD forms. Initially, inoculum composition and moistening media was optimised using a one-factor-at-a-time (OFAT) approach. The influence of different sugars, starches, and nitrogen sources on inoculum development was also examined. Further optimisation of the moistening media was performed using a Plackett-Burman Design (PBD) and a Central Composite Design (CCD). The effects of inoculum quantity and initial moisture content on enzyme activity were also evaluated.

Table 1 Comparative analysis of CGTase production under solid-state fermentation

Microorganism	Substrate	Fermentation conditions	Assay conditions	CGTase activity	Reference
Bacillus circulans	Soybean industrial fibrous residue (SIFR)	48 h at 37°C	Phenolphthalein assay (soluble starch, pH 6.0, 60 °C, 15 min; A_{550})	32,776 $U/g_{(SIFR)}$	Pinto et al. (2007)
Bacillus cereus	Wheat bran	24 h at 37°C	Phenolphthalein assay (soluble starch; A_{550})	59 U/mL	Ramakrishna et al. (1994)
Paenibacillus campinasensis H69-3	Wheat bran + soy bran	72h at 45°C	Phenolphthalein assay (1% soluble starch, 0.1 M acetate buffer, pH 5.5, 55 °C, 10 min, A_{550})	1.5 U/mL	Alves Prado et al. (2007)

MATERIALS AND METHODS

Chemicals

β-CD was purchased from SD Fine-Chem Limited, Mumbai, India. Substrates like wheat and tapioca starch were sourced from Urban Platter, Mumbai, India. Maize starch and potato starch were obtained from Loba Chemie Pvt Ltd, Mumbai, India. Soluble starch and Phenolphthalein (PHP) were purchased from Merck Ltd., Mumbai, India. Media components were procured from Himedia Laboratories, Mumbai, India. Wheat bran was procured from a local flour mill. All the other chemicals used in the experiments were of analytical grade.

Microorganism

Bacillus sp. NCIM 5799 was originally isolated by our laboratory from a soil sample and was used throughout the experiments. The strain was deposited in the National Collection of Industrial Microorganisms (NCIM) under accession number NCIM 5799, and its 16S rRNA sequence is available in NCBI GenBank under the number MN938303.
The bacterial culture was grown on nutrient agar (NA) slants (pH 10.5) and incubated at 30°C for 24 hours. Following incubation, the cultures were stored at 4°C in a refrigerator until further use, with regular sub-culturing performed every 15 days to maintain culture viability and purity.

Selection of suitable inoculum media for CGTase production

Five different inoculum media compositions (A-E) were selected for this study (Table 2), including: Horikoshi medium, as originally described by **Horikoshi (1971)**; a modified version of this medium developed by **Ibrahim *et al.* (2005)**; and OFAT and response surface methodology (RSM) optimised formulations reported by **Solanki *et al.* (2022)**; and, lastly, nutrient broth.
A loopful of bacteria from the NA slant was initially inoculated into nutrient broth (pH 10.5) and incubated for 24 h at 30°C on a gyratory shaker at 150 rpm. Subsequently, 10% v/v of this culture was transferred to each of the different autoclaved inoculum media and incubated under identical conditions for an additional 24 h. The 50% (v/w) of this grown culture was thereafter, used to aseptically inoculate sterilised solid substrate medium (wheat bran moistened with 0.5% sodium carbonate, 50% v/w). In preliminary experiments, the maximum CGTase activity in SSF was observed at 32°C after 72 h incubation; therefore, the enzyme activity was determined and compared at this time point.

Table 2 Composition of different inoculum media

Inoculum Media	Concentration (%)								
	Soluble Starch	Peptone	Yeast Extract	K_2HPO_4	$MgSO_4.7H_2O$	Na_2CO_3	Beef Extract	NaCl	Reference
A	1	0.5	0.5	0.1	0.02	1	-	-	**Horikoshi, 1971**
B	4	2	-	0.1	0.04	1	-	-	**Ibrahim *et al.*, 2005**
C	8	2	-	-	0.06	0.5	-	-	**Solanki *et al.*, 2022**
D	8	3	-	0.1	0.02	0.8	-	-	**Solanki *et al.*, 2022**
E	-	5	1.5	-	-	-	1.5	5	Nutrient broth

Inoculum Optimisation

Once the inoculum medium was selected, the effect of initial pH (8.5-10.5) on CGTase activity was investigated by varying the volume of 1% Na_2CO_3. *Bacillus* is alkaliphilic bacteria, so pH could play an important role. Further optimisation of inoculum medium B involved evaluating alternative carbon sources to identify the most suitable substrate. The sugars tested were glucose, sucrose, maltose, and mannitol , while various starch sources- soluble starch, tapioca starch, wheat starch, potato starch, and maize starch were also assessed for their effect on CGTase production. Following carbon source optimisation, the effect of different nitrogen sources in the inoculum medium was evaluated. Peptone was replaced with different organic nitrogen sources (yeast extract, tryptone, and casein), as well as the inorganic nitrogen sources (potassium nitrate, sodium nitrate, ammonium nitrate, ammonium sulfate, and ammonium chloride).

Table 3 Composition of different moistening media

S. No.	Moistening Media	Composition	Reference
01	Sodium carbonate	0.5% Na_2CO_3	**Alves-Prado *et al.*, 2006**
02	Horikoshi II Medium	Soluble starch (10 g/L), Peptone (5 g/L), Yeast extract (5 g/L), K_2HPO_4 (1 g/L), $MgSO_4.7H_2O$ (0.2 g/L), Na_2CO_3 (10%)	**Horikoshi, 1971**
03	Hoagland solution	$Ca(NO_3)_2.4H_2O$ (472.2 mg/L), KNO_3 (303 mg/L), $(NH_4)H_2PO_4$ (115 mg/L), $MgSO_4.7H_2O$ (69.2 mg/L), KCl (1.8 mg/L), H_3BO_3 (0.8 mg/L), $CuSO_4.5H_2O$ (0.1 mg/L), $H_2MoO_4.H_2O$ (0.1 mg/L), $MnSO_4.4H_2O$ (0.1 mg/L)	**Esperin, 1972**
04	Murashige and Skoog (MS) complete medium	Macronutrients- KNO_3 (1900 mg/L), NH_4NO_3 (1650 mg/L), $CaCl_2.2H_2O$ (440 mg/L), $MgSO_4.7H_2O$ (370 mg/L), KH_2PO_4 (170 mg/L) Micronutrients- $Na_2EDTA.2H_2O$ (37.3 mg/L), $FeSO_4.7H_2O$ (27.8 mg/L), $MnSO_4.4H_2O$ (22.3 mg/L), H_3BO_3 (6.2 mg/L), $ZnSO_4.7H_2O$ (8.6 mg/L), KI (0.83 mg/L), $Na_2MoO_4.2H_2O$ (0.25 mg/L), $CuSO_4.5H_2O$ (0.025 mg/L), $CoCl_2.6H_2O$ (0.025 mg/L)	**Murashige and Skoog, 1962**
05	Murashige and Skoog (MS) micronutrients only medium	$Na_2EDTA.2H_2O$ (37.3 mg/L), $FeSO_4.7H_2O$ (27.8 mg/L), $MnSO_4.4H_2O$ (22.3 mg/L), H_3BO_3 (6.2 mg/L), $ZnSO_4.7H_2O$ (8.6mg/L), KI (0.83 mg/L), $Na_2MoO_4.2H_2O$ (0.25 mg/L), $CuSO_4.5H_2O$ (0.025 mg/L), $CoCl_2.6H_2O$ (0.025 mg/L)	**Murashige and Skoog, 1962**
06	Packaged mineral water	-	-
07	Tap water	-	-

Finally the effect of inoculum quantity on CGTase production was determined, by testing different inoculum concentrations (25%, 50%, 75%, 100%, and 125% v/w). The volume of moistening media was adjusted to maintain uniform final moisture content of 100% v/w across all flasks, except for the 125% inoculum treatment, where the final moisture content was 125%.

Production of CGTase under solid-state fermentation

Experiments were conducted in 250 mL Erlenmeyer flasks containing 5 g of wheat bran, supplemented with 50% (v/w) of various moistening media (Table 3). All flasks were autoclaved at 121°C for 40 min, aseptically inoculated with 50% (v/w) inoculum, and incubated at 32°C in a slanted position, to increase surface area for aeration. The effect of initial moisture content on CGTase production was also investigated by varying the concentrations of moistening media (0%, 25%, 50%, 75%, and 100% v/w). The samples were withdrawn at regular intervals to determine enzyme activity.

Screening of key moistening medium components by Plackett-Burman Design (PBD)

Screening of key moistening medium components was conducted across nine variables ($Na_2EDTA.2H_2O$, $FeSO_4.7H_2O$, $MnSO_4.4H_2O$, H_3BO_3, $ZnSO_4.7H_2O$, KI, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, and $CoCl_2.6H_2O$) using PBD in MS (micronutrients only) medium following standard incubation conditions. The maximum level (+) corresponded to a 10% increase in the original concentration, while the minimum level (–) represented a 10% decrease. The centre point (0) reflected the original, unmodified concentration of each factor. These coded levels were used to construct the experimental matrix, resulting in a total of 48 experimental runs consisting of 16 base runs with three replicates each (Table 4), using MINITAB 18.1 (Minitab LLC, State College, PA, USA).

Additionally, twelve centre points were incorporated to estimate pure experimental error and to assess the presence of curvature in the response. A first-order model was fitted to results, and the equation is given in Eq. (1), where Y is the CGTase activity and A, B, C, D, E, F, G, H, and J are $FeSO_4.7H_2O$, H_3BO_3, $Na_2EDTA.2H_2O$, $MnSO_4.4H_2O$, KI, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, and $ZnSO_4.7H_2O$, respectively. Ct Pt represents the centre-point contrast used to detect curvature in the experimental response. The effect on CGTase activity was analysed using a Pareto graph based on the F- and P-values. Model adequacy was assessed using analysis of variance (ANOVA), and variables with P-values <0.05 were considered significant for CGTase activity and selected for further statistical analysis.

$$Y = 3388.1 - 13.4\,A + 40.8\,B + 2.5\,C + 60.8\,D + 31.5\,E - 55.8\,F + 48.6\,G + 25.3\,H - 24.4\,J + 785.6\,CtPt \quad (1)$$

Table 4 PBD table for screening of key moistening medium components of MS (micronutrients only) medium. A, B, C, D, E, F, G, H, and J are $FeSO_4.7H_2O$, H_3BO_3, $Na_2EDTA.2H_2O$, $MnSO_4.4H_2O$, KI, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, and $ZnSO_4.7H_2O$, respectively. The minimum level (–), centre point (0), and maximum level (+) correspond to –10%, 0%, and +10% variation from the original concentration.

Run	Blk	A	B	C	D	E	F	G	H	J	CGTase activity (U/gds)
1	1	+	+	-	+	+	-	+	-	-	3712
2	1	+	-	+	-	-	-	+	+	+	3416
3	1	-	-	-	+	+	+	-	+	+	3420
4	1	+	-	-	-	+	+	+	-	+	3173
5	1	+	+	-	+	+	-	+	-	-	3721
6	1	+	+	-	+	-	-	-	+	+	3426
7	1	0	0	0	0	0	0	0	0	0	4182
8	1	+	-	+	-	-	-	+	+	+	3373
9	1	-	+	+	-	+	-	-	-	+	3248
10	1	+	-	-	-	+	+	+	-	+	3186
11	1	-	-	-	+	+	+	-	+	+	3425
12	1	+	+	-	+	-	-	-	+	+	3428
13	1	0	0	0	0	0	0	0	0	0	4180
14	1	-	+	-	-	-	+	+	+	-	3473
15	1	-	+	-	-	-	+	+	+	-	3471
16	1	0	0	0	0	0	0	0	0	0	4150
17	1	+	+	+	-	+	+	-	+	-	3250
18	1	0	0	0	0	0	0	0	0	0	4156
19	1	-	+	+	+	-	+	+	-	+	3486
20	1	-	+	+	-	+	-	-	-	+	3571
21	1	0	0	0	0	0	0	0	0	0	4179
22	1	0	0	0	0	0	0	0	0	0	4171
23	1	+	+	-	+	+	-	+	-	-	3624
24	1	0	0	0	0	0	0	0	0	0	4170
25	1	+	+	+	-	+	+	-	+	-	3256
26	1	-	-	+	+	+	-	+	+	-	3541
27	1	-	-	+	+	+	-	+	+	-	3516
28	1	+	+	+	-	+	+	-	+	-	3253
29	1	-	-	+	+	+	-	+	+	-	3542
30	1	+	-	-	-	+	+	+	-	+	3146
31	1	-	+	+	+	-	+	+	-	+	3376
32	1	-	-	-	-	-	-	-	-	-	3159
33	1	+	-	+	+	-	+	-	-	-	3145
34	1	0	0	0	0	0	0	0	0	0	4180
35	1	-	+	+	-	+	-	-	-	+	3543
36	1	+	-	+	+	-	+	-	-	-	3418
37	1	0	0	0	0	0	0	0	0	0	4165
38	1	0	0	0	0	0	0	0	0	0	4179
39	1	-	+	+	+	-	+	+	-	+	3147
40	1	+	+	-	+	-	-	-	+	+	3267
41	1	0	0	0	0	0	0	0	0	0	4181
42	1	-	-	-	-	-	-	-	-	-	3257
43	1	+	-	+	+	-	+	-	-	-	3461
44	1	+	-	+	-	-	-	+	+	+	3489
45	1	0	0	0	0	0	0	0	0	0	4191
46	1	-	-	-	+	+	+	-	+	+	3426
47	1	-	-	-	-	-	-	-	-	-	3158
48	1	-	+	-	-	-	+	+	+	-	3469

Screening for interaction among significant variables using Central Composite Design (CCD)

The interaction among four significant factors was further evaluated using Response Surface Methodology (RSM) employing CCD in MINITAB 18.1. Three blocks were performed; each block was an independently run CCD (16 factorial, 8 axial and 7 centre points; 31 runs per block; total n = 93) (Table 5). The factor levels were coded as –2, –1, 0, +1, and +2, corresponding to –20%, –10%, 0%, +10%, and +20% variation from the original concentration, with α = 2. The quadratic model for predicting the optimal point is given by Eq. (2), where Y is the predicted response (CGTase activity), x_1 is H_3BO_3, x_2 is $MnSO_4.4H_2O$, x_3 is $CoCl_2.6H_2O$, and x_4 is $CuSO_4.5H_2O$.

$$Y = 5282 - 18.4\,x1 + 135.7\,x2 + 57.9\,x3 - 314.0\,x4 - 41.4\,x12 - 61.1\,x22 - 60.4x32 + 138.7\,x42 + 198.8\,x1x2 + 15.4\,x1x3 + 69.4\,x1x4 - 226.9\,x2\,x3 - 98.5\,x2x4 - 147.0\,x3x \quad (2)$$

The statistical significance of the model was assessed using ANOVA. The lack of fit was accounted for in the model estimation. Overall, the model significance was determined using Fisher's *F*-test and its associated probability (*F*). The polynomial model was evaluated using R^2. Three-dimensional response surface plots were used to illustrate the interactions between the responses and the significant variables at their experimental levels.

Table 5 Central Composite Design table for screening for interaction among significant variables. The factor levels were coded as –2, –1, 0, +1, and +2, corresponding to –20%, –10%, 0%, +10%, and +20% variation from the original concentration. A is H_3BO_3, B is $MnSO_4.4H_2O$, C is $CoCl_2.6H_2O$, and D is $CuSO_4.5H_2O$.

Run Order	Pt Type	Blocks	A	B	C	D	CGTase activity (U/gds) (actual)	CGTase activity (U/gds) (predicted)
1	1	2	1	1	1	1	3796.32	4325.23
2	0	2	0	0	0	0	5220.51	4677.07
3	0	2	0	0	0	0	4252.2	4677.07
4	1	2	-1	1	-1	-1	4210.1	5127.48
5	-1	2	2	0	0	0	5346.81	4474.6
6	1	2	1	1	1	-1	5936.24	5305.36
7	1	2	-1	-1	-1	-1	5473.12	4602.88
8	1	2	-1	-1	-1	1	4757.41	4327.17
9	1	2	-1	1	1	-1	6188.84	5052.5
10	0	2	0	0	0	0	5094.21	4677.07
11	1	2	-1	-1	1	1	4715.3	4571.91
12	1	2	1	-1	-1	1	4799.52	4000.59
13	1	2	-1	-1	1	-1	6188.84	5435.49
14	1	2	1	-1	1	1	3115.46	4307.01
15	1	2	1	1	-1	-1	5094.21	5318.65
16	-1	2	0	0	0	2	4462.7	4603.91
17	-1	2	0	2	0	0	4589	4704.05
18	-1	2	0	0	0	-2	5599.42	5859.75
19	0	2	0	0	0	0	4462.7	4677.07
20	0	2	0	0	0	0	5262.62	4677.07
21	1	2	1	-1	-1	-1	4673.2	3998.78
22	-1	2	0	0	2	0	4167.99	4551.06
23	0	2	0	0	0	0	3578.59	4677.07
24	1	2	1	-1	1	-1	5978.35	4893.08
25	-1	2	0	-2	0	0	3115.46	4161.23
26	-1	2	-2	0	0	0	3368.07	4548.32
27	1	2	-1	1	1	1	3831.19	3794.86
28	0	2	0	0	0	0	4414.26	4677.07
29	1	2	1	1	-1	1	4252.2	4926.4
30	-1	2	0	0	-2	0	3452.28	4319.62
31	1	2	-1	1	-1	1	5010.02	4457.71
32	0	3	0	0	0	0	4120.36	3598.32
33	0	3	0	0	0	0	3562.46	3598.32
34	0	3	0	0	0	0	4116.62	3598.32
35	0	3	0	0	0	0	4037.46	3598.32
36	1	3	1	1	-1	-1	3522.88	4239.9
37	0	3	0	0	0	0	2652.06	3598.32
38	1	3	1	-1	1	-1	3324.97	3814.33
39	1	3	-1	1	-1	-1	4354.13	4048.72
40	1	3	-1	1	1	1	2849.97	2716.1
41	-1	3	-2	0	0	0	3087.48	3469.56
42	1	3	-1	-1	-1	-1	3590.18	3524.12
43	1	3	1	-1	-1	1	2612.48	2921.83
44	1	3	-1	-1	-1	1	2335.39	3248.41
45	1	3	-1	1	-1	1	2968.72	3378.95
46	-1	3	2	0	0	0	4156.22	3395.85
47	1	3	-1	-1	1	1	4433.29	3493.15
48	-1	3	0	0	-2	0	3918.71	3240.86
49	-1	3	0	0	0	2	3918.71	3525.16
50	1	3	1	1	1	1	3324.97	3246.48
51	1	3	1	-1	-1	-1	2533.3	2920.03
52	1	3	1	1	1	-1	3404.13	4226.61
53	-1	3	0	2	0	0	3443.73	3625.3
54	0	3	0	0	0	0	3483.3	3598.32
55	1	3	-1	-1	1	-1	3918.71	4356.73
56	0	3	0	0	0	0	4077.04	3598.32
57	1	3	1	1	-1	1	3997.88	3847.64
58	-1	3	0	0	2	0	3997.88	3472.31

59	1	3	1	-1	1	1	4116.62	3228.26
60	-1	3	0	-2	0	0	2968.72	3082.48
61	-1	3	0	0	0	-2	4377.05	4781
62	1	3	-1	1	1	-1	3760.37	3973.74
63	1	1	-1	1	1	1	6437.27	6687.7
64	0	1	0	0	0	0	7220.75	7569.92
65	-1	1	0	0	-2	0	7106.57	7212.46
66	1	1	1	-1	1	-1	6619.27	7785.93
67	1	1	-1	1	-1	1	8157.4	7350.55
68	1	1	-1	-1	1	1	7837.43	7464.75
69	-1	1	0	0	0	-2	9658.24	8752.59
70	1	1	1	1	1	1	6372.85	7218.08
71	1	1	1	1	-1	-1	8074.92	8211.5
72	1	1	-1	-1	-1	1	7280.11	7220.01
73	1	1	1	-1	-1	1	6521.15	6893.43
74	-1	1	-2	0	0	0	7220.75	7441.16
75	1	1	-1	1	-1	-1	8510.33	8020.32
76	0	1	0	0	0	0	6847.85	7569.92
77	-1	1	0	0	2	0	7718.68	7443.91
78	1	1	1	-1	1	1	7124.94	7199.86
79	0	1	0	0	0	0	8470.75	7569.92
80	1	1	1	1	-1	1	8114.5	7819.24
81	-1	1	0	2	0	0	8668.66	7596.9
82	1	1	-1	-1	-1	-1	8233.26	7495.72
83	1	1	1	1	1	-1	8312.41	8198.21
84	0	1	0	0	0	0	7758.26	7569.92
85	1	1	-1	1	1	-1	7283.25	7945.34
86	0	1	0	0	0	0	7481.19	7569.92
87	-1	1	0	-2	0	0	7560.34	7054.08
88	0	1	0	0	0	0	8272.84	7569.92
89	-1	1	2	0	0	0	7639.5	7367.45
90	-1	1	0	0	0	2	7124.94	7496.76
91	1	1	1	-1	-1	-1	6184.11	6891.63
92	1	1	-1	-1	1	-1	7741.62	8328.33
93	0	1	0	0	0	0	6531.18	7569.92

Statistical analysis

All SSF experiments were conducted in triplicate using three independent biological replicates for each condition. Enzyme activity was measured in triplicate for each biological replicate, and the results are expressed as mean ± standard deviation. P values < 0.05 were considered statistically significant at a 95% confidence level. Statistical experimental designs were generated using MINITAB 18.1.

Enzyme assay determination

The enzyme was extracted by adding 150 mL of distilled water to 5 g of fermented solid substrate. The contents were shaken at 100 rpm for 1 h at 32°C. Afterwards, the suspension was filtered through two layers of paper towels and centrifuged at 10,000 rpm for 15 min at 4°C. The resulting supernatant was used to determine enzymatic activity.

CGTase activity was determined using the phenolphthalein (PHP) assay method described by **Goel and Nene (1995)**. The reaction mixture, containing 1 mL soluble starch (1% w/v) prepared in 50 mM phosphate buffer (pH 7.0) and 0.1 mL crude enzyme, was incubated at 60°C for 15 min in a water bath, and the reaction was terminated by heating at 100°C for 5 min.

Subsequently, 4 mL of PHP working solution was added to the enzyme–substrate mixture. The PHP working solution was prepared by adding 4 mL of 95% ethanol and 1 mL of PHP stock solution (4 mM PHP in 90% ethanol) to 100 mL of 125 mM carbonate buffer (pH 10.8). Control was prepared similarly without the enzyme, and absorbance was measured at 550 nm. The basal β-CD present in the fermentation broth was estimated separately and deducted from the final readings to obtain net enzyme activity.

A standard calibration curve for β-CD (20–240 µg) was plotted using a stock solution of β-CD (2 mg/mL) prepared in 50 mM Tris-HCl buffer. The working solution of β-CD was obtained by diluting 1.0 mL of the stock with 9.0 mL of Tris-HCl buffer. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol β-CD per minute under standard assay conditions. Enzyme activity was expressed as units per gram dry substrate (U/gds) in SSF.

RESULTS AND DISCUSSION

Effect of different inoculum media on CGTase production

Selection of an appropriate inoculum medium is essential for bacterial growth. Based on the literature survey, different medium compositions were selected for the study of CGTase production, as described in the Materials and Methods section. Among these, Medium B was identified as the most suitable inoculum medium for enhanced enzyme production. It exhibited the highest enzyme activity (4053.53 U/gds) after 72 h of incubation, followed by media D, A, C, and E, with activities of 3317.04 U/gds, 2612.99 U/gds, 2541.04 U/gds, and 413.39 U/gds, respectively (Fig. 1). The superior performance of medium B can be attributed to the presence of soluble starch together with balanced nitrogen content, creating optimal metabolic conditions for CGTase activity **(Ibrahim *et al.*, 2005)**. Medium C and D, corresponding to OFAT and RSM optimised formulations of medium B **(Solanki *et al.*, 2022)**, showed no significant improvement over medium B when evaluated under SSF conditions. This suggests that media optimised for SmF do not necessarily translate into superior performance in SSF, likely due to differences in microbial physiology, mass transfer, and oxygen availability. Medium E (nutrient broth) reported lowest enzyme activity as it lacks a substantial polysaccharide-based carbon source, such as starch, which is essential not only as an inducer but also as the donor substrate required for CGTase catalysis.

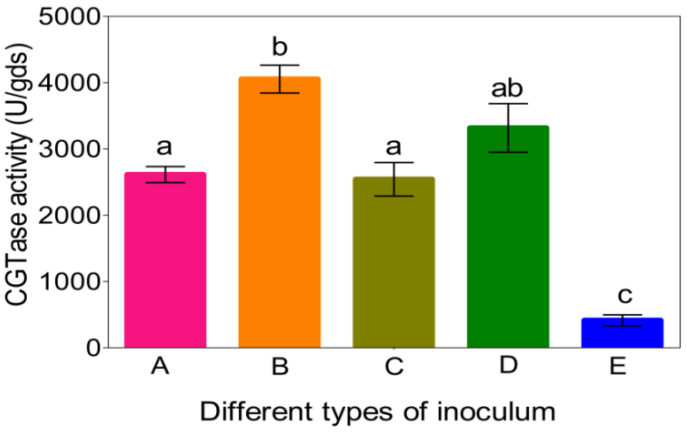


Figure 1 Effect of different inoculum media on CGTase activity by *Bacillus* sp. NCIM 5799 after 72 h of incubation. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, P < 0.05

Effect of different inoculum pH on CGTase production

Medium B was further optimised with respect to pH. A steady increase in CGTase activity was observed with increasing pH in inoculum medium B, followed by a subsequent decline. At pH 8.5, the activity was 1,322.02 U/gds, which increased to 1,830.15 U/gds and 2,354.82 U/gds at pH 9.0 and 9.5, respectively. At pH 10, the activity reached a maximum of 4,087.88 U/gds, beyond which it declined, yielding 3,685.08 U/gds at pH 10.5 (Fig. 2). Most CGTase-producing bacteria, particularly *Bacillus* species, are known to be alkaliphilic and exhibit optimal growth and enzyme activity at elevated pH values between 9.5-10.5 **(Martins and Hatti-Kaul, 2002; Ibrahim et al., 2005; Alves-Prado *et al.*, 2007; Sivakumar and Shakilabanu, 2013)**. However, some studies have reported *Bacillus* strains capable of producing CGTase under mildly alkaline to near-neutral conditions, with optimal activity observed between pH 6.5 and 8.5 **(Rosso *et al.*, 2002; Saleh *et al.*, 2023)**. Taken together, these findings highlight that optimal pH for enzyme activity is strongly strain-dependent. For the strain investigated in this study, pH 10 supported the highest enzyme activity, suggesting that alkaline conditions favour CGTase synthesis up to an optimum, beyond which enzyme production may be inhibited due to metabolic stress or reduced enzyme stability.

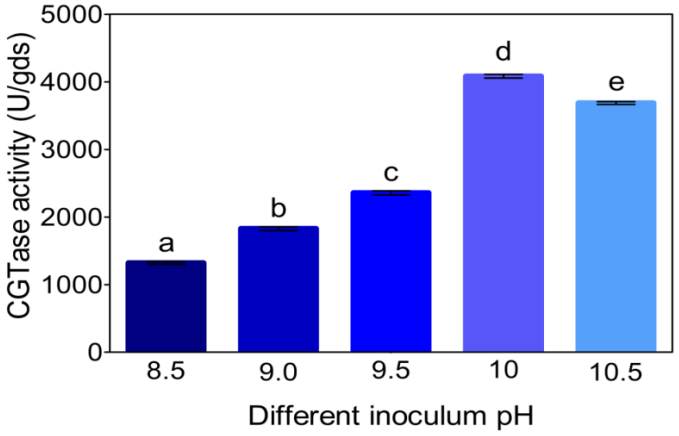


Figure 2 Effect of different inoculum pH on CGTase activity by *Bacillus* sp. NCIM 5799 after 72 h of incubation. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, $P < 0.05$

Effect of different sugar sources in the inoculum

Further optimisation of inoculum medium B involved replacing soluble starch with alternative carbon sources to evaluate their effect on CGTase production at different incubation times (24–120 h) and to identify the time at which each carbon source produced maximum enzyme activity. Among the sugars tested, only sucrose reached its maximum CGTase activity at 48 h, whereas all other carbon sources exhibited peak enzyme production after 72 h of SSF. A decline in CGTase activity was observed beyond the peak point, likely due to nutrient depletion or the accumulation of metabolic heat generated during microbial growth **(dos Santos *et al.*, 2004)**.

Inoculum containing soluble starch reported maximum enzyme activity (4308.63 U/gds), followed by glucose (3606.28 U/gds), maltose (2771.8 U/gds), mannitol (2491.23 U/gds), and sucrose (2300.91 U/gds) (Fig. 3). Similar observation was reported by **Rosso *et al.* (2002)** and **Gawande *et al.* (1998)**, who noted that although simple carbon sources, such as glucose and maltose, supported microbial growth, CGTase activity was detected only when starch based substrates were present. **Go *et al.* (2007)** further demonstrated that starch and maltodextrin are more effective donor substrates for CGTase than low-molecular-weight carbohydrates, such as maltose and dextrin. Because donor substrates must contain at least two glucose units to participate in the CGTase-catalysed transglycosylation reaction, glucose cannot act as a functional donor **(Mathew and Adlercreutz, 2013; Lim et al., 2021)**. Collectively, these findings reinforce that starch-containing media are superior inducers of CGTase synthesis compared with readily metabolizable sugars.

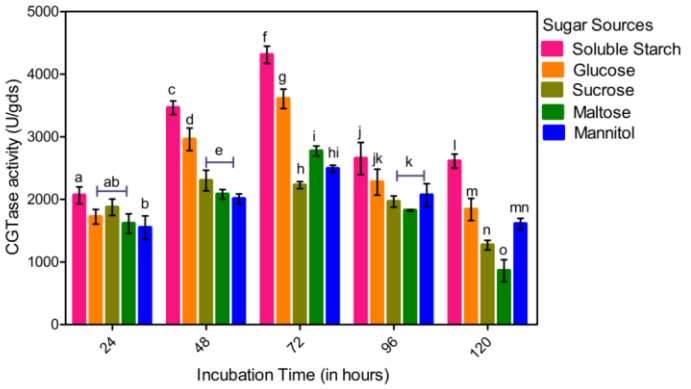


Figure 3 Effect of supplementation of different sugars in inoculum media B on CGTase activity by *Bacillus* sp. NCIM 5799. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, $P < 0.05$. Statistical comparisons were performed only among sugar sources at the same incubation time.

Effect of different starch sources in the inoculum

When different starch sources were evaluated, all alternatives to soluble starch resulted in lower CGTase activity. Inoculum incorporated with soluble starch exhibited the highest enzymatic activity (4308.63 U/gds), followed by inoculum with tapioca, wheat, potato, and maize starch (3805.85 U/gds, 3447.2 U/gds, 3343.65 U/gds, and 3343 U/gds, respectively). For tapioca starch, the maximum enzyme activity was observed at 48 h, whereas all other starch sources reached their peak activity at 72 h (Fig. 4). Our results align with those of **Kim *et al.* (1990)**, who also reported better CGTase yield by *B. stearothermophilus* with soluble starch. Meanwhile, **Kulshreshtha *et al.* (2020)** and **Saini *et al.* (2022)** observed maximum production of beta and gamma CGTase, respectively, with potato starch. **Ibrahim *et al. (2005)*** reported maximum CGTase activity when tapioca was used as a carbon source for *Bacillus* G1. The variations in enzyme activity observed with different starch sources may be attributed to differences in their physical structure, a characteristic that appears to be selectively recognised by the bacterial species.

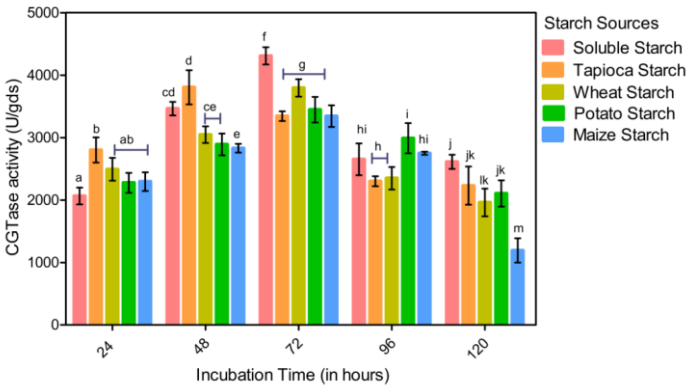


Figure 4 Effect of supplementation of different starch sources in inoculum media B on CGTase activity by *Bacillus* sp. NCIM 5799. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, $P < 0.05$. Statistical comparisons were performed only among starch sources at the same incubation time.

Effect of different nitrogen sources in the inoculum

Inoculum with different organic nitrogen sources showed varying levels of CGTase activity, with peptone yielding the highest activity (5109.65 U/gds), followed by yeast extract (3409.12 U/gds), casein (3290.91 U/gds), and tryptone (2714.8 U/gds) at 72 h (Fig. 5). Meanwhile, all the inorganic nitrogen sources significantly suppressed the CGTase activity with sodium nitrate, potassium nitrate, ammonium sulphate, ammonium nitrate, and ammonium chloride showing activities of 3368.23 U/gds, 1602.04 U/gds, 1002.94 U/gds, 804.3 U/gds, and 530.18 U/gds, respectively. These findings are consistent with previous reports **(Yap *et al.*, 2010; Rajput *et al.*, 2016; Yang et al., 2017)**. However, the results differ from those reported by of **Rosso *et al.* (2002)**, who reported ammonium sulphate (0.5%) to be the optimum nitrogen source for CGTase production by *Bacillus circulans DF 9R* compared with organic nitrogen sources. In the present study, organic nitrogen sources supported enhanced enzyme production, which may reflect limited utilisation of inorganic nitrogen under alkaline conditions. This reinforces the idea that organic nitrogen sources, being more readily assimilable

and metabolically compatible, often provide superior support for enzyme synthesis in alkaliphilic systems.

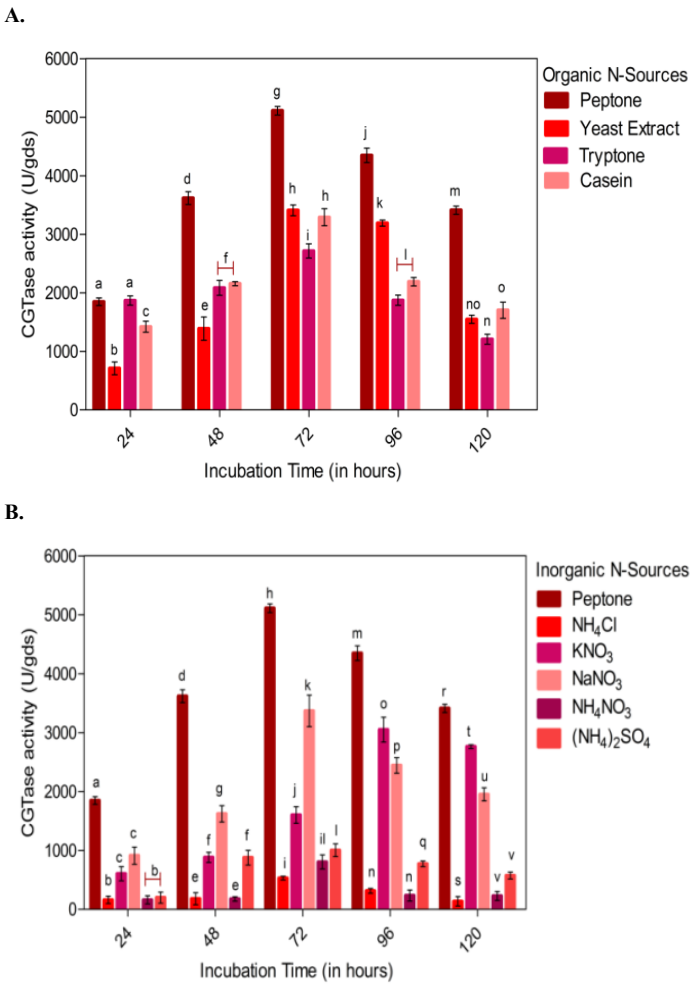


Figure 5 Effect of supplementation of different organic (A) and inorganic (B) N-sources in inoculum media B on CGTase activity by *Bacillus* sp. NCIM 5799. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, $P < 0.05$. Statistical comparisons were performed only among nitrogen sources at the same incubation time.

Effect of different moistening media

Moistening medium plays a crucial role in SSF by influencing microbial growth, nutrient availability, and enzyme secretion. Among the media evaluated, MS based media supported higher CGTase activity than other formulations. MS (micronutrients only) medium showed the highest enzymatic activity at 72 h (4882.68 U/gds), while MS (complete) medium reached its maximum activity at 96 h (4544.35 U/gds); both values exceeded the activity obtained with sodium carbonate (4314.1 U/gds at 72 h), which was used in the preliminary experiments. Peak enzymatic activity for Hoagland solution was observed at 96 h (3874.12 U/gds); for tap and mineral water, the activity was highest at 48 h (3148.11 U/gds and 2971.65 U/gds, respectively). Horikoshi media gave highest enzymatic activity of 1826.96 U/gds after 24 h (Fig. 6). The superior performance of the MS (micronutrients-only) medium may be attributed to the presence of trace metal ions, which play important roles in enzyme activity and cyclisation by maintaining enzyme conformation and promoting microbial growth **(Wang *et* al., 2024).** This is the first report describing the use of MS-based medium for enhanced CGTase production under SSF conditions.

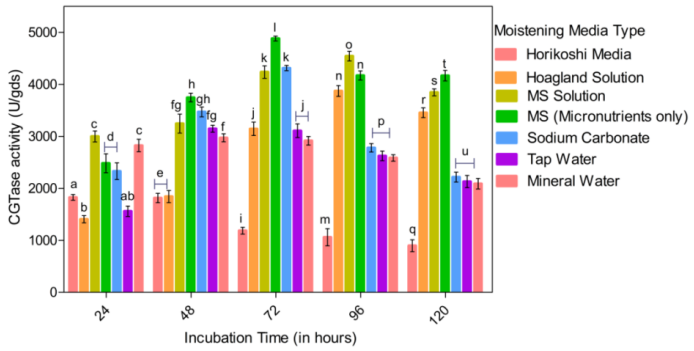


Figure 6 Effect of different moistening media on CGTase activity by *Bacillus* sp. NCIM 5799. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, $P < 0.05$. Statistical comparisons were performed only among moistening media at the same incubation time.

Screening for significant variables using Plackett-Burman Design

Following the identification of MS (micronutrients only) medium as the most suitable moistening medium, statistical optimisation using PBD was performed to determine the key factors influencing CGTase activity. PBD results indicated $MnSO_4.4H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, and H_3BO_3 had statistically significant effects. The positive effect of Mn^{2+}, Co^{2+}, and Cu^{2+} on CGTase activity has also been reported previously **(Pishtiyski *et* al., 2008).** Mn^{2+} is known to play an important role in central carbon metabolism and improve protein stability thereby supporting microbial growth and enzyme production **(Kehres and Maguire 2003; Andreini *et* al., 2008).** Co^{2+} also participates in metabolic reactions related to carbon utilization, which may indirectly promote enzyme synthesis **(Kobayashi and Shimizu, 1999; Andreini *et* al., 2008).**

The experimental design matrix and corresponding results are summarised in Table 4, with CGTase activity ranging from 3145 to 4191 U/gds across 48 experiments. ANOVA (Table 6) revealed that the overall model was highly significant ($F = 50.09$, $P < 0.001$). $MnSO4.4H_2O$ ($P = 0.002$), $CuSO_4.5H_2O$ ($P = 0.004$), $CoCl_2.6H_2O$ ($P = 0.012$), and H_3BO_3 ($P = 0.032$) exhibited statistically significant effects; meanwhile, the remaining factors ($Na_2EDTA.2H_2O$, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, KI, and $Na_2MoO_4.2H_2O$) did not show significant contributions (Fig. 7). The effect of metal ions on enzyme activity seems to depend on the enzyme source **(Wang *et* al., 2004)**.

$MnSO4.4H_2O$, H_3BO_3, and $CoCl_2.6H_2O$ had a positive effect on the process, and $CuSO_4.5H_2O$ had a negative impact. Cu^{2+} are important in enzymes catalyzing oxidoreduction processes and cellular redox homeostasis; however, its beneficial effects are concentration dependent, as excessive Cu^{2+} can induce oxidative stress and inhibit microbial growth **(Rensing and Grass, 2003).**

A first-order model was fitted and described as the Eq (1). Ct Pt represents the centre-point contrast used to detect curvature in the experimental response. The positive and significant Ct Pt coefficient indicates strong curvature, suggesting that the relationship between factors and CGTase production is non-linear. These results justified proceeding to RSM for further optimisation. The model had an R^2 of 0.9812, indicating good model fitness. Adjusted R^2 was 0.9726, and predicted R^2 was 0.9683, which can be concluded as a reasonable agreement of values.

Table 6 ANOVA of PBD

Source	Effect	F-Value	*P*-Value
Model	-	50.09	< 0.001
$FeSO_4.7H_2O$	-26.9	0.54	0.467
H_3BO_3	81.7	4.99	0.032
$Na_2EDTA.2H_2O$	5	0.02	0.892
$MnSO_4.4H_2O$	121.7	11.06	0.002
KI	63	2.97	0.093
$CuSO_4.5H_2O$	-111.7	9.32	0.004
$CoCl_2.6H_2O$	97.2	7.07	0.012
$Na_2MoO_4.2H_2O$	50.6	1.91	0.175
$ZnSO_4.7H_2O$	-48.9	1.79	0.19
Ct Pt	-	785.6	< 0.001
Curvature	-	461.26	< 0.001

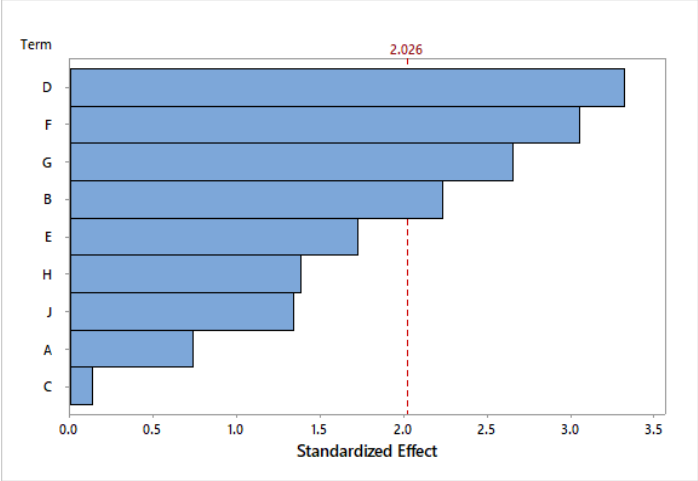


Figure 7 Pareto chart of standardised effects. Factors $FeSO_4.7H_2O$, H_3BO_3, $Na_2EDTA.2H_2O$, $MnSO_4.4H_2O$, KI, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, and $ZnSO_4.7H_2O$ are denoted as A, B, C, D, E, F, G, H, and J, respectively

Central Composite Design for optimisation of significant variables

Based on the results from PBD, CCD was employed to evaluate the interactive effects of the selected four factors (H_3BO_3, $MnSO_4.4H_2O$, $CoCl_2.6H_2O$, and $CuSO_4.5H_2O$) on CGTase activity. Enzyme activity achieved (response and predicted values), with coded variables for each variable concentration and the experimental combinations for the four-factor, five-level experiment design of 93 trials, is shown in Table 5. Although relatively larger deviations between predicted and experimental responses were observed in a few CCD runs, these are likely attributable to inherent experimental variability and the empirical nature of the quadratic model. Nevertheless, ANOVA of the quadratic model (Table 7) demonstrated that the model was highly significant with $F = 38.15$ and $P < 0.001$. The model had an R^2 value of 0.9893, with an adjusted R^2 of 0.966, and predicted R^2 of 0.9308. The non significant lack of fit ($P > 0.05$) further validated that the model adequately represented the experimental data. A second-order polynomial equation was derived to represent the relationship between the response and independent variables (Equation 2); where positive coefficients represent a synergistic impact and negative coefficients denote an antagonistic effect on the response.

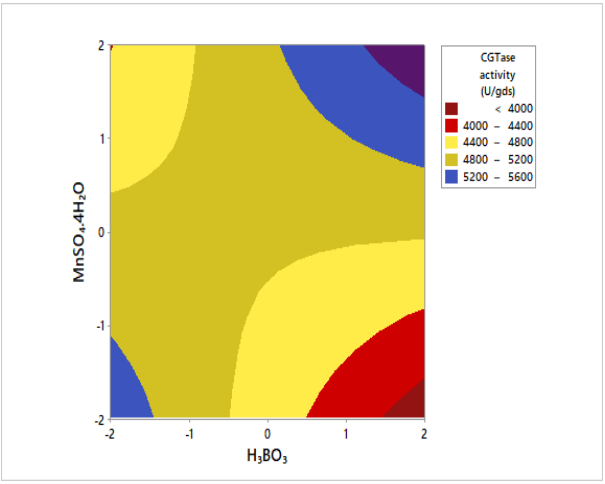
Among the linear terms, $CuSO_4.5H_2O$ showed a strong negative linear effect with $P < 0.001$, whereas the quadratic terms were non-significant ($P = 0.215$). However, the interaction component was significant ($P = 0.049$), with two key interactions influencing the response: H_3BO_3-$MnSO_4.4H_2O$ ($P = 0.045$) showing a synergistic effect, and $MnSO_4.4H_2O$-$CoCl_2.6H_2O$ ($P = 0.023$) showing an antagonistic interaction. Therefore, contour (Fig. 8) and response surface plots (Fig. 9) were generated only for these interactions to visualise their effect on CGTase activity.

Subsequent CCD analyses, supported by graphical evaluation and ANOVA, suggested that H_3BO_3-$MnSO_4.4H_2O$ and $MnSO_4.4H_2O$-$CoCl_2.6H_2O$ constituted key interaction pairs, while $CuSO_4.5H_2O$ emerged as a significant linear factor contributor to enzyme activity. These findings are consistent with those of **Pishtiyski *et al.* (2008)** and **Wang *et al.* (2024)**, who reported that Mn^{2+} ions enhance CGTase activity and the addition of Cu^{2+}markedly reduces extracellular enzyme production, respectively.

Table 7 ANOVA of CCD

Source	DF	Adj SS	Adj MS	F-Value	*P*-Value
Model	16	279011678	17438230	38.15	0.000
Blocks	2	261493905	130746952	286.03	0.000
Linear	4	8688531	2172133	4.75	0.002
H_3BO_3	1	24453	24453	0.05	0.818
$MnSO_4.4H_2O$	1	1325950	1325950	2.90	0.093
$CoCl_2.6H_2O$	1	241058	241058	0.53	0.470
$CuSO_4.5H_2O$	1	7097070	7097070	15.53	0.000
Square	4	2715646	678912	1.49	0.215
H_3BO_3*H_3BO_3	1	147063	147063	0.32	0.572
$MnSO_4.4H_2O$*$MnSO_4.4H_2O$	1	320350	320350	0.70	0.405
$CoCl_2.6H_2O$* $CoCl_2.6H_2O$	1	313315	313315	0.69	0.410
$CuSO_4.5H_2O$*$CuSO_4.5H_2O$	1	1650090	1650090	3.61	0.061
2-Way Interaction	6	6113596	1018933	2.23	0.049
H_3BO_3*$MnSO_4.4H_2O$	1	1897358	1897358	4.15	0.045
H_3BO_3*$CoCl_2.6H_2O$	1	11416	11416	0.02	0.875
H_3BO_3*$CuSO_4.5H_2O$	1	231041	231041	0.51	0.479
$MnSO_4.4H_2O$*$CoCl_2.6H_2O$	1	2471158	2471158	5.41	0.023
$MnSO_4.4H_2O$*$CuSO_4.5H_2O$	1	465846	465846	1.02	0.316
$CoCl_2.6H_2O$*$CuSO_4.5H_2O$	1	1036778	1036778	2.27	0.136
Error	76	34739711	457101	-	-
Lack-of-Fit	58	27640034	476552	1.21	0.339
Pure Error	18	7099677	394426	-	-
Total	92	313751389	-	-	-

A.



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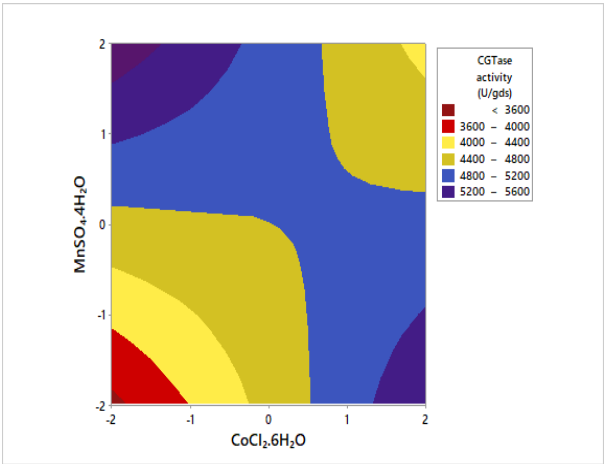
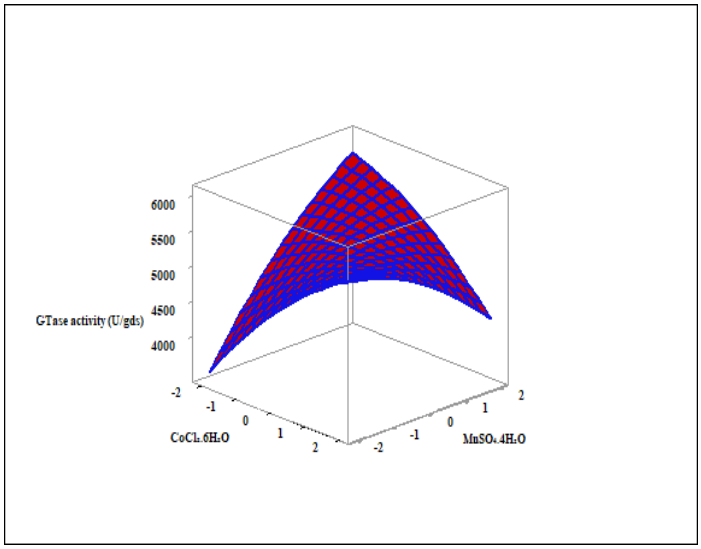
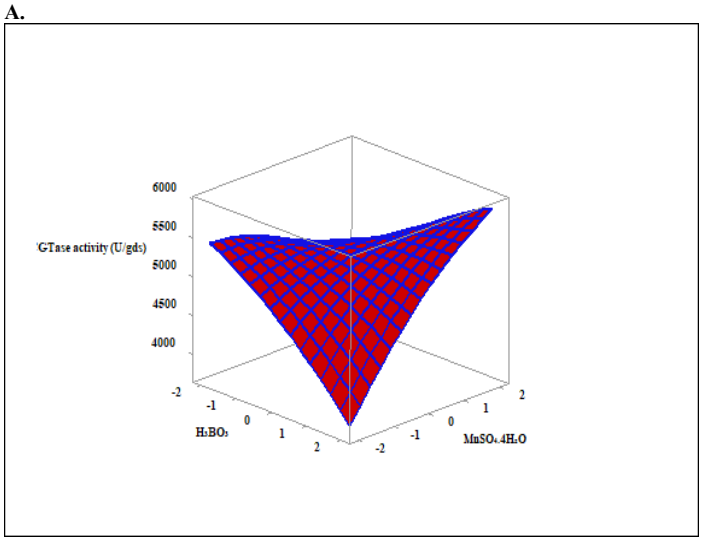


Figure 8 Contour plot of CGTase activity vs. MnSO4.4H2O and CoCl2.6H2O (A) and CGTase activity vs. MnSO4.4H2O and H3BO3 (B)



A.



B.

Figure 9 Response surface graph showing the CGTase activity in the presence of CoCl2.6H2O and MnSO4.4H2O (A) and H3BO3 and MnSO4.4H2O (B)

Validation of the model

For experimental validation, the factor levels were set to the values determined by MINITAB's Multiple Response Optimiser. That is, Co would be set to +2 level and all the other factors at -2 level. The optimised solution predicted an activity of 7806.33 U/gds, while the experimental value was 7804.9 ± 8.15 U/gds. The deviation between the predicted and experimental responses was extremely low, demonstrating excellent agreement between the model and experimental results.

Effect of inoculum quantity

CGTase activity increased with the increase in inoculum quantity up to 75% (v/w), beyond which it remained relatively constant. The enzyme activity at 25% inoculum was 3793.57 U/gds, which increased to 4963.11 U/gds at 50%, and reached 6449.27 U/gds at 75%. Further increase in inoculum size to 100% and 125% resulted in no significant enhancement, yielding 6532.39 U/gds and 6575.92 U/gds, respectively (Fig. 10). The observed result may be because of effect of inoculum size on microbial growth and enzyme production. Insufficient inoculum levels may result in inadequate biomass, thereby reducing enzyme synthesis, whereas excessive inoculum can accelerate nutrient depletion, impair heat and mass transfer, and create metabolic stress, collectively leading to reduced enzyme yields **(Khan *et al.*, 2017)**.

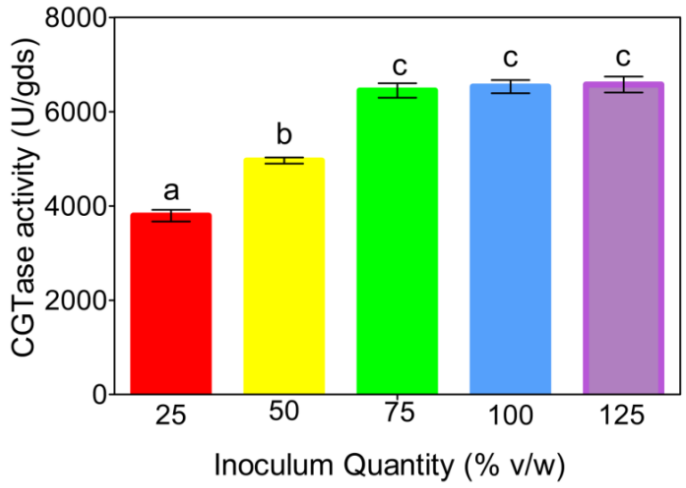


Figure 10 Effect of inoculum quantity on CGTase activity by *Bacillus* sp. NCIM 5799 after 72 h of incubation. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, $P < 0.05$

Effect of moistening media quantity

Solid substrate moistened with increasing volumes of MS (micronutrients-only) medium showed a corresponding rise in CGTase activity up to 48-72 h. When no moistening media was incorporated, CGTase activity was 9354.33 U/gds at 48 h. This activity increased to 10,377.7 U/gds when 25% v/w of moistening medium was supplemented. Increasing it further to 50% v/w elevated the enzyme activity to 12,192.5 U/gds. 75% v/w moistening medium yielded 12,673.4 U/gds, while 100% v/w resulted in the highest activity of 13,653.6 U/gds (Fig. 11).
Low moisture content in SSF limits metabolic activity and restricts the solubility and transport of nutrients needed for growth **(Gervais and Molin, 2003)**. While increasing moisture content enhances nutrient diffusion and metabolic activity, excessive moisture can adversely affect substrate porosity and aeration, rendering the system unsuitable for SSF. The optimum moisture content for microbial growth and substrate utilisation generally lies between 40–70% although this range can vary widely **(Raimbault, 1998)**. For instance, **Sathya *et al.* (2009)** reported optimal enzyme activity at moisture levels as low as 20%, **Mamo *et al.* (2020)** at around 55%, and **Khan *et al.* (2017)** at levels as high as 105%. Such variability arises from differences in the microorganism employed (fungal or bacterial), as well as the physicochemical characteristics of the substrate (porosity, water-holding capacity). In our study, moisture content between 75% and 100% supported progressively higher CGTase activity.

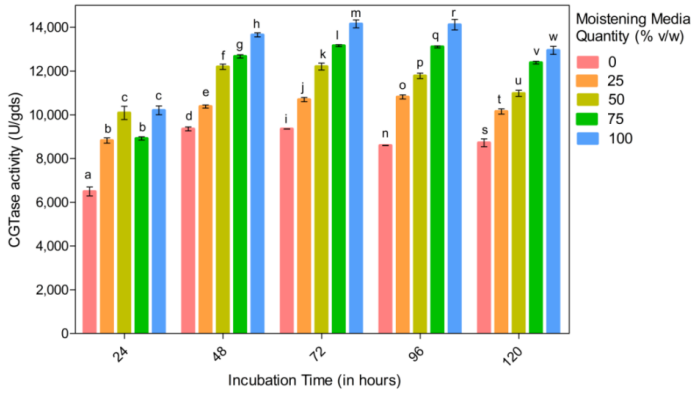


Figure 11 Effect of moistening media quantity on CGTase activity by *Bacillus* sp. NCIM 5799. Data represented as mean ± standard deviation of three independent observations. Symbols having the same alphabet are not significantly different from each other, $P < 0.05$. Statistical comparisons were performed only among moistening media quantity at the same incubation time.

CONCLUSION

The high production costs of CGTase remain a major limitation for the large scale industrial application of β-CD. Therefore, strategies that reduce enzyme production costs are essential for commercial β-CD manufacturing. In this context, SSF emerges as an attractive alternative to conventional SmF methods due to higher product concentration and utilisation of inexpensive agro-industrial residues.

In the present study, the potential of SSF for CGTase production by *Bacillus* sp. NCIM 5799 was successfully demonstrated. Our optimisation study identified a suitable inoculum for enhanced enzyme activity, with pH 10 found to be optimal. Soluble starch identified as the most effective carbon source and peptone as the preferred nitrogen source. MS (micronutrients-only) medium was selected for the moistening medium, and its statistical optimisation showed $CuSO_4.5H_2O$ as the most significant linear factor, while H_3BO_3-$MnSO_4.4H_2O$ and $MnSO_4.4H_2O$-$CoCl_2.6H_2O$ were identified as the most influential factor pair. Using optimised MS (micronutrients-only) moistening medium and inoculum level of 100% (v/w), and an incubation period of 48 h, a maximum activity of 13,653.6 U/gds was achieved.

In addition, the organism used in this study is highly favourable, as it exclusively produces β-CD, thereby reducing downstream separation requirements. To the best of our knowledge, this is the first study to investigate the effect and optimisation of MS (micronutrients-only) medium in an SSF system for CGTase production, where it proved to be highly effective for enzyme enhancement. Furthermore, this study provides a comprehensive evaluation of CGTase production and optimisation under SSF conditions. Although direct comparison between studies is challenging due to variations in assay methods and enzyme activity units, the CGTase activity obtained in the present work compares favourably with those reported for other SSF systems. Future studies should focus on validating the optimized SSF process in larger-scale fermentation systems to assess process reproducibility and scalability. Additionally, the efficiency of downstream β-CD production using CGTase produced under the optimized conditions should be evaluated to establish the industrial applicability of the developed process.

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