PRODUCTION AND OPTIMIZATION OF CYCLODEXTRIN GLUCANOTRANFERASE FROM BACILLUS SP. SS2

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INTRODUCTION

Cyclodextrin glucanotransferase is a versatile enzyme produced by some microorganisms that are capable of acting on starch and related carbohydrates. The enzyme catalyzes reactions such as cyclization, hydrolysis, coupling, and disproportionation. The cyclization reaction is the most beneficial among all the reactions as it produces a non-reducing cyclic molecule known as cyclodextrin (CD). Cyclodextrin is an oligosaccharide having glucopyranose units bonded with α (1,4) linkage. It possesses an external hydrophilic and internal hydrophobic surface conferring it with an ability to encapsulate various guest molecules in its cyclic cavity (Di Cagno, 2017). Due to its inclusion complex forming capability, cyclodextrin is used in multiple industries such as pharmaceutical, cosmetic, agriculture, environmental, plastic, chemical, textile, food, medical for diverse purposes (Sharma and Baldi, 2016). There are three types of cyclodextrins based on the number of glucopyranosyl units. The cyclodextrins which contain 6 glucopyranosyl units are known as α cyclodextrins whereas, 7 and 8 glucopyranosyl comprising cyclodextrins are called as β and γ cyclodextrins respectively (Jansook et al., 2018). The superior intriguing qualities of cyclodextrins are to shelter the guest molecules from biotic and abiotic origins such as heat, light, and microbial degradation, and the capability to transform their properties such as solubility, stability, chemical reactivity, color and odour (Kfoury et al., 2018). Among the three types of cyclodextrins, β-cyclodextrins are routinely utilized for complexing a variety of guest molecules owing to their high stability and low solubility and thus have high commercial value (Upadhay et al., 2018). The CGTase enzyme is known to be produced mostly by bacterial strains such as Bacillus circulans (Costa et al., 2015), Microbacterium terrae (Rajput et al., 2016), Bacillus flexus (Reddy et al., 2017), Bacillus clarkii (Wu et al., 2012) and Amphibacillus sp. (Ibrahim et al., 2012). However, some hyperthermophilic archaea, for instance, Haloflexus mediterranei (Bautista et al., 2012), Thermococcus kodakarenensis (Rashid et al., 2002), and Pyrococcus furiosus (Lee et al., 2007) are also proficient in secreting the enzyme.

The production of cyclodextrins using CGTase enzyme results in a mixture of all the three types of cyclodextrins (α, β and γ). The inability of wild type microorganisms to produce adequate amount of single type of cyclodextrin leads to increased production cost as a result of requirement of additional purification step for extraction of the desired type of cyclodextrin from the mixture of all the cyclodextrins. The problem can be partly overcome by use of strains that produce cyclodextrins in adequate amount so that the production cycle becomes economically feasible. A large number of genetically engineered CGTase overexpressing strains have been developed (Lemhuis et al., 2010) but they suffer from an inherent stability issue for long term application (Qi and Zimmermann, 2005). Looking for novel, high CGTase producing strains from unique environmental niches and optimization of the production medium that may further increase the efficiency of these strains is a more straightforward and simple approach. Moreover, the wild type isolates are expected to retain their characteristics for many generations.

In the present study, we have reported the isolation and characterization of a CGTase producing strain Bacillus sp. SS2. Both conventional and statistical methods were used to optimize the production medium. The enzyme activity was estimated using various starches such as soluble starch, corn starch, potato starch, and rice starch using the conventional method. Further, various operating parameters such as pH, incubation temperature, potato starch concentration, incubation time, and agitation speed were optimized using a combined statistical and mathematical tool known as response surface methodology. This methodology bypasses the conventional single factor optimization and allows study of more than one factor simultaneously. It also generates contour plots showing interaction between the factors and fits the experimental data by least squares technique to calculate the optimal response of the system (Montgomery et al., 2009; Vining and Kowalski 2010). CCD has been used by many researchers to identify optimal reaction conditions (de Oliveira et al., 2019; Sreedharan et al., 2019). The current study was aimed to optimize the CGTase production from a wild type bacterial...
strain using CCD which may reduce the production cost of cycloextrins and may prove as a viable alternative for commercial CGTase production.

MATERIALS AND METHODS

Sample collection and screening of CGTase producing microorganisms

Soil sample was collected from Dumas beach, Surat, Gujarat, India (21° 44’ 45" N, 72° 42’ 55" E) in autoclaved glass jar using sterilized gloves and spatula. 0.1 g of soil sample was added to 500 mL of autoclaved Horikoshi-II medium (Horikoshi, 2003) which was supplemented with 10 g soluble starch, 5 g peptone, 5 g yeast extract, 1 g K2HPO4, and 0.2 g MgSO4.7H2O. 10% Na2CO3 was separately added to the medium after autoclaving to adjust the pH at 9. The soil containing medium was incubated at 37°C for 24 h, 120 rpm. Following incubation, the sample was diluted alternatively in normal saline and spread onto the Horikoshi-II agar plates prepared by adding 2% agar to the liquid Horikoshi-II medium. All the bacterial colonies were separated and screened for CGTase production according to the method described by Park et al. (1989). The screening medium was prepared by adding 0.035 mM phenolphthalein dye, 0.030 mM methyl orange dye (filter sterilized with 0.2 micron filter), and 2% agar to the sterilized liquid Horikoshi-II medium just before pouring of plates. The plates were incubated for 24 to 48 h at 37°C and regularly inspected for the development of halo zones. The bacterial isolates producing yellowish halo zone around the wells were considered as CGTase positive, and its culture supernatant served as crude CGTase enzyme.

CGTase enzyme activity assay

CGTase activity was measured by PHP method described by Goel and Nene (1995). Briefly, 1mL cell-free supernatant of 24 h old bacterial culture grown in SS2 culture grown in 257 U/mL, similar to the supernatant of isolate SS2 produced a yellowish halo ring around the well. The presence of CGTase enzyme was confirmed by performing the bootstrap test with 1000 replicates (Felsenstein, 1985). The data were further analyzed by ANOVA, and the significant p-values (< 0.05) were used for surface response analysis. The optimal values of operating parameters and their interactions were estimated by three-dimensional response surface graphs and 2-dimensional contour graphs. The predicted results generated after the design were verified by conducting experiments under optimal conditions.

RESULTS AND DISCUSSION

Screening of CGTase producing microorganisms

The CGTase producing microorganisms was isolated on Horikoshi-II agar plate supplemented with phenolphthalein and methyl orange dyes. Out of a total of 20 bacterial colonies isolated on the plates, only 1 bacterium (named as SS2) was found to be producing CGTase enzyme. As shown in Figure 1 A, the cell-free supernatant of isolate SS2 produced a yellowish halo ring around the well suggesting the probable presence of β-CGTase (Vinod and More, 2016). The reduction of the red color of phenolphthalein may have occurred due to the formation of cycloextrin and phenolphthalein complex, resulting in an intensified shade of methyl orange. The distilled water was used as negative control for this experiment. The presence of CGTase enzyme was confirmed by spectrophotometric CGTase activity assay (PHP assay) which is predominantly sensitive for β-cycloextrin (Goel and Nene, 1995). The CGTase activity of the crude enzyme (cell-free supernatant) was found to be 3.57 U/mL, similar to the strain Microbacterium terrae KNR 9, having crude CGTase activity of 4.71U (Rajput et al., 2016).

Optimization of CGTase production using Response Surface Methodology

Response surface methodology was used to optimize various factors that are known to affect enzyme production. The optimization of variable factors such as pH, incubation temperature, incubation time, potato starch concentration, and agitation speed was carried out using Central Composite Design of Design-Expert 11.0 software (Trial Version- State Ease, Inc., MN, USA). All the factors were taken as independent variables to study the variations in response (CGTase enzyme activity) which may occur due to the interaction effect of operating parameters. The minimum and maximum values of independent parameters were fixed and entered in the CCD tool which generated a total of 50 runs to analyze the response. According to the design, 50 experiments of different combinations were performed with Horikoshi-II broth. In Horikoshi-II medium, different concentrations of potato starch was added, and the pH of the medium was adjusted according to the specified level. The medium was inoculated with 1% of 24 h old bacterial culture followed by incubation at respective temperatures for a specific time interval at provided agitation speeds. The experiments were conducted in duplicates, and their respective inputs were incorporated in the design for statistical analysis. Further, to develop the mathematical model and to estimate the response of independent variables, following second-order polynomial equation was calculated using an automated statistical tool of software.

\[
Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_7X_7 + \beta_8X_8 + \beta_9X_9 + \beta_{10}X_9X_{10} + \beta_{11}X_8X_{11} + \beta_{12}X_7X_{12} + \beta_{13}X_6X_{13} + \beta_{14}X_5X_{14} + \beta_{15}X_4X_{15} + \beta_{16}X_3X_{16} + \beta_{17}X_2X_{17} + \beta_{18}X_1X_{18} + \epsilon
\]

Where, \(Y\) = Response (Predicted), \(\beta_0\) = Intercept Coefficient, \(\beta_1\), \(\beta_2\), \(\beta_3\), \(\beta_4\), \(\beta_5\), \(\beta_6\), \(\beta_7\), \(\beta_8\), \(\beta_9\), \(\beta_{10}\), \(\beta_{11}\), \(\beta_{12}\), \(\beta_{13}\), \(\beta_{14}\), \(\beta_{15}\), \(\beta_{16}\), \(\beta_{17}\), \(\beta_{18}\) = Linear Coefficients, \(\beta_{19}\), \(\beta_{20}\), \(\beta_{21}\), \(\beta_{22}\), \(\beta_{23}\), \(\beta_{24}\), \(\beta_{25}\), \(\beta_{26}\), \(\beta_{27}\), \(\beta_{28}\) = Interaction Coefficient, \(\beta_{29}\), \(\beta_{30}\), \(\beta_{31}\), \(\beta_{32}\), \(\beta_{33}\), \(\beta_{34}\) = Quadratic Coefficient.

Optimization of CGTase production medium for isolate SS2

CGTase production using various starches

Enzyme production was optimized by measuring the enzyme activity using different types of starches - soluble starch, corn starch, potato starch, and rice starch. 1% of each substrate was added to separate flasks containing 100 mL of Horikoshi-II broth (without dyes) and pH was set to 9 followed by medium sterilization in autoclave at 15 psi for 20 minutes. 1 mL of 24 h old SS2 bacterial culture was inoculated in all the flasks which were further incubated at 37°C for 24 h at 120 rpm. The enzyme activity assay was performed with the cell-free supernatants collected after centrifugation of all the cultures having various starches. The substrate supplemented medium which produced the highest enzyme activity was considered as the best suitable substrate for CGTase production by SS2. The production medium was further optimized using statistical tools taking various operating parameters.

Figure 1 A. CGTase activity plate showing yellow halo zone around the well loaded with crude enzyme, B. Microscopic image of Gram stained SS2 cells
Identification and characterization of CGTase producing isolate SS2

The bacterial isolate SS2 formed white colored opaque colonies with irregular shape and rough surface on nutrient agar medium after incubation for 20±2 h at 30°C. The colony diameter was observed to be 4.6 mm. The bacterium was found to be Gram positive, forming dense rods (Figure 1 B). The biochemical characterization was conducted to decipher the metabolic abilities of the bacterium that may confer a selective advantage to the bacterial isolate, apart from CGTase production. This also provided clues for identification of the isolated strain. As mentioned in Table 1, indole and MR tests produced negative results for SS2 while VP, citrate, urease, catalase, and gelatinase tests were found to be positive. The bacterium can ferment simple sugars such as maltose, fructose, sucrose, dextrose, and lactose but is inefficient for xylose and sorbitol fermentation. The results of biochemical tests along with colony characteristics and Gram’s staining were compared with the related species reported in Bergy’s Manual of Systematic Bacteriology Volume 1 and Volume 3 (Krieg and Holt, 1984; Staley et al., 1989) and it was confirmed that the isolate SS2 belonged to the family Bacillaceae and genus Bacillus. It was presumed from the biochemical tests that the strain might belong to one of the following species – Bacillus thuringiensis, Bacillus mycoides, Bacillus cereus, Bacillus anthracis, Bacillus subtilis, or Bacillus licheniformis. The genetic identification of bacterium was validated by the molecular procedure of 16S rRNA gene sequencing followed by BLAST analysis of the consensus sequence. A BLAST analysis of the consensus sequence revealed that isolate SS2 belongs to the Bacillus cereus group and showed the highest similarity with Bacillus cereus strain D18s-4 (Accession number MK300054.1). The 16S rRNA gene sequence of isolate SS2 (1225 bp) has been deposited to NCBI-Genbank (Accession number - MK389411). The phylogenetic analysis of isolate SS2 carried out using MEGA X software shows that the strain clustered with the different species of Bacillus genus such as Bacillus cereus, Bacillus luteus, Bacillus subtilis, and Bacillus mycoides, and Bacillus tropicalis (Figure 2). Genus Bacillus is known for its biotechnological applications such as hydrolytic enzymes as well as value added products (Kumar et al., 2013; Patel et al., 2019; Porwal et al., 2008). CGTase production from Bacillus genus has been reported previously from the species such as Bacillus clausii (Alves-Prado et al., 2008), Bacillus pseudocaldiphilus (Atanasova et al., 2011), Bacillus clarkii (Wu et al., 2012), Bacillus circulans (Costa et al., 2015) etc. Optimization of CGTase production from SS2

Optimization of CGTase using various starches

The CGTase production was estimated after supplementing the production medium with various starches such as soluble starch, corn starch, potato starch, and rice starch. The maximum CGTase activity was observed in the medium supplemented with the potato starch (14.329±0.14U). However, rice starch showed the second highest CGTase activity of 12.762±0.09U. The medium containing corn starch, and soluble starch showed CGTase activity of 8.42±0.33U and 5.13±0.25U respectively (Table 2). CGTase enzyme production is induced by the starch and amylopectin is highly preferred by the enzyme for cyclodextrin formation (Biewer et al., 2002). Potato starch has clusters of amylopectin which comprises 5-10 amylopectin chains per cluster (BeMiller and Whistler, 2009). Therefore, it seems that potato starch plays a vital role in CGTase production from strain SS2. In a previous literature of Microbacterium terrae, potato starch has been identified as a potent substrate for CGTase production among corn, sago, and soluble starches (Rajput et al., 2016). However, in another study carried out by Elbaz et al. (2015) it was observed that Bacillus lehensis produces maximum CGTase enzyme when cultured in medium supplemented with rice starch. CGTase production was further optimized using other operating parameters along with the potato starch as the main substrate.

Table 2 CGTase activity (U) with various starches

| Starch Type       | CGTase Activity (U) |
|-------------------|---------------------|
| Soluble Starch    | 5.132±0.25          |
| Corn Starch       | 8.42±0.33           |
| Potato Starch     | 14.329±0.14         |
| Rice Starch       | 12.762±0.09         |

Optimization of CGTase production using statistical method (RSM)

Central composite design of RSM was utilized to optimize the CGTase production at various operating parameters. Factors such as pH, incubation temperature (°C), incubation time (h), potato starch concentration (%), and agitation speed (rpm) were determined as independent operating parameters for this study, and CGTase activity was considered as the response. The different levels of these parameters used for optimization are mentioned in Table 3 and the responses (CGTase activity) produced after experimenting the combinations of variables, are described in Table 4.

Table 3 Levels of Independent variables for Central Composite Design

| Independent Variables | -1 Level | 0 Level | +1 Level |
|-----------------------|----------|---------|----------|
| pH                    | 6        | 8       | 10       |
| Incubation temperature (°C) | 25        | 35       | 45       |
| Incubation time (h)    | 24        | 48       | 72       |
| Potato starch concentration (%) | 0.50     | 1.75     | 3.0      |
| Agitation speed (RPM)  | 100      | 200     | 300      |

Figure 2 Phylogenetic tree of isolate SS2 presenting evolutionary association between isolate SS2 and 30 taxa of Bacillus genus. The bootstrap values are displayed next to the branch points. The space bar shows the 0.01 substitution per site. Geobacillus stea rothermophilus NR_040794.1 was used as outgroup.
Table 4: Experimental Central Composite Design having coded values of independent variables along with their respective actual and predicted responses

| Run | Factor | Factor | Factor | Factor | Factor | CGTase activity |
|-----|--------|--------|--------|--------|--------|----------------|
|     | A      | B      | C      | D      | E      |                |
|     | pH     | Temp (°C) | Incubation time (h) | Potato starch conc. (%) | Agitation Speed (rpm) | Actual | Predicted |
| 1   | -1     | +1     | +1     | +1     | +1     | 30.12 ± 0.0832 | 32.55  |
| 2   | 0      | 0      | 0      | 0      | 0      | 39.09 ± 0.0456 | 38.43  |
| 3   | 0      | 0      | 0      | 0      | 0      | 40.21 ± 0.0520 | 41.44  |
| 4   | 0      | 0      | 0      | 0      | -1     | 38.28 ± 0.0789 | 36.88  |
| 5   | 0      | 0      | 0      | 0      | 0      | 39.01 ± 0.0571 | 38.50  |
| 6   | -1     | +1     | -1     | -1     | -1     | 24.32 ± 0.0351 | 25.01  |
| 7   | +1     | -1     | +1     | -1     | +1     | 21.41 ± 0.0411 | 22.69  |
| 8   | 0      | 0      | 0      | -1     | 0      | 36.15 ± 0.0642 | 35.76  |
| 9   | 0      | 0      | 0      | +1     | 0      | 38.25 ± 0.0587 | 39.23  |
| 10  | +1     | -1     | +1     | -1     | -1     | 23.00 ± 0.0935 | 22.56  |
| 11  | +1     | +1     | +1     | +1     | -1     | 26.27 ± 0.0847 | 27.12  |
| 12  | -1     | -1     | -1     | -1     | -1     | 25.19 ± 0.0863 | 24.14  |
| 13  | -1     | +1     | +1     | -1     | -1     | 25.34 ± 0.0572 | 24.95  |
| 14  | -1     | +1     | -1     | +1     | -1     | 24.03 ± 0.0637 | 24.19  |
| 15  | +1     | +1     | -1     | +1     | +1     | 26.53 ± 0.0825 | 25.08  |
| 16  | -1     | +1     | +1     | -1     | +1     | 24.08 ± 0.0790 | 23.72  |
| 17  | +1     | -1     | +1     | +1     | -1     | 25.12 ± 0.1312 | 24.65  |
| 18  | 0      | 0      | 0      | +1     | 0      | 36.43 ± 0.0551 | 35.01  |
| 19  | 0      | 0      | 0      | 0      | 0      | 39.16 ± 0.0645 | 38.27  |
| 20  | -1     | +1     | -1     | +1     | +1     | 29.02 ± 0.0873 | 29.55  |
| 21  | +1     | -1     | -1     | -1     | +1     | 26.35 ± 0.0468 | 26.00  |
| 22  | 0      | 0      | 0      | 0      | 0      | 40.23 ± 0.0388 | 39.89  |
| 23  | +1     | -1     | -1     | -1     | -1     | 27.45 ± 0.0740 | 26.11  |
| 24  | 0      | 0      | 0      | 0      | 0      | 39.36 ± 0.0490 | 38.15  |
| 25  | -1     | -1     | -1     | -1     | +1     | 25.48 ± 0.0217 | 24.23  |
| 26  | 0      | 0      | 0      | 0      | 0      | 40.01 ± 0.0513 | 39.46  |
| 27  | +1     | +1     | -1     | -1     | +1     | 22.35 ± 0.0683 | 23.07  |
| 28  | -1     | -1     | +1     | -1     | -1     | 24.42 ± 0.0879 | 25.32  |
| 29  | -1     | +1     | -1     | -1     | +1     | 27.14 ± 0.0320 | 28.12  |
| 30  | 0      | 0      | -1     | 0      | 0      | 40.12 ± 0.0469 | 39.88  |
| 31  | +1     | -1     | -1     | +1     | -1     | 28.50 ± 0.0553 | 27.78  |
| 32  | 0      | 0      | 0      | 0      | 0      | 41.00 ± 0.1231 | 39.59  |
| 33  | -1     | -1     | -1     | +1     | +1     | 23.23 ± 0.0434 | 24.52  |
| 34  | +1     | +1     | -1     | -1     | -1     | 20.01 ± 0.0659 | 19.00  |
| 35  | +1     | +1     | -1     | -1     | -1     | 21.17 ± 0.0347 | 20.25  |
| 36  | +1     | -1     | -1     | +1     | +1     | 28.25 ± 0.0897 | 29.12  |
| 37  | +1     | +1     | -1     | +1     | -1     | 19.22 ± 0.0923 | 18.77  |
| 38  | +1     | +1     | +1     | +1     | +1     | 24.36 ± 0.0230 | 25.45  |
| 39  | +1     | 0      | 0      | 0      | 0      | 34.43 ± 0.0546 | 35.12  |
| 40  | 0      | +1     | 0      | 0      | 0      | 38.58 ± 0.0681 | 39.95  |
| 41  | -1     | +1     | +1     | +1     | +1     | 28.32 ± 0.1280 | 27.00  |
| 42  | +1     | -1     | +1     | +1     | +1     | 27.23 ± 0.0404 | 26.83  |
| 43  | 0      | -1     | 0      | 0      | 0      | 39.41 ± 0.0361 | 38.65  |
| 44  | +1     | +1     | -1     | +1     | -1     | 25.52 ± 0.0343 | 25.12  |
| 45  | -1     | -1     | -1     | +1     | -1     | 28.34 ± 0.0715 | 29.55  |
| 46  | 0      | 0      | 0      | +1     | 0      | 40.11 ± 0.0591 | 39.43  |
| 47  | -1     | -1     | +1     | +1     | +1     | 29.02 ± 0.0624 | 28.52  |
| 48  | -1     | 0      | 0      | 0      | 0      | 36.53 ± 0.0838 | 38.56  |
| 49  | -1     | -1     | -1     | -1     | +1     | 30.24 ± 0.0449 | 28.65  |
| 50  | -1     | -1     | -1     | +1     | +1     | 31.53 ± 0.0215 | 28.18  |

The statistical and mathematical model fitness was assessed using ANOVA test (Table 5). The inferior probability value (<0.001) and a greater F-value (104.76), along with the decent coefficient of determination value (R²=0.9863) represents that model was highly efficient in predicting response values. As presented in Table 5, all the terms of independent parameters (A to E) including linear, interaction, and square were significant as they possessed the p-values <0.05. The Lack of Fit F-value was not significant which implies that model was of good fit. The observed adjusted and predicted R² values were 0.9769 and 0.9540 respectively suggesting sensible agreement between the experimental and predicted values. The ratio of signal to noise was measured as 32.1999, which denotes that model can potentially circumnavigate the design space.
The enzyme activity decreased at increased temperature and pH (Figure 3A). The interaction between pH and incubation time interval explained that the CGTase production was maximum between 45 to 50 h of inoculation at pH range from 7.5 to 8.5. The long term incubation even at lower or higher pH did not increase the enzyme activity (Figure 3B). As shown in Figure 3C, the potato starch concentration was a significant factor in the interaction of pH and potato starch concentration. The lower concentration of potato starch prohibited the enzyme activity irrespective of pH. The enzyme activity was highest when the substrate concentration was taken in the range of 1.5 to 2.5% and the pH range of 7.5 to 8.5. In the interaction studies between pH and agitation speed, it was observed that, when the isolate was cultured at lower pH with high agitation speed, the enzyme activity was in the increasing mode. However, the maximum level of enzyme activity was obtained when pH was adjusted in the range of 7.5-8.5 with the agitation speed range of 200 to 250 rpm (Figure 3D). Figure 3E, shows that the incubation time interval is a critical operating parameter. The incubation of culture isolate in the temperature range of 30°C to 35°C for 40 to 45 h provides the maximum CGTase activity. The temperature and potato starch concentration interaction disclosed that the CGTase activity was more substrate concentration dependent. At lower substrate concentration the enzyme activity was minimum irrespective of temperature. However, the enzyme activity was maximum in the substrate concentration range of 1.5 to 2.5% and the temperature range of 30°C to 35°C (Figure 3F). As observed in Figure 3G, the high agitation speed increased the enzyme activity in the temperature range of 30°C to 40°C. The lower and higher incubation temperature than the provided, couldn’t actively increase the enzyme activity at lower agitation speed. The interaction between incubation time and potato substrate concentration explained that there was minimal enzyme production at substrate concentration less than 1 irrespective of the incubation time interval. The increasing substrate concentration increased the CGTase activity which reached to the maximum in the time range of 40-50 h and 1.5 to 2.5 % potato starch concentration range (Figure 3H). Figure 3I, shows that the agitation speed in the range from 200 to 250 rpm increased CGTase activity during the incubation time of 40 to 50 h when other factors were set to their standard levels. The interaction analysis of potato starch concentration and agitation speed revealed that the incorporation of 1.5 to 2.5% potato starch to the culture medium followed by adjusting the agitation speed to 200 to 250 rpm could effectively increase the CGTase production (Figure 3J).

The statistical model fitted all the selected operating factors to their optimum level to provide the highest CGTase activity. The final model represented the optimal values of pH, temperature, incubation time, potato starch concentration, and agitation speed to be as 8.8, 38°C, 50 h, 2.2%, and 200 rpm respectively. To validate the method, CGTase production was experimented taking optimum values of the provided factors and the enzyme activity was measured as 38.313U which was similar to the activity calculated by software (39.901) and it was almost 2 folds higher than the conventional medium supplemented with potato starch. The variations in pH affected the enzyme production possibly by transporting chemicals through cell membrane (Sharma et al., 2017). The isolate SS2 produced maximum enzyme at pH 8.8 which means the bacterium is moderately alkaliphilic. At the higher potato starch concentration, enzyme production was not observed as potato starch is viscous in nature and its higher concentration might lead to poor oxygen uptake that may reduce the enzyme production (BeMiller and Whistler, 2009). The enzyme production might also be repressed at higher substrate concentration due to the accumulation of short oligosaccharides and glucose units degraded from potato starch (Elbaz et al., 2015). The bacterium was able to produce enzyme in moderate substrate concentration. The operating parameters of incubation temperature, incubation time, and agitation speed were identified as growth attendant parameters, it was determined that the isolate produced optimal enzyme activity at the log phase which was characterized with the balanced values of these factors. The similar optimization report using CCD was studied before in Bacillus G1 where the optimization of various independent factors increased CGTase activity till 54.9U/mL (Ibrahim et al., 2005). However, the manual optimization of multiple factors in Bacillus cereus RJ30 isolated produced maximum CGTase activity of 54U/mL (Jamuna et al., 1993), and in bacterial isolate Bacillus sp. TRP71H CGTase activity was measured as 30.34U/mL (Ravinder et al., 2014).

The comparative results of CGTase production suggested that the selected factors profoundly influence the enzyme production and their interactions triggered the enzyme activity to reach the optimal level. The optimization study using the statistical approach of CCD generated encouraging and accurate results to enhance the enzyme production in bacterial isolate SS2.

### Table 5 Analysis of variance (ANOVA) for quadratic model

| Source                     | Sum of Squares | Df | Mean Square | F-value | p-value | p<0.05 is considered significant, R2= 0.9863, Adjusted R2= 0.9769, Predicted R2 = 0.9540. |
|----------------------------|----------------|----|-------------|---------|---------|-------------------------------------------------|
| Model                      | 2254.08        | 20 | 112.70      | 104.76  | <0.0001 | significant                                       |
| A-pH                       | 47.06          | 1  | 47.06       | 43.74   | <0.0001 | significant                                       |
| B-Temp                     | 21.44          | 1  | 21.44       | 19.93   | 0.0001  | significant                                       |
| C-Incubation time          | 16.94          | 1  | 16.94       | 15.75   | 0.0004  | significant                                       |
| D- Potato starch concentration | 56.94         | 1  | 56.94       | 52.93   | <0.0001 | significant                                       |
| E-Agitation                | 26.47          | 1  | 26.47       | 24.61   | <0.0001 | significant                                       |
| AB                         | 10.13          | 1  | 10.13       | 9.41    | 0.0046  | significant                                       |
| AC                         | 4.50           | 1  | 4.50        | 4.18    | 0.0500  | significant                                       |
| AD                         | 10.13          | 1  | 10.13       | 9.41    | 0.0046  | significant                                       |
| AE                         | 32.00          | 1  | 32.00       | 29.75   | <0.0001 | significant                                       |
| BC                         | 12.50          | 1  | 12.50       | 11.62   | 0.0019  | significant                                       |
| BD                         | 10.13          | 1  | 10.13       | 9.41    | 0.0046  | significant                                       |
| BE                         | 4.50           | 1  | 4.50        | 4.18    | 0.0500  | significant                                       |
| CD                         | 4.50           | 1  | 4.50        | 4.18    | 0.0500  | significant                                       |
| CE                         | 6.13           | 1  | 6.13        | 5.69    | 0.0238  | significant                                       |
| DE                         | 4.50           | 1  | 4.50        | 4.18    | 0.0500  | significant                                       |
| A²                         | 72.42          | 1  | 72.42       | 67.32   | <0.0001 | significant                                       |
| B²                         | 9.03           | 1  | 9.03        | 8.40    | 0.0071  | significant                                       |
| C²                         | 14.38          | 1  | 14.38       | 13.37   | 0.0010  | significant                                       |
| D²                         | 28.78          | 1  | 28.78       | 26.75   | <0.0001 | significant                                       |
| E²                         | 4.93           | 1  | 4.93        | 4.58    | 0.0409  | significant                                       |
| Lack of Fit                | 27.32          | 22 | 1.24        | 2.24    | 0.1376  | Insignificant                                     |
| Pure Error                 | 3.87           | 7  | 0.5536      |         |         |                                                  |
| Cor Total                  | 2285.28        | 49 |             |         |         |                                                  |
CONCLUSION

The isolate SS2 is a potent CGTase producer which can effectively produce an enhanced amount of enzyme after optimization of the operating parameters. The bacterium can produce the enzyme in a wide thermal and pH range using various substrates. The main feature of isolate is the production of enzyme in alkaliphilic conditions which make it suitable for both research and industrial applications.

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REFERENCES

Alves-Prado, H. F., Carneiro, A. A. J., Pavezzi, F. C., Gomes, E., Boscolo, M., Franco, C. M. L., & da Silva, R. (2007). Production of Cyclodextrins by CGTase from Bacillus clausii Using Different Starches as Substrates. Applied Biochemistry and Biotechnology, 146(1-3), 3–13. https://doi.org/10.1007/s12088-010-0079-y

Atanasova, N., Kitayska, T., Bojadjeva, I., Yankov, D., & Tonkova, A. (2011). A novel cyclodextrin glucanotransferase from alkalophilic Bacillus pseudalcaliphilus 20RF: Purification and properties. Process Biochemistry, 46(1), 116–122. https://doi.org/10.1016/j.procbio.2010.07.027

Battista, V., Esclapez, J., Pérez-Pomares, F., Martínez-Espinosa, R. M., Camacho, M., & Bonete, M. J. (2012). Cyclodextrin glycosyltransferase: a key enzyme in the assimilation of starch by the halophilic archaeon Halofexis mediterranei. Extremophiles, 16(1), 147–159. https://doi.org/10.1007/s00792-011-0414-z

BeMiller, J. N., & Whistler, R. L. (Eds.). (2009). Starch: chemistry and technology. Academic Press. https://doi.org/10.1016/S1082-0132(08)00009-3

Biwer, A., Antranikian, G., & Heinze, E. (2002). Enzymatic production of cyclodextrins. Applied Microbiology and Biotechnology, 59(6), 609–617. https://doi.org/10.1007/s00253-002-1057-x

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K., & Lim, Y.-W. (2007). EZTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. International Journal of Systematic and Evolutionary Microbiology, 57(10), 2259–2261. https://doi.org/10.1099/ijs.0.64915-0

Costa, H., Gastón, J. R., Lara, J., Martínez, C. O., Moriwaki, C., Matioli, G., & Ferrarotti, S. A. (2015). Cyclodextrin glycosyltransferase production by free cells of Bacillus circulans DF 9R in batch fermentation and by immobilized cells in a semi-continuous process. Bioprocess and Biosystems Engineering, 38(6), 1055–1063. https://doi.org/10.1007/s00792-014-1347-6

De Oliveira, C. C., de Souza, A. K. S., & de Castro, R. J. S. (2019). Bioconversion of Chicken Feather Meal by Aspergillus niger: Simultaneous Enzymes Production Using a Cost-Effective Feedstock Under Solid State Fermentation. Indian Journal of Microbiology, 59(2), 209–216. https://doi.org/10.1007/s12088-019-00792-3

Di Cigno, M. P. (2016). The Potential of Cyclodextrins as Novel Active Pharmaceutical Ingredients: A Short Overview. Molecules, 22(1). 1 https://doi.org/10.3390/molecules22010001

Elbaz, A. F., Sobhi, A., & ElMekawy, A. (2015). Purification and characterization of cyclodextrin β-glucosyltransferase from novel alkalophilic bacilli. Bioprocess and Biosystems Engineering, 38(4), 767–776. https://doi.org/10.1007/s00449-014-1318-y

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39(4), 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Goel, A. and Nene, S. (1995). A Novel Cyclomaltodextrin glucanotransferase from Bacillus firmus that Degrades Raw Starch. Biotechnology Letters, 17(4), 411-416. https://doi.org/10.1007/BF00130799

Horikoshi, K. (2003). Alkaliphiles: Alkaline Enzymes and their Applications. Encyclopedia of Environmental Microbiology. John Wiley & Sons, Inc., New York. https://doi.org/10.1002/0471263397.env183

Ibrahim, A.S., Al-Salamah, A.A., El-Tayeb, M.A., El-Badawi, Y.B., Antranikian, G. (2012). A novel cyclodextrin glycosyltransferase from Alkalophilic Amphibacillus sp. NPST-10: purification and properties. International Journal of Molecular Sciences, 13, 10505–10522. https://doi.org/10.3390/ijms131010505

Ibrahim, H.M, Yusoff, W.M.W., Hamid, A.A., Illias, R.M., Hassan, O., Omar, O. (2005). Optimization of medium for the production of β-cyclodextrin
glucanotransferase using Central Composite Design (CCD). Process Biochemistry, 40, 735-758. https://doi.org/10.1016/j.procbio.2004.01.042

Jamuna, R., Saswathi, N., Sheela, R., Ramakrishna, S.V. 1993. Synthesis of cyclodextrin glucosyl transferase by Bacillus cereus for the production of cyclodextrins. Applied Biochemistry and Biotechnology, 43, 163-176. https://doi.org/10.1007/BF02916450

Jansook, P., Ogawa, N., Loftsson, T. (2018). Cyclodextrins: structure, physicochemical properties and pharmaceutical applications. International Journal of Pharmaceutics, 535(1-2), 272-284. https://doi.org/10.1016/j.ijpharm.2017.11.018

Kfouri, M., Loumès-Hadj, S.A., Boudon, N., Laruelle, F., Fontaine, J., Auezova, L., Greige-Gerges, H., and Fourmentin, S. (2016). Solubility, photostability and antifungal activity of phenylpropanoids encapsulated in cyclodextrins. Food Chemistry, 196, 518-525. https://doi.org/10.1016/j.foodchem.2015.09.078

Kimura, M. A. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, 16, 111–20. https://doi.org/10.1007/BF01731581

Kreig, R. N., & Holt, G. J. (1984). In Bergey’s Manual of Systematic Bacteriology, William and Wilkins Co.

Kumar, P., Patel, S.K., Lee, J.K. and Kalia, V.C. (2013). Extending the limits of Bacillus for novel biotechnological applications. Biotechnology Advances, 31(8), 1543-1561. https://doi.org/10.1016/j.biotechadv.2013.08.007

Lee, M.H., Yang, S.J., Kim, J.W., Lee, H.S., Kim, J.W., Park, K.H. (2007). Characterization of a thermostable cyclodextrin glucanotransferase from Pyrococcus furiosus DSM638. Extremophiles, 11, 537-541. https://doi.org/10.1007/s00792-007-0061-6

Leemhuis, H., Kelly, R. M., & Dijkstra, L. (2009). Engineering of cyclodextrin glucanotransferases and the impact for biotechnological applications. Applied Microbiology and Biotechnology, 85(4), 823–835. https://doi.org/10.1007/s00253-009-2221-3

Montgomery, D.C., Runger, G.C. and Hubele, N.F. (2009). Engineering statistics. John Wiley & Sons.

Park, C.S., Park, K.H., Kim, S.H. (1989). A rapid screening method for alkaline β-cyclodextrin glucanotransferase using phenolphthalein-methyl orange containing solid medium. Agricultural and Biological Chemistry, 53, 1167–1169. https://doi.org/10.1080/00021369.1989.10869443

Patel, S.K.S., Ray, S., Prakash, J. et al. (2019). Co-digestion of Biowastes to Enhance Biological Hydrogen Process by Defined Mixed Bacterial Cultures. Indian Journal of Microbiology, 59, 154–160. https://doi.org/10.1007/s12088-018-0077-8

Porwal, S., Kumar, T., Lal, S., Rani, A., Kumar, S., Cheema, S., Purohit, H.J., Sharma, R., Patel, S.K.S. and Kalia, V.C. (2008). Hydrogen and polyhydroxybutyrate producing abilities of microbes from diverse habitats by dark fermentative process. Bioresource Technology, 99(13), 5444-5451. https://doi.org/10.1016/j.biortech.2007.11.011

Qi, Q., and Zimmermann, W. (2004). Cyclodextrin glucanotransferase: from gene to applications. Applied Microbiology and Biotechnology, 66(5), 475–485. https://doi.org/10.1007/s00253-004-1781-5

Rajput, K.K., Patel, K.C., Trivedi, U.B. (2016). β-cyclodextrin production by cyclodextrin glucanotransferase from an alkaliphile Microbacterium terrae KNR 9 using different starch substrates. Biotechnology Research International, 7, 1–7. https://doi.org/10.1155/2016/2034359

Rashid, N., Cornista, J., Ezaki, S., Fukui, T., Atomi, H., Imanaka, T. (2002). Characterization of an archaeal cyclodextrin glucanotransferase with a novel C-terminal domain. Journal of Bacteriology, 184, 777-784. http://www.ncbi.nlm.nih.gov/pubmed/1126548

Ravinder, K., Prabhalakar, T., Prashanthkumar K, Venuka N. (2014). Optimization of Process Parameters of Cyclodextrin Glycosyltransferase isolated from Novel Mutated Bacillus Sp. TPR71HNA6 by Taguchi Orthogonal Array Method. Research in Biotechnology, 5(3), 10-20.

Reddy, S.V., More, S.S., Annappa, G.S. (2017). Purification and properties of β-cyclomaltoolxtranzyme glucanotransferase from Bacillus flexus SV1. Journal of Basic Microbiology, 57, 974-981. https://doi.org/10.1002/jobm.201700270

Saitou, N. and Nei, M. (1987). The neighbour-joining method: a new method for constructing phylogenetic trees. Molecular Biology and Evolution, 4, 406-425. https://doi.org/10.1093/molbev/smb012

Sharma, N. and Baldi, A. (2016). Exploring versatile applications of cyclodextrins: an overview. Drug Delivery, 23, 729–47. https://doi.org/10.1155/2017/93889

Sharma, K. M., Kumar, R., Panwar, S., Kumar, A. (2017). Microbial alkaline proteases: Optimization of production parameters and their properties. Journal of Genetic Engineering and Biotechnology, 15(1), 115–126. https://doi.org/10.1016/j.jeb.2017.02.001

Staley, J. T. (1989). Bergey’s manual of systematic bacteriology. Williams & Wilkins.