CONVENTIONAL PRODUCTION OPTIMIZATION OF CYCLODEXTRIN GLUCOSYL TRANSFERASE BY A NOVEL ISOLATE OF BACILLUS SP. PBS1 FROM POTATO RHIZOSPHERE

Preetibala Solanki*, Pushpendra Awadhiya¹ and Tushar Banerjee¹

Address(es): Preetibala Solanki ¹ School of Life Sciences, Devi Ahilya Vishwavidhyalaya Indore, India.

*Corresponding author: preetisolanki2604@gmail.com

ARTICLE INFO

Received 5. 8. 2021
Revised 24. 3. 2022
Accepted 7. 4. 2022
Published 1. 8. 2022

ABSTRACT

The cyclodextrin glucosyltransferase enzyme (CGTase) is an industrially crucial enzyme for the production of β-cyclodextrin (β-CD). CGTase has a high propensity to produce a mixture of cyclodextrins (CDs). From the industrial perspective, a CGTase that produces only one type of CD is of critical importance. Bacillus sp. PBS1 produced CGTase that converts starch solely into β-CD. The isolated strain PBS1 was found to close similarity with alkalophilic Bacillus sp. based on biochemical, morphological, and phylogenetic analysis of its 16S rRNA gene sequencing. The selection and optimization of media ingredients are warranted for the best possible production of β-CD. These steps were carried out by conventional optimization strategies. The presence of glucose, maltose, lactose, sucrose, galactose, mannitol, nitrates, urea, metal salts, and K_2HPO_4 led to the suppression of CGTase production. The improved enzyme production was observed in peptone, soluble starch, magnesium sulfate, and Na_2CO_3. The organism produces maximum CGTase (93.42 ± 2.4 U/ml) at 96-hour incubation in the optimized production medium containing 8% starch, 2% peptone, 0.06% MgSO_4.7H_2O, 0.5% Na_2CO_3, and having pH of 9.3. The optimization of the medium led to ~16% improvement in CGTase production by Bacillus sp. PBS1.

Keywords: CGTase, Bacillus sp. PBS1, β-cyclodextrin, Production optimization

INTRODUCTION

Cyclodextrins are known for their torus-shaped structure having a hydrophilic surface and hydrophobic cavity. As the name suggests, cyclodextrins have a cyclic arrangement of α-(1,4) linked glucose units. The diameter of the hydrophobic cavity of β-CD (7.8 Å) enables it to form water-soluble inclusion complexes with many hydrophobic compounds. This unique property of cyclodextrins makes them potentially valuable for pharmaceutical, medical, cosmetic, agricultural, environmental, textile and food industries (Sun et al., 2011; Zhang et al., 2019; Buschmann et al., 2002; Fenyesi E., 2011; Bezerra et al., 2020; Maskooki et al., 2013). Cyclodextrins are natural, non-reducing, cyclic malto-oligosaccharides (Sabioni et al., 1992). Depending on glucose residues, cyclodextrins are categorized into three major types; α-CD having six glucose units, β-CD having seven glucose units, and γ-CD having eight glucose units (Van der Veen et al., 2000). Cyclodextrins are produced by the cyclodextrin glucosyltransferase enzyme (CGTase, EC 2.4.1.19). To produce CD, partial degradation of starch and cyclization of the oligomer is catalyzed by the CGTase. CGTase is a member of the amylolytic glucosylase family that catalyzes intramolecular transglycosylation, cyclization as well as reversible intermolecular transglycosylation including coupling, and disproportionation of malt-oligosaccharides, at the same time has a weak starch hydrolyzing activity (Tomocovo et al., 1998). The CGTase enzyme is thought to be evolved from the α-amylase family by specific mutations in the substrate-binding site (Kelly et al., 2009). CGTase enzyme is produced by several genera of bacteria such as B. licheniformis, B. firmus, B. circulans, B. clausii, Brevibacillus brevis, B. steaothermophilus, Klebsiella pneumoniae, and Microbacterium terrae (Bonilha et al., 2006; Gawande and Pathkar 2001; Rosso et al., 2002; Kim et al., 1997; Alves Prado et al., 2008; Chung et al., 2007; Burhan et al., 2005; Rajput et al., 2016). The most prominent CGTase producers are alkalophilic Bacillus sp. (Tomocova et al., 2000) [Table I]. The majority of CGTase produces β-CD as the core product with a low concentration of α and γ-CD. Conventionally, depending on the significant product of CGTase, the enzyme is named α, β, and γ-CGTase, respectively (Vazquez et al., 2016). If the same organism produces them, it would be necessary to separate all the three cyclodextrins from the reaction mixture which might be tedious and costly. To avoid expensive separation and purification steps, a strain that produces a single type of CD (Thattai et al., 1999). Microbial enzyme production is influenced by several factors, such as; medium ingredients, pH, presence of inducers and metal ions, etc. Screening and incorporation of appropriate carbon, nitrogen, and other nutrient sources is warranted for designing an efficient and cost-effective production medium (Wang et al., 2018).

The presented work describes the screening and isolation of a CGTase producing organism that produces only β-CD. Biochemical identification of isolated organisms was followed by attesting by an automated VITECK 2 compact system and molecular identification by 16s-rRNA sequencing. To assess the effect of various ingredients and optimize the production of CGTase, classical, one factor at a time approach was adopted. Which the authors believe is a pre-requisite for any optimization effort for a novel isolate.

Table 1 Some of the important natural key CGTase producer organisms and their enzyme activity

| CGTase producer strain | CGTase activity (U/ml) | References |
|------------------------|------------------------|------------|
| Bacillus firmus         | 0.77                    | Silva et al. (2021) |
| Bacillus macerans       | 3.53                    | Dalmotra et al. (2016) |
| Bacillus megaterium     | 57.75                   | Vazquez et al. (2016) |
| Bacillus lehensis       | 1.9                    | Santos et al. (2013) |
| Bacillus firmus strain 37 | 0.22                  | Santos et al. (2013) |
| Bacillus sp. TS1        | 18.9                   | Yap et al. (2010) |
| Bacillus G1             | 54.9                   | Zain et al. (2006) |
| Bacillus circulans DF 9R | 5.8                    | Rosso et al. (2002) |

MATERIAL AND METHODS

Chemicals

All the chemicals used in the experiments were of analytical grade. β-CD was purchased from SD Fine Chem Limited, Mumbai, India. Phenolphthalein (PH) and soluble starch were from Merck Ltd. Mumbai, India. Media components were procured from Himedia Laboratories, Mumbai, India. Substrates like tapioca, wheat, and rice starch were procured from Urban Platter, Mumbai, India. Potato starch and maize starch were sourced from Loba Chemie Pvt Ltd, Mumbai, India.

Isolation and screening of CGTase producer strain

The screening was preceded by an enrichment process. One gram of soil was added in 10 ml normal saline (0.85% NaCl), and then 0.1 ml was transferred into two 150 ml Erlenmeyer flask containing 30ml of enrichment medium (soluble starch 1%, peptone 0.5%, yeast extract 0.5%, K_2HPO_4 0.1%, MgSO_4 0.02%, and Na_2CO_3 1% (separately autoclaved). Flasks were incubated at 28°C, 130 rpm on a gyratory
Table 2 Production medium used for CGTase production

| S.N | Contains | Medium concentrations (%) |
|-----|----------|---------------------------|
| 1   | Soluble Starch | A  2  1  0.75  2  2  4  3 | |
| 2   | Peptone | -  0.50  0.50  1  5  2  0.50 | |
| 3   | Trptone | -  -  0.50  -  -  -  -  - | |
| 4   | Yeast extract | 1  0.50  -  0.50  5  -  0.50 | |
| 5   | KHPO4 | 0.10  0.10  0.10  -  0.10  0.10  - | |
| 6   | MgSO4 | 0.02  0.02  0.01  -  0.02  0.04  0.02 | |
| 7   | NaHPO4 | -  -  -  -  -  -  -  - | |
| 8   | Na2CO3 | 1  1  1  1  1  1  - | |

Media optimization for CGTase production by isolated Bacillus sp.

Once the production medium was selected, the effect of different starch sources, sugars, a sugar alcohol (mannitol), organic and inorganic nitrogen sources, and different metal ions was studied. For these studies, single-point sampling at 96 h was done. Starch sources included in the study were soluble starch, rice starch, tapioca starch, wheat starch, potato starch, maize starch at 4% concentration. The effect of sugars (glucose, sucrose, maltose, lactose) and mannitol were studied at 0.1M concentration. Organic nitrogen sources (peptone, yeast extract, malt extract, tryptone, casein, and corn steep liquor) were studied at a 2% concentration. Inorganic sources (potassium nitrate, sodium nitrate, ammonium nitrate, ammonium sulfate, ammonium chloride, and urea) were added in the medium at the same nitrogen content as peptone. The presence of metal ions has been reported to improve enzyme production (Wang et al., 2018). The effect of various metal ions viz., MnSO4, MgSO4·7H2O, KCl, FeSO4, CaCl2, CoCl2, CuSO4, were studied at 5 mM concentration. Other important medium components like K2HPO4, Na2CO3, and their concentration variations were studied. Statistical analysis

All the studies were conducted in triplicate, and the data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests using Graphpad Prism version 5.00 for windows. The data is graphically presented as mean ± SD of triplicates (n = 3).

RESULTS AND DISCUSSION

Screening of CGTase producer

Soil samples were collected from potato cultivation fields of the Malwa region of Madhya Pradesh. The highest zone was observed in soil sample IV (rhizosphere soil of spoiled potato) after 24 hours of incubation. This culture was purified by the standard microbial protocol using Horikoshi (PHP) screening medium. The isolated bacterium was found to be Gram-positive, rod-shaped, motile, and capulated. The discerning biochemical tests were performed [Table 3]. The isolated organism has creamy circular colonies with an entire margin and smooth surface on nutrient agar pH 10. The organism was inspected by a preliminary screening of α and γ-CGD production as described by Menocci et al. (2008). No clear zone was observed in both screening mediums. This result concluded that the organism is unable to form an inclusion complex with methyl orange and bromocresol green to produce α and γ-CGD [Figure 1]. A similar observation was noted for Bacillus sp. BACAR produces only β-CGD (Menocci et al., 2008). This result confirms the findings of Gawande and Patkar (2001) that α and γ-CGD producer strains are rare. The formation of CDs appear to be dependent on a variety of factors such as amino acid composition of the enzyme, their orientation and sequence, further the type of CD formed is also found to be dependent on the incubation conditions and time of incubation. It has been found that initially the enzyme based on the substrate concentration produces a wide variety of CDs while after incubation of sufficient duration the larger CDs are rapidly reused via intermolecular transglycosylation reactions to produce a preferred / typical mix of CDs (Terada et al., 1997 and Qi et al., 2005). The exact sequence or conditions required for the production of one type of CDs, still remains elusive despite some limited success. Our study has consisted with previous reports that the enzyme from the alkaliphilic bacterial strain produces preferentially β-CD (Akselyan et al., 2002). The isolated organism was found to predominantly produce β-CD; hence it was classified as a β-CGTase producer.
VITECK 2 compact biochemical analysis and 16s rRNA phylogenetic analysis of isolated strain

VITECK 2 system is utilized for fast, reliable microbial identification and to detect antibiotic sensitivity of desired microorganisms. In Gram’s staining organism was found to be Gram-positive bacilli, so for VITECK 2 biochemical characterization, a Bacillus identification card (BCL) was used. That included 46 biochemical tests, e.g., carbon source utilization, enzymatic degradation, and antibiotic resistance (Table 4). However, the VITECK 2 compact analysis did not show a biochemical pattern similar to other known Bacillus sp. present in the BCL card. Thus advanced characterization was done using the molecular approach. The 16s rRNA gene sequence used for identification was 1373 bp long. On BLASTn analysis of Bacillus sp. PBS1 on NCBI showing ~98.91 to 98.69% similarity with Alkalihalobacillus lehensis and Alkalihalobacillus hunanensis. The Bacillus sp. PBS1 found to more closely to Alkalihalobacillus lehensis strain JO-26 with 98.91% similarity (GeneBank accession No. MF321817). The most similar sequence was retrieved from NCBI for the identification of evolutionary history between our isolated and known organisms. The 16S rRNA phylogenetic tree of alkaliphilic Bacillus sp. PBS1 showed significant similarity with Alkalihalobacillus lehensis by sharing the same clad in phylogenetic tree [Figure 2]. Based on morphological, biochemical, and phylogenetic analysis, the isolated organism was identified as Bacillus sp. and the isolated organism was designated as Bacillus sp. PBS1. The gene sequence of the 16s rRNA gene was submitted to the NCBI Gene bank database with accession number MN938303. In addition, culture was deposited NCIM (CSIR NCL Pune).

Table 3 Biochemical characterization of CGTase producer isolated from potato rhizosphere

| Tests            | Results       |
|------------------|---------------|
| Motility         | Motile        |
| Nitrate Reductase| -             |
| Indole Synthesis | +             |
| Urease           | +             |
| Catalase         | +             |
| Gelatin hydrolysis| -            |
| Casein hydrolysis| +             |
| Starch hydrolysis| +             |
| Growth in 6.5% NaCl| +         |

* - negative; + positive

Results

A. Bacillus sp. PBS1 and closely related Bacillus species with 1000 bootstraps replicate. Numbers at nodes represent bootstrap values (%). Scale bar indicates the genetic distance of 0.001.

Table 4 VITECK 2 Compact biochemical characteristics of CGTase producer

| Carbon Utilization | Result | Enzyme activities | Result |
|--------------------|--------|-------------------|--------|
| Cyclodextrin       | -      | Beta-xyllosidase  | +      |
| D-galactose        | -      | L-lysin arylamidase| -      |
| Glycogen           | +      | L-aspartate arylamidase| +    |
| Myo-inositol       | +      | Leucine-arylamidase| +      |
| Ellman             | -      | Phenylalanine arylamidase| +  |
| Methyl-d-xylloside | -      | L-proline arylamidase| -    |
| Maclorotose        | +      | Beta-galactosidase| -      |
| D-mannitol         | +      | L-pyrrolidinyl arylamidase| + |
| D-mannose          | +      | Alpha-galactosidase| +      |
| D-melezitose       | -      | Alanine arylamidase| +      |
| N-acetyl-d-         | +      | Tyrosine arylamidase| +    |
| Palatinose         | +      | Beta-n-acetyl-glucosaminidase| + |
| L-ramnosode        | -      | Ala-pha-pro arylamidase| +  |
| Pyruvate           | +      | Methyl-A-D-    | acidification| - |
| D-tagatose         | -      | Alpha-mannosidase| -      |
| D-trehalose        | +      | Glycine acylamidase| +      |
| Inulin             | +      | Beta-glucosidase| +      |
| D-glucose          | +      | Beta-mannosidase| -      |
| D-ribose           | +      | Phosphoryl choline| +      |

Petruscin assimilation - Alpha-glucosidase -

Enucle hydrolysis +

Antibiotic Resistance +

Kanamycin resistance -

Oleandomycin resistance +

Polymixin B resistance +

* - negative; + positive

Selection of production medium

The selection of an appropriate production medium is a must for optimal enzyme production. After the literature survey, different medium compositions were selected for the study of CGTase production by Bacillus sp. PBS1. The compositions of the different mediums have been described in the materials and method section. Among the seven tested medium compositions, medium F supported maximum CGTase production being 77.13 ± 0.14 U/ml [Figure 3]. Medium F contained the highest amount of substrate (4% soluble starch), organic nitrogen (2% peptone), and magnesium sulfate (0.04%) as compared to other media included in the study. Next to medium F, medium D and B have also supported CGTase production being 60.71 ± 0.62 and 55.6 ± 0.74 U/ml. Deficient CGTase production was observed in medium G, which could be attributed to a neutral pH of the medium. The effect of different medium compositions on CGTase was in the order of F>D>B>E>A>C>Г. Medium F was previously optimized by Ibrahim et al. (2004) for Bacillus G1 and reported 54.9 U/ml CGTase production. Medium F was selected for further optimization studies.

Figure 1 Isolation and screening for CGTase activities a. Methyl orange (0.035mM) agar plate for α-CGTase showing no reduction of orange colour by the isolated organism, b. PHP (0.94mM) agar plate for β-CGTase showing a reduction in pink colour around the colonies due to inclusion complex formation between PHP and β-CD and c. Bromocresol green (0.02mM) plate for γ-CGTase showing no reduction in green colour.

Figure 2 Neighbor-joining phylogenetic tree showing evolutionary relationship between Bacillus sp. PBS1 and closely related Bacillus species with 1000 bootstraps replicate.
The isolated strain was compared for CGTase production with a known CGTase producer, *Cytobacillus firmus* NCIM 5119 (Gawande et al., 1998). Medium F was used for the comparison. *Bacillus* sp. PBS1 produced a maximum of 81.69 ± 2.26 U/ml CGTase [Figure 4] whereas the *Cytobacillus firmus* NCIM 5119 yielded 60.49 ± 2.17 U/ml CGTase. In our studies *Bacillus* sp. PBS1 exhibited higher CGTase production as compared with the known producer, being 21% more.

**Figure 3** Effect of different fermentation mediums for CGTase production by isolated *Bacillus* sp. PBS1. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Comparison with a known CGTase producer**

The effect of sugars on CGTase production, as compared with the known producer, being 21% more.

**Figure 4** Comparison of CGTase production between *Cytobacillus firmus* NCIM 5119 and *Bacillus* sp. PBS1 in F medium P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Screening of Medium Components for CGTase Production**

**Effect of sugars**

Carbon source is considered to be one of the most important constituents for enzyme production. Some carbon sources (glucose, sucrose, maltose, mannitol, and lactose) were supplemented to the medium F to evaluate their effect on CGTase production. All of them resulted in suppression of CGTase production [Figure 5]. Maltose and glucose were found to exert the highest suppressive effect (~70-80% decline) when used along with starch. The enzyme production was recorded to be 9.53 ± 1.05 and 18.54 ± 1.59 U/ml in the presence of maltose and glucose, respectively. This observation is supported by the findings ofLetsiddi et al. (2011); Gawande et al. (1998); Higuti et al., (2004); Elbaz et al. (2015) and Ramli et al. (2010) reported that the production of CGTase was suppressed when simple sugars were present in the medium. Probably, the presence of an easily utilisable carbon source suppresses the ability of an organism to catabolize starch.

Toncová (1998) and Wang et al. (2006) assumed that glucose and maltose might possess a catabolic repression effect. Contrary to that, Jamuna et al. (1993) reported maximum CGTase production by *B. cereus* in a medium supplemented with glucose and xylose. The effect of simpler sugars on CGTase production appears to be an organism-dependent phenomenon.

**Figure 5** Effect of supplementation of different sugars on CGTase production by *Bacillus* sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Effect of starch sources**

The effect of starch sources viz. rice starch, tapioca starch, wheat starch, potato starch, maize starch, and soluble starch were compared for the CGTase production. Starches were supplemented (4%) one at a time in medium F. Soluble starch was found to be most effective for CGTase production (61.56 ± 0.67 U/ml) [Figure 6]. Soluble starch has been reported for CGTase production by *Microbacterium terra KN9* (Rajput et al., 2016). *Bacillus lehensis* S8 (Vidya et al., 2012), and *Bacillus* GI (Ibrahim et al., 2005). Next to soluble starch, tapioca starch and rice starch were found to support 51.71 ± 5.4 U/ml and 50.20 ± 5.5 U/ml enzyme production, respectively. Maize and wheat starch were least supportive for CGTase production (33.29 ± 0.32 U/ml and 33.79 ± 4.1 U/ml). Gawade et al. (1998) found corn starch to be the best substrate for CGTase production by *Bacillus firmus* at 2.1%. The difference in enzyme production in different starches could be attributed to the difference in the physical nature of starches (Ibrahim et al., 2005).

The utilizability of starch for CGTase production appears to depend on its physical and chemical structure.

**Figure 6** Effect of various starches on CGTase production by isolated *Bacillus* sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Effect of nitrogen source (inorganic)**

To evaluate the effect of inorganic nitrogen sources on CGTase production, peptone was replaced with various inorganic nitrogen sources (potassium nitrate, sodium nitrate, ammonium nitrate, ammonium sulfate, ammonium chloride, and urea) in medium F. All the inorganic nitrogen sources suppressed the CGTase production. 49-60% reduction in CGTase production was observed when peptone was replaced by an inorganic nitrogen source. Control having the organic nitrogen source (peptone) showed maximum CGTase production 89.61 ± 2.9 U/ml [Figure 7]. Among all the tested inorganic nitrogen sources, the best CGTase production was observed in the presence of sodium nitrate (40.85 ± 2.35 U/ml).

Yang et al. (2017) have also reported that NH₄⁺, NO₃⁻, and urea have an inhibitory effect on CGTase production. In contrast, Rasso et al. (2002) reported ammonium sulphate (0.5%) to be the optimum nitrogen source for CGTase production (3.06 U/ml) by *B. circulans* DP-F9R compared to organic nitrogen sources.

The organism *Bacillus* sp. PBS1 is urease positive but probably due to the alkaline pH of the medium organism might not utilize urea. There is a possibility of the breakdown of urea in the form of ammonia at alkaline pH. The organism is nitrate negative; thus nitrates cannot be utilized efficiently.
Our study confirms the finding of previous studies that inorganic nitrogen sources are not suitable for CGTase production (Yang et al., 2017, Rajput et al., 2016 and Yap et al., 2010). It can be concluded that CGTase production would be higher when the production medium is supplemented with peptone as a nitrogen source.

Figure 7 Effect of different inorganic nitrogen sources on CGTase production by Bacillus sp. PBS1 in medium F. Peptone considered as control. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Effect of organic nitrogen source**

Organic nitrogen has been reported to be essential for growth and CGTase production (Wang et al., 2018). Six different organic nitrogen sources were compared to find out the best for CGTase production. Peptone, yeast extract, malt extract, tryptone, casein, and corn steep liquor were supplemented with FeSO₄ at a time in medium F. The concentration of various organic nitrogen sources was adjusted so as to equate the nitrogen content available in 2% peptone, 2% w/v peptone was found to be the best nitrogen source for CGTase production. It supported 92.37 ± 6.16 U/ml CGTase enzyme production [Figure 8]. Next to peptone, tryptone (68.21 ± 7.73 U/ml) and corn steep liquor (52.53 ± 2.84 U/ml) were found to be good for CGTase production. The use of malt extract as a nitrogen source resulted in very low enzyme production (18.28 ± 1.41 U/ml). Ibrahim et al. (2005) reported that peptone significantly enhanced the CGTase production amongst yeast extract, soybean, and glutamate tested. However, the maximum production was only 17.05 U/ml. While on the contrary, Yang et al. (2017) found tryptone as the best organic nitrogen source (3.13 U/ml). Gawande et al. (1998) reported that when peptone was used in combination with corn steep liquor it gave the highest CGTase production (24.51 U/ml).

Figure 8 Effect of various organic nitrogen sources on CGTase production by Bacillus sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Effect of metal salts**

To observe the effect of metal ions SnM concentration, MnSO₄, FeSO₄, CuSO₄, CoCl₂, CaCl₂, and KCl were added in medium F. 1.62 mM MgSO₄ being an ingredient in medium F was treated as control. In the presence of MgSO₄ (control), 88.03±3.43 U/ml enzyme was produced [Figure 9]. All the other tested metal ions had shown a deleterious effect on CGTase production. The enzyme production in the presence of other metal ions was in decreasing order as follows MnSO₄ < KCl < FeSO₄ < CuCl₂ < CoCl₂ < CuSO₄. However, no significant difference between MnSO₄, KCl, FeSO₄ was found. Several studies revealed that Mg²⁺ is essential for accelerating the CGTase production, being a cofactor of CGTase (Blanco et al., 2009; Mora et al., 2012; Yang et al., 2017). Various studies showed that Ca²⁺ is helpful for active enzyme conformation and stabilizing thermal stability. However, in our study, Ca²⁺ was found to inhibit enzyme production slightly. Rosso et al. (2002); Yang et al. (2017) also reported that Ca²⁺ has no positive effect on CGTase production.

Figure 9 Effect of various metal salts on CGTase production by Bacillus sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Concentration Variation of Selected Production Medium Ingredients**

**Effect of starch concentration variation**

The concentration variation of medium ingredients found to enhance CGTase production was studied further. Substrate concentration affects enzyme production. CGTase is an inducible enzyme (Gawade et al., 1998); thus, changing starch concentration is expected to affect enzyme production. In this case, soluble starch was found the best amongst all the tested starch sources. To evaluate the effect on CGTase production, different concentrations of soluble starch (2%, 4%, 6%, 8%, 10%, 12%, and 14%) were added to medium F. Maximum CGTase production was recorded at 8% (91.63 ± 2.48 U/ml) concentration. Further increase in soluble starch concentration led to a decrease in CGTase production [Figure 10]. Similar results were also observed by Rakmai and Cheirsilp (2015) that increasing starch concentration beyond 10% led to a decline in CGTase production. The possible causes of inhibition of enzyme production at high substrate concentrations can be attributed to the increased viscosity resulting in the reduced mass transfer of nutrients and metabolites (Yap et al., 2010; Zain et al. (2007); Elbaz et al. (2015) reported that the presence of higher starch concentration leads to high glucose accumulation, which in turn causes suppression of CGTase production.

Figure 10 Effect of various concentrations of soluble starch on CGTase production. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

**Effect of peptone concentration variation**

Organic nitrogen (peptone) was found to increase CGTase production. A 2% concentration of peptone led to the highest CGTase production (93.42 ± 2.4 U/ml) [Figure 11]. Increasing the peptone concentration from 0.5 to 2% led to the concomitant increase in CGTase production (53.86 ± 2.6 to 93.42 ± 2.4 U/ml). Further increase in peptone concentration led to a dose-dependent decrease in CGTase production (30.11 ± 0.07 U/ml). Ibrahim et al. (2005) reported 55.3 U/ml CGTase productions at 2% peptone. Other authors have also found that higher concentrations of organic nitrogen sources inhibit CGTase production (Ibrahim et al., 2005 and Elbaz et al., 2015). The presence of a higher amount of complex
nitrogen source might trigger the secretion of proteases, which could degrade CGTase (Yang et al., 2017).

**Effect of dipotassium hydrogen phosphate concentration variation**

No positive effect of K$_2$HPO$_4$ addition on CGTase production could be detected at any of the tested concentrations (0.5, 1.0, 1.5, 2.0, and 2.5). Furthermore, an increase in K$_2$HPO$_4$ concentration led to a dose-dependent decline in CGTase production [Figure 12]. At 2.5% concentration of K$_2$HPO$_4$, 62% reduction was observed as compared to control. Control which was devoid of K$_2$HPO$_4$ showed maximum CGTase production (82.17 ± 2.34 U/ml). Mahat et al. (2004) also reported that K$_2$HPO$_4$ had no significant effect on CGTase production. These results differ from Wang et al. (2018), where increased production (3230 U/ml) was observed in medium supplemented with 0.04% MgSO$_4$; 0.02% K$_2$HPO$_4$, and 0.02% magnesium sulfate. As shown in Figure 13, 0.08% concentration, production was declined slightly. Likewise, K$_2$HPO$_4$ was reported as a crucial factor for influencing CGTase production by Bacillus megaterium NCR; the pH of the medium was 7. (Ahmed and Refai 2010). The pH of the medium might affect the role of phosphate metabolism in the bacteria.

To verify the negative effect of the K$_2$HPO$_4$ experiment was repeated several times. It was found that the Bacillus sp. PBS1 produces CGTase in the limited environment of potassium and phosphate. The absence of K$_2$HPO$_4$ favours good CGTase production.

**Effect of magnesium sulfate concentration**

The different concentrations tested ranged from 0.02 to 0.08% (with an increment of 0.02%) added to the production medium F. As shown in figure 13, 0.06% concentration was found to enhance the CGTase production significantly. At the same time, no significant difference was observed in 0.02 to 0.04% concentrations. At 0.08% concentration, production was declined slightly. For Bacillus G1, 0.04% MgSO$_4$ was found to be suitable leading to 17.48 U/ml enzyme production (Ibrahim et al., 2005). Wang et al., (2018) observed that 0.02% magnesium sulfate (3.05 U/ml) was significant in promoting CGTase production. Bacillus sp. PBS1 required a slightly higher concentration of MgSO$_4$ as compared to known CGTase producers.

**Effect of sodium carbonate concentration**

Na$_2$CO$_3$ is used to adjust pH and as a source of Na$^+$. The effect of initial pH was evaluated by varying the concentration of Na$_2$CO$_3$. Na$_2$CO$_3$ was added in medium F after sterilization (separately autoclaved). At varying concentrations as, 0%, 0.5, 1% (Control), 1.5%, 2%, 2.5% and their respective pH were 6.95, 9.83, 10.68, 11, 11.30 and 11.46. The 0.5% concentration had a pH of 9.3 ± 0.1 showed the highest production of 82.32 U/ml [Figure 14]. Comparatively low enzyme production (zero concentration) was observed without Na$_2$CO$_3$, possibly due to the neutral pH of the medium. Further increase in Na$_2$CO$_3$ concentration from 1% to 2.5% led to a 53% reduction CGTase production. Higher pH might lead to cell lysis, which results in reduced enzyme production. B. firmus (Gawande et al., 1998), Bacillus G1 (Ibrahim et al., 2005) and Bacillus lehensis S8 (Yap et al., 2010) were reported to produce maximum CGTase production at 1% Na$_2$CO$_3$ concentration.

**CONCLUSION**

Isolated organism, Bacillus sp. PBS1 deposited to NCIM and assigned culture accession number NCIM 5799. Phylogenetic and molecular characterization of Bacillus sp. PBS1 (NCIM 5799) revealed it to be a novel CGTase producer strain. Moreover it has capability to produce 21% higher CGTase as compared to Cytobacillus firmus NCIM 5119. The medium optimized for CGTase production contains 8% soluble starch, 2% peptone, 0.06% magnesium sulfate, and 0.5% sodium carbonate, having final pH of 9.3. This modified medium F was found to be optimal for the production of CGTase enzyme using Bacillus sp. PBS1. The medium optimization process led to an overall 16% improvement (from 77.29 to 93.42 U/ml) in enzyme production.

**Acknowledgement:** Financial support from the Ministry of Tribal Affairs, New Delhi, India, (Ref. No. F117.1/2014/15/RGNF/201415STMAD/699008/ (SAIL/Website) for a Senior Research Fellowship (SRF) to Preetibala Solanki is gratefully acknowledged.
isolated bacillus sp. Tp71h by conventional method. *International Journal of Advanced Biotechnology and Research, 3* (2) 578-584.
Rosso, A. M., Ferrarotti, S. A., Krymkiewicz, N., & Nudel, B. C. (2002). Optimisation of batch culture conditions for cyclodextrin glucanotransferase production from Bacillus circulans DF 9R. *Microbial cell factories, 3* (1), 3. https://doi.org/10.1186/1475-2859-1-3
Sabioni, J.G., Park Y.K. (1992). Production and characterization of cyclodextrin glucanotransferase from Bacillus lentus. Starch *44* (4) 225–229 https://doi.org/10.1002/star.199204404067
Silva, L., Mattoli, G., Zaman, G. & Moraes, F. (2021). Batch CGTase Production with Free and Immobilized Bacillus firmus Strain 37 in Bovine Bone Charcoal. *Advances in Chemical Engineering and Science 11* (1) 91-104. https://www.scirp.org/journal/aces
Sun, H., Seshadri, M., Lingard, S., Monaghan, W., Faoagali, J., Chan, E., McDonald, H., Houston, T., King, M., Peak, I., Wilson, J. C., Haywood, A., Spencer, B., Dunn, P. & Grant, G. D. (2011). Antibacterial Activity of β-Cyclodextrin and 2-Hydroxypropyl-β-Cyclodextrin Trimethoprim Complexes. *Current Research in Microbiology, 2*(1), 1-8. https://doi.org/10.3844/ajmsp.2011.1.8
Thombre, R.S, Kanekar, P.P. & Rajwade, J.M. (2013) Synthesis of β-cyclodextrin by CGTase produced by Bacillus licheniformis MCM-B-1010. *International Journal of Pharma and Bio Sciences 4* (1) 515 – 523.
Tonkova, A. (1998). Bacterial cyclodextringlu cotransferase. *Enzyme Microbial Technol 22* (8) 678-86. https://doi.org/10.1016/S0141-0229(97)00263-9
Van der Veen BA, Uitdehaag, J. C., Dijkstra, B. W., & Dijkhuizen, L. (2000). Engineering of cyclodextrin glycosyltransferase reaction and product specificity. *Biochimica et biophysica acta, 1543* (2), 336–360. https://doi.org/10.1016/S0006-3002(00)00273-8
Vidya, A.S., More, V.S. & More, S.S. (2012). Isolation, purification and characterization of a novel CGTase from alkalophilic Bacillus lehensis SV1. *Biotechnology, 6* (7),226-234
Wang, H., Zhou, W., Li, H., & Bu, R. (2018). Optimization of the fermentation conditions for the mutant strain of β-cyclodextrin glycosyltransferase H167C to produce cyclodextrins. *3 Biotech, 8* (3), 165. https://doi.org/10.1007/s13205-018-1182-6
Yang, Y. N., Shan, W. X., & Wang, P. W. (2017). Upscale production of a recombinant cyclodextrin glycosyltransferase from Paenibacillus macerans in Escherichia coli. *3 Biotech, 7*(3): 207. https://doi.org/10.1007/s13205-017-0838-y
Yup, W.P., Ariffin, B.A., Woo, K.K., & Hii, L.S., (2010). Production of Cyclodextrin Glycosyltransferase (CGTase) by Bacillus lehensis S8 using Sago Starch as Carbon Source. *Journal of Biological Sciences,10*:676-681. https://scialert.net/abstract/?doi=jbs.2010.676.68
Zain, W. S. W. M., Illias, R. M., Salleh, M. M., Hassan, O., Rahman, R. A., & Hamid, A. A. (2007). Production of cyclodextrin glucanotransferase from alkalophilic Bacillus sp. TS1-1: Optimization of carbon and nitrogen concentration in the feed medium using central composite design. *Biochemical Engineering Journal, 33*(1), 26–33. https://doi:10.1016/j.bej.2006.09.024
Zhang, Y. M., Xu, X., Yu, Q., Yu, H. J., & Liu, Y. (2019). Drug Displacement Strategy for Treatment of Acute Liver Injury with Cyclodextrin-Liposome Nanassembly. *Science, 15*, 223–233. https://doi.org/10.1016/j.isci.2019.04.029