JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

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

Preetibala Solanki[1*], *Pushpendra Awadhiya*[1] *and Tushar Banerjee*[1]

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

*Corresponding author: preetisolanki2604@gmail.com

https://doi.org/10.55251/jmbfs.5130

ARTICLE INFO

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

Regular article

OPEN ACCESS

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 alkaliphilic *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; Fenyvesi 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 malto-oligosaccharides, at the same time has a weak starch hydrolyzing activity **(Tonocovo *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. stearothermophilus, Klebsiella pnemoniae, and Microbacterium terrae* **(Boniha *et al.*, 2006; Gawande and Patkar 2001; Rosso *et al.*, 2002; Kim *et al.*, 1997; Alves Prado *et al.*, 2008; Chung *et al.*, 1998 Burhan *et al.*, 2005; Rajput *et al.*, 2016)**. The most prominent CGTase producers are alkalophilic *Bacillus* sp. **(Tonocova *et al.*, 2000) [**Table1]. 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 **(Thatai *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	0.45	**Elbaz *et al.* (2015)**
Bacillus firmus strain 37	0.22	**Santos *et al.* (2013)**
Bacillus lehensis	18.9	**Yap *et al.* (2010)**
Bacillus sp. TS1	78.05	**Zain *et al.* (2006)**
Bacillus G1	54.9	**Ibrahim *et al.* (2005)**
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 (PHP) 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 28^0C, 130 rpm on a gyratory

shaker for 24 hours. 0.1ml. sample from these enrichment flasks was inoculated in modified Horikoshi screening medium plates containing 0.94 mM phenolphthalein **(Geetha *et al*., 2010)**. The plates were incubated at 28^{0}C for 48 hours. After the incubation, clear zones were developed due to the complexation of phenolphthalein inside the hydrophobic core of β-CD, due to its equivalent size with the hydrophobic core. To avoid false positives due to acid production, the plates were covered with 1N NaOH before zone size measurement. The colonies with the largest clear zone were selected for CGTase production. This clear zone was compared with known CGTase producer *Cytobacillus firmus* NCIM 5119.

Screening of α and γ-CGTase producer on the agar plate

The detection of α and γ-CGTase production by the isolated β -CD producer strain on Horikoshi screening medium containing methyl orange (0.035mM) for α-CD and bromocresol green (5mM) for γ-CD was used **(Menocci *et al*., 2008)**. Methyl orange and bromocresol green have appropriate molecular sizes to fit in the hydrophobic cavity of α and γ-CGTase, respectively **(Makela *et al*., 1990)**.

Characterization and identification of isolated microorganism

The isolate was characterized by following the steps of Bergey's manual **(1957)** of determinative bacteriology. Gram staining and biochemical identification were performed. Further identification by VITECK 2 compact system analysis (Biomerieux Diagnostics) and molecular 16s rRNA sequencing at National Chemical Laboratory (NCIM), Pune was done. The DNA sequences were deposited to NCBI GenBank through the BankIt procedure. The 16s rRNA sequence was matched with NCBI data base trough BLASTn program. The alignment of nucleotide sequence of similar sequence was done by ClustalW. The evolutionary history was inferred by the neighbor-joining method. Phylogenetic tree was constructed using MEGAX software (10.0.5). The bootstrap analysis was based on 1000 resembling.

Maintenance of Microorganism

Isolated organisms were grown on nutrient agar (NA) slants of pH 10.5 and incubated at 28^{0}C for 24 hours. After incubation and confluent growth, nutrient agar slants were maintained at 4^{0}C. Sub-culturing was done every 15 days on the same medium.

CGTase Assay

To assay CGTase, 2 ml. samples were centrifuged (10000 rpm for 20min) to obtain cell-free supernatant. The assay mixture consisted of 1% soluble starch in 50mM phosphate buffer having pH 7. The diluted (based on initial activity) enzyme (0.1ml) was added to the assay mixture and incubated at room temperature for 5 minutes. Final incubation was carried out in a water bath for 15min at 60^{0}C. The reaction was stopped by increasing the temperature to 100^{0}C for 5 minutes **(Goel and Nene, 1995)**. Control was prepared similarly without enzyme.

The amount of β-CD present in the fermentation broth (basal β-CD) was also estimated and deducted from the final readings. One unit of enzyme activity was defined as the amount of enzyme required for the production of 1 µmol β-CD per minute under standard assay conditions.

The standard curve for β-CD was plotted in the range from 20 to 240µg. β-cyclodextrin stock was prepared in 50mM Tris-HCl buffer (2mg/ml), and PHP stock (4mM) was prepared in ethanol. The working solution was prepared by diluting 0.5ml of stock in 4.5ml of Tris-HCl buffer. PHP working solution was prepared by adding 1ml of 4mM stock solution in 4ml of ethanol (95%) for 100ml of 125mM carbonate buffer (pH 10.8). Colour reduction of PHP stock may cause false detection of β-CD; thus calibration curve was drawn in every experiment.

Selection of suitable production medium for CGTase

Seven different media compositions described by **Zain *et al*. (2007); Horikoshi *et al*. (1982); Blanco *et al*. (2009); Thombre *et al.* (2013); Mahat *et al*. (2004); Ibrahim *et al.* (2004); and Ravinder *et al*. (2012)** were selected for the study [Table 2]. To prepare inoculum, a loop full of isolated organism was inoculated in nutrient broth having pH 10.5 and incubated at 30^{0}C at 130 rpm on a gyratory shaker for 24 hours. 10% inoculum was added to the production media and incubated for 24 hours at 30^{0}C, 130 rpm. Samples were drawn aseptically at 96 hours. Further parameter optimization studies were carried out only to the selected production medium.

Table 2 Production medium used for CGTase production

S.N	Contains	Medium concentrations (%)						
		A	B	C	D	E	F	G
1	Soluble Starch	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	K_2HPO_4	0.10	0.10	0.10	-	0.10	0.10	-
6	$MgSO_4$	0.02	0.02	0.01	-	0.02	0.04	0.02
7	Na_2HPO_4	-	-	-	-	-	-	0.10
8	Na_2CO_3	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) was 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., $MnSO_4$, $MgSO_4.7H_2O$, KCl, $FeSO_4$, $CaCl_2$, $CoCl_2$, $CuSO_4$. were studied at 5mM concentration.

Other important medium components like K_2HPO_4, Na_2CO_3 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 microbiological protocol using Horikoshi (PHP) screening medium [Figure 1]. The isolated bacterium was found to be Gram-positive, rod-shaped, motile, and capsulated. 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-CD production as described by **Menocci *et al*. (2008).** No clear zone was observed in both screening mediums. This result concluded that the organism was unable to form an inclusion complex with methyl orange and bromocresol green to produce α and γ-CD [Figure 1]. A similar observation was noted for *Bacillus* sp. BACAR produces only β-CD **(Menocci *et al*., 2008)**. This result confirms the findings of **Gawande and Patkar (2001)** that α and γ-CD 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 **(Abelyan *et al*., 2002)**. The isolated organism was found to predominantly produce β-CD; hence it was classified as a β-CGTase producer.

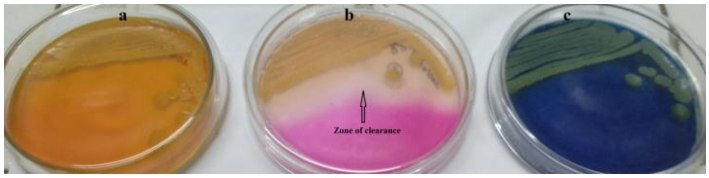

Figure 1 Isolation and screening for CGTase activities **a.** Methyl orange (0.035mM) agar plate for α-CGTase showing no reduction of orange colour by the isolated organism, **b.** PHP (0.94mM) agar plate for β-CGTase showing a reduction in pink colour around the colonies due to inclusion complex formation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

Table 3 Biochemical characterization of CGTase producer isolated from potato rhizosphere

Tests	Results[a]
Motility	Motile
Nitrate Reductase	-
Indole Synthesis	+
Urease	+
Catalase	+
Gelatine hydrolysis	-
Casein hydrolysis	-
Starch hydrolysis	+
Growth in 6.5% Nacl	+

[a] – negative; + positive

VITECK 2 compact 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).

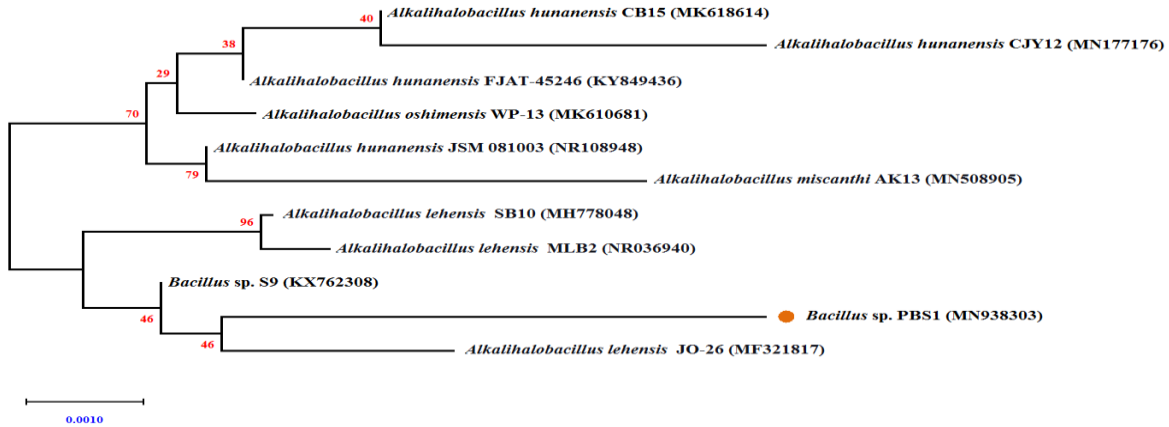

Figure 2 Neighbor-joining phylogenetic tree showing evolutionary relationship between *Bacillus* sp. PBS1 and closely related *Bacillus* species with 1000 bootstraps replicate. Numbers at nodes represent bootstrap values (%). Scale bar indicates the genetic distance of 0.001.

Table 4 VITECK 2 Compact biochemical characteristics of CGTase producer

Carbon Utilization	Result[a]	**Enzyme activities**	Result[a]
Cyclodextrin	-	Beta-xylosidase	+
D-galactose	-	L-lysine arylamidase	-
Glycogen	+	L-aspartate arylamidase	+
Myo-inositol	+	Leucine- arylamidase	+
Ellman	-	Phenylalanine arylamidase	+
Methyl-d-xyloside	-	L-proline arylamidase	-
Maltotriose	+	Beta-galactosidase	-
D-mannitol	+	L-pyrrolidinyl arylamidase	+
D-mannose	+	Alpha-galactosidase	+
D-melezitose	-	Alanine arylamidase	+
N-acetyl-d-glucosamine	+	Tyrosine arylamidase	+
Palatinose	+	Beta-n-acetyl-glucosaminidase	+
L-rhamnose	-	Ala-phe-pro arylamidase	+
Pyruvate	+	Methyl-A-D-glucopyranosidase acidification	-
D-tagatose	-	Alpha-mannosidase	-
D-trehalose	+	Glycine acylamidase	+
Inulin	+	Beta-glucosidase	+
D-glucose	+	Beta-mannosidase	-
D-ribose	+	Phosphoryl choline	+
Putresicin assimilation	-	Alpha-glucosidase	-
Esculin hydrolysis	+		
Antibiotic Resistance			
Kanamycin resistance	-		
Oleandomycin resistance	+		
Polymixin B resistance	+		

[a] – 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 5 ± 074 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>G. 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.

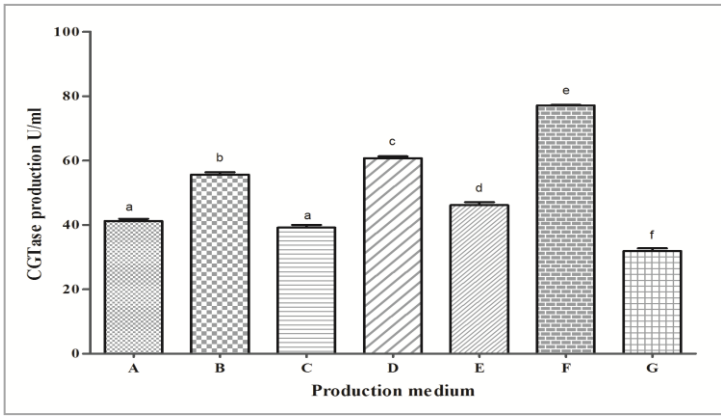

Figure 3 Effect of different fermentation mediums for CGTase production by isolated *Bacillus sp.* PBS1. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

Comparison with a known CGTase producer

The 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.

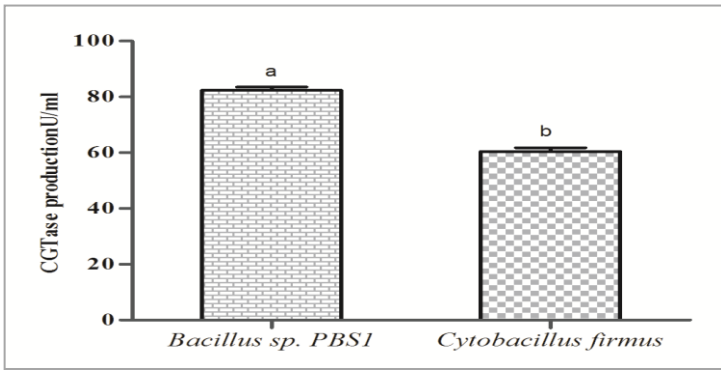

Figure 4 Comparison of CGTase production between *Cytobacillus firmus* NCIM 5119 and *Bacillus* sp. PBS1 in F medium P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

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 of **Letsididi *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 utilizable carbon source suppresses the ability of an organism to catabolize starch. **Tonocova (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.

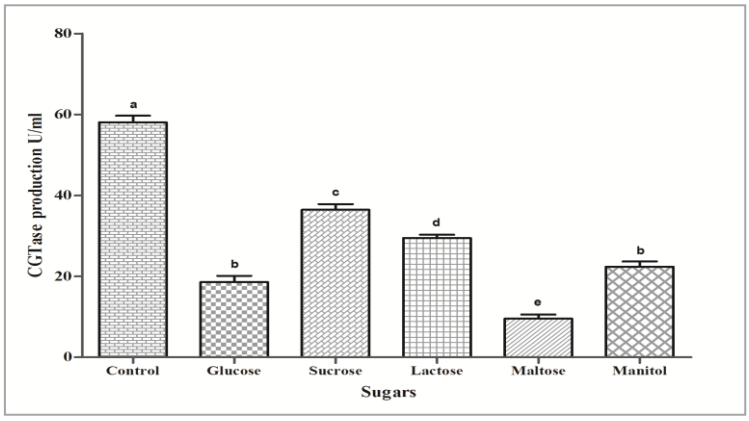

Figure 5 Effect of supplementation of different sugars on CGTase production by *Bacillus* sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

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* KNR9 **(Rajput *et al.*, 2016)**, *Bacillus lehensis* S8 **(Vidya *et al.*, 2012)**, and *Bacillus* G1 **(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.

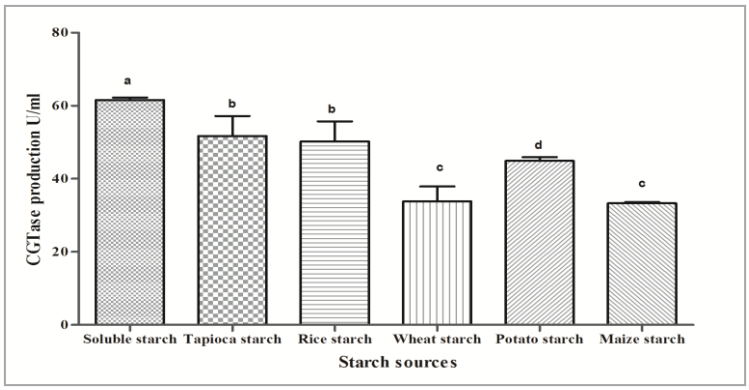

Figure 6 Effect of various starches on CGTase production by isolated *Bacillus* sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

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 NH_4^+, NO_3^-, 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* DF 9R 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.

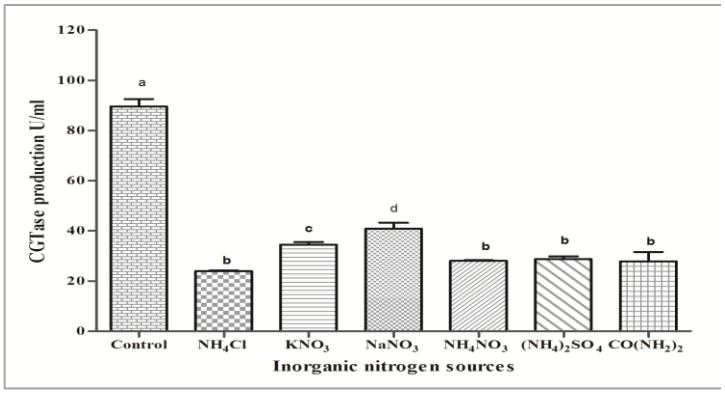

Figure 7 Effect of different inorganic nitrogen sources on CGTase production by *Bacillus* sp. PBS1 in medium F, peptone considered as control. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

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 one 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).

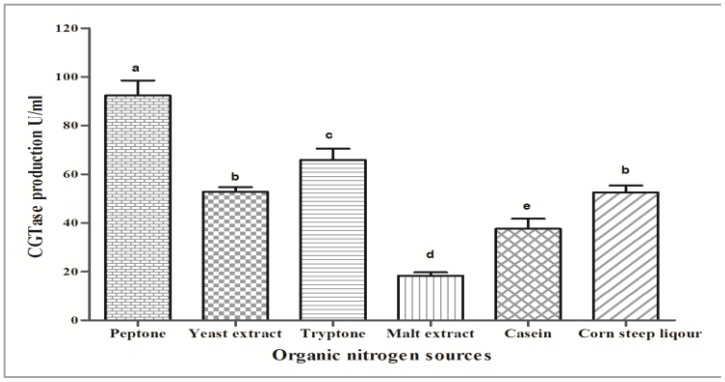

Figure 8 Effect of various organic nitrogen sources on CGTase production by *Bacillus* sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

Effect of metal salts

To observe the effect of metal ions 5mM concentration, $MnSO_4$, $FeSO_4$, $CuSO_4$, $CoCl_2$, $CaCl_2$, and KCl were added in medium F. 1.62 mM $MgSO_4$ being an ingredient in medium F was treated as control. In the presence of $MgSO_4$ (control), 88.03±4.38 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 $MnSO_4$< KCl< $FeSO_4$ < $CaCl_2$ < $CoCl_2$ < $CuSO_4$. However, no significant difference between $MnSO_4$, KCl, $FeSO_4$ was found. Several studies revealed that Mg^{2+} 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 Ca^{2+} is helpful for active enzyme conformation and stabilizing thermal stability. However, in our study, Ca^{2+} was found to inhibit enzyme production slightly. **Rosso *et al*. (2002); Yang *et al*. (2017)** also reported that Ca^{2+} has no positive effect on CGTase production.

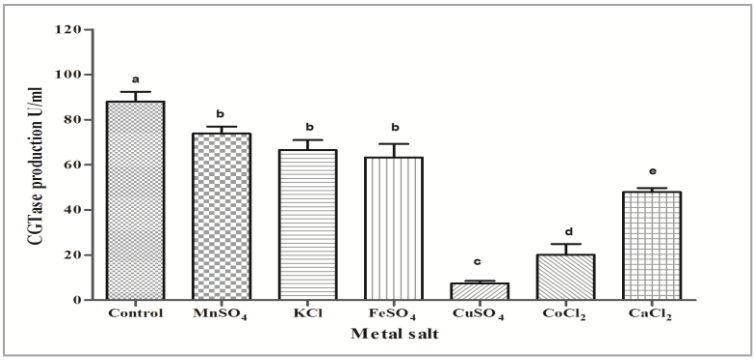

Figure 9 Effect of various metal salts on CGTase production by *Bacillus* sp. PBS1 in medium F. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

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.

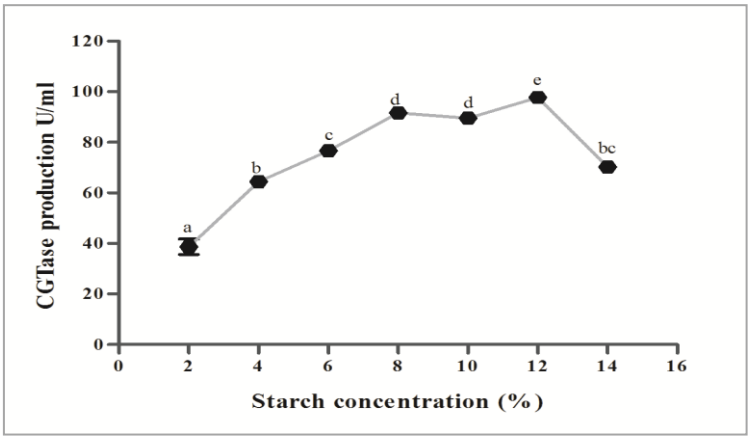

Figure 10 Effect of various concentrations of soluble starch on CGTase production. Symbols having the same alphabets are not significantly different from each other P < 0.05. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

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)**.

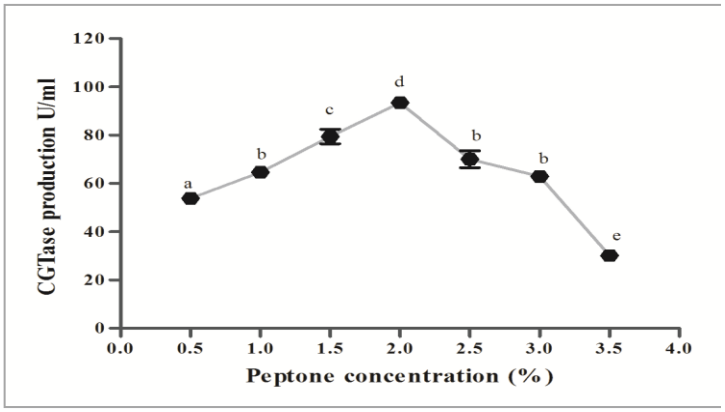

Figure 11 Effect of various concentrations of peptone on CGTase production. Symbols having the same alphabets are not significantly different from each other $P < 0.05$. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

Effect of dipotassium hydrogen phosphate concentration variation

No positive effect of K_2HPO_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_2HPO_4 concentration led to a dose-dependent decline in CGTase production [Figure 12]. At 2.5% concentration of K_2HPO_4, 62% reduction was observed as compared to control. Control which was devoid of K_2HPO_4 showed maximum CGTase production (82.17 ± 2.34 U/ml). **Mahat *et al.* (2004)** also reported that K_2HPO_4 had no significant effect on CGTase production.
These results differ from **Wang *et al.* (2018)**, where increased production (3230 U/ml mutant strain of β-CGTase H163C) was observed when K_2HPO_4 was used. Likewise, K_2HPO_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_2HPO_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_2HPO_4 favours good CGTase production.

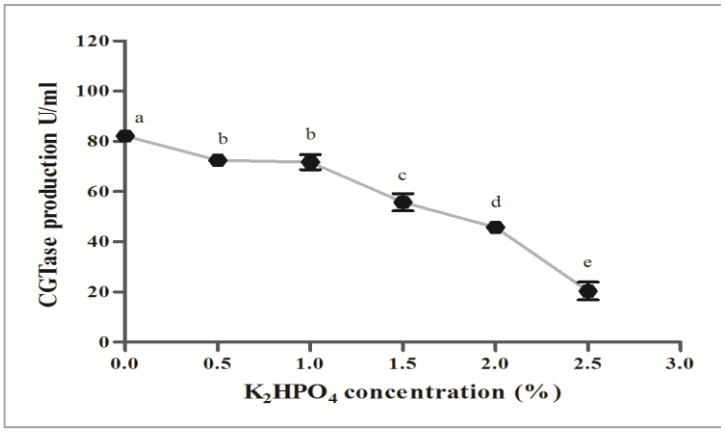

Figure 12 Effect of different concentrations of dipotassium hydrogen phosphate on CGTase production. Symbols having the same alphabets are not significantly different from each other $P < 0.05$. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

Effect of 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 to17.48 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.

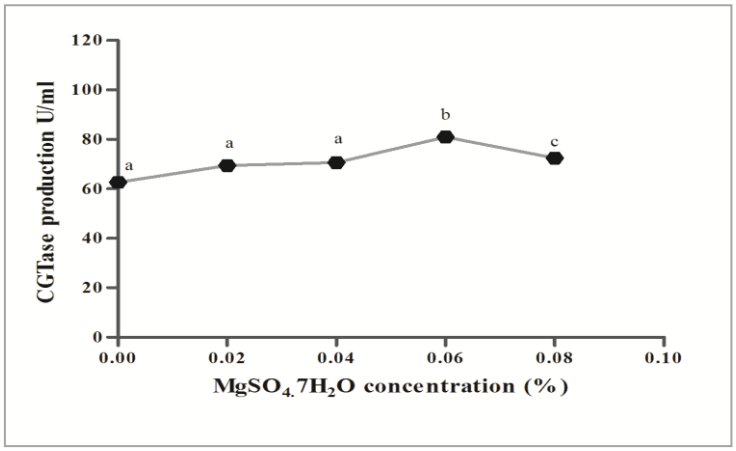

Figure 13 Effect of magnesium sulfate heptahydrate concentration on CGTase production. Symbols having the same alphabets are not significantly different from each other $P < 0.05$. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

Effect of sodium carbonate concentration

Na_2CO_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_2CO_3. Na_2CO_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 having a pH of 9.3 ± 0.1 showed the highest production of 82.32U/ml [Figure 14]. Comparatively low enzyme production (zero concentration) was observed without Na_2CO_3, possibly due to the neutral pH of the medium. Further increase in Na_2CO_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_2CO_3 concentration.

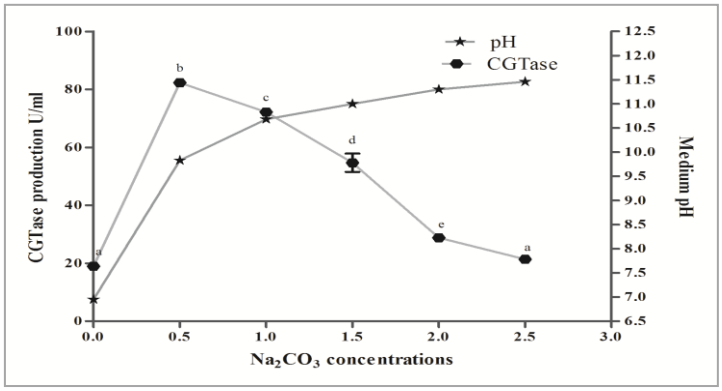

Figure 14 Effect of various concentrations of sodium carbonate and respective pH on CGTase production. Symbols having the same alphabets are not significantly different from each other $P < 0.05$. Standard deviation (Mean ± SD, n = 3) is represented by the bars.

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. F117.1/201415/RGNF201415STMAD69908/ (SAIII/Website) for a Senior Research Fellowship (SRF) to Preetibala Solanki is gratefully acknowledged.

REFERENCES

Abelyan, V. A., Balayan, A. M., Ghochikyan, V. T., & Markosyan, A. A. (2004). Transglycosylation of Stevioside by Cyclodextrin Glucanotransferases of Various Groups of Microorganisms. *Applied Biochemistry and Microbiology*, 40(2), 129–134. https://doi:10.1023/b:abim.0000018914.08571.50

Ahmed, E. M., & El-Refai, H. A. (2010). Cyclodextrin glucosyltransferase production by Bacillus megaterium NCR: evaluation and optimization of culture conditions using factorial design. I*ndian Journal of Microbiology*, 50(3), 303–308. https://doi:10.1007/s12088-010-0009-x

Alves-Prado, H. F., Carneiro, A. A., Pavezzi, F. C., Gomes, E., Boscolo, M., Franco, C. M., & da Silva, R. (2008). 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/s12010-007-8093-z

Arce-Vázquez, M. B., Ponce-Alquicira, E., Delgado-Fornué, E., Pedroza-Islas, R., Díaz-Godínez, G., & Soriano-Santos, J. (2016). Integral Use of Amaranth Starch to Obtain Cyclodextrin Glycosyltransferase, by *Bacillus megaterium*, to Produce β-Cyclodextrin. *Frontiers in microbiology*, *7*, 1513. https://doi.org/10.3389/fmicb.2016.01513

Bergey, D.H., Robert, S.B., (1957). Bergey's Manual of Determinative Bacteriology. *Williams & Wilkins Co: Baltimore*. https://doi.org/10.5962/bhl.title.10728

Bezerra, F. M., Lis, M. J., Firmino, H. B., Dias da Silva, J. G., Curto Valle, R. de C. S., Borges Valle, J. A., & Tessaro, A. L. (2020). The Role of β-Cyclodextrin in the Textile Industry. *Review Molecules*, 25(16), 3624. https://doi:10.3390/molecules25163624

Blanco, K. C., De Lima, C. J. B., De Oliveir, P. A. P. L. V., Piao, A. C. S., & Contiero, J. (2009). Cyclodextrin Glycosyltransferase Production by the Bacillus sp., Subgroup alcalophilus using a Central Composite Design. *Research Journal of Microbiology*, 4(11), 450–459. https://doi:10.3923/jm.2009.450.459

Bonilha, P. R. M., Menocci, V., Goulart, A. J., Polizeli, M. de L. T. de M., & Monti, R. (2006). Cyclodextrin glycosyltransferase from Bacillus licheniformis: optimization of production and its properties. *Brazilian Journal of Microbiology*, 37(3), 317–323. https://doi:10.1590/s1517-83822006000300022

Burhan, N., Sapundzhiev, T. and Beschkov, V. (2005) Mathematical Modelling of Cyclodextrin-Glucanotransferase Production by Batch Cultivation. *Biochemical Engineering Journal*, 24, 73-77. https://doi.org/10.1016/j.bej.2005.02.007

Buschmann, H. J., & Schollmeyer, E. (2002). Application of cyclodextrins in cosmetic products: A review. *Journal of cosmetic science*, 53(3), 185–191. https://pubmed.ncbi.nlm.nih.gov/12053209/

Chung, H.J., Yoon, S.H., Lee, M.J., Kim, M.J., Kweon, K.S., Lee, W., Wan Kim J.W, Oh, B.A, Lee H.S., Spiridonova V.A., & Park K.H. (1998). Characterization of a Thermostable Cyclodextrin Glucanotransferase Isolated from Bacillus stearothermophilus ET1. *Journal of Agricultural and Food Chemistry* 46 (3), 952−95 https://doi.org/10.1021/jf970707d

Dalmotra, N., Tripathi, A. D., Srivastava, S. K., Arya, S. K., & Naik, B. (2016). Statistical optimization of cyclodextrin glycosyltransferase (CGTase) production from Bacillus macerans in batch cultivation and its purification. *International Journal of Food and Fermentation Technology*, 6(2), 261. https://doi:10.5958/2277-9396.2016.00049.0

Elbaz, A. F., Sobhi, A., & ElMekawy, A. (2014). Purification and characterization of cyclodextrin β-glucanotransferase from novel alkalophilic bacilli. *Bioprocess and Biosystems Engineering*, 38(4), 767–776. https://doi:10.1007/s00449-014-1318-y

Fenyvesi, Eva. (2011) 25: 1-7. *CycloLab R&D Laboratory Ltd*., Hungary.

Gawande, B. N., & Patkar, A. Y. (2001). Purification and properties of a novel raw starch degrading-cyclodextrin glycosyltransferase from Klebsiella pneumoniae AS- 22. *Enzyme and Microbial Technology*, 28(9-10), 735–743. https://doi:10.1016/s0141-0229(01)00347-7

Gawande, B. N., & Patkar, A. Y. (1999). Application of factorial designs for optimization of cyclodextrin glycosyltransferase production from Klebsiella pneumoniae pneumoniae AS-22. *Biotechnology and bioengineering*, *64*(2), 168–173. https://doi.org/10.1002/(SICI)1097-0290(19990720)64:2%3C168::AID-BIT5%3E3.0.CO;2-5

Gawande, B. N., Singh, R. K., Chauhan, A. K., Goel, A., & Patkar, A. Y. (1998). Optimization of cyclomaltodextrin glucanotransferase production from Bacillus firmus. Enzyme and Microbial Technology, 22(4), 288–291. https://doi:10.1016/s0141-0229(97)00184-1

Geetha, R., & More, S. (2010). Isolation and Characterization of Cyclodextrin Glucanotransferase from Soil Bacterium. *Research Journal of Biological Sciences*, 5(10), 699–707. https://doi:10.3923/rjbsci.2010.699.707

Goel, A., & Nene, S. N. (1995). Modifications in the Phenolphthalein Method for Spectrophotometric Estimation of Beta Cyclodextrin. *Starch - Stärke*, 47(10), 399–400. https://doi:10.1002/star.19950471006

Higuti, H.I., Silva, P.A., Papp J., Okiyama, V.M., Andrade, E. A., Marcondes, A.A., & Nascimento A.J. (2004). Colorimetric Determination of α and β-cyclodextrins and studies on Optimization of CGTase Production from *B. firmus* Using Factorial Designs. *Brazilian archives of biology and technology*, 47 (6), 837-841. https://doi.org/10.11590/S01516-89132004000600001

Horikoshi K., Nakamura N., Matsuzawa N., & Yamamoto M. (1982) Industrial Production of Cyclodextrins. In: Szejtli J. (eds) Proceedings of the First International Symposium on Cyclodextrins. *Advances in Inclusion Science*, vol 1. Springer, Dordrecht. https://doi.org/10.1007/978-94-009-7855-3_3

Ibrahim, H. M., Yusoff, W. M. W., Hamid, A. A., & Omar, O. (2010). Enhancement of Cyclodextrin Glucanotransferase Production by Bacillus G1 using Different Fermentation Modes. *Biotechnology* 9(4), 506–512. https://doi:10.3923/biotech.2010.506.512

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(2), 753–758. https://doi: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(3), 163–176. https://doi:10.1007/bf02916450

Kamble, R., and Gupte, A. (2014). Cyclodextrin glycosyltransferase production by alkaliphilic *bacillus sp.* isolated from rice cultivated soil and media optimization using taguchi method. *International Journal of pharmaceutical sciences and Research*, 7 (5), 2754-2762. http://dx.doi.org/10.13040/

Kelly, R. M., Dijkhuizen, L., & Leemhuis, H. (2009). The evolution of cyclodextrin glucanotransferase product specificity. *Applied microbiology and biotechnology*, *84* (1), 119–133. https://doi.org/10.1007/s00253-009-1988-6

Kim, M.H., Sohn C.B., Lim, Y.H., Oh, T.K., (1997). Purification and characterization of cyclodextrin glycosyltransferase from *Bacillus brevis* CD162. *Agricultural Chemistry and Biotechnology*, 40 (6), 465-471.

Letsididi, R., Sun, T., Mu, W., Kessy, N. H., Djakpo, O., & Jiang, B. (2011). Production of a Thermoactive B-cyclodextrin Glycosyltransferase with a High Starch Hydrolytic Activity from an Alkalitolerant Bacillus Licheniformis Sk 13.002 Strain. *Asian Journal of Biotechnology*, 3 (3), 214–225. https://doi:10.3923/ajbkr.2011.214.225

Mahat, M. K., Illias, R. M., Rahman, R. A., Rashid, N. A. A., Mahmood, N. A. N., Hassan, O., & Kamaruddin, K. (2004). Production of cyclodextrin glucanotransferase (CGTase) from alkalophilic Bacillus sp. TS1-1: media optimization using experimental design. *Enzyme and Microbial Technology*, 35(5), 467–473. https://doi:10.1016/j.enzmictec.2004.07.008

Mäkelä, M. J., Paavilainen, S. K., & Korpela, T. K. (1990). Growth dynamics of cyclomaltodextrin glucanotransferase producing *Bacillus circulans* var. *alkalophilus. Canadian Journal of Microbiology* 36, 176–182. https://doi: 10.1139/m90-031

Maskooki, A. M., Beheshti, S. H. R., Valibeigi, S., & Feizi, J. (2013). Effect of Cholesterol Removal Processing Using β-Cyclodextrin on Main Components of Milk. *International Journal of Food Science*, 2013,1–6. https://doi:10.1155/2013/215305

Menocci, V., Goulart, A. J., Adalberto, P. R., Tavano, O. L., Marques, D. P., Contiero, J., & Monti, R. (2008). Cyclodextrin glycosyltransferase production by new Bacillus sp. strains isolated from brazilian soil. *Brazilian Journal of Microbiology*. 39 (4), 682–688. https://doi.org/10.1590/S1517-83822008000400016

More, S.S., Niraja, R., Chris, E. C., Byadgi, A.M., Shwetha, V. & Mangaraj S.D. (2012). Isolation, Purification and Biochemical Characterization of CGTase from *Bacillus halodurans. Croatian Journal of Food Technology Biotechnology and Nutrition* 7, 90-97. https://hrcak.srce.hr/84932

Moriwaki, C., Costa, G. L., Pazzetto, R., Zanin, G. M., Moraes, F. F., Portilho, M., & Matioli, G. (2007). Production and characterization of a new cyclodextrin glycosyltransferase from Bacillus firmus isolated from Brazilian soil. *Process Biochemistry*, 42(10), 1384–1390. https://doi:10.1016/j.procbio.2007.07.007

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

Rajput, K. N., Patel, K. C., & Trivedi, U. B. (2016). A novel cyclodextrin glucanotransferase from an alkaliphile Microbacterium terrae KNR 9: purification and properties. *3 Biotech*, *6*(2), 168. https://doi.org/10.1007/s13205-016-0495-6

Rajput, K. N., Patel, K. C., & Trivedi, U. B. (2016). Screening and Selection of Medium Components for Cyclodextrin Glucanotransferase Production by New Alkaliphile Microbacterium terrae KNR 9 Using Plackett-Burman Design. *Biotechnology Research International*, 2016, 1–7. https://doi:10.1155/2016/3584807

Rakmai, J., & Cheirsilp, B. (2016). Continuous production of β-cyclodextrin by cyclodextrin glycosyltransferase immobilized in mixed gel beads: Comparative study in continuous stirred tank reactor and packed bed reactor. *Biochemical Engineering Journal*, 105, 107–113. https://doi:10.1016/j.bej.2015.09.011

Ramli, N., Abd-Aziz, S., Hassan, M. A., Alitheen, N., & Kamaruddin, K. (2010). Potential cyclodextrin glycosyltransferase producer from locally isolated bacteria. *African Journal of Biotechnology*, *9* (43), 7317-7321. https://doi.org/10.5897/AJB10.081

Ravinder, K., Prabhakar, T., & Bhavanidevi, R. (2012). Optimization of process parameters for the production of cyclodextrin glycosyltransferase by newly

isolated bacillus sp. Tpr71h 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*, *1*(1), 3. https://doi.org/10.1186/1475-2859-1-3
Sabioni, J.G., Park Y.K. (1992). Production and characterization of cyclodextrin glucanotrasferase from *Bacillus lentus*. *Starch* 44 (6) 225–229 https://doi.org/10.1002/star.19920440607
Silva, L., Matioli, G., Zanin, 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
Terada, Y., Yanase, M., Takata H., Takaha, T., & Okada S. (1997) Cyclodextrins are not the major cyclic alpha-1,4-glucans produced by the initial action of cyclodextrin glucanotransferase on amylose. *Journal Biological Chemistry*, 272 (25) 15729–15733. https://doi.org/10.1074/jbc.272.25.15729
Thatai, A., Kumar, M., & Mukherjee, K. J. (1999). A single step purification process for cyclodextrin glucanotransferase from a Bacillus sp. isolated from soil. *Preparative biochemistry & biotechnology*, *29*(1), 35–47. https://doi.org/10.1080/10826069908544691
Thombre, R.S, Kanekar, P.P. & Rajwade, J.M. (2013) Synthesis of β-cyclodextrin by CGTase produced by Bacillus lichenoformis MCM-B-1010. *International Journal of Pharma and Bio Sciences* 4 (1) 515 – 523.
Tonkova, A. (1998). Bacterial cyclodextringlucotransferase. *Enzyme Microbia 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/s0167-4838(00)00233-8
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
Yap, W.P., Ariff, 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 Nanoassembly. *iScience*, *15*, 223–233. https://doi.org/10.1016/j.isci.2019.04.029