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EXPLORING THE POTENTIAL OF SHOUCHELLA OSHIMENSIS P-106 FOR CYCLODEXTRIN GLUCANOTRANSFERASE PRODUCTION

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Abstract

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an enzyme that utilizes starch by promoting an intramolecular transglycosylation reaction to produce cyclodextrins. An alkaliphilic, spore-forming, Gram-positive, potential CGTase producing bacteria was isolated from maize field and was identified as Shouchella

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oshimensis P-106 by 16S rDNA sequencing. The isolate P-106 efficiently produced 1.37 U/ml of β-CGTase as a major product and showed little existence of α- and γ-CGTase production. Optimization by one-factor-at-a-time (OFAT) approach significantly enhanced the β-CGTase production to 2.31 U/ml showed 1.68-fold increase.

Keywords: *Starch, CGTase, cyclodextrin, Shouchella oshimensis P-106, one-factor-at-a-time*

INTRODUCTION

Cyclodextrin glycosyltransferases commonly known as CGTases (EC 2.4.1.19) signify one of the most useful families of microbial amylolytic enzymes with a broad spectrum of activities to cyclize, disproportionate, hydrolyze, and couple substrates. CGTases play their main role in the industrial production of cyclodextrins (CDs) by the conversion of starch and related oligo- and polysaccharides by intramolecular transglycosylation (cyclization). Cyclodextrins exist in three fundamental structural forms that differ based on the number of glucose units bound by α-1,4 glycosidic linkages: α (cyclohexamilose cG6), β (cycloheptamilose cG7), and γ (cyclooctamilose cG8). The main difference between these types is the variance in the volume of their apolar cavities and their hydrophilicity (Pardhi et al., 2023).

The CGTase producing microorganisms mostly produce a fusion of α-, β-, and γ-CDs in different proportions depending on the reaction environments. Generally, β-CD covers about 80% of this mixture of CDs produced by microbial sources as a major product (Rabadiya et al., 2024). Literature revealed *Bacillus* species as the foremost supplier of CGTase, and the involvement of alkaliphilic *Bacillus* spp. shown accountable production of CGTase at industrial stage. Some other species such as *Paenibacillus*, *Klebsiella*, *Microbacterium*, *Brevibacterium*, *Micrococcus*, and *Thermoactinomyces* has been introduced in recent years as a potential CGTase producers (Pardhi et al., 2023).

The CD production using a microbial approach can be economic by utilizing an agro-industrial wastes as a substrate. Different types of starch viz., soluble, potato, corn, sago, tapioca, and rice starch may be used as a substrate for CGTase production (Kaur *et al*., 2014). Present study deals with the isolation, screening, and optimization of CGTase production by *Shouchella oshimensis* P-106.

MATERIALS AND METHODS

2.1. Materials

Soluble starch and α/β/γ-cyclodextrins were procured from ThermoFisher Scientific (India) and CycloLab (Hungary). The additional chemicals and reagents used were analytical grade (Himedia Pvt. Ltd., India),

2.2. Isolation and identification of bacteria

The CGTase producers are selectively obtained using Horikoshi agar medium supplemented with phenolphthalein (0.03%) and methyl orange (0.01%). The composition of Horikoshi medium is (w/v) soluble starch (1.0%), yeast extract (0.5%), peptone (0.5%), K_2HPO_4 (0.1%), and $MgSO_4 \cdot 7H_2O$ (0.02%). Sterile 1.0% Na_2CO_3 was used to adjust the media pH to 10.5 ± 0.2. Next, the soil samples collected from different farms (paddy, maize, potato, millet, sorghum) of Dadra & Nagar Haveli, Ahmedabad, and Mumbai were serially diluted and spread on the Horikoshi agar medium. The plates were observed for bacterial isolate showing yellow zone of clearance around the colony after incubation at 37 °C for 72-96 hrs (Rajput et al., 2016). The colony showing utmost yellow zone was selected as potential CGTase producer and maintained by sub-culturing. Colonial and morphological characterisation, along with motility and biochemical tests (using VITEK 2 system; Neuberg Supratech, India), were performed for the selected isolate. Additionally, the selected bacterial isolate was identified by 16S rDNA sequencing (Eurofins Scientific, India), and a phylogenetic analysis was carried out through BLAST, NCBI GenBank.

2.3. Screening

2.3.1. Primary screening

The plate assay was carried out for primary screening of *Shouchella oshimensis* P-106 CGTase to produce various cyclodextrins by spot inoculation on Horikoshi agar medium supplemented with respective indicator dye i.e., α-CGTase (0.03% (w/v) methyl orange, pH 7.0), β-CGTase (0.03% phenolphthalein, 0.01% methyl orange, pH 10.5), and γ-CGTase (0.001% (w/v) Xylene cyanole FF, 0.01% (w/v) Congo red, pH 10.5); incubated at 37 °C for 72 hrs (Higuti et al., 2004; Rajput et al., 2016; Matioli et al., 1996). The plates were regularly observed for the zone of clearance around the colony and the ratio of colony:zone of clearance was measured.

2.3.2. Secondary screening

Shouchella oshimensis P-106 was further screened to determine the production of α-, β-, and γ-CGTase using shake flask method. Fifty millilitre Horikoshi basal media (pH >10.5) was inoculated with 2.0% (v/v) *S. oshimensis* P-106 and incubated at 37 °C, 130 rpm. Cell-free supernatant was collected at 24-hour intervals up to

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96 hours by centrifugation at 10,000 rpm (4 °C) for 10 min and used as a crude enzyme for CGTase assay. One unit of CGTase is the amount required to produce one µmole of cyclodextrin per minute at standard conditions.

α-CGTase. The α-CGTase assay was conducted according to Higuti et al. (2004) with slight modifications. 0.1 ml of diluted crude enzyme was incubated with 1.0 ml of 1% (w/v) soluble starch prepared in 50 mM potassium phosphate buffer (pH 7.0) at 40 °C for 10 min. The reaction was stopped by immediate cooling in an ice bath and the addition of 0.1 ml of 1N HCl. Then 1.0 ml of 0.035 mM methyl orange solution was added and tubes vortexed for 1 min. Tubes were further incubated at 15 °C for 30 min, then absorbance was measured at 507 nm. α-CD amount was calculated from 0-1000 µg/ml α-CD standard curve.

β-CGTase. The β-CGTase activity was determined by the phenolphthalein assay of Goel and Nene (1995) with slight modifications. 0.1 ml of diluted crude enzyme was incubated with 1.0 ml of 1% (w/v) soluble starch in 50 mM potassium phosphate buffer (pH 7.0) at 60 °C for 20 min. The reaction was stopped by immediate cooling in an ice bath. Lastly, 4.0 ml of working phenolphthalein solution (1.0 ml of 4 mM phenolphthalein in 125 mM Na_2CO_3 containing 4% (v/v) methanol) was added and vortexed for 1 min. Its absorbance was recorded at 550 nm. The β-CD content was determined from a 0-100 µg/ml β-CD standard curve.

γ-CGTase. The γ-CGTase assay was performed according to Wang et al. (2004) with some modifications. 0.1 ml of diluted crude enzyme was incubated with 1.0 ml of 1% (w/v) soluble starch in 50 mM potassium phosphate buffer (pH 6.0) at 40 °C for 10 min. The reaction was terminated by ice bath cooling. Then, 0.1 ml of bromocresol green solution (0.5 mM in 20% ethanol) was added and vortexed for 1 min. The mixture was allowed to stand at room temperature for 20 min. Subsequently, 2.0 ml of citric acid buffer (50 mM, pH 4.0) was added, and absorbance was recorded at 630 nm after stirring for 1 min. The amount of γ-CD was determined from a calibration curve of 0-1000 µg/ml γ-CD.

2.4. Optimization by One-Factor-at-a-Time

The physical and chemical parameters were tested for optimizing the CGTase production by *Shouchella oshimensis* P-106, the response was measured as mentioned in 2.3.2.

Shake flask culture. One loopful of *S. oshimensis* P-106 was inoculated in sterile Horikoshi basal medium and cultured on an orbital shaker at 37 °C for 16-24 hrs. On the next day, the absorbance of culture was measured at 600 nm to reach OD 1.0, which was considered as an inoculum (%, v/v). The freshly prepared inoculum was inoculated in 50 ml of the media taken in a 250 ml Erlenmeyer flask and incubated at 37 °C for 72-96 hrs on an orbital shaker, 150 rpm. Each optimal factor was incorporated in the subsequent experiment.

2.2.1. Physical factors

pH effect. To check the ideal pH for CGTase production, Horikoshi basal media was initially set with different pH i.e., 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0 using 1% (w/v) sterile Na_2CO_3.

Effect of inoculum size. To find out the optimal inoculum size to produce CGTase, 2%, 4%, 6%, 8%, and 10% (v/v) inoculum were checked.

Effect of temperature. To determine the optimal temperature for the CGTase production, 25, 30, 37, and 45 °C were used.

Effect of incubation time. To maximize harvesting time of CGTase production, the crude enzyme was collected after every 24-hr interval up to 96 hrs.

2.5.2. Chemical factors

Effect of Carbon Source. The effect of different carbon sources i.e., soluble starch, potato starch, maize starch, sago starch, rice flour, wheat flour, refined wheat flour, millet flour, water chestnut flour, and sorghum flour (purchased from local rice mill) on CGTase production was measured.

Effect of carbon source concentration. The optimized carbon source was further checked for its optimum concentrations supporting maximum CGTase production with a range of 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5% (w/v).

Effect of nitrogen sources. To evaluate the effect of nitrogen sources on CGTase production, both organic (1.0%; w/v) i.e., peptone + yeast extract, yeast extract, peptone, soybean chunk crush, casein, groundnut crush, and inorganic nitrogen (0.1%; w/v) i.e., sodium nitrate, ammonium sulfate, potassium nitrate, urea) sources were checked.

Effect of minerals. The effect of different minerals ions like $MgSO_4$, Na_2SO_4, K_2SO_4, $MnSO_4$, $FeSO_4$, $CuSO_4$, $ZnSO_4$, NaCl, $CaCl_2$, and $MgCl_2$ were assessed for CGTase production.

Effect of phosphorous. To measure the effect of phosphorous on CGTase production, different phosphorous sources i.e., K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, and $(NH_4)_2PO_4$ were used with 0.1% (w/v).

RESULTS AND DISCUSSION

The CGTase producing alkaliphilic bacteria P-106 was isolated from soil sample of maize field from Dadra & Nagar Haveli, India on Horikoshi agar medium. The P-106 produced large, round, slightly convex, regular margin, smooth, opaque, motile, and off-white colonies on Horikoshi agar (Fig. 1a). The isolate P-106 was found Gram positive, long rod-shaped bacteria present singly and in chains (Fig. 1b) with spore forming ability (Fig. 1c).

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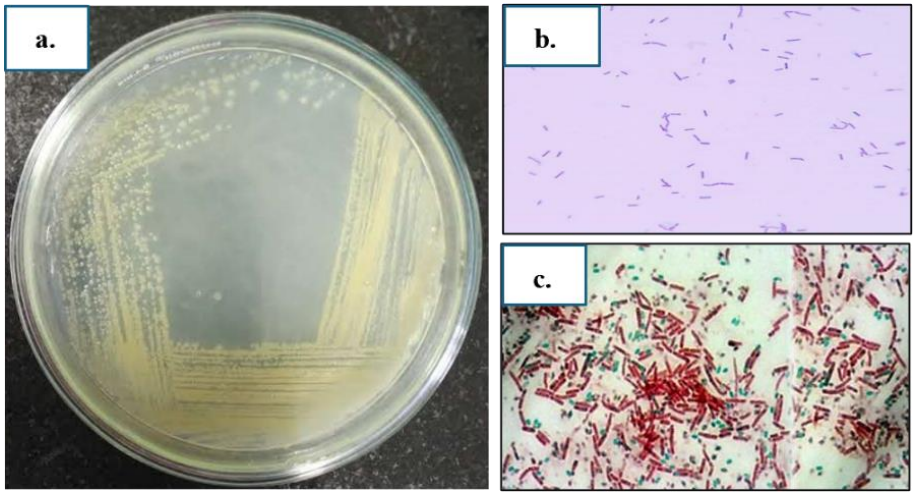

Figure 1. Colonial and morphological characteristics of P-106: **a.** growth on Horikoshi agar medium, **b.** Gram staining, and **c.** spore staining

The biochemical tests of P-106 with the VITEK 2 system are summarized in Table 1. The P-106 can produce enzymes like tyrosine arylamidase, α-galactosidase, α-mannosidase, gelatinase, catalase, oxidase, and lipase that support diverse biological processes and industrial applications. The isolate uses D-glucose, D-mannitol, D-galactose, D-lactose, and D-mannose (Table 1) as substrates for fermentation, which makes it useful in dairy farms. P-106 has potential applications in food processing, and more studies need to be conducted to enhance its impact on biotechnology practice.

Table 1. Biochemical tests of P-106.

No.	Enzyme Production	Response	No.	Sugar Fermentation	Response
1.	β-Xylosidase	(-)	31.	Methyl-D-Xyloside	-
2.	β-Galactosidase	-	32.	Maltotriose	-
3.	Ala-Phe-Pro-Arylamidase	(-)	33.	D-Ribose	-
4.	β-Mannosidase	-	34.	Melezitose	-
5.	L-Lysine Arylamidase	-	35.	Palatinose	-
6.	L-Aspartate Arylamidase	-	36.	L-Rhamnose	-
7.	Leucine Arylamidase	-	37.	D-Tagatose	-
8.	L-Phenylalanine Arylamidase	-	38.	D-Trehalose	-
9.	L-Proline Arylamidase	-	39.	D-Glucose	+
10.	L-Pyrrolydonyl Arylamidase	-	40.	D-Galactose	+
11.	Alanine Arylamidase	-	41.	D-Mannitol	+
12.	Tyrosine Arylamidase	(+)	42.	D-Mannose	+
13.	β-N-Acetyl-Glucosaminidase	-	43.	D-Lactose	+
14.	α-Galactosidase	+	44.	Glycogen	-
15.	α-Mannosidase	+	45.	Inositol	-
16.	β-N-Acetyl-Galactosaminidase	-	46.	Methyl-A-D-Glucopyranoside	-
17.	Glycine Arylamidase	-	**Antibiotic Resistance**		
18.	β-Glucosidase	(-)	47.	Kanamycin	-
19.	α-Glucosidase	-	48.	Polymixin B	-
20.	Amylase	+	49.	Oleandomycin	-
21.	Catalase	+	**Other components**		
22.	Oxidase	+	50.	Cyclodextrin	+
23.	Gelatinase	+	51.	Ellman	-
24.	Lipase	+	52.	Phosphoryl choline	-
IMViC Test			53.	Pyruvate	-
25.	Indole Production	-	54.	Inulin	-
26.	Methyl Red	-	55.	Putrescine	-
27.	Voges-Proskauer	+	56.	Growth in 6.5% NaCl	+
28.	Citrate Utilization	+	57.	Esculin hydrolysis	-
29.	H_2S Production	-			
30.	Triple Sugar Iron Agar	-			

Note: The reaction results that are shown in parentheses "(–)" or "(+)" indicate weak reactions that are near the threshold values.

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The bacterial isolate P-106 was characterized by 16S rDNA sequencing. The sequence had 100% similarity with *Shouchella oshimensis* strain K11 (Accession no. AB188090) in the NCBI GenBank database. Therefore, 16S rDNA sequence of P-106 is submitted to NCBI and was designated as *Shouchella oshimensis* P-106 (Accession No. PV291702). The *S. oshimensis* P-106's phylogenetic relationship with other strains is illustrated in Fig. 2. *S. oshimensis* P-106 is closely related to *Bacillus lehensis* but carries a unique metabolic pathway. To best of our knowledge, this is the first report of CGTase production by *S. oshimensis* P-106, other reported CGTase producers involves *B. lehensis* (Elbaz et al. 2015), *Bacillus* sp. T1 (Liu et al. 2022), *B. cereus* SS2 (Kulshreshtha et al. 2020), *B. subtilis* WB800 (Crozatti et al. 2023).

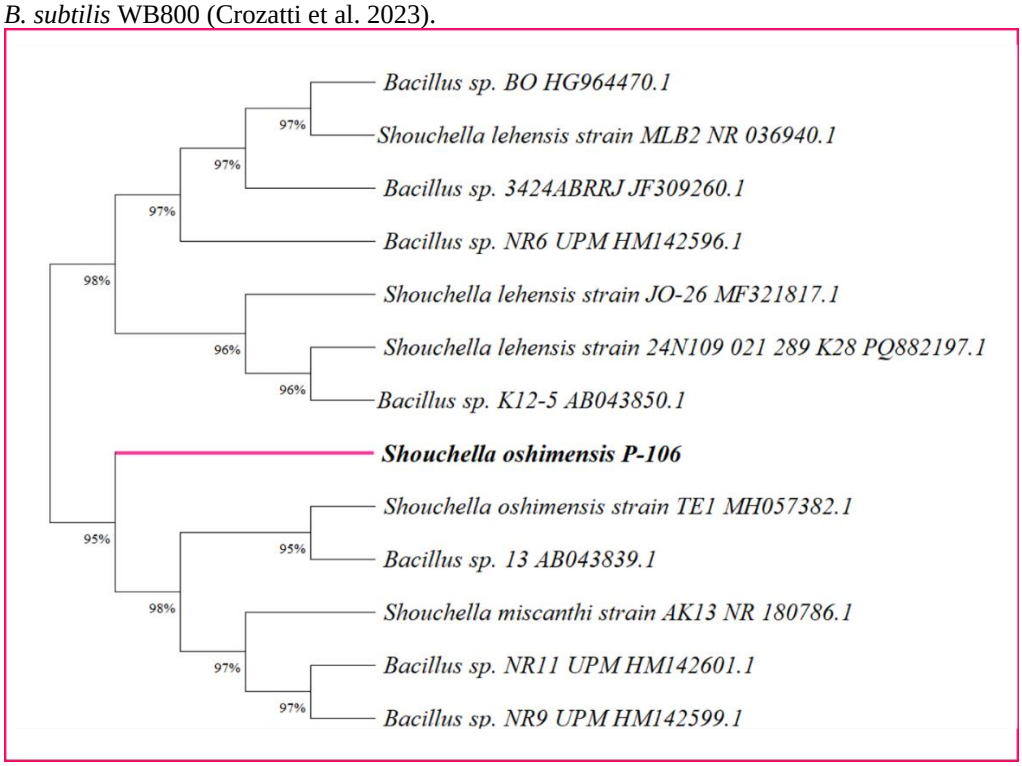

Figure 2. Phylogenetic analysis of *Shouchella oshimensis* P-106

Primary screening of *S. oshimensis* P-106 employed Horikoshi agar plate with indicator dye for α-, β-, and γ-CGTase. The colony:zone of clearance ratio for α-, β-, and γ-CGTase were 2.04, 2.5, and 1.71, respectively (Fig. 3). P-106 was chosen for secondary screening based on colony:zone of clearance ratio > 2.0. Secondary screening of P-106 showed 0.05, 1.37, and 0.067 U/ml for α-, β-, and γ-CGTase, respectively. This represents that *S. oshimensis* P-106 produced β-CGTase efficiently as the main product, with minimal presence of α- and γ-CGTase. *B. agaradhaerens* KSU-A11 and *B. firmus* NCIM 5119 were documented for their ability to produce α-, β-, and γ-CGTase (Ibrahim et al., 2011; Gawande and Patkar, 2001).

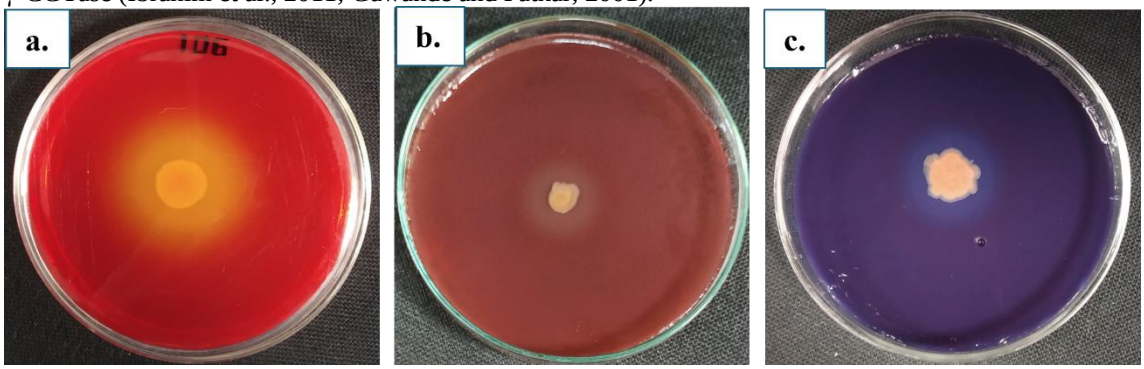

Figure 3. Screening of *S. oshimensis* P-106: **a.** α-CGTase, **b.** β-CGTase, and **c.** γ-CGTase

Bacterial growth and enzyme secretion are greatly affected by medium pH. The *S. oshimensis* P-106 grows in alkaline pH (10-12), and it has optimal production of β-CGTase (1.10 U/ml) at pH 11 ± 0.2 (Fig. 4a). There is no enzyme production at pH 7.0 and 8.0, mild production at pH 9.0, which indicates P-106 performs worse in neutral to slightly alkaline pH.

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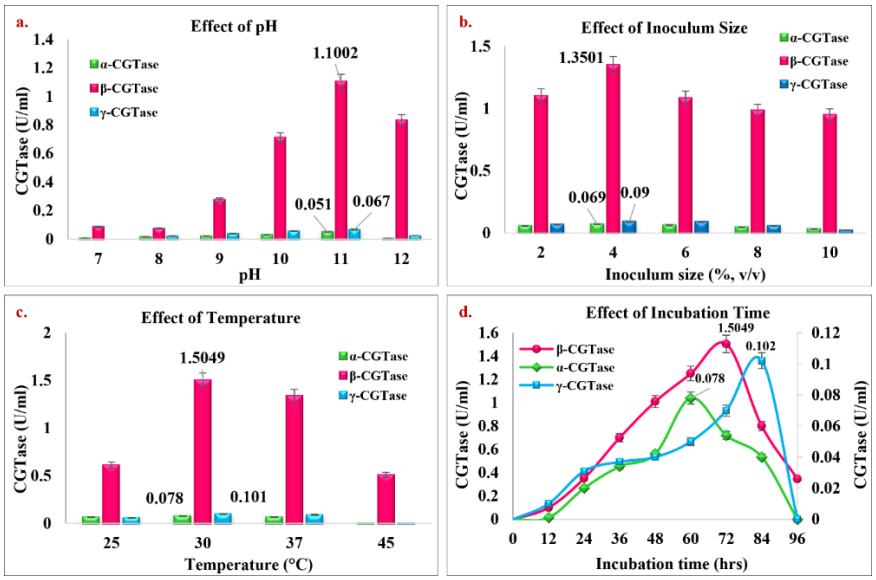

Figure 4. Optimization of physical production conditions by One-factor-at-a-time: **Effect of (a)** pH, **(b)** inoculum size (%, v/v), **(c)** temperature (°C), and **(d)** incubation time (hrs)

Optimal inoculum size is important for microbial growth and enzyme production. The testing of 2-10% (v/v) inoculum sizes revealed higher biomass and CGTase production from 2% to 4%, but no corresponding returns from 6% to 10% (Fig. 4b). The maximum β-CGTase production (1.35 U/ml) at 4% (v/v) inoculum indicates an optimal balance between microbial density and prevention of possible inhibitory effects.

The *S. oshimensis* P-106 produced highest levels of β-CGTase (1.50 U/ml) at 30 °C (Fig. 4c) with respect to optimal conditions for enzymatic reactions. Production at 37 °C was moderate, demonstrating strain adaptability. Metabolism was lesser in lower (25 °C) and greater (45 °C) temperatures because of reduced metabolic rates and stress conditions, respectively.

Type-specific incubation time effects on CGTase production were observed. β-CGTase production began at 48 hours, with a peak at 72 hours (1.50 U/ml). α- and γ-CGTase production reached peaks at 60 (0.078 U/ml) and 84 (0.102 U/ml) hours, respectively (Fig. 4d). The time corresponds to the nutrient consumption of the strain during its exponential growth phase. Production decreased considerably after 84 hours, which may be due to nutrient limitation, inhibitory metabolite buildup, or shift in metabolism from log to stationary phase.

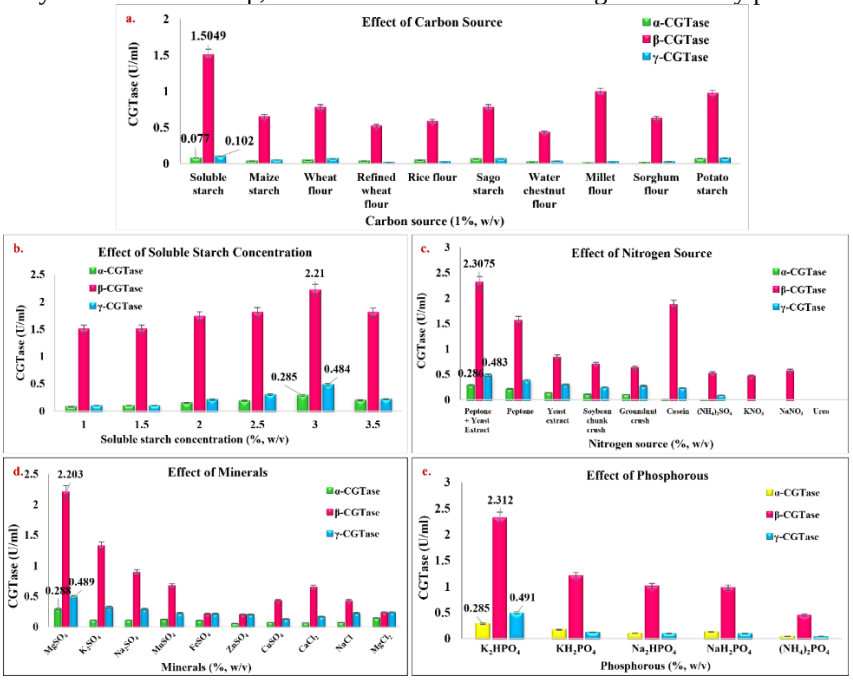

Figure 5. Optimization of chemical production conditions by One-factor-at-a-time: **Effect of (a)** different carbon source (%, w/v), **(b)** soluble starch concentration (%, w/v), **(c)** nitrogen source (%, w/v), **(d)** mineral source (%, w/v), and **(e)** phosphorous source

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Carbon source selection also affects CGTase production drastically as shown in Fig. 5a. Soluble starch has been shown to be the most appropriate carbon source for *S. oshimensis* P-106, while millet flour and potato starch also shown lead to the significant production of the enzyme. Conversely, rice flour, refined wheat flour, and water chestnut flour gave the lowest yields. The soluble starch concentration is important (Fig. 5b), with a peak β-CGTase yield of 2.21 U/ml at 3% (w/v). In addition to carbon sources, a mixture of peptone and yeast extract gives the best β-CGTase production (2.3 U/ml) as an optimal nitrogen source, showing a synergistic effect that assists microbial growth and enzyme production (Fig. 5c).

Mineral supplementation also affects CGTase production (Fig. 5d). Magnesium sulfate ($MgSO_4$) is quite effective in inducing yields, purportedly because of its function as a cofactor in enzymic reactions. Others such as $ZnSO_4$, $FeSO_4$, and $MgCl_2$ were not only ineffective in promoting production but were detrimental, indicating the finicky nature of some strains regarding certain minerals. In general, physical, and nutritional optimization is very important for efficient CGTase production and consumption of renewable sources. *S. oshimensis* P-106 showed maximum yield of 2.312 U/ml β-CGTase production with K_2HPO_4 as a phosphate source (Fig. 5e), which was superior to other phosphates such as KH_2PO_4 and sodium-based salts. On the other hand, $(NH_4)_2PO_4$ proved to be inhibitory due to its ammonium content to microbial growth.

The one-factor-at-a-time (OFAT) approach significantly optimized the CGTase production by *S. oshimensis* P-106 with parameters such as an initial pH of 11.0, a 4% inoculum, incubation at 30°C, 3% soluble starch, a mix of 0.5% peptone and yeast extract, 0.02% $MgSO_4$, and 0.1% K_2HPO_4. Interestingly, optimum incubation durations were different for various CGTase types: 60 hours for α-CGTase, 72 hours for β-CGTase, and 84 hours for γ-CGTase. After optimization, β-CGTase production increased by 1.68-fold, while α and γ-CGTase production were elevated by 5.7-fold and 7.3-fold, respectively. These results reflect the significance of specific conditions in maximizing enzyme production and the feasibility of eco-friendly production methods. Other isolates with optimal fermentation conditions are brief in Table 2.

Table 2: Fermentation conditions for CGTase production.

Microorganism	Optimal fermentation conditions				CGTase Produced	Reference
	Substrate	Temp. (°C)	pH	Time (h)		
Shouchella oshimensis P-106	Soluble starch	30	11	72	α/β/γ	Present study
B. subtilis WB800	Corn starch	37	8	24	β	Crozatti et al. 2023
Bacillus sp. T1	Soluble starch	37	-	72	α/β	Liu et al. 2022
B. cereus SS2	Potato starch	38	8.8	24	β	Kulshreshtha et al. 2020
B. lehensis	Rice starch	37	10.5	24	β	Elbaz et al. 2015
B. halodurans	Soluble starch	37	10.5	72	β	More et al., 2012
B. pseudalcaliphilus 20RF	Raw starch	60	6/9	-	β/γ	Atanasova et al. 2011
B. agaradhaerens KSU-A11	Potato starch	37	10	48	α/β/γ	Ibrahim et al. 2011
B. pseudalcaliphilus 8SB	Soluble starch	40	9.8-10	24	β/γ	Kitayska et al., 2011
B. sphaericus 41	Soluble starch	37	10.3	48	β	Moriwaki et al., 2009
B. circulans ATCC 21783	Soluble starch	40	9.8-10	24	β	Vassileva et al., 2007
B. firmus 7B	Soluble starch	37	10.3	120	β	Cao et al., 2005
B. agaradhaerens LS-3C	Soluble starch	37	9	30	α/β	Martins and Hatti-Kaul, 2002
B. halophilus BIO-12H	Potato starch	37	7-7.2	24-30	β/γ	Abelyan et al., 2002
B. firmus NCIM 5119	Raw starch	65	5.5/8.5	-	α/β/γ	Gawande and Patkar, 2001
B. macerans 15	Potato starch	37	7	48	α	Abelyan et al., 2000

Previously studied *Bacillus* sp. PBS1 produced maximum CGTase at pH 9.5, 10% inoculum, and after 96 hours of incubation (Solanki et al., 2022). *Bacillus* sp. TS1-1 also produced CGTase under optimized conditions at pH

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10.32 and 37 °C after 20 hours (Mahat et al., 2004). *B. stearothermophillus* HR1 had optimal production at pH 7.54 and 55 °C after 24 hours (Abd Rahman et al., 2004). Besides, *Bacillus* sp. TPR71HNA6 gave the most CGTase under conditions that were optimized to pH 7.5, 30 °C, and incubation for 48 hours (Ravinder et al., 2014).

CONCLUSION

Present study deals with the production of cyclodextrin glucanotransferase from a new alkaliphilic bacterial isolate *Shouchella oshimensis* P-106. The *S. oshimensis* P-106 produced β-CGTase as a key product, and showed slight production of other two CGTases α and γ. The production conditions were optimized via one-factor-at-a-time to improved CGTase production up to 0.285, 2.312. and 0.491 U/ml for α-, β-, and γ-CGTase, respectively. Overall, the results highlight the suitability of *S. oshimensis* P-106 for CGTase production with cost-effective substrate and suggests the incorporation of statistical approach for further optimization as well as protein engineering study. This isolate is a good candidate for CGTase production for biotechnological applications in starch processing industries.

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