

Biosynthesis of raw starch degrading β -cyclodextrin glycosyltransferase by immobilized cells of *Bacillus licheniformis* using potato wastewater

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Abstract: The study was sought to enhance the synthesis of thermal stable β -cyclodextrin glycosyltransferase (β -CGTase) using potato wastewater as a low-cost medium and assess the degree to which it is efficient for industrial production of β -cyclodextrin (β -CD) from raw potato starch. Thermophilic bacteria producing β -CGTase was isolated from Saudi Arabia and the promising strain was identified as *Bacillus licheniformis* using phylogenetic analysis of the 16S rRNA gene. Alginate-encapsulated cultures exhibited twice-fold of β -CGTase production more than free cells. Scanning electron microscopy (SEM) of polymeric capsules indicated the potential for a longer shelf-life, which promotes the restoration of activity in bacterial cells across semi-continuous fermentation of β -CGTase production for 252 h. The optimal conditions for β -CGTase synthesis using potato wastewater medium were at 36 h, pH of 8.0, and 50°C with 0.4% potato starch and 0.6% yeast extract as carbon and nitrogen sources, respectively. The purified enzyme showed a specific activity of 63.90 U/mg with a molecular weight of ~84.6 kDa as determined by SDS-PAGE analysis. The high enzyme activity was observed up to 60°C, and complete stability was achieved at 75°C. High levels of activity and stability were shown at pH 8.0, and the pH range from 7.0–10.0, respectively. The enzyme has an appreciable affinity for raw potato starch with a K_m of 5.7×10^{-6} M and a V_{max} of 87.71 $\mu\text{mol/mL/min}$. β -CD production was effective against 25 U/g of raw potato starch. The outcomes demonstrated its feasibility to develop a fermentation process by integrating the cost-effective production of β -CGTase having distinctive properties for β -CD production with eco-friendly utilization of potato wastewater.

Introduction

Cyclodextrins (CDs) are cyclic molecules synthesized by the intramolecular transglycosylation reaction of microbial cyclodextrin glucanotransferase (CGTase) on starch forming a hydrophilic external surface and a hydrophobic internal cavity (Rakmai and Cheirsilp, 2016). The prominent group of glycoside hydrolase is the family 13 (GH13) that catalyzes the conversion of starch to malto-oligosaccharides (Stam *et al.*, 2006). The CGTases (EC 2.4.1.19) and α -amylases

(EC 3.2.1.1.) are members of the GH13 family. Dependent on their protein sequence in the Carbohydrate-Active Enzyme (CAZY) database, GH13 family is divided into 43 subfamilies (Lombard *et al.*, 2014). The CGTases are the main members of the subfamily GH13_2, while α -amylases (3.2.1.1.) are members of other subfamilies.

Because CDs are shaped like rings, they can contain guest particles and produce inclusion complexes with both inorganic and organic compounds.

As a result, their applications are widespread in various industries; pharmaceutical, food, cosmetics, chemical, agriculture, emulsifiers, nanotechnology, and biomedical (Rakmai and Cheirsilp, 2016). The CGTase acts explicitly on α -glycosidic bonds in starch to produce various types of

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CDs through transglycosylation reaction relying upon the enzyme sources (Amiri *et al.*, 2015). The CGTase cleaves α -1,4 glycosidic bond to 6, 7, or 8 glucose units of the non-reducing end of starch. The formed reducing end glucose was then transferred to the hydroxyl group of the non-reducing end glucose unit of the same chain of glucose residue to form a cyclic compound of α -, β - or γ - CDs with 6, 7, or 8 glucose units, respectively (Janeček *et al.*, 2019). Various industries are interested in the use of β -CGTase due to its ability to produce large quantities of β -CD from starch as well as the preparation of inclusion complexes that can straightforwardly take place, and their low solubility offers genuine steadiness (Wang *et al.*, 2018).

The highly β -CGTase production can take place using specific bacteria, including *Bacillus* spp. (*B. macerans*, *B. megaterium*, *B. circulans*, *B. stearothermophilus*, *B. ohbensis*), *Klebsiella oxytoca*, *Micrococcus luteus*, and *Thermococcus* sp. (Martins and Hatti-Kaul, 2002; Rajput *et al.*, 2016b). The main challenge for β -CD production is a commercial one, which stems from the high cost of enzymes used, and physical treatments for starchy substrates, and the feedback inhibition associated with the end products (Moriwaki *et al.*, 2007). Subsequently, the search for thermophilic bacteria that produce high amounts of the enzyme has been driven by the industrial demand for thermostable β -CGTase (Wang *et al.*, 2017).

When fermentation processes are designed, it is possible to use immobilized bacterial cells several times with a reduced impact of inhibitory compounds and nutrient exhaustion. Also, the dense cell culture, and straightforward nature of product separation, which gives rise to an efficient bioprocess (Eş *et al.*, 2016; Ravinder *et al.*, 2012a; Ravinder *et al.*, 2012b). For almost all β -CGTase, it is only possible to convert gelatinized starch to β -cyclodextrin, and the physical treatments have been employed to improve the efficacy of the enzyme. β -CGTase from some microbial sources has proven viable to degrade raw starch, thus avoiding physical treatment and increasing the efficiency of CD production (Gawande and Patkar, 2001; Rajput *et al.*, 2016a). The considerable promise is associated with the use of starchy residues in fermentation procedures to improve the enzyme production cost and productivity, as well as a notable rise in environmental issue (Coelho *et al.*, 2016).

Potato wastewater is one of the most prevalent starch-rich by-products of potato industrial processes (Guo *et al.*, 2018). It is an obvious candidate for use in the production of high-value components given its presence of inorganic and organic compounds, e.g., proteins, starch, ammonium, and phosphate (Yuliani *et al.*, 2011). The large quantities of potato wastewater are produced from the potato-chips factories in Saudi Arabia that can be used for the fermentation process such as lactic acid (Huang *et al.*, 2003), bio-flocculants (Guo *et al.*, 2018), and activated sludge system (Azab, 2008).

With these issues in mind, the purpose of the present study was to isolate thermophilic bacteria that produce β -CGTase and to develop a fermentation process employing potato wastewater in a semi-continuous fermentation process. The properties of β -CGTase and their activity concerning gelatinized and raw starch were examined.

Materials and Methods

Sample collection

Soil samples were collected from two unexplored areas of the southern region, Saudi Arabia: Abha city (18°13'1"N 42°30'19"E, 21°C), and Jazan city (16°53'21"N 42°33'40"E, 44°C). The potato wastewater was collected after the peeling, slicing, and washing process of potato from the Saudi Snack Foods Company, Jeddah, KSA. The same batch of wastewater was used to carry out the experiments. The chemical analysis of potato wastewater was performed for dry matter, nitrogen, starch, reducing sugars, and total ash according to Baird *et al.* (2017) and Evers *et al.* (1984).

Isolation and screening of β -CGTase-producing bacteria

A 1.0-g soil sample underwent serial dilution (10^{-1} – 10^{-5}), 0.1 mL of which was spread onto the agar plates medium containing soluble starch (2%), peptone (0.5%), yeast extract (0.5%), $MgSO_4 \cdot 7H_2O$ (0.02%), K_2HPO_4 (0.1%), phenolphthalein (0.03%), methyl orange (0.01%), and agar (1.5%) at pH 7.5 (Salva *et al.*, 1997). The plates were incubated at 50°C for 48 h. The potential colonies surrounded by the clear zone in the rapid plate assay method were selected for further investigation based on enzymatic activity index, a ratio of the zone of clearance diameter to colony diameter (More *et al.*, 2012).

Molecular identification and phylogenetic tree construction

The growth of the promising bacterial isolate took place for 24 h in a nutrient broth medium, after which centrifuging was undertaken for 20 min at $6573 \times g$ (Hermle, Z 326 K, Germany). To facilitate genomic DNA extraction, QIAamp DNA Mini kit (Qiagen Inc., Valencia, CA) was employed (Neilan, 1995).

The PCR amplification of 16S rRNA was performed by PCR Master Mix kit (TAKARA, Japan) using the universal primers 27 F (5'-CCA GCA GCC GCG GTA ATA CG-3') and 1492 R (5'-ATC GG(C/T) TAC CTT GTT ACG ACT TC-3'). The PCR components were incubated at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 54 s, followed by a final extension step at 72°C for 10 min. Using 1.5% agarose gel in a 1 \times TBE running buffer with an electrophoresis unit, the migration of the PCR product was observed. The confirmation of the presence of the PCR product was facilitated after 30 min and shot-utilizing gel documentation was undertaken (BIORAD, USA). The 16S rRNA gene PCR product was sequenced by Macrogen, Korea. The obtained sequence was aligned and contrasted with the genes saved in GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In turn, accommodation in GenBank was facilitated using an explicit accession number. A phylogenetic tree was constructed with MEGA v. 5.1 (Abdel-Razek *et al.*, 2020). The phylogenetic relationship among the sequenced gene and other related genes in the GenBank database was constructed depending on the neighbor-joining method and based on the Jukes-Cantor distances of the used software.

Immobilization of bacterial cell using sodium alginate

The bacterial cell immobilization was carried out according to Ravinder *et al.* (2012a). The bacterial cells of the free-cell

inoculum were centrifuged at $6573 \times g$ for 20 min, and mixed with 10.0 mL 2% (w/v) sodium alginate, then stirred for 10 min. This enabled the achievement of a consistent texture. The drop-wise extrusion of the mixture then took place using 2 mL syringe into 25 mL of 3.5% (w/v) CaCl_2 solution. The hardening of the alginate drops was observed after contacting the solution, thereby leading to the formation of capsules that entrapped the bacterial cells. The solidification of the capsules occurred for 30 min, after which excess Ca^{2+} and cells were eliminated through washing in a sterile saline solution several times. The capsules were preserved in a refrigerator using CaCl_2 solution. The immobilization process was carried out in an aseptic manner in the laminar flow unit.

β -CGTase production

The free cells inoculum was prepared in nutrient broth medium at 50°C, 200 rpm, for 24 h. Erlenmeyer flasks (250 mL) containing 50 mL of potato wastewater medium (pH 7.5) were inoculated with 5.0 mL (1×10^8 CFU/mL) to produce the β -CGTase by free cell cultures. This amount of inoculum was equal to that used in immobilized cultures (4.0 g alginate gel capsules). The production of β -CGTase by free and immobilized cultures were performed at 50°C, 200 rpm for 48 h. Cultures were centrifuged ($6573 \times g$ for 20 min) and the supernatant was used as the enzyme source. The biomass of free and immobilized cells was determined as cell dry weight at 70°C (Costa *et al.*, 2015). The optimization of β -CGTase production was carried out by changing single factors, including time (12–96 h), initial pH (6.0–10), temperature (35–65°C). Different potato starch concentrations (0.1–0.8%) were added to potato wastewater. The control was the native potato starch in potato wastewater. Also, various nitrogen sources (yeast extract, peptone, ammonium sulfate, ammonium nitrate, ammonium chloride, sodium nitrate, and potassium nitrate) were added to the potato wastewater medium to fortify the medium for β -CGTase production in the same nitrogen concentration of 0.06% (w/v).

Scanning electron microscopy (SEM)

In line with Costa *et al.*'s description (Costa *et al.*, 2015), energy-dispersive analysis (Joel Jsm 6360 LA, Japan) was used to characterize the highly amplified morphological appearance of the alginate capsules and the immobilized bacterial cells. After solidification of the prepared capsules, some plain and immobilized capsules were divided into two hemispheres using sharp razor. Both whole and divided spheres were kept at room temperature for 24 h until dryness. The capsules were then submitted for the coating process followed by photographing using scanning electron microscope with multiple magnifications.

Semi-continuous fermentation

The fermentation process was executed under optimal conditions for 10 cycles to achieve semi-continuous fermentation. The recovery of the production medium was undertaken once each cycle had finished. The curing of the immobilized cells occurred with 2% (w/v) CaCl_2 following the end of every cycle, after which a new production medium was inserted to continue the fermentation procedure (Abdel-Naby *et al.*, 2011).

Purification of β -CGTase by gel-filtration chromatography

The enzyme protein of the cell-free extract was precipitated overnight by 70% $(\text{NH}_4)_2\text{SO}_4$. The precipitated mixture after centrifugation ($8586 \times g$ for 20 min) was resolved in 0.1 M phosphate buffer and then dialyzed at 4°C. The dialyzed fraction was loaded onto a Sepharose 6B column and the fractions were collected and estimated for protein and enzyme activity (Laszlo *et al.*, 1981). The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% SDS polyacrylamide gel and run at 150 V for 3.0 h at 25°C (Laemmli, 1970; Guru *et al.*, 2012). The extracted protein was stained with Coomassie Blue, and the gel was treated by de-staining solution to remove unbound dye and was imaged by a Gel Documentation System (BIORAD, USA).

Kinetics properties of β -CGTase

The effect of pH and temperature on β -CGTase activity was investigated under several pH and temperature conditions (from pH 4.0 to 12.0 and 40–90°C, respectively). The following buffers were used: 50 mM sodium acetate (pH 4.0–6.0); 50 mM Tris-HCl (pH 7.0–8.0); 50 mM glycine-NaOH buffer (pH 9.0–10.0); 50 mM carbonate-bicarbonate buffer (pH 11.0–12.0). An assessment of the thermo-stability of β -CGTase occurred through the pre-incubation of the enzyme in 50 mM Tris-HCl (pH 8.0) at 40–90°C for 60 min. Additionally, pH stability was evaluated through the incubation of the enzyme in various buffers for 60 min. The relative activity of β -CGTase (%) was expressed as the ratio of the enzyme activity at different parameters (pH, temperature) to the activity of the control sample under standard assay conditions. The kinetic parameters of β -CGTase (K_m and V_{max}) were determined using potato starch as a substrate at concentrations ranging from 0.0 to 0.1 M. The Michaelis–Menten parameters were determined from Lineweaver–Burk plots using the equation derived from linear regression analysis of the curve using Sigma Plot ver. 14.0 (Mora *et al.*, 2012).

β -cyclodextrin production

The production of β -CD was studied under various β -CGTase concentrations with gelatinized and raw potato starch as the substrates. The gelatinized potato starch was prepared by heating 10 mL starch (20 g/L) in 50 mM phosphate buffer (pH 8.0) in a water bath for 10 min. The reaction mixture of 100 g/L substrate in 10 mL phosphate buffer (50 mM, pH 8.0) and 100 μL of different enzyme concentrations were incubated for 1 h at 60°C. The samples were inactivated by boiling for 5 min, then centrifuged at $9693 \times g$ for 10 min. The β -CD was assessed with the phenolphthalein method (Kaneko *et al.*, 1987) based on the standard curve of 0–0.8 g/L β -CD. 0.5 mL of 0.02% (w/v) phenolphthalein in 0.005 M Na_2CO_3 was added. The absorbance was measured using a double beam UV/Vis scanning spectrophotometer (Model: Shimadzu, 1601PC) at 550 nm.

Analytical assays

The phenolphthalein method was employed to estimate β -CGTase activity (Kaneko *et al.*, 1987). The reaction mixture containing 1.0 mL of 40 mg soluble potato starch in 0.1 M phosphate buffer (pH 8.0), and 0.1 mL enzyme was

incubated at 50°C for 10 min in a water bath. The reaction was stopped by adding 3.5 mL of 0.03 M NaOH solution. A 0.5 mL of 0.02% (w/v) phenolphthalein in 0.005 M Na₂CO₃ was added to the reaction mixture. The decrease in color intensity was assessed after 15 min using a spectrophotometer at 550 nm after 15 min. The unit of enzyme activity was defined as the rate of the enzyme catalyzing the production of 1.0 μmol of β-CD/min under the reaction conditions. The protein concentration was estimated using Folin-Ciocalteu reagent (Lowry *et al.*, 1951). All experiments were performed in triplicate.

Statistical analysis

Data were first investigated for their normal distribution of errors with Shapiro-Wilk's *W* test, and Levene's test was employed to facilitate an investigation of the homogeneity of variances. One-way analysis of variance (ANOVA) was then used to examine the significance of variation. To examine significant differences between the means of the treatments, the least significant difference (LSD) was employed, where *P* < 0.05 was used as the cut-off for statistical significance.

Results

Isolation and screening of thermophilic β-CGTase-producing bacteria

Out of 112 thermophilic bacterial isolates were isolated from the soil samples of the two regions: Jazan and Abha, Saudi Arabia. All purified bacterial isolates were screened for β-CGTase production by rapid plate assay method. Forty-three of β-CGTase-producing bacterial isolates (38.39%) were detected by a clearance zone around the colonies. The highest number of β-CGTase-producing bacteria was isolated from the Jazan region (N = 32), and the lowest isolates were from Abha region (N = 11). Only ten promising isolates showed a high clear zone around the colony in rapid plate assay reached 1.8 cm for promising bacterial isolate, JA-16 (Fig. 1A). The quantitative screening of ten potentially effective isolates for β-CGTase production was undertaken with submerged fermentation. The large spectrum of enzyme

production ability was detected by the tested isolates (Fig. 1B). The optimum β-CGTase production (9.6 U/mL) was obtained by bacterial isolate, JA-16 strain isolated from the Jazan region.

Molecular identification of potential β-CGTase-producing bacterial isolate

A 99% of the promising isolate, JA-16 was comparable to *Bacillus licheniformis*, as revealed by the sequencing and amplification of the 16S rRNA gene. The isolate's sequence was submitted to GenBank with an explicit accession number (MK693006.1). The isolate's phylogenetic relationship to the other species was identified, and contemporary taxonomy for distinguishing between rarely seen bacterial strains relied on the use of molecular techniques (foremost among which was the examination of the 16S rRNA gene (Fig. 2).

Effect of incubation time on β-CGTase production with free and immobilized cells

The immobilized cells of *Bacillus licheniformis* were more effective for β-CGTase production that gave about two-fold higher than the free cells (Fig. 3). A significant relationship was observed between β-CGTase production and bacterial growth, where the highest level of enzyme production occurred during the late exponential phase and, after this, a reduction in bacterial growth took place. A high rate of cell growth was observed in the primary 12 h of free cell cultures, and once 48 h had elapsed, a cell concentration of 3.6 mg/mL was associated with optimal enzyme production (9.6 U/mL). On the other hand, the rapid increase in biomass contained in gel capsules occurred over 36 h, thereby reaching the highest level of growth, and enzyme production reached to 4.5 mg/mL and 17.2 U/mL, respectively (Fig. 3). The subsequent fermentation experiments were performed by immobilized cells of *Bacillus licheniformis*.

Scanning electron microscopy (SEM)

SEM was used to examine the internal and external appearances of the cells immobilized in alginate capsules.

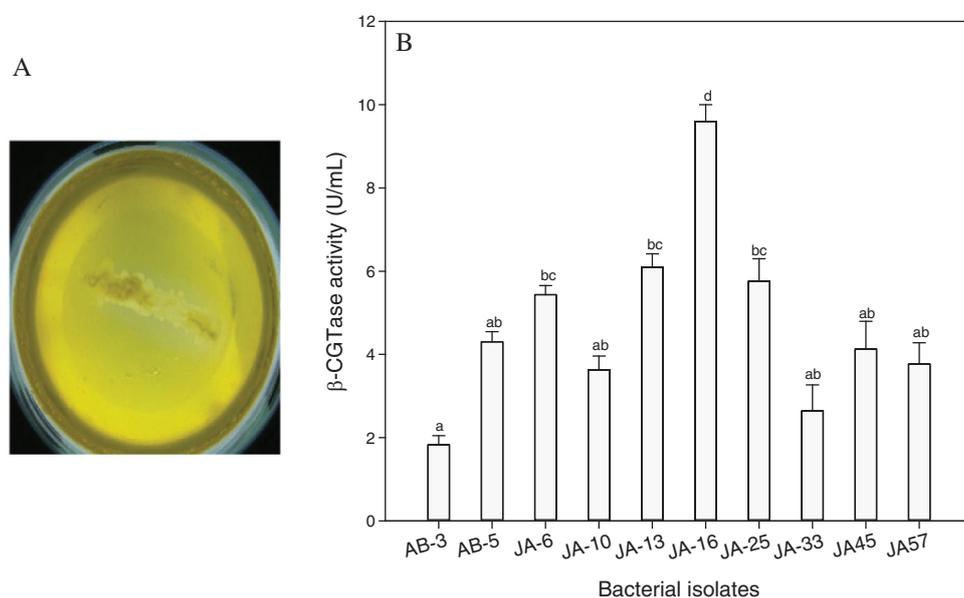


FIGURE 1. Rapid plate assay for promising bacterial isolate, JA-16 for β-CGTase production (A), and quantitative screening of promising bacterial isolates for β-CGTase production at pH 7.5, 50°C, 200 rpm, and 48 h (B).

Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at *P* < 0.05. Bacterial isolates were collected from Abha (AB), Bacterial isolates were collected from Jazan (JA).

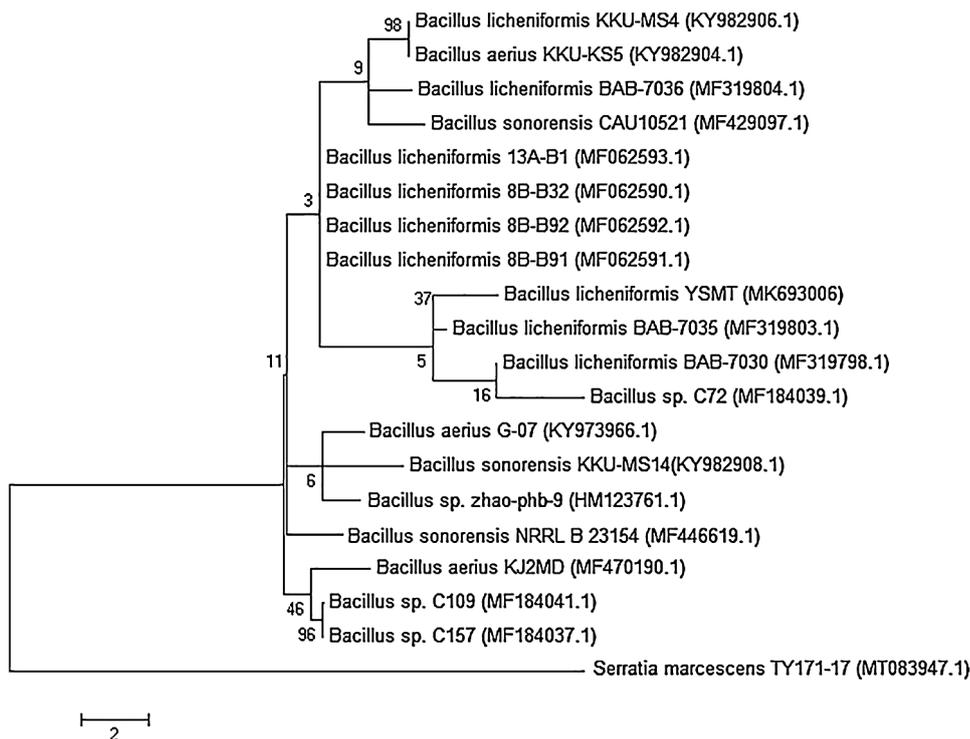


FIGURE 2. Phylogenetic relationship of *Bacillus licheniformis* YSMT and other related 16S rRNA gene sequences of published strains generated by the Neighbour-Joining method using MEGA 5.1 software. The bootstrap values (%) at the nodes. The accession number in GenBank put in parentheses.

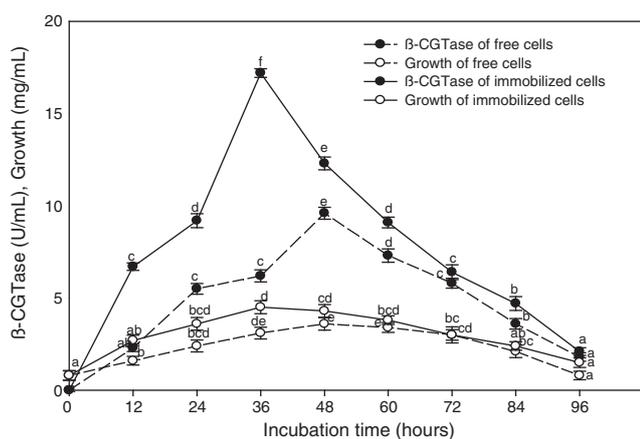


FIGURE 3. Time course of β -CGTase production using free and immobilized cells of *Bacillus licheniformis* YSMT at pH 7.5, 50°C, and 200 rpm.

Mean \pm SEM (N = 3) was presented. Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at $P < 0.05$.

The dried alginate capsule displayed a solid, elongated structure while, under standard conditions, the alginate capsules were consistently round and hollow in terms of their structure (Fig. 4A). The alginate gel was encapsulated and safeguarded the bacterial cells that enabled nutrients from the outside to enter and exit the capsules without restriction. Furthermore, the encapsulated bacterial cells were specified as intact bacilli shapes that appeared not to have sustained damage, thereby suggesting that alginate capsules had been safeguarded from extreme environmental conditions (Fig. 4B).

Effect of initial pH and incubation temperature on β -CGTase

The highest level of β -CGTase production was observed at an initial pH of 8.0 reaching 25.40 U/mL (Fig. 5A). The reduction

in enzyme activity in the alkaline conditions was not high compared to acidic conditions. The decrease of 44.09% in β -CGTase production was observed at pH 9.0, whereas a greater reduction of 91.33% was related to pH 6.0. In respect to the effect of the incubation temperature, a positive correlation was observed between temperature and β -CGTase production up to 50°C (Fig. 5B). The incubation temperature changes on both sides of the optimal value resulted in a decrease in β -CGTase production. The rapid reduction in β -CGTase production was noticed at temperature conditions that exceeded 60°C.

Effect of potato starch concentration on β -CGTase production

The chemical analysis of potato wastewater showed beneficial compounds for the fermentation process, including dry matter (2.87%), nitrogen (0.33%), starch (1.94%), reducing sugars (0.1%), and total ash (0.29%). Various potato starch concentrations were added to potato wastewater to elucidate the best concentration for maximum bacterial growth and β -CGTase production. The control is the native potato starch in potato wastewater. The results showed that adding 0.4% of potato starch to potato wastewater medium gave the highest β -CGTase production (Fig. 6). Further increasing the amount of starch was unfavorable to produce β -CGTase.

Effect of nitrogen sources on β -CGTase production

Different organic and inorganic nitrogen sources were added to the potato wastewater medium in the same nitrogen concentration to support the enzyme production. The results refer to that organic nitrogen sources promoted β -CGTase production and bacterial growth than inorganic sources (Fig. 7A). The high bacterial performances for both growth and β -CGTase production were noticed using yeast extract as the optimum nitrogen source with a 17.33% increase compared to the control medium. The inorganic nitrogen sources had a reductive impact on β -CGTase production. The

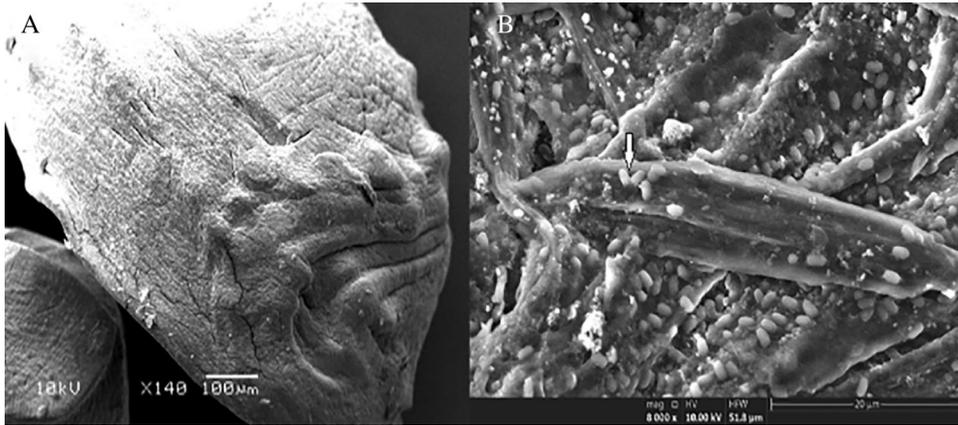


FIGURE 4. SEM of alginate capsules externally (A), and internally (B).

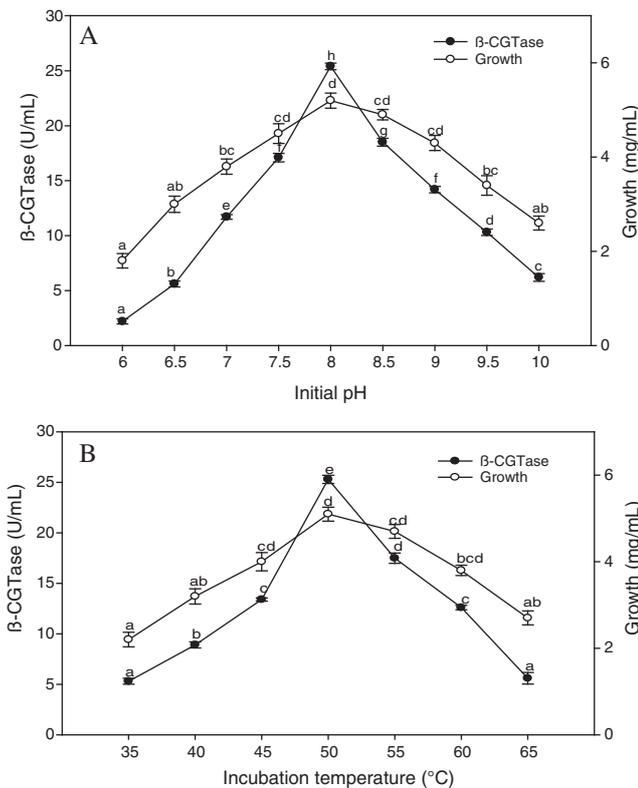


FIGURE 5. Effect of initial pH on β-CGTase production at 50°C, 200 rpm, and 36 h (A), the effect of incubation temperature on β-CGTase production at pH 8.0, 200 rpm, and 36 h (B). Mean ± SEM (N = 3) was presented. Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at P < 0.05.

nitrate salts lead to a decrease in β-CGTase production (47.13% to 70.26%), while ammonium salts led to a reduction from 28.01% to 54.21%. Furthermore, the effect of different yeast extract concentrations, as an ideal nitrogen source on β-CGTase production was evaluated. The high enzyme production was obtained by adding 0.6% yeast extract (Fig. 7B).

Semi-continuous β-CGTase production

In repeated batch cultures, the immobilized cells were transferred from the fermented medium into a sterile medium once a 36-hour period had elapsed, and the process was carried out for ten cycles. The culture activity was attained for β-CGTase production until the seventh cycle

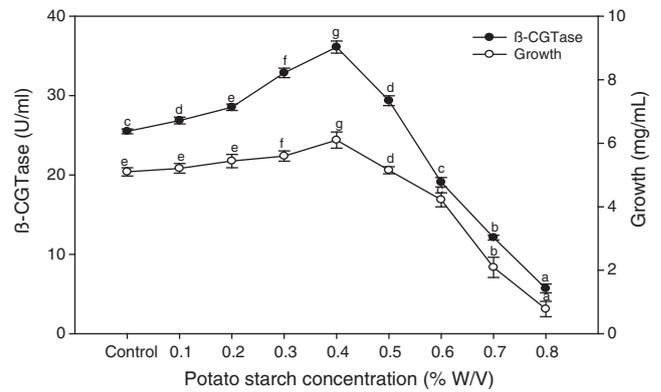


FIGURE 6. Effect of potato starch concentration on β-CGTase production at pH 8.0, 50°C, 200 rpm, and 36 h. Mean ± SEM (N = 3) was presented. Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at P < 0.05. (Control is the potato wastewater without additions).

since enzyme production remained between 40.10–50.60 U/mL over the entire optimum period of operation (Fig. 8). The β-CGTase production remained stable in a semi-continuous fermentation over a period of 252 h, then the enzyme production decreased extraordinarily. The positive relationship with the biomass content of the beads and β-CGTase production was observed when biomass concentration in the beads increased with the first few batches which remained almost steady for the subsequent batches.

Purification and kinetic properties of β-CGTase

The enzyme was purified by (NH₄)₂SO₄ precipitation, dialysis, and gel filtration chromatography using a Sepharose 6B column. The purification results of each step were as shown in Tab. 1. One portion from 21 fractions showed β-CGTase activity. After purification to homogeneity, the specific activity was 63.90 U/mg and the fold purification and yield were 19.84 and 41.77%, respectively.

The molecular weight of the purified enzyme was determined by SDS-PAGE analysis. The single protein band with a molecular weight of ~84.4 kDa was detected (Fig. 9).

The activity and stability of β-CGTase were studied as an effect of pH and temperature. The enzyme activity was observed in the neutral-to-alkaline pH (7.0 to 11.0), with the highest level at pH 8.0, while 85.8% of the enzyme’s activity was retained until pH 10 (Fig. 10A). A broad range of pH

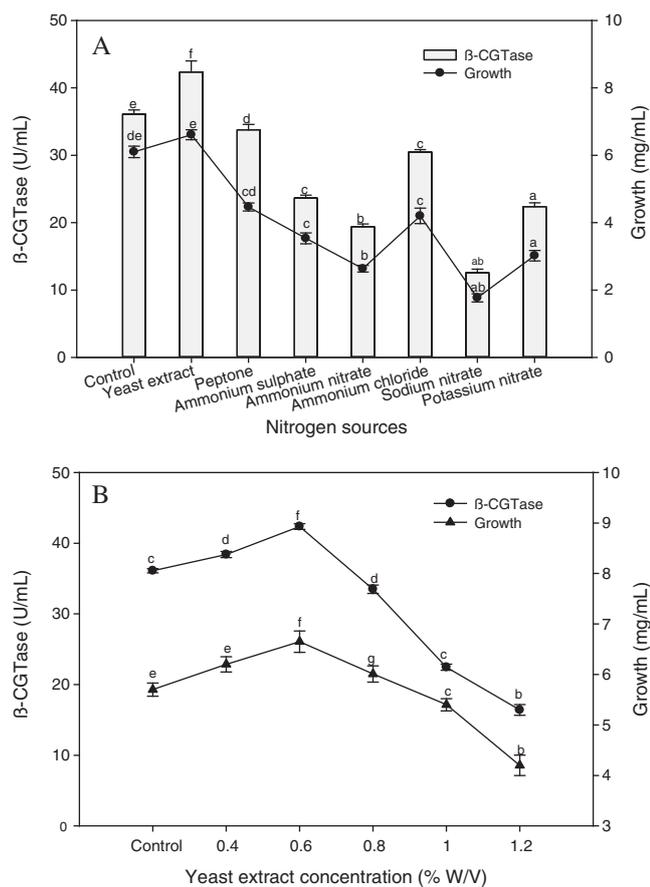


FIGURE 7. Effect of nitrogen sources on β -CGTase production at pH 8.0, 50°C, 200 rpm, 36 h, and 4.0% (w/v) potato starch (A), effect of yeast extract concentration at pH 8.0, 50°C, 200 rpm, 36 h and 4.0% (w/v) potato starch (B).

Mean \pm SEM (N = 3) was presented. Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at $P < 0.05$. (Potato wastewater without additions of nitrogen source was used as control).

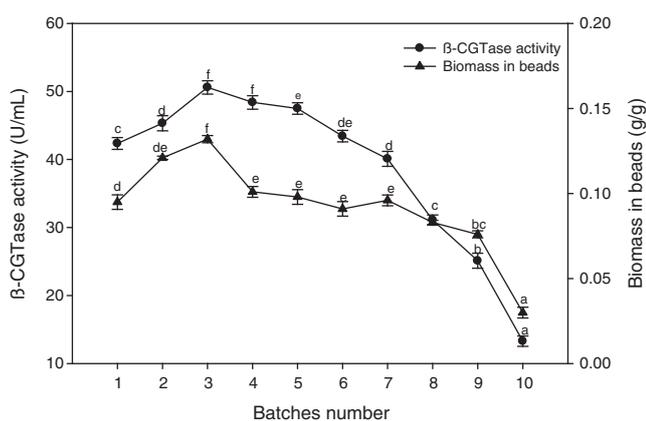


FIGURE 8. Semi-continuous β -CGTase production by repeated batch culture for 10 batches.

Mean \pm SEM (N = 3) was presented. Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at $P < 0.05$.

values (7.0 to 11.0) for 1.0 h was associated with maintenance of the enzyme’s stability (91–100%). Under acidic conditions, the stability of the enzyme was lower, with residual activity of 62.16% and 75.16% at pH 5.0 and 6.0, respectively. In respect

TABLE 1

Purification profile of results of β -CGTase of *Bacillus licheniformis*

Purification step	Total β -CGTase activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	4237	1317	3.22	1	100
Ammonium sulfate /Dialysis	3050	421	7.24	2.25	71.98
Sepharose 6B	1770	27.7	63.90	19.84	41.77

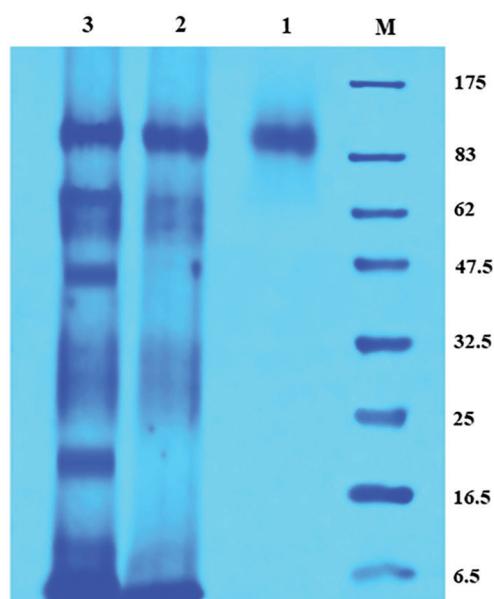


FIGURE 9. SDS-polyacrylamide gel electrophoresis of the purified enzyme.

M: Protein marker; 1: Sepharose 6B purified enzyme; 2: $(\text{NH}_4)_2\text{SO}_4$ precipitated enzyme; 3: Cell-free crude enzyme.

to the temperature effect on enzyme activity and stability, the temperature from 40°C to 60°C was significant for β -CGTase activity. The highest level of enzyme activity was obtained at 50–55°C, which reduced to 93.4% at 60°C, and fell to 45.83% with an increase in the reaction temperature to 70°C (Fig. 10B). The thermal stability of the enzyme was completely retained in terms of initial activity following thermal treatment to 75°C for 1 h, while at 80°C retained about 93.26% of the activity. The K_m and V_{max} were estimated using the Michaelis-Menten equation and the Lineweaver-Burk plot. The β -CGTase has a high affinity toward the potato starch, with a lower K_m value of 5.7×10^{-6} M with a V_{max} of 87.71 $\mu\text{mol}/\text{mL}/\text{min}$ (Fig. 10C).

β -cyclodextrin production with raw and gelatinized potato starch
The purified β -CGTase was used to produce β -CD using both raw and gelatinous potato starch as the substrate to evaluate the enzyme action. The raw potato starch granules were

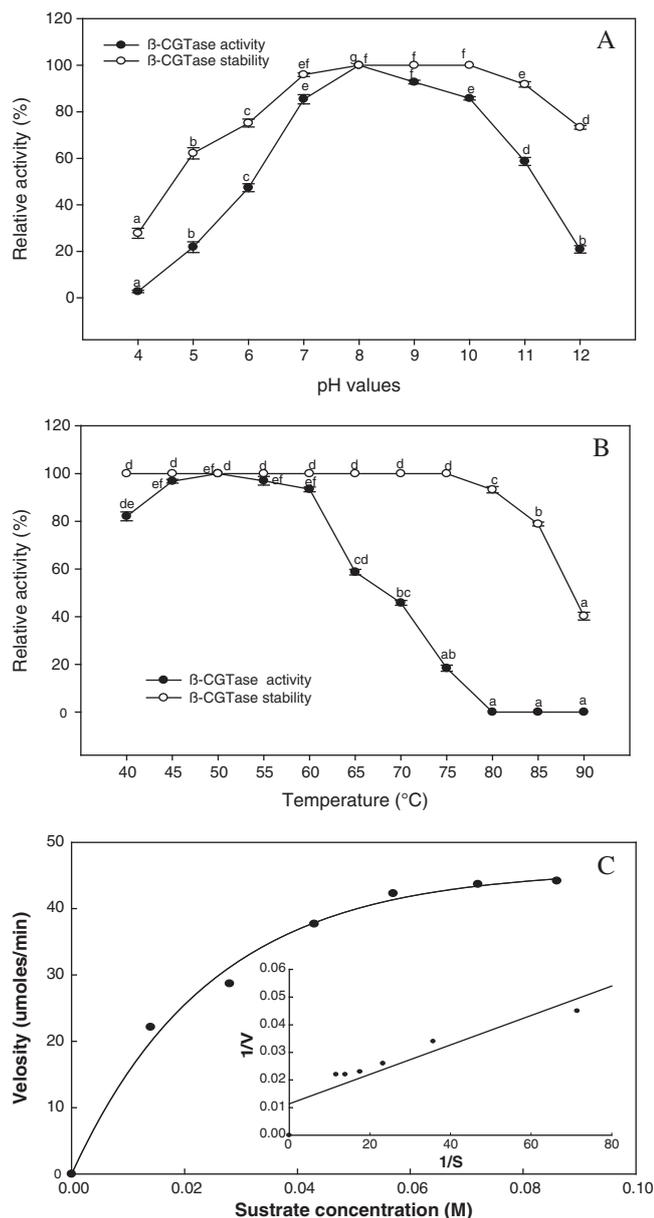


FIGURE 10. Kinetic parameters of purified β -CGTase. Effect of pH value from 4.0 to 12 (A) and temperature from 40°C to 90°C (B), on β -CGTase activity and stability. Mean \pm SEM (N = 3) was presented. Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at $P < 0.05$. The plot of the reaction velocities (V) vs. substrate concentration fitted to the Michaelis-Menten equation and determination of the K_m and V_{max} values of the purified enzyme by Lineweaver-Burk plot (C).

optimal for β -CD production than gelatinous form. The raw potato starch at an enzyme concentration of 25 U/g achieved 33.3 g/L β -CD while using gelatinous potato starch granules, 17.60 g/L β -CD was obtained at an enzyme concentration of 15 U/g (Fig. 11).

Discussion

The thermophilic β -CGTase -producing bacterial isolates were isolated from soil samples of Abha and Jazan regions, Saudi Arabia. The phenolphthalein inclusion with the production of cyclodextrin due to microbial efficiency in

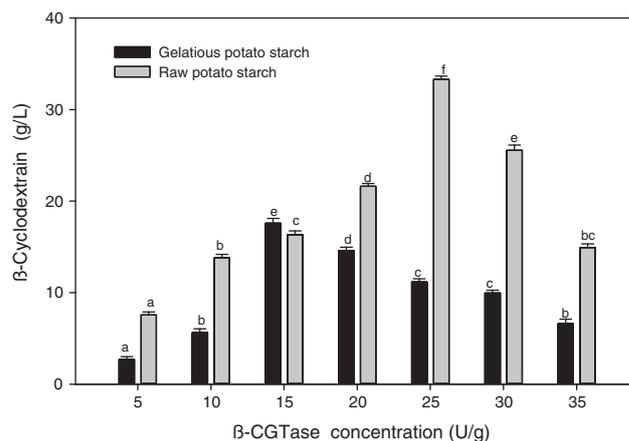


FIGURE 11. Effect of β -CGTase concentration on β -CD production using raw and gelatinized potato starch. Mean \pm SEM (N = 3) was presented. Vertical bars indicate the standard errors of the means. Means followed by different letters are significantly different at $P < 0.05$.

producing β -CGTase was indicated by the observation of a clearance zone around the colonies (Kitcha et al., 2008). The acclimatization of bacterial strains in the hot region is one of the ways to ensure their survival, which are regarded as the optimal areas for their isolation. This may explain the increase in the number of isolates in the Jazan region (44°C) compared to the Abha region (21°C). Despite various research reported the existence of thermophilic bacteria in permanently hot habitats, thermophilic bacteria have been isolated from a range of temperate and cold soil environments worldwide (Marchant et al., 2002). The diversity of these bacteria and their geochemical impact on their environment need more investigation where it may have an effective role in humus hydrolysis accompanied by exothermic reactions (Rahman et al., 2004). The soil properties and environmental conditions can influence the presence of bacterial isolates in a particular area (Bibi et al., 2017). The thermophilic bacteria usually produced distinctive enzymes that operate in the context of extreme conditions, which are analogous to industrial conditions relating to specific processes (Letsididi et al., 2011). Among ten promising isolates examined by submerged fermentation, the JA-16 strain obtained from the Jazan region was associated with the greatest rate of β -CGTase production (9.6 U/mL). This isolate was comparable for β -CGTase production when compared with the strains that have already been documented (Amiri et al., 2015; Costa et al., 2015).

The molecular identification of the promising isolate, JA-16 by the sequencing and amplification of the 16S rRNA gene proved high genetic similarity to *Bacillus licheniformis*. The widely recognized genetic markers were used due to their appropriate length (1500 bp), which includes steady locals that sign in the planning of amplification primers (Bibi et al., 2017). Various *Bacillus* strains producing β -CGTase have been identified using molecular basis as *Bacillus* sp N-227 (Wang et al., 2017); *Bacillus cereus* NRC7 (Abdel-Naby et al., 2011); *Bacillus circulans* DF 9R (Costa et al., 2015), and *Bacillus firmus* (Higuti et al., 2004).

The fermentation procedure for enzyme production has oscillated between immobilized and free cells techniques that

play the main role in the high production of β -CGTase. The immobilized cells of *Bacillus licheniformis* were more active for β -CGTase production in an abbreviated cultivation time (36 h) compared with free cells (48 h), where gave two-fold higher during the late exponential phase. The same results were obtained previously by Eş *et al.* (2016). The reduction in bacterial growth and enzyme production after the optimum fermentation period was due to the consumption of nutrients (Rajput *et al.*, 2016b). The presence of *Bacillus* sp. cells within the alginate gel capsules enhanced the efficacy of β -CGTase production reached 68.50 U/mL at 36 h (Ravinder *et al.*, 2012a). The positive change in enzyme synthesis kinetics in the immobilized state can be due to the modifications in macro-environmental conditions and stress conditions (Costa *et al.*, 2015).

Scanning electron microscopy (SEM) for alginate capsules indicates the dried capsule displayed a solid, elongated structure. This can be attributed to the dryness of the air and the coating parameters needed for a valid SEM evaluation. The alginate capsules safeguarded the bacterial cells and enabled nutrients to enter without restriction. The immobilized cells appeared not to have sustained damage, thereby suggesting that alginate capsules had been safeguarded from extreme environmental conditions. The same trend was mentioned in previous studies where SEM has been employed to verify the accuracy and efficient immobilization of *Bacillus* sp. (Eş *et al.*, 2016), and *Bacillus firmus* (Pazzetto *et al.*, 2012).

The optimum β -CGTase production was obtained at an initial pH of 8.0 (25.40 U/mL), which was following *Bacillus licheniformis* BS 7 (Sivakumar and Banu, 2011). However, the optimum pH value for β -CGTase production by *Bacillus licheniformis* strains were varied from pH 6.0–9.0 (Letsididi *et al.*, 2011). The pH exhibits a potent effect on enzyme production by influence the bioavailability of the trace elements and permeability of nutrients which vary depending on the microbial strain (Coelho *et al.*, 2016). A significant correlation was observed between incubation temperature and β -CGTase production up to 50°C. Based on the bacterial strain, the ideal temperature range for β -CGTase production was varied, at 37°C for *Bacillus circulans* DF 9R (Costa *et al.*, 2015), and *Bacillus licheniformis* (Sivakumar and Banu, 2011), while at 60°C for *Geobacillus thermoglucosidans* (Jia *et al.*, 2018) and 65°C for *Thermoanaerobacter kivui* (Avci and Dönmez, 2009). The incubation temperature changes other than optimal value resulted in a decrease in β -CGTase production due to the reduced activity of the metabolic enzymes (Eş *et al.*, 2016).

The highest β -CGTase production was achieved by supporting the potato wastewater medium with 0.4% of potato starch. These results may be explained by the result of potato wastewater analysis which found that it had a lot of remaining starch (1.94%). These results were in accordance with those of Azab (2008), Yuliani *et al.* (2011), and Guo *et al.* (2018). They reported that the useful compounds for the fermentation process were detected in potato wastewater as organic and inorganic compounds, e.g., proteins, starch, ammonium, and phosphate. Different starch concentrations were reported for high β -CGTase productivity; 1.5% for *Bacillus halodurans* (More *et al.*, 2012), and *Bacillus*

alkalophilic CGII (Freitas *et al.*, 2004), while at 3.0% the efficient enzyme production was accomplished by *Bacillus* sp. TPR71H (Ravinder *et al.*, 2012c). Also, Nishida *et al.* (1997) found that the high β -CGTase production was achieved in the presence of starch, while the β -CGTase gene (*cgt*) was completely repressed by glucose. On the contrary, Wang *et al.* (2018) mentioned that β -CGTase synthesis was not dependent on the presence of an explicit inducer which asserts that the metabolic synthesis of β -CGTase varied among microbial strains. The decline of enzyme production with a high concentration of starch could be explained by the medium's high viscosity which influenced the accessibility of oxygen required in the bacterial growth (Sivakumar and Banu, 2011).

The production of β -CGTase is a process affected strongly by nitrogen because it plays a main role in protein and cell wall component synthesis (Wang *et al.*, 2018). Based on the results, yeast extract promoted β -CGTase production and bacterial growth than inorganic sources. Consistent results have been noted that yeast extract incorporates the basic micronutrients; minerals, vitamins, amino acids, and nitrogen components that are required to initiate bacterial growth and β -CGTase production (Yap *et al.*, 2010). Nevertheless, the current study found that inorganic nitrogen sources, nitrates, and ammonium salts had a reductive impact on β -CGTase production.

Despite comparable results regarding the impact of inorganic nitrogen sources (Amiri *et al.*, 2015), the difference between inorganic and organic nitrogen sources on β -CGTase production was negligible in other reports (Higuti *et al.*, 2004). On the other hand, the enzyme production was optimum with 0.6% yeast extract which attributed to the nature of potato wastewater used as a basic medium contains about 0.33% nitrogen. The results agreed with those of Yuliani *et al.* (2011), they found out that the potato wastewater contained about 3.47 g/L total nitrogen and could be exploited for the production of industrial enzymes. Also, the availability of minerals and nitrogen in potato wastewater was essential to promote enzyme production (Huang *et al.*, 2003). The maximum β -CGTase production by *Bacillus licheniformis* (4.5 U/mL) (Sivakumar and Banu, 2011), *Bacillus* sp. TPR71H (30.34 U/mL) (Ravinder *et al.*, 2012c), and *Bacillus* sp. C26 (25.7 U/mL) (Eş *et al.*, 2016) were obtained using 0.4%, 0.5% and 1.0% (w/v) yeast extract, respectively. The decline in β -CGTase production at high yeast extract concentration could be explained by its effects on the surface charge, hydrophobicity, and the bacterial cell wall's carbon/nitrogen ratio (Schär-Zammaretti *et al.*, 2005).

The ability of immobilized cells to produce β -CGTase in a stable manner in the semi-continuous fermentation is the essential advantage. The culture activity was attained for β -CGTase production until the seventh cycle since enzyme production remained between 40.10–50.60 U/mL over the entire optimum period of operation for about 252 hours. Both Ravinder *et al.* (2012b) and Costa *et al.* (2015) reported comparable results, demonstrating that *Bacillus* sp. and *Bacillus circulans* immobilized cells had greater efficacy in β -CGTase production for repeated batch fermentation up to 6 and 12 batches, respectively. Another study reported that β -CGTase production by immobilized *Bacillus firmus*

and *Bacillus sphaericus* gave the higher rates of β -CGTase production for three cycles (Moriwaki *et al.*, 2014). The reduction in β -CGTase production from cycle to cycle can be clarified by referencing the diminishing availability of nutrients and product transport and capsule mechanical degradation (Rakmai and Cheirsilp, 2016).

The specific activity of the purified enzyme was about 63.90 U/mg with the fold purification and yield of 19.84% and 41.77%, respectively. Considering the previous studies of purification techniques, it was established that the yield gained was appreciable. The purification yield of CGTase from *Bacillus halodurans* by starch adsorption was 49.44% (More *et al.*, 2012), while from *Bacillus circulans* by Sepharose 6B was 55.14% (Guru *et al.*, 2012), besides, a yield of 36.9% for CGTase of *Geobacillus thermoglucosidans* by Sephadex G-100 (Jia *et al.*, 2018). The molecular weight of the purified enzyme was determined by SDS-PAGE analysis and the single protein band with a molecular weight of \sim 84.4 kDa was detected. The result was in accordance with β -CGTase produced by *Bacillus licheniformis* with one protein of 85.2 kDa (Bonilha *et al.*, 2006). The molecular weights of β -CGTase vary according to the bacterial species, 83 kDa for *Bacillus circulans* (Guru *et al.*, 2012), and 33 kDa for *Bacillus halodurans* (More *et al.*, 2012).

The highest level of enzyme activity was observed at a pH 8.0 with a broad range of pH stability (7.0–11.0) with optimum activity at 50–60°C, and the thermal stability was completely retained at 75°C. Consistent results have been reported by Bonilha *et al.* (2006). A broad pH range of 5.0–11.0 and temperature of 45–75°C was mentioned for CGTase of *Amphibacillus* sp. NPST-10 (Ibrahim *et al.*, 2012). Similarly, the highest β -CGTase activity of *Bacillus* sp. N-227 was at pH 6.0 and 70°C, with stability maintained at a range of pH 5.0–7.0 (Wang *et al.*, 2017). The β -CGTase obtained in the present study was associated with higher thermal stability than the β -CGTase of *Klebsiella pneumoniae* AS-22 (Gawande and Patkar, 2001) and *Bacillus agaradhaerens* (Martins and Hatti-Kaul, 2002), in both cases, the highest level of stability observed was 40°C. The stability of β -CGTase at alkaline pH and high temperatures are favorable for β -CD production in industrial settings. The enzyme's thermal stability can support its reaction with raw starch, and alkaline pH can lower the viscosity problem with elevated concentrations of substrate, thereby promoting the cost-effectiveness of cyclodextrin (Jia *et al.*, 2018).

The K_m and V_{max} of purified enzyme were estimated using Michaelis-Menten equation and Lineweaver-Burk plot (Mora *et al.*, 2012). The purified β -CGTase has a high affinity toward the raw potato starch, with a lower K_m value of 5.7×10^{-6} M with a V_{max} of 87.71 μ mol/mL/min (Fig. 10C). These results can be efficient compared with K_m and V_{max} values for β -CGTase from *Bacillus licheniformis* (Bonilha *et al.*, 2006), *Bacillus firmus* (Moriwaki *et al.*, 2007).

Different industries are concerned about the use of β -CGTase due to its ability to produce a high amount of β -CD from starch, preparation of inclusion complexes, and their low solubility that offers genuine steadiness (Wang *et al.*, 2018). β -CD production with raw and gelatinized potato starch using diverse β -CGTase concentrations was

evaluated. Raw potato starch granules were optimal for β -CD production, offering a 1.89-fold advantage over gelatinous potato starch. The β -CD production using raw potato starch offers benefits at high enzyme concentrations (25 U/g) where the weak binding affinity of β -CGTase for raw potato starch is a possibility that accounts for the relevance of high enzyme concentrations (Ibrahim *et al.*, 2013). Also, the raw potato starch was the optimal substrate for β -CD production (13.46 g/L) with β -CGTase of *Microbacterium terrace*, which bypassed the need to gelatinize starch (Rajput *et al.*, 2016b). Another study demonstrated that raw corn starch played a key role in elevated β -CD production with the β -CGTase of *Bacillus megaterium*, noting that α -amylase pre-treatment was unnecessary (Pishtiyski and Zhekova, 2006). Besides, the unfavorable effect of physical treatment on the starch structure at temperatures above 75°C adversely affects β -CD production (Kim *et al.*, 1997). Also, Jia *et al.* (2018) stated that the thermal stability of β -CGTase could support its interaction with raw starch which improves β -CD production through efficient use of β -CGTase without degradation of the produced β -CD and avoid uses of physical treatment of starch substrates. These notices confirm our results as the obtained β -CGTase has thermal stability at 75°C which supports its interaction with raw potato starch.

Conclusions

The results indicated that potato wastewater could be used as a new inexpensive medium to produce of β -CGTase by *Bacillus licheniformis* YSMT. When compared to free cells, alginate encapsulated cells were associated with higher enzyme production at the optimized conditions of 36 h, pH 8.0, and 50°C, with adding the potato starch (0.4%) and yeast extract (0.6%) to the potato wastewater medium. The seventh-batch semi-continuous repeated cultivation process is utilized for maximized the cost-effectiveness of β -CGTase. The highest level of purified enzyme activity was observed for the enzyme at 50°C and pH 8.0, and full stability was retained until 75°C. Additionally, it was effective when considered in relation to raw potato starch. The potato wastewater as a cost-effective fortify medium for β -CGTase production and the high thermal stability with an efficiency of the enzyme for β -CD synthesis from raw starch is intrinsic for industrial issues.

Author Contribution: Conceptualization, (YSM and SAA); Data curation, (SAA, MH, MM); Formal analysis, (SAA, THT, LIF, MH); Funding acquisition, (YSM and SAA); Methodology, (THT, LIF, SA and MH.); Writing–Original draft, (SAA, YSM, MM, and MH); Writing–Review and editing, (YSM, SA and SAA). All authors have read and agreed to the published version of the manuscript.

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