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Original Article

Cyclodextrin glycosyltransferase from a newly isolated alkalophilic *Bacillus* sp. C26

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Abstract

A cyclodextrin glycosyltransferase (CGTase) producing bacterium was isolated from a farm soil sample and identified as *Bacillus* sp. C26. The highest CGTase production by *Bacillus* sp. C26 was achieved using 1% (w/v) sago starch and 1% (w/v) yeast extract as carbon source and nitrogen source, respectively with an initial pH of 10 and a temperature of 37ºC. Other carbon sources such as soluble starch and rice starch were almost as good as sago starch but tapioca and corn were poor substrates for CGTase production. There was very little difference between the various N-sources tested i.e. peptone, tryptone and yeast extract. Under the optimal conditions, the specific growth rate and CGTase production rate of *Bacillus* sp. C26 were 0.193 h^{-1} and 5.94 U mg^{-1} h^{-1}, respectively. The partially purified CGTase from *Bacillus* sp. C26 exhibited 2 peaks of optimum pH at 6.0 and 8.5 and had an optimum temperature of 65ºC. The enzyme was stable from pH 7.0 to 9.0 and retained its high activity up to 50ºC.

Key words: alkalophilic bacterium, *Bacillus* sp., cyclodextrin, CGTase, sago starch

1. Introduction

Cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19), is a unique extracellular enzyme that can degrade starch and related substrates to form cyclodextrins (CD) as products. Cyclodextrins are oligosaccharides consisting of 6 to 12 glucose units joined by a-1,4 glucosidic linkages. The 3 most common forms of cyclodextrin are α-CD, β-CD and γ-CD consisting of six, seven and eight glucose molecules, respectively shaped into a conical doughnut shape. Their hydroxyl groups face the outside making them water soluble while the interior cavity is relatively apolar and hydrophobic so they can easily form inclusion complex with many organic hydrophobic substances. This alters the physicochemical properties of the guest molecule; thus increasing its water solubility and stability. These benefits are of use in a wide variety of areas such as in the food, chemical, pharmaceutical, analytical, diagnostic, cosmetic, agriculture, and as stabilizing agents in the plastic industries, emulsifiers, antioxidants and other industrial areas (Hedges, 1992; Cao *et al*., 2005). Most CGTases produce β-CD as the main product and this is more suitable for industrial use since stable inclusion complexes can be easily prepared due to the low solubility of β-CD in water. β-CD is most easily obtained by CGTase activity and the α-CD and γ-CD are relatively rare (Gawande and Patkar, 2001).

Bacteria are regarded as important sources of CGTases. Some known CGTase producers are *Klebsiella pneumoniae* AS-22 (Gawande and Patkar, 2001), *Micrococcus* sp., *Thermoanaerobacterium thermosulfurigenes*, *Bacillus alkalophilic* CGII (Freitas *et al*., 2004), *Bacillus* G1 (Ibrahim *et al*., 2005), *Bacillus* sp., *Thermoanaerobacter* sp. 501 (Alcalde *et al*., 2001), and others. The genus *Bacillus* is the main source of bacterial CGTase. CGTase producing bacteria can be isolated from various sources, for example *Bacillus* G1 was isolated locally from a soil sample in Malay-

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sia (Ibrahim *et al*., 2005), alkalophilic *Bacillus* sp. G1 was isolated from soil samples collected from rubber plantations in Pekan Nenas Johor (Illias *et al*., 2002), *Bacillus circulans* DF 9R was isolated from rotten potatoes (Rosso *et al.,* 2002) and an alkalophilic *Bacillus* sp. TS1-1 was isolated from the soil (Mahat *et al.,* 2004).

This study focuses on the optimization of medium composition for CGTase production by a bacterium, isolated locally from farm soil. The optimal conditions for producing CGTase, including pH and temperature optima were also investigated. Finally, a study was made of some of the primary characteristics of the enzyme.

2. Material and Methods

2.1 Screening and isolation of bacteria

Bacteria were isolated from soil collected from corn plantations at Prince of Songkla University, Thailand, in 2007. Soil samples were suspended in normal saline, serially diluted and then plated on a Horikoshi II agar plate containing (w/v) 1.0% soluble starch, 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, 0.02% $MgSO_4 \times 7H_2O$, 0.02% phenolphthalein, 1.0% Na_2CO_3 and 1.5% agar, pH 10 (Illias *et al.,* 2002). Plates were incubated at 37°C for 24 h. The bacterium colony that produced the largest clear halo zones and highest CGTase activity was selected and identified by 16S rDNA for further studies.

2.2 Preparation of bacterium inoculum for CGTase production

A loopful of fresh bacterial culture was inoculated into 5 mL Horikoshi II broth and incubated at 37°C, with shaking at 200 rpm for 24 h. Five percent of the cell suspension with an optical density of about 1.0 (660 nm) was then inoculated into a 250 mL conical flask containing 50 mL medium and again incubated at 37°C, 200 rpm for 48 h. Experimental enzyme studies were carried out on a cell-free supernatant after cultivated cells were sedimented at 8,000 rpm for 15 min. The supernatant was used as a crude enzyme solution for assaying enzyme activity.

2.3 Partial purification of CGTase

The culture broth harvested after 48 h growth was centrifuged at 8,000 rpm for 15 min to sediment the bacteria, and solid ammonium sulfate was added to the supernatant at 4°C, to 70% saturation and the precipitate obtained was collected by centrifugation. The precipitates were dissolved in 20 mM phosphate buffer (pH 7.0 at 4°C) and the solution was dialyzed by using a membrane with an 8,000 Da molecular cut-off at 4°C in the same buffer. The partial purified enzyme thus obtained was employed for further studies.

2.4 Activity and stability of CGTase

The effect of pH on the activity of the enzyme was measured at pH values from 4.0 to 10.0 at 60°C using 0.1 M sodium acetate buffer (pH 4.0-5.0), 0.1 M sodium phosphate buffer (pH 6.0-8.0) and 0.1 M glycine-NaOH buffer (8.5-10). The effect of temperature on the enzyme activity was also measured at temperatures between 30-90°C.

The pH stability of the enzyme was also determined by incubating the enzyme at 60°C for 1 h using 0.1 M sodium acetate buffer (pH 4.0-5.0), 0.1 M sodium phosphate buffer (pH 6.0-8.0) and 0.1 M glycine-NaOH buffer (8.5-10), then assaying for remaining activity. The enzyme was incubated at temperatures from 30 to 80°C for 1 h, then tested for remaining activity to estimate its thermal stability.

2.5 Analytical methods

Cell growth was followed by measuring cell protein content. Cell protein was extracted by hydrolyzing the cell pellet with 1 N NaOH at 100°C for 10 min. The extracted cell protein content was determined according to the method of Lowry *et al*. (1951) using dried bovine serum albumin (BSA) as a standard.

CGTase activity was assayed by the method of based on the reduction in the colour intensity of phenolphthalein after complexing with -CD (Illias *et al.*, 2002). The CGTase activity was initially measured by mixing 0.5 mL of 4% soluble starch in 0.1 M phosphate buffer pH 8.0 and 0.5 mL of CGTase solution, and incubating at 60°C for 10 min. The reaction was stopped by boiling for 5 min. Then 0.5 ml of 0.02 % phenolphthalein in 0.005 M Na_2CO_3 was added. The reduction in the colour intensity was measured at 550 nm. One unit of CGTase was defined as the amount of enzyme that formed 1 mmol of β-CD min^{-1} under standard conditions. Standard graph was plotted with β-CD. The CGTase yield was calculated from CGTase activity produced by 1 mg of cell protein.

To measure β-CD by a HPLC method, the filtrate obtained from the ultrafiltration unit was then treated with glucoamylase to convert any remaining linear dextrins into glucose. This was then filtered through a 0.45 mm cut-off microfilter (Millipore) and injected (sample-loop size = 20 μL) into the HPLC system to determine the concentrations of cyclodextrin (Kinalekar *et al.,* 2000). β-CD were determined by a HPLC method under the following conditions: Column, Zorbax Carbohydrate 4.6x150 mm, 5 mm; mobile phase, acetonitrile:water (70:30); flow rate, 1.5 ml min^{-1}; column temperature, 35°C; Refractive Index Detector (Agilent 1100 series HPLC).

All experiments were performed in two or three replicates. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference ($p \leq 0.05$) was used to separate means, using the SPSS software.

3. Results and Discussion

3.1 Isolation of CGTase producing bacteria

During the selection of CGTase producer microorganisms from soil collected from a corn plantations, serial dilutions were plated and incubated at 37°C for 24 h. 76 bacterial colonies that were surrounded by a clear halo were treated to isolate a pure bacterial culture and the pure isolates from each colony were tested for CGTase production. Thirty three isolates produced CGTase and 3 of them that produced highest activity when grown in Horikoshi II broth (identified as C7, C26 and C28) were selected for further study. As a result, isolate C26 that produced the most CGTase was then selected and identified. Based on the partial 16S rDNA sequence data (536/538 bp), isolate C26 was identified as belonging to the genus *Bacillus* with the similarity of 99%.

Figure 1 shows chromatograms of standard β-CD (a) and the product from the enzymatic reaction of *Bacullus* sp. C26 (b). The retention times of both CD products were the same. This result confirmed that *Bacillus* sp. C26 could produce CGTase.

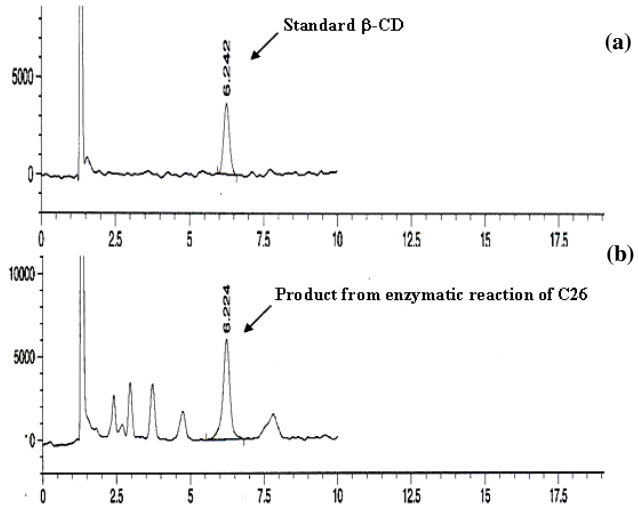

Figure 1. HPLC chromatogram of (a) standard β-cyclodextrin and (b) the product from an enzymatic reaction of *Bacillus* sp. C26 after ultrafiltration and glucoamylase treatment.

3.2 Optimization of CGTase production from *Bacillus* sp. C26

3.2.1 Effect of carbon source

The effect of different carbon sources on cell growth and the CGTase production of *Bacillus* sp. C26 were assayed by substituting the soluble starch in the basal medium with other carbon sources. The highest CGTase activity was achieved using rice starch as a carbon source (Table 1). However, the highest CGTase yield (134.22 U/mg-cell protein) was obtained with sago starch. Soluble starch was almost as good as sago starch but tapioca and corn starches were poor substrates for CGTase production. It has been reported that some starches may contain an inducer for CGTase production. The difference in CGTase activity obtained with different starches may be due to the differences in their physical structures and chemical properties (Ibrahim *et al.,* 2005). In other studies, *Bacillus* G1 produced its highest CGTase activity when tapioca starch was used as a carbon source (Ibrahim *et al*., 2005), while maximum CGTase production of *Bacillus strearothermophilus* was achieved with soluble starch (Jin-Bong *et al*., 1990). In this study, since sago starch is a local agricultural product in southern Thailand and it also gave the highest yield of CGTase, it was selected as the optimal carbon source for *Bacillus* sp. C26.

Figure 2(a) demonstrates the effect of sago starch concentrations on growth and CGTase production by *Bacillus* sp. C26 after cultivation for 48 h. *Bacillus* sp. C26 could produce maximum CGTase activity at a low sago starch level of 1%. Maximum CGTase production by *B. circulans* DF 9R was obtained with 1.5% cassava starch (Rosso *et al.,* 2002) and by the alkalophilic *Bacillus* sp. TS1-1 and *Bacillus stearothermophilus* HR1 with 1.48% and 1.5% of sago starch, respectively (Mahat *et al.,* 2004; Rahman *et al*., 2004).

3.2.2 Effect of nitrogen source

The effect of nitrogen source on cell growth and CGTase production was investigated using sago starch at 1%. It was found that 1% yeast extract gave the highest CGTase yield (139.57 U/mg-cell protein) (Table 2), and this was selected as the optimal nitrogen source for CGTase produc-

Table 1. Effect of carbon sources on cell protein content, CGTase activity and CGTase yield of *Bacillus* sp. C26

Carbon source (1 %)	Cell protein content (mg/mL)	CGTase activity (U/mL)	CGTase yield (U/mg-cell protein)
Soluble starch	$0.211\pm0.003^{a*}$	19.05 ± 0.62^{c}	90.16 ± 3.68^{c}
Tapioca starch	0.165 ± 0.003^{b}	3.03 ± 0.30^{d}	18.37 ± 0.79^{e}
Corn starch	0.154 ± 0.006^{c}	4.27 ± 0.63^{d}	27.78 ± 4.04^{d}
Sago starch	0.167 ± 0.003^{b}	22.37 ± 0.12^{b}	134.22 ± 4.28^{a}
Rice starch	0.217 ± 0.001^{a}	25.12 ± 0.33^{a}	115.69 ± 1.14^{b}

* Different letters in the same column indicate significant differences ($p<0.05$).

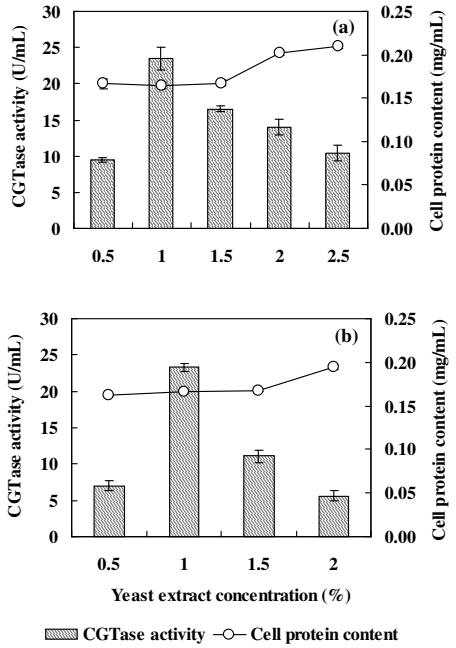

Figure 2. Effects of sago starch concentrations (a) and yeast extract concentrations (b) on cell growth and CGTase production from *Bacillus* sp. C26 at 37°C after cultivation for 48 h.

tion from *Bacillus* sp. C26. However, there was no significant difference in cell growth and CGTase production between peptone, yeast extract and a mixture of yeast extract and peptone (1:1). Ibrahim *et al*. (2005) investigated the influence of organic and inorganic nitrogen sources on CGTase production from *Bacillus* G1. They found that CGTase production was higher when organic nitrogen source was present in the medium. Moreover, the highest activity was obtained when peptone was used in the medium. Gawande and Patkar (2001) also observed a maximum production of CGTase when peptone was used as nitrogen source for *K. pneumoniae* AS-22, while urea and NH_4NO_3 inhibited the growth of bacteria and therefore no enzyme was secreted.

Figure 2(b) shows the effect of yeast extract concentrations on cell growth and CGTase production from *Bacillus* sp. C26 after cultivation for 48 h. There was no significant difference in cell growth of *Bacillus* sp. C26 at 0.5-1.5% of yeast extract. Although *Bacillus* sp. C26 grew well at a high yeast extract content of 2%, the CGTase activity decreased. This result was different from the CGTase production of alkalophilic *Bacillus* sp. TS1-1 as the highest activity of CGTase was observed at high concentration of yeast extract of 1.89% (Mahat *et al*., 2004). Rahman *et al*. (2004) also reported that maximum production of CGTase from *B. stearothermophilus* HR1 was obtained at high concentration of peptone of about 2%.

3.2.3 Effect of initial growth pH

The effect of the initial pH of the growth medium on cell growth and CGTase production was studied by varying the Na_2CO_3 (as buffer) to give different initial pH values of 7, 8, 9, 10 and 11. As shown in Figure 3(a), the optimal initial pH for maximum cell growth and CGTase production was 10. At an initial pH lower or higher than 10, CGTase production decreased. This result indicated that *Bacillus* sp. C26

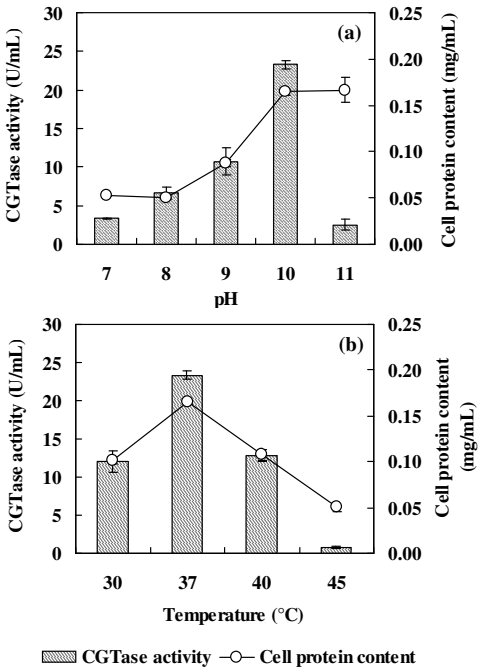

Figure 3. Effects of initial pH (a) and temperature (b) on cell growth and CGTase production from *Bacillus* sp. C26 at 37°C after cultivation for 48 h.

Table 2. The effect of nitrogen sources on cell protein content, CGTase activity and CGTase yield of *Bacillus* sp. C26

Nitrogen source (1 %)	Cell protein content (mg/mL)	CGTase activity (U/mL)	CGTase yield (U/mg-cell protein)
Peptone	0.172±0.001[a*]	23.49±0.59[a]	136.58±3.68[a]
Yeast extract	0.167±0.003[a]	23.31±0.56[a]	139.57±0.26[a]
Peptone+Yeast extract	0.165±0.001[a]	22.39±0.62[a]	134.22±4.74[a]
Tryptone	0.125±0.002[b]	12.52±0.59[b]	99.82±1.40[b]

* Different letters in the same column indicate significant differences ($p<0.05$).

was an alkalophilic bacterium. Similar results have also been reported for *Bacillus* G1 (Ibrahim *et al.,* 2005) while CGTase production from *B. circulans* DF 9R in cultures was maximal at a lower initial pH of 8.3 (Rosso *et al*., 2002).

3.2.4 Effect of incubation temperature

Bacillus sp. C26 was cultured at various incubation temperatures 30, 37, 40 and 45°C as shown in Figure 3 (b). The optimal temperature for cell growth and CGTase production was at 37°C. At temperature lower or higher than 37°C, cell growth and CGTase decreased. A similar result for cell growth and CGTase activity of *B. circulans* was reported by Rosso *et al*. (2002).

Figure 4 shows the cell growth and the CGTase production profile of *Bacillus* sp. C26 growing under the optimal conditions of 1% sago starch, 1% yeast extract, initial pH 10 and incubation temperature of 37°C. The CGTase production increased significantly during the log phase. The maximum CGTase concentration of 25.7 U mL^{-1} was obtained during the decline phase of the microbial growth. This level of CGTase activity was relatively higher than that of *B. stearothermophilus* HR1 and *Bacillus* sp. G1 which produced lower CGTase activities of 14.20 U mL^{-1} (Rahman *et al*., 2004) and 19 U mL^{-1} (Illias *et al*., 2002), respectively. The specific growth rate and production rate of *Bacillus* sp. C26 under these optimal conditions are 0.193 h^{-1} and 5.94 U mg^{-1} h^{-1}, respectively.

3.3 Effect of pH and temperature on CGTase activity and stability

3.3.1 Effect of pH on enzyme activity and stability

The activity of partially purified CGTase was measured at pH values ranging from 4-10 at 60°C. Optimal activity showed two peaks at pH 6.0 and 8.5 as shown in Figure 5(a). This could be explained by the presence of different kinds of CGTase (neutral and alkaline). Cao *et al.*

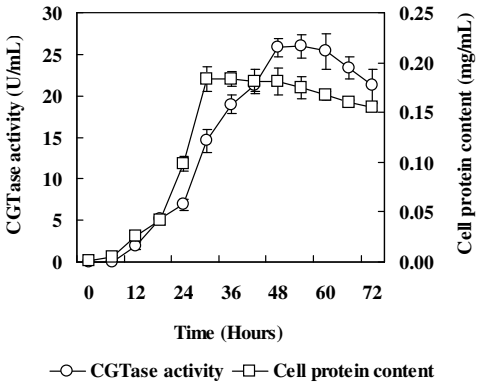

Figure 4. Cell growth and CGTase production profiles of *Bacillus* sp. C26 in basal medium: 1% sago starch, 1% yeast extract, initial pH 10.0 and 37°C.

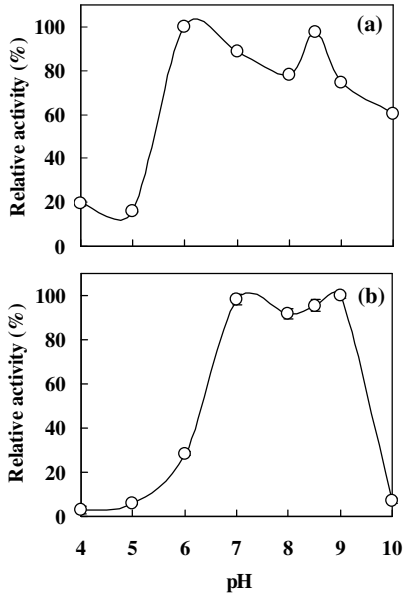

Figure 5. Effects of pH on CGTase activity (a) and stability (b). CGTase was incubated at 60°C for 10 min for activity and 1 h for stability studies at different pH values.

(2005) also found that the CGTase activity from *Bacillus* sp. showed two peaks at pH 5 and 8.5 on the pH activity curve. The effect of pH on the stability of CGTase is shown in Figure 5 (b). At 60°C, the enzyme was stable over the wide pH range of 7-9. However, the enzyme activity decreased drastically beyond that range with almost no activity below pH 6.0 and above pH 10.0. Gawande and Patkar (2001) reported that CGTase from *K. pneumoniae* AS-22 was most stable in the pH range of 6-9, with maximum stability at pH 7.5.

3.3.2 Effect of temperature on enzyme activity and stability

The effect of temperature on CGTase activity was investigated for 10 min at temperatures between 30-80°C. The result showed that CGTase from *Bacillus* sp. C26 was most active in the temperature range of 50-70°C with the optimum temperature at 65°C, pH 8.5 (Figure 6 a). This was higher than the optimum temperature of *K. pneumoniae* AS-22 CGTase at 45°C (Gawande and Patkar, 2001), *B. alkalopfilic* CGII at 55°C (Freitas *et al.,* 2004), *Bacillus* sp. G1 (Sian *et al*., 2005) and *Bacillus* sp. 7-12 (Cao *et al*., 2005) at 60°C. It is important that the reaction temperature for cyclodextrin production from starch should be moderately high. At high temperatures starch is converted into a better structure for enzyme attack and the reaction rate can be improved. Although Illias *et al*. (2002) reported that the optimum temperature of CGTase from *Bacillus* sp. G1 was 70°C, its thermal stability has not been tested. When kept in buffer at pH 8.5 for 1 h, the CGTase from *Bacillus* sp. C26 showed a wide thermal stability from 30 to 50°C, although the enzyme rapidly lost its activity above 55°C and only 6% activity remained at 70°C as shown in Figure 6(b).

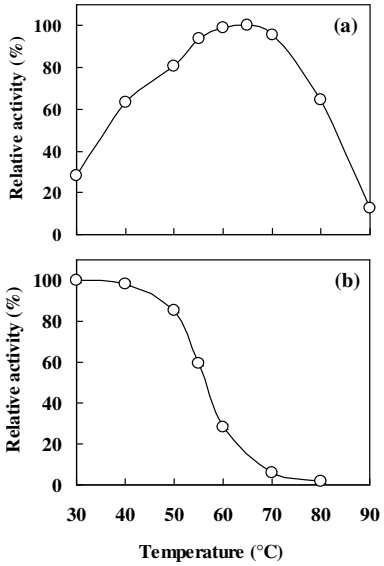

Figure 6. Effects of temperature on CGTase activity (a) and stability (b). CGTase was incubated in 0.1 M glycine–NaOH buffer pH 8.5 for 10 min for activity and 1 h for stability studies at various temperatures.

4. Conclusion

The CGTase producing alkalophilic *Bacillus* sp. C26 was successfully isolated and characterized. The optimal nutritional and culture conditions for CGTase production from *Bacillus* sp. C26 were in good agreement with many other CGTase enzymes studied by other research works. However, the CGTase from *Bacillus* sp. C26 in this study exhibited the highest maximum activity at a higher temperature than the others. This property of the CGTase could enhance cyclodextrin production from sago-starch at a high temperature of 60°C.

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