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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 5(6): 688-692, 2002

Cyclodextrin Glucanotransferase Producing Alkalophilic *Bacillus* sp. G1: its Cultural Condition and Partial Characterization of the Enzyme

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Abstract: A cyclodextrin glucanotransferase producing bacteria was isolated from soil sample and identified as *Bacillus* sp. G1. Crude CGTase production was observed to be maximum after 28 h incubation at 37°C with CGTase activity reading 19 U/ml. The enzyme production was shown to be growth associated and maximum CGTase production was detected during the decline phase. The effect of nutritional requirements on the CGTase production was carried out in this study. Commercial grade tapioca starch and peptone were found to be the best carbon and nitrogen sources respectively for production of CGTase from *Bacillus* sp. G1. Effect of mineral salts on the CGTase production showed that $MgSO_4$ gave the highest activity. However nutrient such as urea, $CuSO_4$ and NH_4NO_3 were found to inhibit the production of CGTase. Characterization of the crude CGTase showed that the optimum temperature and pH for the crude enzyme activity were 70°C and 6.0, respectively.

Key words: Cyclodextrin glucanotransferase, *Bacillus* sp., characterization, alkalophilic

Introduction

Cyclodextrin glucanotransferase (1,4-α-D-glucan: 1,4-α-D-glucopyranosyltransferase) (CGTase) is a unique extracellular enzyme which can degrade starch and related substrates to form cyclodextrin (CD) as products. Cyclodextrin are oligosaccharides consisting of 6 to 12 glucose units joined by α - (1, 4) – linkages. Cyclodextrin exists in three major forms, which are α-CD, β-CD and γ-CD consisting of six, seven and eight glucose molecules respectively. Due to the distribution of hydroxyl groups, the interior cavity is relatively apolar compared with water and this characteristic make cyclodextrin easy to form an inclusion that complexes with a variety of poorly water soluble compound. Therefore cyclodextrin have wide variety of application such as stabilizer and carrier in pharmaceuticals, cosmetic, household products, food and agrochemical industries. Cyclodextrin glucanotransferase (CGTase) is one of the most unusual members of the amylolytic glucosylase family which have strong hydrolytic and synthetic capabilities (Tao *et al*., 1991). This enzyme (CGTase) not only can hydrolyze starch and related substrate but also performs cyclizing, coupling and dispropotionation activity towards glucose residues (Tonkova, 1998). A number of CGTases from microorganisms have been described. These enzymes are derived from several different bacterial sources and vary in properties such as pH optimum, temperature optimum, stability and the relative properties of α, β and γ cyclodextrin produced. In most organisms, CGTase are produced extracellularly. Most of the CGTase isolated from bacteria form α and β-cyclodextrin with small amounts of γ-CD. Bacteria are the only known sources of CGTase. Some known sources of CGTase producers are *Bacillus macerans* (Pocsi *et al*., 1998), *Bacillus stearothermophilus* (Stefanova *et al*., 1999), *Bacillus circulans* (Bovetto *et al*., 1992), *Bacillus firmus* (Goel and Nene, 1995), *Brevibacterium* sp. (Mori *et al*., 1995), *Klebsiella oxytoca* (Lee *et al*., 1992), *Klebsiella pneumoniae* (Gawande and Patkar, 1999) and *Thermoanaerobacterium thermosulfurigenes* (Wind *et al*., 1998). The genus *Bacillus* is the main source of the bacterial cyclodextrin glucanotransferase. The present paper describes the isolation of CGTase producing bacterium from Malaysia soil, growth and effect of nutrient supplement on CGTase production and finally primary characterization of the crude enzyme.

Materials and Methods

Screening and Isolation of bacteria: Bacteria were isolated from soil samples collected from rubber plantations in Pekan Nenas Johor, Malaysia in 1998. Samples were suspended in sterile water, serial diluted and then plated on 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 % $MgSO_4.7H_2O$, 0.02 % phenolphthalein and 1.0 % Na_2CO_3 (autoclave separately). Plates were incubated at 37 °C for 24 h. Bacterial colonies which produced the largest clear halo zones were selected for further studies.

Characterization and identification of bacteria: The bacteria were identified based on the microbiological and biochemical characteristics done according to the method of Bergey's Manual Determinative Bacteriology (Holt *et al*., 1994). The bacteria were maintained on Horikoshi II agar slant without the addition of phenolphthalein.

Preparation of bacterium inoculum: A loopful of fresh bacterial culture was inoculated into a 250 ml conical flask containing 20 ml medium: 1.0 % (w/v) soluble starch, 0.5 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.1 % (w/v) KH_2PO_4, 0.02 % (w/v) $MgSO_4.7H_2O$, and 1 % (w/v) Na_2CO_3 and incubated with shaking (200 rpm) for 18 h. The cells were then washed once with normal saline. Ten percent of the cells with optical density of about 0.5 (550nm) were then inoculated into a 500ml conical flask containing 100 ml medium and incubated at 37°C at a speed of 200 rpm for 24 to 48 h.

The cyclization activity of CGTase: Enzyme assay was carried out according to the method of Kaneko (1987). After incubation for 24 to 48 h, the culture was centrifuged at 5000 rpm for 2 min. Crude enzyme solution (0.5ml) was added to 1.0 ml of 0.04 g soluble starch in 1.0 ml of phosphate buffer (pH 6.0). After incubation at 70°C for 10 min in water bath, 0.03M NaOH was immediately added to the solution to stop the reaction. Then 0.5 ml of 0.02 % phenolphthalein in 0.005M Na_2CO_3 was added. The reduction in the colour intensity was measured at 550nm using a Hitachi spectrophotometer. One unit enzyme activity was defined as 1μmol of β-CD formed per minute. Standard graph was plotted with β-CD.

Growth curve: Ten percent of the bacterial inoculum was inoculated into eight 500 ml conical flasks, each with 200 ml of Horikoshi II medium. The culture was incubated at 37°C in an orbital shaker at a speed of 200 rpm for 48 h. In the first 24 h, 3.7 ml aliquots of the culture were withdrawn at two hourly intervals and in the next 24 h aliquots were withdrawn every four hourly. The optical density at 550nm, was determined and enzyme assays and viable counts were carried out.

Effect of the nutrient supplement on CGTase production

Effect of different types of carbon sources on CGTase production: Standard Horikoshi II liquid medium which normally contain soluble starch as carbon source was replaced by other types of carbon sources such as corn starch, wheat starch, rice starch, sago starch, tapioca starch and potato starch at a concentration of 1 % (w/v). After 24 h of incubation, the culture was then centrifuged at 5000rpm for 2 min. The supernatant was assayed for CGTase activity.

Effect of different nitrogen source on CGTase production: Various types of nitrogen source such as tryptone, polypeptone, soya bean meal, corn steep liquor, urea and casein at a concentration of 0.5 % (w/v) were used to replace peptone as in the standard Horikoshi II broth for CGTase production. After 24 h of incubation the culture was assayed for CGTase production following centrifugation.

Effect of mineral salts on CGTase production: $MgSO_4$ normally added in the standard Horikoshi medium was replaced by various mineral salts such as $MgCl_2$, $FeCl_2$, $FeCl_3$, $FeSO_4$, KCl, NaCl, $CaCl_2$, K_2SO_4, $MnSO_4$, $CuSO_4$ and $ZnSO_4$ at a concentration of 0.02 % (w/v). For control, medium without any mineral salts added was used. Following inoculation with bacterial sample, the medium was incubated at 37 °C. After 24 h of incubation, the culture was centrifuged at 5000 rpm for 2 min. Enzyme assays were carried out on the crude CGTase to determine the CGTase activity.

Effect of pH and temperature on enzyme activity: The effect of pH and temperature on enzyme activity was studied. The effect of pH was monitored where reaction mixture containing soluble starch as substrate with the addition of enzyme solution in a 0.1M buffer solution with varying pH ranging from 4.0 to 10.0 (pH 4.0 and 5.0 using potassium acetate buffer; pH 6.0, 7.0 and 8.0 using potassium phosphate buffer; pH 9.0 and 10.0 using 0.2 M glycine-NaOH). The reaction mixture was incubated for 10 min at 70°C. To determine the optimum temperature for optimal CGTase activity, the reaction mixture containing soluble starch as substrate in 0.1 M potassium phosphate buffer (pH 6.0) was incubated with crude enzyme solution at various temperatures ranging from 20 to 80°C for 10 min.

Results and Discussion

Isolation and some characteristics of CGTase producing bacteria: After incubation at 37°C for 24 h, clear halos were observed around nine bacteria of which an isolate designated G1 gave the highest activity when grown in Horikoshi broth. This isolate was further studied for its potential in CGTase production. Strain G1 is rod-shaped bacteria 3.0-3.3µm and a width of 0.7-0.9µm. Strain G1 formed oval-shaped endospores. G1 grew well between 25 to 40°C in Horikoshi medium. Due to the alkalophilic nature of strain G1, it can only grow on alkaline medium with pH above 8.0. No growth was detected when grown in medium with pH lower than 7.0. The detail characteristics of G1 are listed in Table 1. It could be derived as belonging to the genus *Bacillus* and therefore designated *Bacillus* sp. G1.

Table 1: Some characteristics of alkalophilic *Bacillus* sp. G1

Shape	Rod
Cell width, µm	0.5
Cell length, µm	3.0
Gram staining	+
Sporulation	+
Spore shape	Oval
Motile	+
Growth temperature, °C	25 - 40
Growth pH	7.8 - 11
Hydrolysis of starch	+
Gelatin liquefaction	+
OF test	+
VP reaction	-
Methyl red reaction	-
Production of indole	+
Utilization of citrate	-
Reduction of nitrate	-
Production of urease	-
Catalase	+
Oxidase	+
Growth on NaCl (% w/v)	
5	+
7	+
10	+
Acid produced from utilization of glucose, lactose, sucrose and xylose.	+

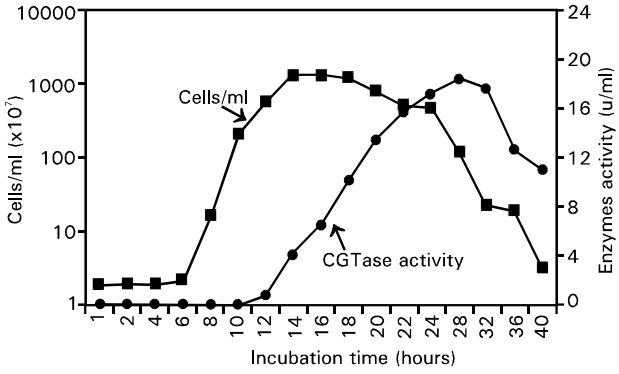

Fig. 1: Growth and CGTase production profile of alkalophilic *Bacillus* sp. G1

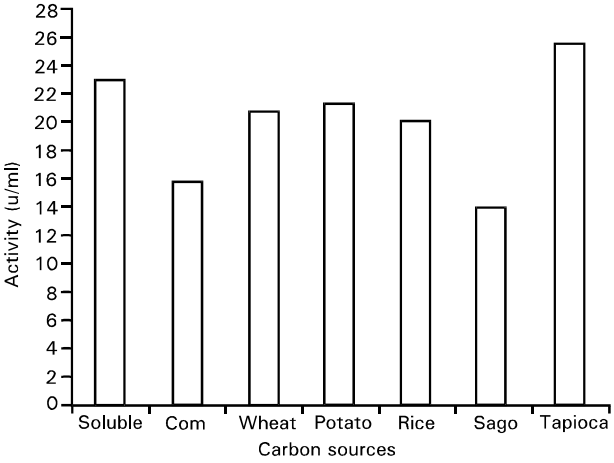

Fig. 2: Effect of carbon sources on CGTase production

Growth curve: The growth was assessed by the total number of cells via viable count. The data obtained suggests a typical growth curve with a typical lag, log, stationary and death phase (Fig. 1). Following inoculation with fresh culture, a slow lag period of about 6 h was observed before the beginning of the log phase. The long lag phase could mean that cells were taking time to adapt to a new environment. A *Bacillus* sp. isolated from soil by Thatai *et al.* (1999) also had a lag phase of about 5 h before it began its log phase. However they managed to reduce the lag phase from 5 h to 2 h by adding a small amount of carbon source such as

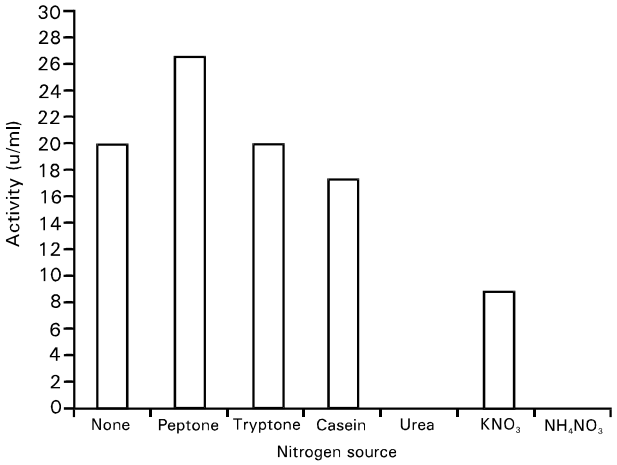

Fig. 3: Effect of nitrogen sources on CGTase production

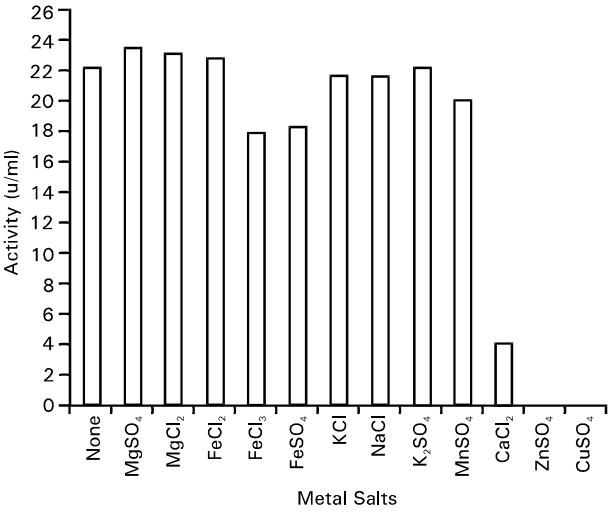

Fig. 4: Effect of minerals on CGTase production

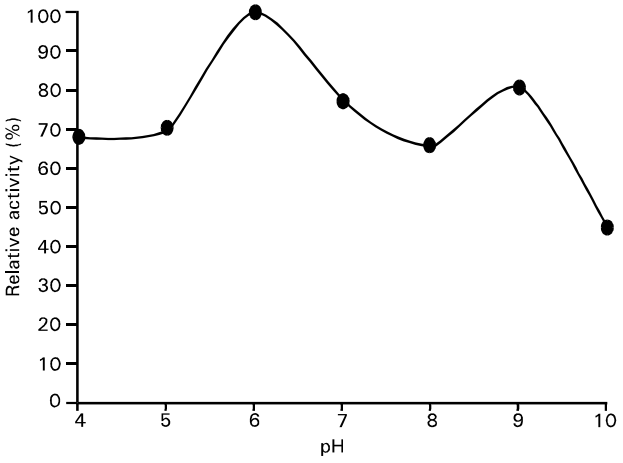

Fig. 5: Effect of pH on CGTase activity at 70°C

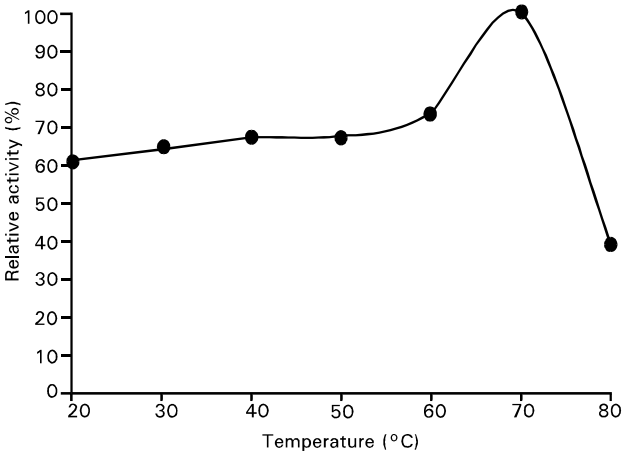

Fig. 6: Effect of temperature on CGTase activity at pH 6.0

glucose. This was also observed by Nogrady *et al.* (1995) where in the presence of glucose the lag phase was significantly shorter than with starch as the carbon source. During the log phase, *Bacillus* sp. G1 grew logarithmically and reached maximum growth (1.24×10^{10} cells per ml) in about 14 h before it entered stationary phase. During log phase the growth rate of cells gradually increased, therefore cells grew at a constant, maximum rate. The duration of log phase varies depending on the organism and the composition of the medium. For *Bacillus* sp. G1 the specific growth rate is $\mu = 1.1369$ with a doubling time *td* of 0.61 h. The cells then entered stationary phase after 14 h of incubation. For *Bacillus* sp. G1 the stationary phase lasted for 4 h only and this is relatively short. The number showed rapid viable cells decreased after 18-20 h of incubation.

CGTase production was significant during log phase and it continued to increase (Fig. 1). The maximum yield obtained was 19 U/ml achieved after 28 h of incubation, which is during the decline phase of the microbial growth. Similar trends were also observed during the production of CGTase by *Bacillus* sp. 5-6 (Jun *et al.* 1998) and *Bacillus* sp. (Varavinit *et al.*, 1997). They observed that CGTase was also produced during log phase and continued into the death phase where the maximum yield of CGTase was obtained. Nogrady *et al.* (1995) and Pocsi *et al.* (1998) reported that extracellular CGTase may not be involved in the degradation of starch during the exponential phase growth of the bacteria, because the enzyme is only released into the culture medium after all the starch has been consumed. The reason behind this is because the enzyme is anchored to the cell membrane during the exponential (log) phase and retained in between the cell membrane and the cell wall during the early stationary phase. The enzyme is only released into the culture medium during the late stationary phase and during cell lysis. After 24 h of incubation, the viable cell number declines as the culture enters the death phase (Fig. 1). Prolonged incubation may reduce enzyme production probably due to the accumulation of toxic products in broth cultures that inhibit the cells from growing as suggested by Stanbury and Whitaker (1984).

Effect of carbon sources on enzyme production: *Bacillus* sp. G1 could grow well on most of the carbon sources tested. All of the carbon sources used were raw starch without pretreatment. A maximum activity of 25.63 U/ml was obtained when industrial grade tapioca starch was used as carbon source. Other starches were used such as soluble starch, potato starch, wheat starch, rice starch, corn starch and sago starch and their CGTase activities

are shown in Fig. 2. The difference in activity obtained with different sources of starches may be due to the differences in their physical structure. The supplemented starch in the medium serves as an inducer for CGTase production. Gawande and Patkar (1999) observed maximum CGTase production with dextrin as carbon source for *Klebsiella pneumoniae* AS-22 while Do *et al.* (1993) and Pocsi *et al.* (1998) found that soluble starch is the best carbon source for *Bacillus firmus* and *Bacillus macerans* respectively. In our study, glucose was not tested for CGTase production. However studies carried out by Stefanova *et al.* (1999) found that at 0.5 % (w/v) glucose was found to be the most suitable substrate for CGTase production. Contradicted results by Varavinit *et al.* (1997) showed that the production of CGTase by *Bacillus* sp. MP523 was repressed significantly by glucose.

Effect of nitrogen sources on enzyme production: CGTase production was higher when an organic nitrogen source was present in the medium. Enzyme production using inorganic nitrogen sources was found to be low compared with organic nitrogen source (Fig. 3). The best nitrogen source for the production of CGTase was peptone since it gives the highest CGTase activities. Gawande and Patkar (1999) also observed a maximum production of CGTase when peptone was used as nitrogen source for *Klebsiella pneumoniae.* A 0.5 % (w/v) of urea and NH_4NO_3 was found to inhibit the growth of bacteria G1 and therefore no enzyme was secreted. However, Thatai *et al.* (1999) observed that urea concentration at 10mM was able to increase the CGTase activity by more than 200 % for *Bacillus* sp. Casein gave no effect on the production of CGTase since CGTase activity detected was about the same compared to that when no nitrogen source was added.

Effect of minerals on enzyme production: The effect of minerals on the production of CGTase was studied by substituting $MgSO_4$ with other minerals at 0.02 % (w/v). The enzyme activity was assayed after 24 h of incubation. $MgSO_4$ gave the highest activity at 26.14 U/ml and may be important in the production of CGTase, whereas, $CuSO_4$ and $ZnSO_4$ totally inhibits cell growth therefore no CGTase activity was detected (Fig. 4). CGTase from *Bacillus autolyticus* exhibits 92% of relative activity when 2mM $CuSO_4$ was used (Tomita *et al.*, 1993). Thatai *et al.* (1999) reported that Zn^{2+}, a known inhibitor enhanced the activity by 150 % and the presence of $CaCl_2$ increased the production of CGTase. Nevertheless, in this work $CaCl_2$, seemed to depress the production of CGTase. In most cases, calcium ions needed to stabilize CGTase at higher temperature where it will react as a protective agent but are not involved in increasing the reaction rate of the enzyme. However the concentration of $CaCl_2$ might affect the CGTase production and therefore a suitable concentration should be used if increase in activity is to be achieved. The other minerals were ranked in descending order as follows: $MgSO_4$ > $MgCl_2$ > $FeCl_2$ > none > KCl > NaCl > K_2SO_4> $MnSO_4$ > $FeSO_4$ > $FeCl_2$ > $CaCl_2$ > $ZnSO_4$ and $CuSO_4$.

Effect of pH and temperature on enzyme activity: The CGTase activity of *Bacillus* sp. G1 was found to be optimal at pH 6 (Fig. 5). Assays were done in phosphate buffer and incubation was for 10 min at 70 °C. Though pH 7 was found to be optimum, another peak was observed at pH 9. The enzyme activity declined sharply at pH above 9. The trend of having more than one pH optimum for CGTase activity was also observed by Kaneko *et al.* (1987) for crude CGTase from an alkalophilic *Bacillus* sp. In their case, the enzyme exhibited 3 distinct pH optimums at pH 4.5, 6.0 and 8.5. However, they gave no explanation for such an occurrence.
Turnes and Bahar (1996) also observed similar optimum results on CGTase from another alkalophilic *Bacillus* sp. The crude CGTase from this bacterium gave three optimum pH values at 5.0, 7.0 and 9.0. Their rationale for the 3 peaks is the presence of different kind of CGTase (acid, neutral and alkaline) in the culture filtrate. Our result is almost similar to that of Gawande and Patkar (1999) as crude CGTase from *Klebsiella pneumoniae* AS-22 exhibited two peaks, a small are at pH 7.5-8.0 and a larger one at pH 9.5.
The effect of different temperatures on the activity of CGTase was done by incubating the reaction mixture at temperatures ranging from 20- 80°C. Optimum temperature for maximum CGTase activity was found to be 70 °C as shown in Fig. 6. At temperature higher than 70 °C, activity of CGTase declines sharply may be because the CGTase could be denatured above 70 °C. Relative activity also decreased by about 60% at 80 °C. However the crude CGTase from *Bacillus* sp. G1 was found to be quite stable at temperature ranging between 20 to 60°C.
We have successfully isolated and characterize CGTase producer microorganism as *Bacillus* sp. G1 in our laboratory at Department of Bioprocess Engineering. The optimal pH and temperature for the CGTase activity were in good agreement with many CGTase studied by other researchers. Medium formulation screening have improved the production of the CGTase form *Bacillus* sp. G1.

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