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Enhancement of *Amphibacillus* sp NPST-10 Cyclodextrin Glucanotransferase Production by Optimizing Physio-Environmental Factors

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The present study was conducted to optimized culture conditions and environmental factors that enhance cyclodextrin glucanotransferase (CGTase) production by a previously isolated, new alkaliphilic *Amphibacillus* sp. NPST-10. Production of CGTase was growth-associated, with enzyme synthesis starting during the early exponential-growth phase and increasing as the cells grew exponentially, reaching the highest concentration levels (0.6 U/ml) at the end of the stationary growth phase (38 h) and remaining constant during prolonged incubation up to 60 h. While mono- and disaccharides supported *Amphibacillus* sp. NPST-10 growth, maximal CGTase production was detected using various types of starch as the main carbon source, with maximum enzyme production observed with 1.75% soluble starch. Organic nitrogen sources enhanced CGTase production, with a mixture of peptone, yeast extract, and casamino acids (1.2% w/v) supporting maximal production. In addition, *Amphibacillus* sp. NPST-10 was able to grow in a wide range of NaCl concentrations up to 15%, with maximal growth and CGTase production at 6% and 3%, respectively, indicating the halophilic nature of this bacterium. Furthermore, maximal *Amphibacillus* sp. NPST-10 growth and CGTase production were detected at pH 9.5 and 9.0, respectively, indicating that *Amphibacillus* sp. NPST-10 is alkaliphilic in nature. Moreover, maximum enzyme production was observed at 35°C with aeration by shaking at 150 rpm. Addition of Zn^{2+}, Cu^{2+}, Co^{2+}, or Ba^{2+} ions led to reductions both in bacterial growth and CGTase production, particularly at a high metal concentration (10 mM), by approximately 83.6%, 72.7%, 57.6%, and 31.6%, respectively. However, inclusion of Ca^{2+} ions in the culture medium led to a significant increase in CGTase production of approximately 16%. These results indicated that optimizing the culture medium and environmental conditions increased *Amphibacillus* sp. NPST-10 CGTase production by approximately two fold.

Key words: Cyclodextringlucanotransferase, *Amphibacillus* sp, Alkaliphiles, Optimization, Production.

Cyclodextringlucanotransferase (CGTase; EC 2.4.1.19) is an extracellular enzyme that converts starch and related polysaccharides, such as dextrins, amylose, and amylopectin, to cyclodextrins (CDs) [Lo *et al*., 2007]. CDs are grouped into three main types, α-, β-, and γ-CDs, which are composed of six, seven, and eight α-(1,4)-linked glycosyl units, respectively. CD is a doughnut-shaped molecule that has a hydrophobic internal cavity and hydrophilic external surface. With this structure, CDs can accommodate various organic molecules without forming covalent bonds. In addition, the physical properties of CDs can be altered by chemical modifications to enhance their inclusion ability [Loftsson and Duchene, 2007]. These properties lead to broad applications for CDs in various settings and industries, including food, pharmaceuticals, cosmetics, environmental protection, analytics, bioconversion, fermentation, and textiles [Astray *et al*., 2009; Otero-Espinar *et al*., 2010]. Because the guest molecule is

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individually surrounded by a CD (or derivative), the molecule is micro-encapsulated from the eternal environment. This encapsulation may lead to favorable changes in the chemical and physical properties of the guest molecule, including fixation of volatile substances, solubility improvement, protection of oxygen-sensitive substances, protection against microorganismal degradation, and stabilization against light [Szerman *et al*., 2007; Valle *et al*., 2009].

The production of CGTases has been reported for several bacterial sources, including *Bacillus* spp [Kunamneni *et al*., 2007; Kitcha *et al*., 2008; Moriwaki *et al*., 2009; Gastón *et al*., 2009], *Klebsiella* spp. [Gawande *et al*., 2001& 2003], *Brevi bacterium* [Morio *et al*., 1995], and thermophilic bacteria and archaea [Thiemann *et al*., 2004; Lee *et al*., 2007; Avci and Donmez, 2009]. Most CGTases convert starch into a mixture of α-, β-, and γ-CDs with different ratios, depending on the enzyme source [Goh *et al*., 2007; Leemhuis *et al*., 2010]. Among the three types of CDs, β-CD is of great interest due to the favorable size of its non-polar cavity, which is suitable for encapsulating several guest molecules, and its low solubility in water, which facilitates its separation from the reaction mixture. Moreover, β-CD inclusion complexes are easily prepared and stable [Valle *et al*., 2009; Otero-Espinar *et al*., 2010]. Because the separation of different CDs is costly and time-consuming, CGTases that predominantly synthesize one type of CD are of great interest [Biwer *et al*., 2002; Thiemann *et al*., 2004; Kitcha *et al*., 2008]. We previously isolated and characterized a novel CGTase from alkaliphilic *Amphibacillus* sp. NPST-10, which was isolated from a hyper saline soda lake located in northern Egypt, that produces β-CD as the major CD type [Ibrahim *et al*., 2012]. Because CGTase production depends on the reaction of the producer organisms with the substrate, altering environmental factors, such as nutrient type and concentration in the medium, can lead to increased production of CGTases [AbdRahman *et al*., 2004]. In this work, we report the optimization of CGTase production by alkaliphilic *Amphibacillus* sp. NPST-10 by manipulating physio-environmental factors, such as cultivation conditions and nutrient concentration and composition in the production media.

MATERIALS AND METHODS

Strain and culture conditions

The alkaliphilic, CGTase-producing *Amphibacillus* sp. NPST-10 used in this study was previously isolated from hyper saline soda lakes located in the Wadi El-Natrun valley in northern Egypt [Ibra him*et al*., 2012]. The alkaline agar medium (pH 10.5), which was modified from Horikoshi II medium [Horikoshi, 1999], contained soluble starch (10 g/l), yeast extract (5 g/l), casamino acids (5 g/l), peptone (5 g/l), NaCl (50 g/l), Na_2CO_3 (15 g/l), and agar (15 g/l). Na_2CO_3 and trace elements solutions were autoclaved separately before addition to the medium.

Enzyme assay

The cyclization activity of CGTase was measured as -CD-forming activity according to a method previously described, with some modifications [Martins *et al*., 2002]. A 750-µl aliquot of 1% (w/v) starch solution prepared in 50 mMTris-HCl buffer (pH 8) was pre-incubated at 50°C for 5 min. A 100-µl enzyme sample was added to the reaction mixture, and the mixture was then incubated for 20 min at 50°C. The reaction was then quenched by adding 375 µl of 0.15 M NaOH. Subsequently, 100 µl of 0.02% (w/v) phenolphthalein prepared in 5 mM Na2CO3 was added. The mixture was incubated at room temperature for 15 min, and the color intensity was measured at 550 nm. One unit of CGTase activity was defined as the amount of enzyme releasing 1µmol of β-CD per min under the defined assay conditions. A calibration curve was made using 0.001-0.5 µmol of β-CD in 50 mMTris-HCl (pH 8). The protein concentration was determined according to the method described by Bradford, with bovine serum albumin as the standard [Bradford, 1976].

Production of CGTase by *Amphibacillus* sp NPST-10

Colonies of the selected strain were transferred to 250-ml Erlenmeyer flasks containing 50 ml of alkaline liquid culture medium with the same composition as the solid medium (except for the presence of agar) and incubated overnight at 30°C with orbital shaking (120 rpm). This culture was used to inoculate (2%) a 1-l Erlenmeyer flask containing 250 ml of the same medium, which was cultivated under the same conditions. Samples (2 ml) were withdrawn at 2-h intervals for a total of 60

h to measure growth and CGTase activity. The collected samples were centrifuged at 10000 × g at 4°C, and the pellets were washed twice using Tris buffer (pH 7) and resuspended in 1 ml of the same buffer. Absorbance was measured at 600 nm using Trisbuffer (pH 7) as blank and reported as bacterium growth. Triplicate measurement were taken at each time point to calculate growth. The CGTase activity and protein content in the cell-free supernatants were measured as described above.

Optimization of CGTase production

Effect of carbon source

The effects of different carbon sources on the production of CGTaseby *Amphibacillus* sp. NPST-10 were studied. The soluble starch in Horikoshi II medium was substituted with other carbon sources. The carbon sources used in this study were monosaccharides (glucose, fructose, ribose, xylose, galactose, and arabinose), disaccharides (maltose, cellobiose, lactose, sucrose, and trehalose), tri-saccharides (raffinose), and polysaccharides (soluble starch, potato starch, sago starch, wheat starch, rice starch, tapioca, pectin, and xylan), in addition to some industrial byproducts (molasses and whey). The various carbon sources were autoclaved separately and added to the medium on an equal carbon basis. Furthermore, the effects of different concentrations of the best carbon source were also investigated [Rahman *et al*., 2004; Zain *et al*., 2007; Yap *et al*., 2010].

Effect of nitrogen source

The effects of different nitrogen sources on the production of CGTase and the growth of *Amphibacillus* sp. NPST-10 were studied. The mixture of peptone and yeast extract in Horikoshi II medium was substituted with different sources of organic nitrogen (peptone, yeast extract, casamino acids, gelatin, casein, tryptone, and urea) and inorganic nitrogen (ammonium chloride and ammonium sulfate), in addition to soybeans, baker's yeast, wheat bran, and corn steep liquor. The sources were added to the production medium on an equal nitrogen basis. Furthermore, the effects of various concentrations of the best nitrogen source were investigated.

Table 1. Effects of various carbon sources on growth andCGTase production by *Amphibacillus sp*. NPST-10. Standard deviationranged from 0.2 to 3.0%

	Carbon Source	Growth (OD600)	CGTase specific activity (U/mg protein)
	Glucose	3.60	0.70
	Fructose	2.40	0.80
Monosaccharide	Ribose	2.30	0.41
	Xylose	1.66	0.61
	Galactose	2.50	0.60
	Arabinose	3.30	0.77
	Maltose	3.30	0.55
	Cellobiose	1.80	0.20
Disaccharides	Lactose	2.22	0.80
	Sucrose	3.30	0.90
	Trehalose	2.44	0.60
Tri-saccharides	Raffinose	2.00	0.18
	Soluble Starch	3.00	4.20
	Potato starch	3.20	3.70
	Sago starch	2.96	3.81
Polysaccharides	Wheat Starch	3.11	3.92
	Rice Starch	3.20	3.88
	Tapioca	3.20	4.00
	Pectin	1.10	0.17
	Xylan	1.00	0.22
Industrial waste	Molasses	3.44	1.00
	Whey	2.22	0.76

In addition to carbon and nitrogen source testing, the effects of NaCl concentration (0-15%), metal ion concentration (Mg^{+2}, Mn^{+2}, Zn^{+2}, Ca^{+2}, Cu^{+2}, Co^{+2}, K^{+}, Fe^{+2}, and Ba^{+2}), initial pH of the culture medium (pH 4-12), incubation temperature (25-60°C), and aeration level (0-350 rpm) were also investigated [De Freitas *et al*., 2004; Kitcha *et al*., 2008; Ravinder *et al*., 2012].

RESULTS AND DISCUSSION

An experiment to observe *Amphibacillus* sp. NPST-10 growth and CGTase production over time in rich alkaline production medium was conducted.The production of CGTase was found to be growth-associated, with enzyme synthesis beginning during early exponential growth (6 h) and increasing as the cells grew exponentially. CGTase reached its highest level (0.6 U/ml) at the end of the stationary growth phase (38 h), and it remained constant during prolonged incubation up to 60 h. This result was similar to the production profile of CGTase by *Bacillus* sp. TPR71H [Ravinder *et al*., 2012]. However, CGTase production by *Amphibacillus* sp. NPST-10 was different from the data reported by Jamuna *et al*. (1993) on the pattern of CGTase production by Bacillus cereus. In the case of B. cereus, CGTase was produced during early exponential growth and reached its maximum level during the mid-sporulating stage (16 h). Production of CGTase by *Bacillus circulans* var. alkalophilus reached its maximum level after a longer incubation period of 48-50 h [Paloheimo *et al*. 1992] *Amphibacillus* sp. NPST-10 CGTase activity was found to be extracellular throughout growth; no cell-associated CGTase activity was detected. Most bacterial CGTases are extracellular enzymes [Cao *et al*., 2005; Ong *et al*., 2008; Li *et al*., 2010; Atanasova *et al*., 2011].

Table 2. Effects of various nitrogen sources on CGTase production by *Amphibacillus sp*. NPST-10. Standard deviation ranged from 0.0 to 4.0%.

Nitrogen Source	Growth (OD600)	CGTase specific activity (U/mg protein)
Peptone	2.88	3.1
Yeast extract	3.2	3.88
Casamino acids	2.86	3.66
Peptone, yeast extract, and casamino acids	3.25	4.65
Gelatin	1.77	2.25
Casein	2.3	2.5
Tryptone	2.95	4
Soybean	2.88	3.3
Baker's yeast	2.66	2.4
Wheat bran	2.8	3.44
Corn steep liquor	2.72	3.1
Urea	1.88	2
Ammonium chloride	1.2	3.1
Ammonium sulfate	1.35	2.86
Sodium nitrate	0.9	0.65
Potassium nitrate	0.8	0.71

Table 3. Effects of various metal ions on cell growth and CGTase production. Standard deviation ranged from 0.0 to 7.0%.

Metal	1 mM		5 mM		10 mM	
	Growth (OD600)	CGTase-specific activity (U/mg protein)	Growth (OD600)	CGTase-specific activity (U/mg protein)	Growth (OD600)	CGTase-specific activity (U/mg protein)
None	3.13	5.5				
Mg	3.2	5.6	3.12	5.7	2.88	5.8
Mn	3.1	5.7	3.33	5.8	2.9	5.7
Zn	2.6	3.5	2.22	2.77	1.44	0.9
Ca	3.31	6.25	3.26	6.4	3.2	6.35
Cu	2.5	3.45	2.31	2.11	1.88	1.5
Co	2.8	4.8	2.65	4.66	2.65	2.61
K	3.22	5.4	3.15	5.3	3.15	5
Fe	3	4.11	2.88	3.15	2.7	2.33
Ba	3.21	4.88	2.88	4.76	3	3.76

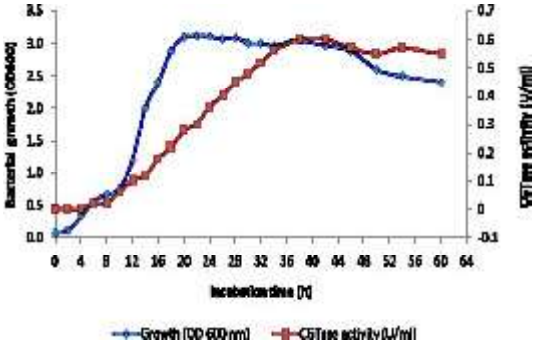

Fig. 1. Growth and CGTase production by *Amphibacillus* sp. NPST-10. Bacteria weregrown in alkaline production medium at pH 10.4 for 60 h at 30°C and 100 rpm, andgrowth and CGTase activity were determined. Standard deviation values ranged from 0.0 to 0.03 U/ml

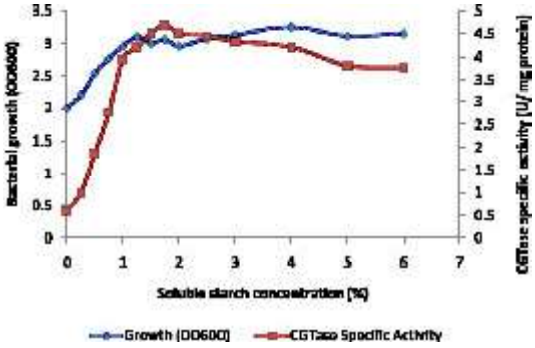

Fig. 2. Effect of soluble starch concentration on CGTase production by *Amphibacillus* sp. NPST-10. Standard deviation ranged from 0.2 to 4.0%

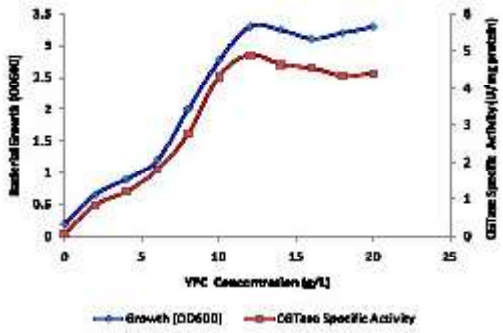

Fig. 3. Effect of different concentrations of a peptone, yeast extract, and casamino acid mixture on bacterial growth and CGTase production by *Amphibacillus* sp. NPST-10. Standard deviation ranged from 0.0 to 6.0%

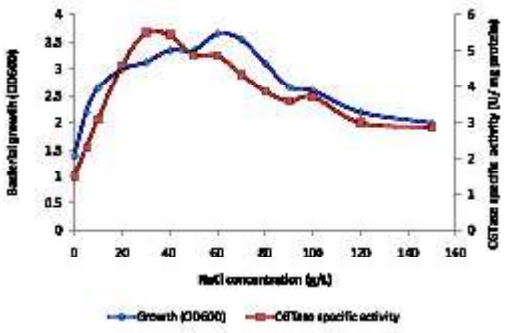

Fig. 4. Effect of NaCl concentration on cell growth and CGTase production by *Amphibacillus* sp. NPST-10. Standard deviation ranged from 0.0 to 5.0%.

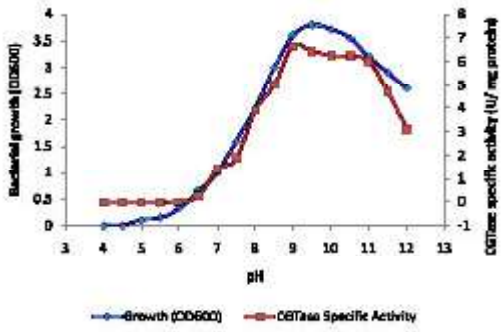

Fig. 5. Effect of pH on growth and CGTase production by *Amphibacillus* sp. NPST-10. Standard deviation ranged from 0.0 to 7.0%.

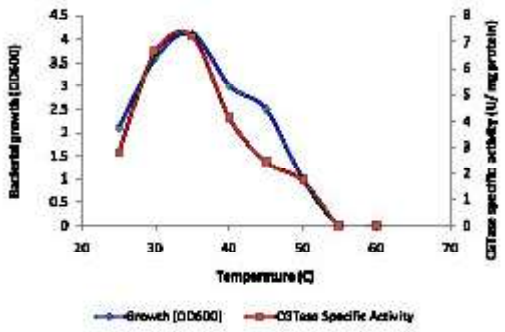

Fig. 6. Effect of incubation temperature on growth and CGTase production by *Amphibacillus* sp. NPST-10. Standard deviation ranged from 0.0 to 6.5%.

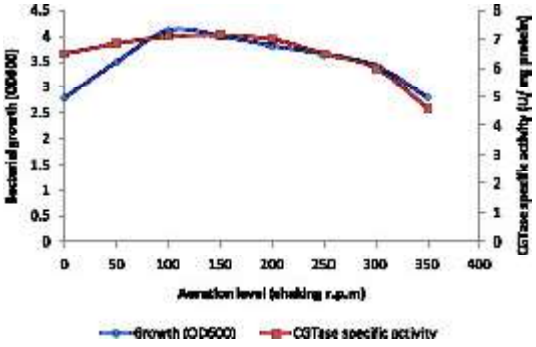

Fig. 7. Effect of aeration level on growth and CGTase production by *Amphibacillus* sp. NPST-10. Standard deviation ranged from 0.0 to 6.5%

Optimization of CGTase production

Effect of carbon source on growth and CGTase production

The carbon source is known to be a determining factor in CGTase synthesis rate. Therefore, the effects of different carbon sources, including mono-, di- and polysaccharides, on the production of CGTase by *Amphibacillus* sp. NPST-10 were investigated. The results presented in Table 1 show a significant effect of the carbon source on bacterial growth and CGTase production. While most of the mono- and disaccharides supported *Amphibacillus* sp. NPST-10 growth, the greatest CGTase production was found using various types of starch as the main carbon source, with soluble starch performing best (4.2 U mg-1 protein). These results were in agreement with those reported forCGTase production by *Bacillus* sp. TPR71H [Ravinder *et al*., 2012] and *Bacillus macerans* [Pocsi *et al*., 1998], in which soluble starch was the best carbon source for CGTase production. The differences in CGTase activity obtained with different starches may be due to differences in their physical structures and chemical properties (Ibrahim *et al*., 2005). In addition, it has been reported that some starches may contain an inducer for CGTase production [Kitcha *et al*., 2008]. Maximum CGTase production by *Bacillus* sp. TS1-1 and *Bacillus* G1 was observed using tapioca starch [Ibrahim *et al*., 2005; Zain *et al*., 2007], while sago starch was the best carbon source for CGTase production by *Bacillus stearothermophilus* HR1 and *Bacillus lehensis* S8 [Rahman *et al*., 2004; Yap *et al*., 2010]. Gawande *et al*. (1998) reported that corn starch showed maximum CGTase yield by *Bacillus firmus* and that dextrin was the best carbon source for CGTase production by *Klebsiella pneumoniae* AS-22 [Gawande *et al*., 2003]. However, there was no significant difference in CGTase production by *Bacillus firmus* with various starch sources [Higuti *et al*., 2004]. The concentration of carbon sources is another very important parameter for enzyme production by many organisms, especially when a carbon source is required to induce production of the enzyme (Gawande *et al*., 2003). Therefore, the effect of soluble starch concentrations on CGTase production by *Amphibacillus* sp. NPST-10 was further investigated. As shown in Figure 2, both growth and CGTase production were increased by increasing the soluble starch concentration, with maximal bacterial growth and CGTase production (4.7 U mg-1 protein) observed at concentrations of 1.25% and 1.75%, respectively. Further increases in starch concentration did not increase enzyme production, and CGTase production began to decrease at higher concentrations. These results were relatively consistent with previous reports. Maximum CGTase yield was observed with 1.5% starch as a carbon source by *B. circulans* DF 9R (Rosso *et al*., 2002), *Bacillus stearothermophilus* HR1 (Rahman *et al*., 2004), *Bacillus alkalophilic* CGII (De Freitas *et al*., 2004), and *Bacillus* sp. TS1-1 (Mahat *et al*., 2004). However, 4% (w/v) carbon source was reported as the optimal concentration for enzyme production in other bacteria (Gawande *et al*., 2003; Ibrahim *et al*., 2005). The reduction of CGTase production with higher starch concentrations is due to catabolite repression that occurs above a certain concentration of carbon substrate [Gawande*et al*. 2003; Ai-Noi *et al*., 2008; Blanco *et al*., 2009] and because of the high viscosity of media with high starch concentrations, which causes poor oxygen uptake [Zain *et al*., 2007].

Effect of nitrogen source on growth and CGTase production

The effects of different organic and inorganic nitrogen sources on the growth and production of CGTaseby *Amphibacillus sp*. NPST-10 were studied. Generally, organic nitrogen sources supported both bacterial growth and CGTase production better than inorganic nitrogen sources (Table 2). Maximal bacterial growth and CGTase production (4.65 U mg-1 protein) were detected using a mixture of peptone, yeast extract,

and casamino acids as the nitrogen source. The effect of the concentration of the peptone, yeast extract and casamino acid mixture on bacterial growth and CGTase production was further investigated. The results in Fig 3 showed that both growth and CGTase production by *Amphibacillus sp*. NPST-10 were increased by increasing the nitrogen concentration. Maximal enzyme production (of 4.9 U mg-1 protein) was observed at a nitrogen concentration of 12g/l, and itremained constant with further increases in nitrogen concentration. These results are similar to those reported for CGTase production by *Bacillus alkalophilic* CGII, in which maximum enzyme production was detected using 1.5% of a mixture of yeast extract and peptone [De Freitas *et al*., 2004]. Different types of microbial strains require different nitrogen levels to support the production of CGTase. Kitcha *et al*. (2008) reported maximum CGTase production by *Bacillus* sp. C26 using 1% yeast extract. In addition, that study reported no significant differences in cell growth or CGTase production when cells were grown on peptone, yeast extract, or a 1:1 mixture of yeast extract and peptone. Moreover, yeast extract was the best nitrogen source for CGTase production by several other bacteria, including *B. lehensis* S8 [Yap *et al*., 2010], alkaliphilic *Bacillus* sp. C26 [Kitcha *et al*., 2008], and alkaliphilic *Bacillus* sp. TS1-1 [Mahat *et al*., 2004]. This may be because yeast extract contains some essential micronutrient or inducer capable of enhancing CGTase production [Gawande and Patkar, 1999]. Peptone has been reported as the best nitrogen source for maximum CGTase production by other bacteria [Gawande and Patkar, 2001; Ibrahim *et al*., 2005]. However, an inorganic nitrogen source (0.4% w/v ammonium sulfate) was reported to support maximum CGTase yield by *B. circulans* DF 9R [Rosso *et al*., 2002].

Effect of sodium chloride on growth and CGTase production

The results presented in Fig 4 show the influence of NaCl concentration on cell growth and CGTase production by *Amphibacillus* sp. NPST-10. The results indicated that *Amphibacillus* sp. NPST-10 can grow over a wide range of NaCl concentrations from 0 to 15%; its maximal growth at 6% demonstrates the halophilic nature of this bacterium [Horikoshi, 2011]. However, maximum CGTase production was detected with 3%NaCl.

Effect of metal ions on growth and CGTase production

Different concentrations of various metal ions were added to the culture medium, and their effects on cell growth and CGTase production were investigated. Addition of Zn^{2+}, Cu^{2+}, Co^{2+}, and Ba^{2+} ions reduced bacterial growth, and CGTase production was reduced, particularly at a high concentrations (10 mM), by approximately 83.6%, 72.7%, 57.6%, and 31.6%, respectively (Table 3). Addition of Mg^{2+} and Mn^{2+} ions caused a slight enhancement of CGTase production. However, including Ca^{2+} ions in the culture medium significantly increased CGTase production by approximately 16%. Enhancement of CGTase activity by Ca^{2+} ions appears to be a characteristic feature of different bacterial CGTases [Hirano *et al*., 2006; Alves-Prado *et al*., 2007]. The effects of metal ions on CGTase production by *Amphibacillus* sp. NPST-10 are relatively similar to those reported for the CGTase of *Bacillus macorous* [Wang *et al*., 2004], except that Zn^{2+} increased CGTase production by *B.macorous*.

Effect of initial pH on growth and CGTase production

Generally, medium pH plays a vital role in microorganismal growth and the production of extracellular enzymes. The influence of pH on growth and CGTase production by *Amphibacillus* sp. NPST-10 was investigated using culture media with various initial pH values ranging from 4 to 12. The results presented in Fig. 5 show that *Amphibacillus* sp. NPST-10 could grow and produce CGTase over a wide pH range from 6 to 11. Maximal growth and enzyme production were observed at pH 9.5 and 9.0, respectively, demonstrating the alkaliphilic nature of this bacterium. At an initial pH other than 9, CGTase production decreased. Similar results have also been reported for *Bacillus* G1 [Ibrahim *et al*., 2005], *B. lehensis* S8 [Yap *et al*., 2010], B. alkalophilic CGII [De Freitas, *et al*., 2004], and *B. circulans* DF 9R [Rosso *et al*., 2002].

Influence of temperature on growth and CGTase production

Bacterial growth and CGTase production by *Amphibacillus* sp. NPST-10were studied at various temperatures (25 to 60°C). Bacterial growth and CGTase production increased with increasing incubation temperatures to 35°C, which appears

to be the optimum temperature for both *Amphibacillus* sp. NPST-10 growth and CGTase production (Fig 6). In addition, maximum bacterial growth and CGTase production occurred at incubation temperature up 50°C, and growth ceased at higher temperatures. Temperature optima in the range of 55 to 65°C have been reported for CGTases from alkaliphiles [Cao *et al*., 2005; Sian *et al*., 2005; Hirano *et al*., 2006; Alves-Prado *et al*., 2007; Atanasova *et al*., 2011]. CGTases from thermophilic and hyperthermophilic bacteria and archaea usually show higher thermostability [Tachibana *et al*., 1999 and Rashid *et al*., 2002].

Influence of aeration level on growth and CGTase production

Culture aeration is one of the most important parameters affecting microbial growth and enzyme production. The effect of aeration level on bacterial growth and CGTase production by *Amphibacillus* sp. NPST-10 was investigated by incubating the cultures at various shaking speeds ranging from 0 to 350 rpm. As shown in Fig 7, both bacterial growth and CGTase production increased with increasing aeration up to 150 rpm. Increasing the aeration level to 350 rpm led to slight decreases in both growth and CGTase production by *Amphibacillus* sp. NPST-10.

CONCLUSION

The present study aimed to investigate the effect of the culture medium composition and other physiological factors on cell growth and CGTase production by a previously isolated alkaliphilic *Amphibacillus*, sp. NPST-10, to optimize CGTase production. Maximal CGTase production by *Amphibacillus* sp. NPST-10 was detected when the bacteria were cultured in medium containing 1.75% soluble starch, 1.2% peptone, yeast extract and casamino acid mixture, 3% NaCl, and 5mM $CaCl_2$ and incubated at35°Cand pH 9.0 with aeration at 150 rpm. Addition of Zn^{2+}, Cu^{2+}, Co^{2+}, or Ba^{2+} ions led to reductionsin both bacterial growth and CGTase production, particularly at high metal concentrations. However, addition of Ca^{2+} ionsto the culture medium significantly increasedCGTase production. CGTase production by *Amphibacillus* sp. NPST-10 was enhanced by approximately two fold by manipulating physio-environmental factors, such as medium composition, nutrient concentrations,and cultivation conditions.

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