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Potential cyclodextrin glycosyltransferase producer from locally isolated bacteria

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Cyclodextrin glycosyltransferase (CGTase) is one of the most important groups of microbial amylolytic enzymes that have been used for degradation of starch to yield cyclodextrin (CD) via cyclization reaction. The increasing demand for CD in industrial application has led to an extensive study about CGTase which begin with screening, isolation and characterization of CGTase-producing bacteria. The identification of CGTase producer involves the use of solid media containing phenolphthalein and methyl orange as indicators that was detected by the colour changes. The formations of clearance zone around the bacterial colony in the starch-containing medium were observed and the diameters were measured to gauge the hydrolytic efficiency of the bacteria. Out of 65 soil bacterial samples screened, *Bacillus* sp. NR5 UPM was identified as the most prolific CGTase producer, which produced highest CGTase activity (11.709 U/ml) and highest β-CD concentration (2.504 mg/ml) with α-CD:β-CD:γ-CD ratio was 0.5:91.1:8.4 when using raw soluble starch as a substrate. It also showed as the best CGTase producer when using sago starch as a substrate (15.514 U/ml). This isolate was known as a raw starch-degrading enzyme producer since it has the capability to use raw starch as a substrate. Thus, in the future, this new isolate perhaps can share the biggest market in industrial application.

Key words: Cyclodextrin, cyclodextrin glycosyltransferase, starch.

INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19) is a multifunctional enzyme which catalyzes four related reactions: cyclizing, coupling, disproportionation and hydrolysis. By means of the cyclizing activity, CGTases convert starch and related substrates into cyclodextrins (CDs) (Ishii et al., 2000). The production of CD that was catalysed by CGTase are in the mixture of α-CD, β-CD and γ-CD, containing 6, 7 and 8 glucose residues, respectively. However, CGTase also is capable of synthesizing other types of CDs in very small amounts, which contain nine-, ten-, eleven-, or twelve-membered rings (Szejlti, 1998). The CGTase producer came from various species such as *Bacillus, Klebsiella, Micrococcus, Brevibacterium, Thermoactinomyces* and *thermophilic archea* are the major producer. Soluble starch (Yampayont et al., 2003), potato (Avci and Donmez, 2009), corn (Kim et al., 1995, 1997), wheat starch (Gawande et al., 1999; Gawande and Patkar, 2001), etc can be used as a sole source of carbon for the production of CD with the sago starch as a potential alternative substrate (Nisanart et al., 2003; Charoenlap et al., 2004). The unique hydrophobic interior cavity and hydrophilic surface of CDs enable it to

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Abbreviations: CGTase, Cyclodextrin glycosyltransferase; **CD,** cyclodextrin; **HPLC,** high performance liquid chromatography.

be widely used in encapsulating a wide range of organic and inorganic compounds, thereby changing their physical and chemical properties of the inclusion complex formed within them (Gawande et al., 1998). The conversion of starch into CDs gives many benefits to industrial application. Thus, as a result, CDs find increasing application in biotechnology, food, pharmaceutical, cosmetic, agricultural, medicine and chemical industries (Szejtli, 1998; Li et al., 2007).

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade, unless stated otherwise. Soluble starch was purchased from Merck. Sago starch is of industrial grade. Yeast extract and peptone were purchased from BDH. Phenolphthalein was purchased from Merck. Dipotassium hydrogen phosphate (K_2HPO_4), magnesium sulfate ($MgSO_4$) and sodium carbonate (Na_2CO_3) was purchased from Merck. Cyclodextrin standards, α-, β- and γ-CD and sugar standards were purchased from Sigma-Aldrich and BDH Laboratory.

Screening and isolation of the bacterial strains from soil

Soil samples were collected from various places such as corn, potato and tapioca plantation in Malaysia. Criteria for soil selection were mainly based on its soil's pH (pH 5 - 6). Collected soil samples were suspended in sterile saline and the solid particles were allowed to settle. Plating was done at high pH (pH 7- 9) to discourage the growth of fungal species. Screening for β-CD producers was done according to Park et al. (1989). In primary selection, soil samples were diluted and streaked onto the isolation media. The plates were incubated at 37°C for 24 - 48 h. Bacterial colonies with yellowish clearance zone that shows the biggest diameter were selected and streaked onto Horikoshi-Phenolphthalein (PHP) and Horikoshi-Phenolphthalein-methyl orange plates for several times until uniform colonies were formed. Suspensions of vegetative cells were grown in Horikoshi Broth (HB) for 24 h at 37°C, mixed with sterilised glycerol (20% v/v) and kept in 1 ml aliquots at -80°C until further use.

Morphological characterization

The morphological characteristics of the isolates namely size, shape and colour of colonies were examined from the 24 h cultures on Horikoshi agar (HA) using Gram staining method. The shapes of cells, characteristic arrangements of cells in groups and colour of colonies were microscopically examined.

Identification of isolated strain

The identification of isolated strain was performed by using 16S rRNA analysis. Ish-Horowicz and Burke, (1981) method was used for the purpose of genomic DNA extraction. The 16S rRNA was amplified by PCR with 5'-AGAGTTTGATCCTGGCTAG-3' as forward primer and 5'-AAGGAGGGATCCAGCCGCA-3' as reverse primer. The PCR conditions were performed as denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with final extension for 5 min at 72°C. DNA sequencing was performed by First Base Laboratories, Biosyntech Malaysia on the ABI PRISM

377 DNA sequencer. The 16S rRNA sequence of strain NR5 was compared with all deposited nucleotide sequences in the GenBank database by using Blast 2 options. Close relatives were chosen as the reference and aligned with each other using Clustal W 1.8 program.

Preparation of bacterial inoculums

Newly isolated strains were grown in 50 mL Horikoshi medium (Park et al., 1989) containing the following solutions after autoclaving. Solution 1: Starch 1.0% (w/v), peptone 0.5% (w/v), yeast extracts 0.5% (w/v), K_2HPO_4 0.1% (w/v), $MgSO_4$ 0.02% (w/v). Solution 2: Na_2CO_3 1.0% (w/v). The culture was incubated at 37ºC, agitated at 200 rpm for 18 h. Cells were harvested by centrifugation at 5000 rpm for 5 min and washed once with normal saline solution (0.85% w/v NaCl) and were then suspended in normal saline solution to give an optical density reading of 0.5 at 660 nm, using a UV-spectrophotometer.

Production of crude CGTase

Ten percent (v/v) of bacterial inoculums was inoculated into 100 ml of Horikoshi Medium II in a 250 ml conical shale flask. The bacteria culture was incubated at 37°C in an incubator shaker for 24 h. At the end of the incubation period, 1.0 ml of the culture was removed and separated by centrifugation at 8000 rpm for 10 min at 4°C. The supernatant was assayed for CGTase activity and used as crude enzyme solution.

CGTase assay

CGTase activity was determined using phenolphthalein assay (Kaneko et al., 1998). Reaction mixture containing 1 ml of 40 mg of soluble starch in 100 mM phosphate buffer (pH 6.0) and 0.1 ml enzyme solution. The mixture was incubated at 60ºC for 10 min in a water bath. Then, 3.5 ml of 30 mM NaOH was added to stop the reaction. Subsequently, 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 solution was then added to the reaction mixture and mix well. After 15 min, the reduction in colour intensity was measured at 550 nm. Blanks lacking CGTase were analysed simultaneously with each batch of samples. As a standard, the soluble starch and enzyme were replaced by 0.5 mg of β-CD and 0.1 ml of water, respectively. A calibration curve was made using β-CD in 100 mM phosphate buffer at pH 6.0. One unit of enzyme activity was defined as the amount of the enzyme that formed 1 µmol β-CD per min under the conditions above.

Determination of cyclodextrin

The cyclodextrin concentration was determined using HPLC. Samples were first centrifuge to remove suspended solids and biomass. Centrifugation was done at 12,000 rpm for 10 min. The supernatant was then filtered through a nylon filter paper with 0.45 µm pore size. The liquid chromatograph comprised of a Jasco-PU 980 pump (Jasco, Japan) and a differential refractive index detector (Perkin Elmer LC-25, USA) with a sensitivity of $5 \times 10\text{-}5$ RIU. Data integration was done using Borwin Software package V1.21 for liquid chromatography integration. The column used was Rezex RSO-oligosaccharide Ag+4% column (5 µm, 250 x 4.6 mm). Injections were carried out using a 20 µl sample loop at room temperature (25 - 28°C) with 75% acetonitrile as the mobile phase with the flow rate of 1.0 ml/min. Samples (optimised crude enzyme) and cyclodextrin standard were prepared using deionised water.

Table 1. CGTase activity by isolated bacteria using soluble starch as a substrate in raw and gelatinized form.

Isolate	Enzyme activity (U/ml) at 24 h*		Enzyme activity (U/ml) at 48 h*	
	Raw soluble starch	Gelatinized soluble starch	Raw soluble starch	Gelatinized soluble starch
NR1	7.333	6.020	6.404	6.227
NR2	6.345	7.274	8.233	9.355
NR3	7.628	7.983	8.101	9.001
NR4	10.461	9.975	10.417	10.565
NR5	10.702	9.680	11.709	7.089
NR6	7.431	7.890	6.438	11.906
NR7	4.948	6.372	6.161	7.422
NR8	10.083	9.469	10.969	10.631
NR9	6.193	5.279	5.770	5.566
NR10	7.530	5.265	6.539	6.616
NR11	6.261	7.184	7.584	8.980

* Duration of fermentation.

RESULTS AND DISCUSSION

Soil samples were collected in various places to be screened for α-CD, β-CD and γ-CD producer. The phenolphthalein and methyl orange were used to detect the presence of CGTase activity. However, this modified method is only a qualitative indicative measure of excretion of CGTase enzyme. In this study, the total of 65 isolates has been successfully isolated. However, out of 65 isolates, only eleven isolates have been chosen for further study according to the biggest diameter of isolates which ranged from 4.3 to 5.5 cm following a 24 h of incubation at 37ºC.

The morphological characterization showed that the 24 h old cells under microscope are straight rods, gram negative and non-spore forming. The additional characterization by 16S rRNA analysis has been carried out for further identification and classification of the NR5 isolate. The alignment of the 1511 bp correspondent sequence revealed 99% homology with the 16S rRNA sequence of *Bacillus* sp. 17-1 (accession number AB043843), *Bacillus* sp. B001 (accession number GQ383919), *Bacillus lehensis* strain MLB2 (accession number AY793550), *Bacillus* sp. G1 (accession number AY754340) and *Bacillus* sp. 13 (accession number AB043839). Based on these results, the strain was classified as *Bacillus* sp. NR5 UPM. The 16S rRNA sequence has been deposited in the GenBank database under the accession number HM142595.

Further quantitative methods which are the study for the reduction of phenolphthalein using spectrophotometric analysis and the CD production using HPLC have been carried out. The eleven isolated strains, namely NR1 to NR11 were further characterised using the modified phenolphthalein method (Kaneko et al., 1998). The CGTase activity was determined on the basis of the reduction of the colour intensity of phenolphthalein under alkaline conditions. The soluble starches in raw and gelatinized form were used as a substrate in determining the CGTase activity. Among 11 isolates, *Bacillus* sp. NR5 UPM shows the best CGTase producer in raw form at 48 h of fermentation (11.709 U/ml) (Table 1). Due to the efficiency of this isolate to use raw starch as a substrate, it can be considered as a raw starch-degrading enzyme producer. The compact crystalline structure of raw starch makes the starch harder to be attacked by CGTase. However, the use of raw starch for CD production is preferred because the energy-intensive gelatinization or physical treatment steps can be avoided. Hence, the raw starch-degrading CGTase became Industrially important as the cost involved in gelatinization of starch can be reduced or eliminated (Gawande et al., 1999; Gawande and Patkar, 2001). The production of CGTase involved the specific reaction between the microbe and carbon source (Ibrahim et al., 2005) and it depends mostly on starch as a substrate, hence did not require the presence of specific inducer. The bacteria can grow well on various type of starch. In this study, sago starch has been chosen as a substrate for further study for the production of enzyme. The sago starch became a potential substrate for the production of CD due to high yield and low cost of production. As reported by Abd-Aziz (2002), the functions of sago starch as an alternative cheap carbon source to be used in the fermentation process make it attractive for both economic and geographical considerations. Besides, the sago palm itself is economically acceptable, environmentally friendly and promotes a socially stable agroforestry system as it serves as a countermeasure to the green house effect (Singhal et al., 2008). Out of eleven isolates, five best isolates have been choosen for further study for the production of CGTase using sago starch as a substrate, namely NR2, NR3, NR5, NR8 and NR10. Among these isolates, *Bacillus* sp. NR5 UPM shows the highest enzyme production at 32 h of fermentation (15.514 U/ml) using 3.34% of sago starch (Figure 1). The enzyme production was also slightly higher compared to
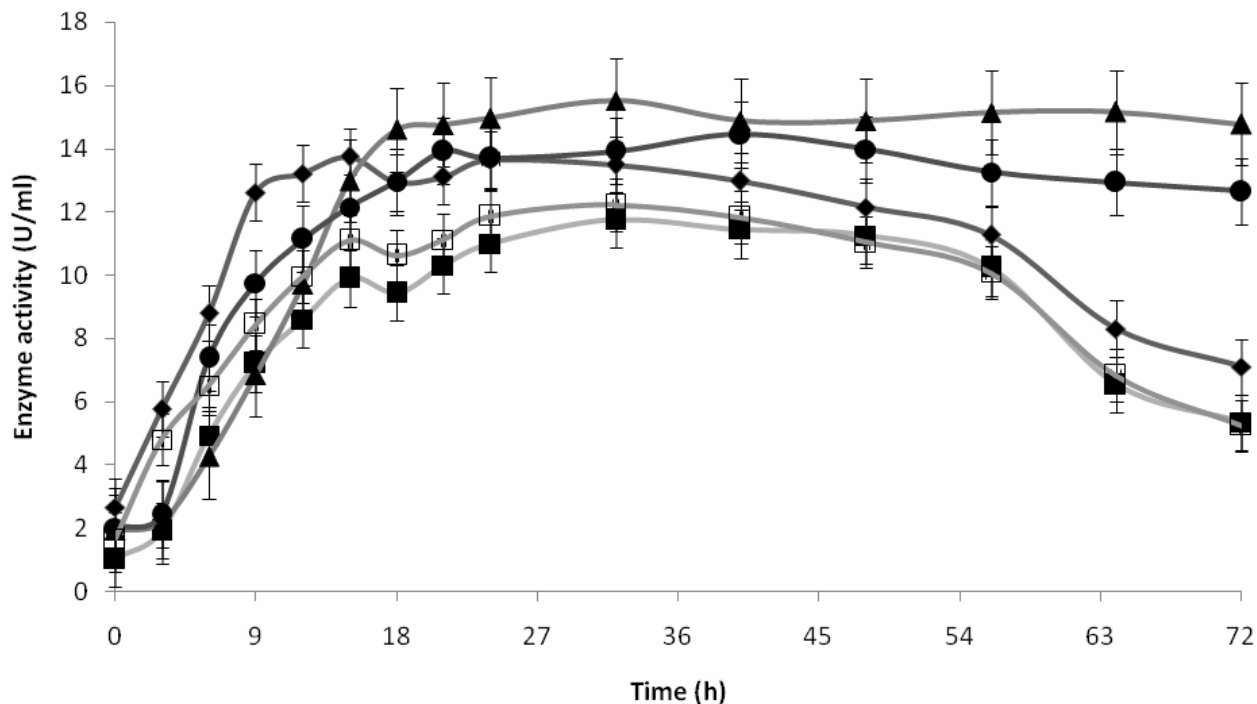
Figure 1. Profile of enzyme production by five isolates using sago starch as a substrate: -♦- NR2, -■- NR3, -▲- NR5, -●- NR8, and -□- NR10.

Table 2. CD production (mg/ml) by isolated bacteria using raw and gelatinized soluble starch as a substrate at 24 h of fermentation.

Isolate	Raw soluble starch			Gelatinized soluble starch		
	α-CD	β-CD	γ-CD	α-CD	β-CD	γ-CD
NR1	-	1.054	0.092	-	0.501	0.060
NR2	-	0.694	0.073	-	0.628	0.078
NR3	-	1.185	0.108	-	0.593	0.068
NR4	-	1.440	0.116	-	1.063	0.102
NR5	0.011	2.504	0.188	-	1.756	0.142
NR6	0.002	1.278	0.102	0.007	0.373	0.054
NR7	-	0.885	0.071	-	0.743	0.079
NR8	-	1.478	0.139	0.013	1.248	0.104
NR9	0.005	0.960	0.100	0.004	0.544	0.063
NR10	-	1.157	0.104	-	0.607	0.076
NR11	0.004	1.483	0.125	-	0.816	0.091

CGTase activity using raw soluble starch (11.709 U/ml). The study also has been carried out by Abd Rahman et al. (2004) using 1.6% of sago starch as a substrate and the result shows that the enzyme production by *Bacillus stearothermophilus* HR1 was slightly lower than *Bacillus* sp. NR5 UPM which is 14.80 U/ml.

The production of CD was further investigated by using HPLC as a quantitative method, with raw and gelatinized soluble starch as a substrate (Table 2). Out of eleven isolates, *Bacillus* sp. NR5 UPM shows the highest pro-duction of CD using raw soluble starch as a substrate at 24 h of fermentation. The ratio of α-CD: β-CD:γ-CD obtained was 0.49:91.12:8.39 with the concentration of α-CD, β-CD and γ-CD produced were 0.011, 2.504 and 0.188 mg/ml, respectively. The result shows the produc-tion of CGTase was highly specific for β-CD formation. Thus, *Bacillus* sp. NR5 UPM can be used preferentially for producing β-CD as a main product. This isolate will be favoured for the production of β-CD since the enzymes that are capable of predominantly producing the particular

type of CD are commercially desired due to its ability to reduce subsequent purification cost in the CD production. The study that has been carried out by Nisanart et al. (2004) using sago starch as a substrate also shows specific for β-CD formation with the distribution of α-CD, β-CD and γ-CD obtained by *B. circulans* was 7, 65 and 28%, respectively. Besides, *B. circulans* 251 showed α-CD as a major product (van der Veen et al., 2000), whereas a novel mutant enzyme namely H43T CGTase constructed from *Bacillus* sp. G1 showed γ-CD as a major product (Goh et al., 2007).

Conclusion

Eleven isolated strains namely NR1 to NR11 have been successfully isolated from local soil using modified Horikoshi medium type II with different indicator to detect the presence of different CGTase producer. The modified phenolphthalein assay and HPLC were used as a quantitative method to measure the CGTase activity and the production of CD, respectively as it has high reproducibility and significant operational advantages. Among eleven isolates, *Bacillus* sp. NR5 UPM shows the highest CGTase activity (11.709 U/ml) and CD production using raw soluble starch as a substrate with the concentration of α-CD; β-CD and γ-CD produced were 0.011, 2.504 and 0.188 mg/ml, respectively. The experiment using sago starch as a substrate also has been carried out and the result also shows *Bacillus* sp. NR5 UPM has the highest CGTase activity (15.514 U/ml) at 32 h of fermentation. In the future, this isolate has a probability to share the biggest market in industrial application since it's capable of predominantly producing β-CD as a main product and has the capability to use raw starch as a substrate, thus known as a raw starch-degrading enzyme producer.

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