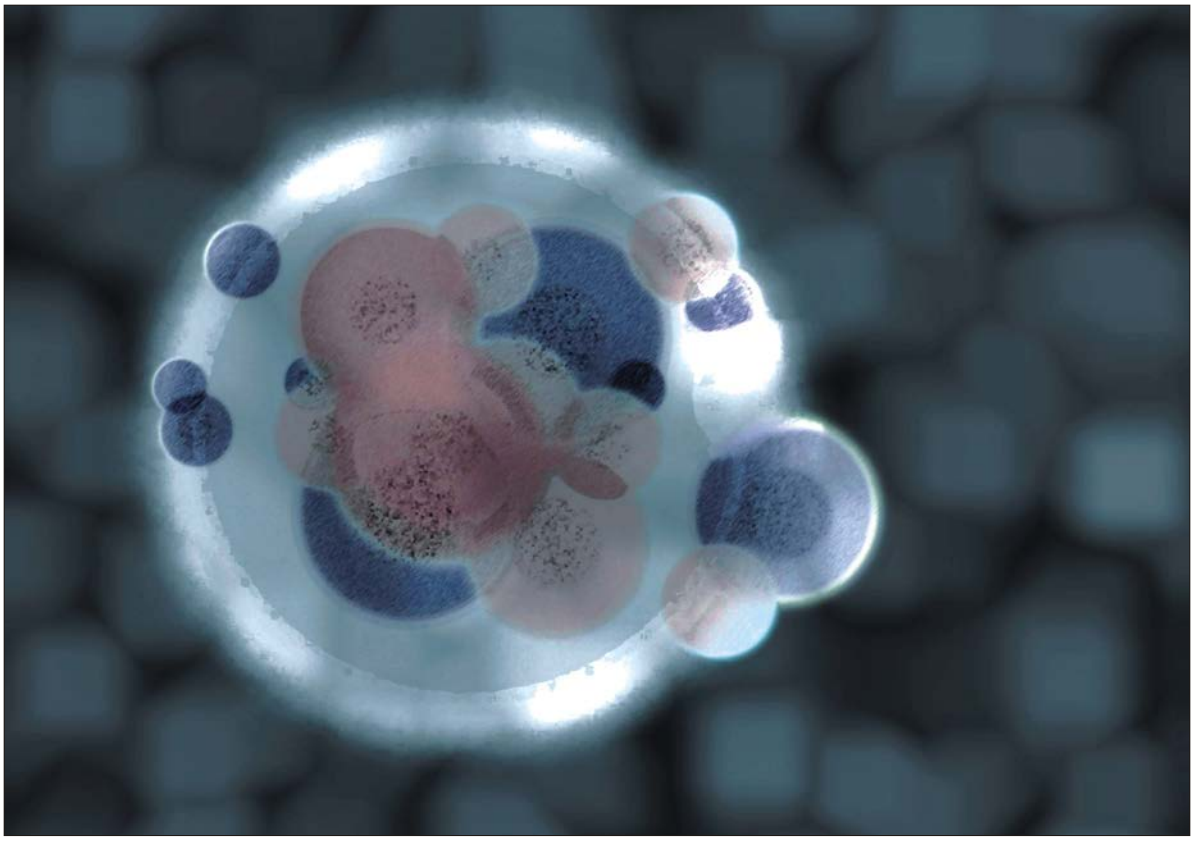

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Production of Cyclodextrin Glycosyltransferase (CGTase) by *Bacillus lehensis* S8 using Sago Starch as Carbon Source

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Abstract: Production of cyclodextrin glycosyltransferase (CGTase) is influenced by the reaction of the CGTase-producing strain towards various types of substrates. Variations in environmental factors such as concentrations of carbon and nitrogen sources possess significant effects on CGTase production. The present study was conducted with the prime purpose to optimise the cultivation medium in enhancing the CGTase production by a locally isolated alkalophilic *Bacillus* sp. The CGTase fermentation processes were performed in 250 mL Erlenmeyer flasks containing 200 mL of production medium with continuous shaking at 200 rpm and 37°C. Optimisation process was conducted by using change-a-factor-at-a-time method. From the study, an indigenous Malaysian carbon source, i.e., sago starch was found capable in improving the CGTase production with the CGTase yield of 18452 U g^{-1} at 0.1% w/v of starch. In addition to that, by using yeast extract as the sole nitrogen source in the medium, the CGTase excretion by the isolate is greatly enhanced as compared to the basal medium which employed two types of nitrogenous compounds. The optimised growth medium that has been successfully developed for high level of CGTase production by using the locally isolated *Bacillus lehensis* in 250 mL Erlenmeyer flask is comprised of (% w/v): 0.1% sago starch, 1% yeast extract, 1% sodium carbonate, 0.009% magnesium sulphate and 0.1% di-potassium hydrogen phosphate.

Key words: Cyclodextrin glycosyltransferase, *Bacillus* sp., optimisation, sago starch, alkalophilic

INTRODUCTION

Bacterial cyclodextrin glycosyltransferases (CGTase, EC 2.4.1.19) are mostly extracellular enzyme which produced by *Bacillus* sp. such as *B. circulans* DF 9R (Szerman *et al*., 2007), *B. macorous* (Wang *et al*., 2006), *B. firmus* (Higuti *et al*., 2003; Moriwaki *et al*., 2007) and *B. agaradhaerens* (Martins and Hatti-Kaul, 2002). Besides, *Paenibacillus pabuli* US132 (Jemli *et al*., 2007), *Klebsiella pneumoniae* AS-22 (Gawande and Patkar, 1999) and *Micrococcus luteus* (Tonkova, 1998) are also reported as potential CGTase producers. CGTase degrades starches to produce cyclodextrin (CD) ring through the cyclization process (intramolecular transglycosylation). Besides, CGTase catalyzes intermolecular transglycosylation (coupling and disproprtionation) as well as hydrolyzation reaction (Tonkova, 1998; Wang *et al*., 2006). In general, products contain mixture of α-, β- and γ-CD in different proportion based on the cultural conditions and origin of CGTase. Thus, CGTase is classified as α-, β- and γ-CGTase according to the major CD produced (Rahman *et al*., 2004).

Cyclodextrins (CD) are doughnut-shaped molecules with hydrophilic outer surface and hydrophobic cavity. The molecular encapsulation alters the physicochemical properties of the guest molecule. Thus, cyclodextrins are widely used in food, pharmaceutical, agricultural, chemical and cosmetic industries (Martin Del Valle, 2004; Moriwaki *et al*., 2007; Szerman *et al*., 2007). Because of the broad industrial application of cyclodextrins, improvement in production of microbial CGTase is of great interest. Production of CGTase could be enhanced by varying physio-environmental factors such as the concentrations and compositions of nutrient in the medium (Mahat *et al*., 2004). Starch is reported as the main carbon source for CGTase production. By using starch as carbon substrate, higher enzyme activity was detected compared to those cultures containing simple sugars (Ibrahim *et al*., 2005;

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Szerman *et al*., 2007; Tonkova, 1998). In the present study, productions of CGTase by an indigenous *Bacillus* sp. by using different types of raw starch as carbon source were conducted. Besides, effects of several types of nitrogen source on CGTase production were evaluated. Upon that, concentrations of chosen carbon and nitrogen source as well as concentration of sodium carbonate were also optimised with the purpose to enhance CGTase production.

MATERIALS AND METHODS

Materials: Soluble starch was purchased from Scharlau (Spain) in 2007 while sago starch was supplied by Seng Ngeng Sago Ind. (EM) Sdn Bhd, Sibu, Sarawak, Malaysia, between year 2007 and 2009. Cornstarch, potato starch and tapioca starch were purchased from a local hypermarket. Peptone (neutral bacteriological grade) and agar powder were purchased from R and M chemical (England). Yeast extract was purchased from Oxoid (England). Magnesium sulphate ($MgSO_4$), di-potassium hydrogen phosphate (K_2HPO_4), phenolphthalein, methyl orange and sodium carbonate (Na_2CO_3) were purchased from Merck (Germany).

Strain and basal medium composition: A locally isolated CGTase-producing strain designated as *Bacillus lehensis* S8 was used in this study. Basal Horikoshi (II) medium consists of (% w/v): soluble starch (1%), peptone (0.5%), yeast extract (0.5%), $MgSO_4$ (0.009%), K_2HPO_4 (0.1%) and Na_2CO_3 (1%) (autoclaved separately), was used in the cultivation of bacteria. Samples were harvested at predetermined time interval for the measurement of cell growth and CGTase activity. The results presented were the mean value of triplicate experiments and analysed using analysis of variance (ANOVA).

Biomass determination: Cell concentration was determined by using filtration and oven drying method. Commercial α-amylase, Termamyl 120 L (0.1 mL) was added to 1 mL of sample solution prior to incubation at 65°C for 30 min to hydrolyse the starch residues. The sample was then centrifuged at 10000 rpm for 5 min. The cell pellet was washed twice with distilled water and then absorbance was measured at 550 nm. Then, the cells suspension were filtered through a pre-weighted filter paper and dried in an oven at 90°C until constant weight. The dry weight of filter paper with cells after cooling process in desiccators was measured.

Assay of CGTase activity: One milliliter of culture supernatant was added to 1 mL of 0.04 g sago starch in 0.1 M phosphate buffer (pH 6). The reaction mixture was incubated for 10 min at 60°C, followed by adding of 3.5 mL of 0.03 M NaOH to stop the reaction. A 0.5 mL of 0.02% (w/v) phenolphthalein in 0.005 M Na_2CO_3 was added to the mixture. The mixture was left at room temperature for 15 min before absorbance was read at 550 nm. For control tube, 1 mL of supernatant was boiled for 5 min before adding to the starch solution. One unit of CGTase activity was defined as the amount of enzyme producing 1 μmol of β-cyclodextrin per minute under standard assay conditions.

Effect of carbon and nitrogen sources: Effect of carbon sources (C) on growth and CGTase production was investigated by substituting soluble starch (1%) in basal medium with different types of starches (potato starch, corn starch, sago starch and tapioca starch). Following that, effect of nitrogen sources (N) was evaluated by replacing peptone and yeast extract in the basal medium with different types of nitrogen sources. Nitrogen content applied is equivalent to number of N present in basal medium. The most suitable carbon and nitrogen source in production of CGTase were chosen for the subsequent experiments.

Effect of concentration of C, N and sodium carbonate: By maintaining the concentration of other ingredients constant in the medium, the effect of concentration of chosen carbon and nitrogen source on the CGTase production was evaluated. Effect of sodium carbonate concentration on CGTase production was also studied.

RESULTS

Effect of carbon and nitrogen sources: As shown in Table 1, maximum CGTase activity (18.9 U mL^{-1}), CGTase productivity (0.6 U/mL.h) and CGTase yield (1894.1 U mL^{-1} of starch) were achieved when sago starch was supplemented as sole carbon source in the medium.

By using sago starch as carbon source, effects of nitrogen sources on CGTase production were also evaluated. As observed in Table 2, CGTase production was low when inorganic nitrogen source, i.e., ammonium nitrate, was used in the production medium. In the present study, the maximum CGTase activity attained (19.1 U mL^{-1}) by using yeast extract as sole nitrogen source was comparable to those in basal medium (18.9 U mL^{-1}) which contains two types of nitrogen sources (yeast extract and peptone). A significantly lower CGTase production was observed in medium without supplementation of yeast extract.

Table 1: Comparison of the performance and kinetic parameter value of CGTase fermentation by *Bacillus lehensis* S8 in shake flask using different types of starch (1% w/v)

	Type of starch				
Parameters	Soluble	Sago	Tapioca	Potato	Corn
Maximum CGTase activity, A_{max} (U mL^{-1})	17.5^{b}	18.9^{a}	16.2^{c}	16.3^{c}	16.0^{c}
CGTase productivity, P (U/ml.h)	0.5^{b}	0.6^{a}	0.5^{b}	0.5^{b}	0.5^{b}
CGTase yield, $Y_{P/S}$ (U CGTase/g starch)	1729^{b}	1894^{a}	1619^{c}	1630^{c}	1603^{c}

$^{a-c}$Mean values in the same row not followed by the same letter(s) are significantly different ($p<0.05$)

Table 2: Comparison of the performance and kinetic parameter value of CGTase fermentation by *Bacillus lehensis* S8 in shake flask using different types of nitrogen sources

	Types of nitrogen sources							
Parameters	P + YE	P	YE	T	ME	SP	PP	AN
Maximum CGTase activity, A_{max} (U mL^{-1})	18.9^{a}	16.1^{c}	19.1^{a}	15.5^{c}	17.2^{b}	17.9^{b}	17.6^{b}	3.9^{d}
CGTase productivity, P (U/ml.h)	0.6^{a}	0.5^{c}	0.6	0.5	0.5^{d,c}	0.5^{b,a,c}	0.5^{b,a,c}	0.2^{e}
CGTase yield, $Y_{P/S}$ (U CGTase/g starch)	1894^{a}	1613^{c}	1913^{a}	1553^{c}	1721^{b}	1788^{b}	1763^{b}	1227^{d}

$^{a-e}$Mean values in the same row not followed by the same letter(s) are significantly different ($p<0.05$). P: Peptone; YE: Yeast extract; T: Tryptone; ME: Meat extract; SP: Soy peptone; PP: Polypeptone; AN: Ammonium nitrate

Table 3: Effect of different sago starch concentration on performance of CGTase fermentation by *Bacillus lehensis* S8 in shake flask

	Concentrations of sago starch (% w/v)							
Parameters	0	0.1	1	2	3	4	5	10
Maximum CGTase activity, A_{max} (U mL^{-1})	6.0^{e}	18.5^{a}	18.5^{a}	15^{b}	14.7^{c}	14.4^{c}	13.7^{d}	13.6^{d}
CGTase productivity, P (U/ml.h)	0.3^{c}	0.6^{b,a}	0.5^{b,a,c}	0.6^{b,a}	0.7^{a}	0.7^{a}	0.4^{b,c}	0.5^{b,a}
CGTase yield, $Y_{P/S}$ (U CGTase/g starch)	0^{h}	18452^{a}	186^{b}	766^{e}	497^{d}	378^{g}	289^{f}	142^{g}

$^{a-g}$Mean values in the same row not followed by the same letter(s) are significantly different ($p<0.05$)

Table 4: Effect of different yeast extract concentration on performance of CGTase fermentation by *Bacillus lehensis* S8 in shake flask

	Concentrations of yeast extract (% w/v)						
Parameters	0	1	2	4	8	12	16
Initial pH of medium	11.2	10.1	9.9	9.4	8.8	8.5	8.3
Maximum CGTase activity, A_{max} (U mL^{-1})	1.2^{f}	18.5^{a}	18.5^{a}	17.6^{b}	15.2^{c}	8.3^{d}	6.4^{e}
CGTase productivity, P (U/ml.h)	0.1^{d}	0.6^{a}	0.6^{a}	0.6^{a}	0.5^{b}	0.3^{c}	0.3^{c}
CGTase yield, $Y_{P/S}$ (U CGTase/g starch)	6313^{b}	18389^{a}	18227^{a}	17752^{a}	14664^{a}	8436^{b}	6486^{b}

$^{a-f}$Mean values in the same row not followed by the same letter(s) are significantly different ($p<0.05$)

Table 5: Effect of different sodium carbonate concentration on performance of CGTase fermentation by *Bacillus lehensis* S8 in shake flask

	Concentrations of sodium carbonate (% w/v)					
Parameters	0	1	2	4	8	12
Initial pH value	7.4	10.1	10.6	10.8	10.9	11.0
Maximum CGTase activity, A_{max} (U mL^{-1})	7.0^{b}	18.5^{a}	1.7^{c}	1.4^{c}	0.9^{c}	1.2^{c}
CGTase productivity, P (U/ml.h)	0.20^{b}	0.60^{a}	0.06^{c,b}	0.13^{c,b}	0.08^{c,b}	0.04^{c}
CGTase yield, $Y_{P/S}$ (U CGTase/g starch)	10365^{b,a}	18453^{a}	7368^{b,c}	6732^{b,c}	N.D.	ND

$^{a-d}$Mean values in the same row not followed by the same letter(s) are significantly different ($p<0.05$). ND: Not determined

Effect of concentration of C, N and sodium carbonate: The effect of different concentration of sago starch on CGTase production was observed. As observed from Table 3, a significantly high CGTase production was achieved in medium containing 0.1 and 1% (w/v) sago starch while significantly low enzyme was detected in medium without any supplementation of sago starch. Nevertheless, CGTase activities decreased when sago starch concentration exceeded 2% (w/v). From the present study, sago starch with low concentration (0.1% w/v) was found sufficient to achieve significantly high CGTase yield (18453 U CGTase/g starch).

The effects of different concentrations of yeast extract, in the range of 0% (w/v) to 16% (w/v), on CGTase fermentation process by *B. lehensis* S8 were evaluated. With concentration of yeast extract within 1% (w/v) to 4% (w/v), high CGTase activity, productivity and enzyme yield was achieved (Table 4). Low enzyme production and cell growth were observed without the addition of yeast extract in the medium. From Table 4, it can be seen that CGTase activity decreased with yeast extract concentration exceeded 4% (w/v) and increment of acidity in the medium was observed. Thus, from the present study, 1% w/v of yeast extract was the optimum nitrogen concentration for significantly high CGTase production.

As observed from Table 5, by varying the concentration of sodium carbonate, different initial pH of medium was attained and hence affects the production

of CGTase (Table 5). In the present study, the most suitable concentration of sodium carbonate to be used was 1% w/v which provided the initial pH of cultivation medium at around 10.1 and yielded 18.5 U mL^{-1} of CGTase activity. A significantly lower CGTase activity was observed with concentration higher or lower than 1% (w/v) of sodium carbonate.

DISCUSSION

B. lehensis S8 grew well on all the starches tested. Sago starch was found to be a suitable carbon source for maximum CGTase production by *B. lehensis* S8 in the present study. Sago starch was also reported by Ai-Noi *et al.* (2008) as a potential carbon source for qualitative indicative measurement of CGTase excretion in the medium. The variations in CGTase production contributed by different starches in medium might be due to the differences in their physical properties (Gawande and Patkar, 1999; Illias *et al.*, 2002; Rosso *et al.*, 2002). Through literature survey, different types of starches were used as the carbon source for CGTase production. Pocsi *et al.* (1998) noticed that soluble starch was suitable for maximum CGTase production for *B. macerans* while Gawande *et al.* (1998) reported that corn starch provided the most suitable condition for the CGTase production by *B. firmus*. Gawande and Patkar (1999) mentioned that dextrin was the best carbon source for CGTase production by *Klebsiella pneumonia* AS-22. Besides, tapioca starch was applied as carbon source to obtain maximum CGTase production by using *Bacillus* sp. G1 (Ibrahim *et al.*, 2005; Illias *et al.*, 2002).

By using sago starch as carbon source, effects of nitrogen sources on CGTase production were also evaluated. Table 2 shows that a significantly low CGTase production when inorganic nitrogen source, i.e., ammonium nitrate, was used in the production medium, which agreed to the findings reported by Illias *et al.* (2002). In the present study, results suggested that a maximum CGTase activity could be attained (19.14 U mL^{-1}) by using yeast extract as sole nitrogen source. Moreover, a lower CGTsae production was observed if the medium lack of yeast extract was used. This was supported by the report of Gawande and Patkar (1999) which stated that yeast extract might contains some essential micronutrient or inducer to enhance CGTase production. Through literature survey, several types of organic nitrogen sources were found capable of enhancing CGTase production. In the finding of Yim *et al.* (1997), corn steep liquor provided the highest enzyme production for *B. firmus* while Gawande *et al.* (1998) reported that plant protein sources were the best nitrogen source for CGTase production. Illias *et al.* (2002) and Ibrahim *et al.* (2005) reported that basal medium containing both yeast extract and peptone was suitable for the highest enzyme production by *Bacillus* sp. G1.

Concentration of carbon source is highly important in enzyme production by many organisms especially when carbon source is required in the enzyme induction (Gawande *et al.*, 2003). In view of that, effects of sago starch concentration on CGTase production by *Bacillus lehensis* S8 were evaluated. From our findings, low concentration of sago starch (0.1% w/v) provided higher CGTase production. This was similar to the report of Stefanova *et al.* (1999), who reported that a high α-CGTase production was observed by using concentration of starch as low as 0.5% (w/v). Blanco *et al.* (2009) stated that low level of cassava starch (0.75% w/v) contributed higher CGTase activity than the higher level of starch. Besides, in our study, CGTase activities decreased when sago starch concentration exceeded 2% (w/v) and this phenomenon might due to the fact that high viscosity of medium at high starch concentration, which in turn causes poor oxygen uptake (Zain *et al.*, 2007). Maximum CGTase yield was observed with 1.5% starch as carbon source by using *B. circulans* DF 9R (Rosso *et al.*, 2002), *B. stearothermophilus* HR1 (Rahman *et al.*, 2004), *B. alkalophilic* CGII (De Freitas *et al.*, 2004) and *Bacillus* sp. TS1-1 (Mahat *et al.*, 2004). Meanwhile, 4% (w/v) carbon source was set as optimal concentration for enzyme production (Gawande *et al.*, 2003; Ibrahim *et al.*, 2005). According to Gawande *et al.* (2003), high concentration of carbon source reduced the CGTase production which is due to catabolite repression occurred above a certain concentration of carbon substrate when other nutrients are kept constant.

Evaluation on effect of concentration of yeast extract on CGTase production showed that low CGTase production and slow cell growth were reported in the medium without yeast extract. This further agreed that yeast extract contains some essential micronutrient or inducer capable of enhancing CGTase production (Gawande and Patkar, 1999). Besides, same phenomenon was also reported by De Freitas *et al.* (2004) in which increment of acidity in the medium and reduction in CGTase activity was obtained with yeast extract concentration exceeded 4% (w/v). A low level of nitrogen source (0.75% w/v) was also reported as optimum level for higher CGTase production than the higher level of nitrogen source concentration (Blanco *et al.*, 2009). Different types of microbial strain require different level of nitrogenous concentration to support the production of CGTase. In our study, 1% (w/v) yeast

extract was set as the optimum concentration for CGTase production by *B. lehensis* S8. Through literature survey, it was found that a 0.4% w/v ammonium sulphate was reported as optimal concentration for CGTase production (Rosso *et al*., 2002), while 1.5% of nitrogenous concentration (yeast extract and peptone) was used to achieve high CGTase level at shorter period of cultivation (De Freitas *et al*., 2004). Also, concentration of yeast extract at 1.89% w/v was used as optimum concentration for maximum CGTase activity by Mahat *et al*. (2004). Rahman *et al*. (2004) and Ibrahim *et al*. (2005) reported the use of the 2% w/v of nitrogen source for maximum CGTase production.

pH of cultivation medium was reported important for cell growth and enzyme production (Rahman *et al*., 2004). In our study, 1% (w/v) sodium carbonate (pH 10.07) contributed to the highest CGTase production. Similarly, Gawande *et al*. (1998), De Freitas *et al*. (2004) and Ibrahim *et al*. (2005) also reported that 1% sodium carbonate was the optimum concentration for CGTase production. In addition, the best initial pH of medium for the CGTase production was around pH 10 and it might be due to the alkalophilic nature of isolated microorganisms (Ai-Noi *et al*., 2008; Illias *et al*., 2002).

In conclusion, types and concentrations of carbon and nitrogen sources, as well as concentration of sodium carbonate, significantly affected the CGTase production by *Bacillus lehensis* S8. From the present study, a cheaper carbon source, sago starch could be used to replace the soluble starch in basal medium, for comparable level of CGTase production. In addition, by comparing with the basal medium, the optimised medium is more economical viable by employing only one nitrogenous compound for production of CGTase with the locally isolated *Bacillus lehensis* S8. The final composition of optimised cultivation medium was as follow (% w/v): sago starch, 0.1; yeast extract, 1; sodium carbonate, 1; magnesium sulphate, 0.009 and di-potassium hydrogen phosphate, 0.1.

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