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Enzymatic Characterization of Recombinant Cyclodextrin Glycosyltransferase from *Bacillus* sp. A2-5a using Sagoo Starch as Substrate

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Cyclodextrin (CD) is a cyclic oligosaccharide molecule and depending on the number of glucose molecules, three types of CDs are commonly used, α-CD, β-CD, and γ-CD. CDs can be produced enzymatically using starch as substrate catalyzed by CD glycosyltransferase (CGTase). In current research, recombinant CGTase production from the synthetic gene was optimized for its production using three growth media and two induction temperatures. The highest yield was obtained in Luria Bertani medium at 25 °C. The rCGTase protein was affinity purified as a 76.39 kDa protein which showed β-cyclization and starch hydrolysis activities using zymography method. The optimum temperature, pH, and incubation time was 55 °C, 6, and 24 h, respectively. The enzyme was stable at a wide pHs in the range of 5-10, retained its half activity at 56 °C for 30 min and had cyclization ratio for α-CD: β-CD: γ-CD was 4 : 81 : 15. An amount of 542 mg β-cyclodextrin was produced from 100 mL reaction of 1% (b/v) sagoo starch using 38.4 µg rCGTase in optimum condition. This work reports for the first time the character of rCGTase from *Bacillus* sp. A2-5a using sagoo starch as a substrate.

Key words: *Bacillus* sp. A2-5a, β-cyclodextrin, characterization, rCGTase, sagoo starch

Siklodekstrin (CD) merupakan molekul oligosakarida siklik. Berdasarkan jumlah molekul glukosa yang dikandungnya, tiga tipe CD yang biasa digunakan yaitu α-CD, β-CD, dan γ-CD. CD dapat diproduksi melalui konversi enzimatik pati menggunakan siklodekstrin glikosiltransferase (CGTase). Pada penelitian ini dilakukan optimasi jenis medium dan suhu induksi yang digunakan untuk produksi CGTase rekombinan dari gen sintetik. Rendemen rCGTase tertinggi didapatkan dengan penggunaan medium Luria Bertani (LB) pada suhu 25 °C. Protein rCGTase dimurnikan menggunakan kolom afinitas dan menghasilkan protein berukuran 76,39 kDa yang menunjukkan aktifitas siklisasi-β dan hidrolisis dengan metode zimografi. Suhu, pH, dan waktu inkubasi optimum aktivitas rCGTase berturut-turut adalah 55 °C, 6, dan 24 jam. Enzim memiliki stabilitas pada rentang pH 5,0-10,0 dan mempertahankan 50% aktivitasnya pada 56 °C selama 30 menit. Rasio siklisasi α-CD, β-CD, dan γ-CD pada penggunaan pati sagu berturut-turut adalah 4 : 81 : 15. CD sebanyak 542 mg dihasilkan dari 100 mL reaksi antara pati sagu 1% (b/v) terpregelatinasi dengan 38,4 µg rCGTase pada kondisi optimum. Penelitian ini melaporkan untuk pertama kali karakter rCGTase dari *Bacillus* sp. A2-5a dengan pati sagu sebagai substrat.

Kata kunci: *Bacillus* sp. A2-5a, karakterisasi, pati sagu, rCGTase, siklodekstrin-β

Cyclodextrin (CD) is a cyclic oligosaccharides enzymatically derived from starch. Three kinds of CD have been identified, namely α-CD, β-CD and γ-CD which consists of six, seven, eight glucose molecules, respectively (Endo and Ueda 2004). In the CD molecules, glucopyranose units are linked by 1,4 glycosidic bonds which are unique characteristic of linier starch molecule. CD forms a structure with hydrophilic property at outer surface and hydrophobic property at inner cavity, which makes CD is used for many applications, such as in food, chemistry, pharmacy, analytical, diagnostics, cosmetics and agriculture (Szejtli 1997). It works by trapping the non polar molecule with suitable size through non covalent interaction. Amongst the three CD molecules, β-CD has been used mostly at industrial scale due to its inner cavity size which is more suitable for many applications.

Due to the need of CD at industrial level, cyclodextrin glycosyltranferase (CGTase, EC 2.4.1.19) has been studied for many years. CGTase is an extracellular enzyme that converts starch and β-glucan with 1,4 glycosidic bonds to CD. CGTase belongs to family 13 glycoside hydrolase. CGTase that catalyzes the conversion of starch to CD with β-CD as main products is called β-CGTase. The β-CGTase gene from *Bacillus* sp. A2-5a with accession number of AB015670 (GenBank, NCBI) has been cloned and expressed in *Bacillus subtilis* ANA-1 (Ohdan *et al*. 2000). The CGTase gene has 2115 bp which encodes a 704 amino acids preprotein (Accession number

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BAA31539, GenBank, NCBI) with the first 29 amino acids predicted as a signal sequence. The native CGTase has an optimal pH of 5.5 and an optimal temperature of 50-55 °C with product specificity of CD-α : β : γ with ratio of 5 : 77 : 18 and the conversion rate was 50% from soluble starch (Kelly *et al*. 2009; Ohdan *et al*. 2000).

In our previous research, the CGTase gene of *Bacillus* sp. A2-5a was codon optimized for high level expression in *Escherichia coli* and synthetically constructed. Sagoo starch was the best substrate in an assay using Horikoshi medium. This research was aimed to optimize the rCGTase overproduction and characterize the enzyme using sagoo starch as substrate.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition. *E. coli* BL21(DE3)/pJExpress_401_cgtase (www.dna20.com) was grown in Luria Bertani (LB) medium containing 25 μg mL^{-1} kanamycin at 37 °C. All experiments were done using sagoo starch from Riau Island, Indonesia as substrate.

Overproduction and Purification. Three growth media, namely Luria Bertani (LB), Terrific Broth (TB) and Super Optimum Broth (SOB) were used to overproduce rCGTase at two temperatures, 16 and 25 °C, in the presence of 0.1 mM of IPTG for 6 h. The rCGTase was affinity purified using resin in column contain nickel and Tris (carboxymethyl) Ethilene Diamine (TED) (Protino, Germany). First, the column was equilibrated with 4 bed volumes of lysis-equilibration-wash buffer, then allow the column to drain by gravity. The supernantant was added to the pre equilibrated column and allow the column to drain by gravity. The column was washed twice with 4 bed volumes of lysis-equilibration-wash (LEW) Buffer and allow column to drain by gravity. The rCGTase was eluted in for fractions. Elution was done by the addition of 4 x 3 bed volumes of Elution Buffer containing 250 mM imidazole, pH 8 and fractions were collected separately. The overproduction and purification processes were monitored by 10% SDS-PAGE analysis. rCGTase was separated on 10% polyacrilamide gels for 60 minutes at 125 V. The gel was flooded by Coomassie blue staining and destained with destaining solution to visualize rCGtase band on gel. The concentration of rCGtase was determined by densitometry method compared to Bovine Serum Albumin (BSA) concentration using ImageJ (Gallagher 2010). The purified rCGTase was used in the enzyme characterization.

Activity Assays. The hydrolysis and cyclization activity were monitored by zymography (Pakzad *et al*. 2004). Native PAGE was performed with 10% polyacrylamide gels. Three wells were used to separate 10 μL rCGTase each. One half of the gel was incubated in 3% soluble starch at 37 °C for 30 min. The gel was washed with distilled water, and stained with a solution containing 0.1% I_2 in 1% KI. The clear band in the blue context of the gel was indicated of amylolytic activity. For phenolphthalein indicator gel method, the indicator gel was prepared by mixing 0.24 g soluble starch, 0.14 g agar in 16 mL of 0.2 M phosphate buffer (pH 8). After the mixture, 0.5 mL of 0.4% phenolphthalein was added and the whole mixture was cooled about 50 ºC. The indicator gel was poured on the second half of the polyacrylamide gel. After a 5 min incubation at 37 °C, the indicator gel was flooded with a 0.1% sodium carbonate solution until the context of the gel turned into red. After visualization of β-CGTase activity, the third half polyacrylamide gel was subjected to conventional Coomassie blue staining method.

The β-cyclization activity of rCGTase was measured by spectrophotometer using phenolphtalein method (Goel and Nene 1995). The reaction mixtures containing 1% (w/v) gelatinized sagoo starch in various pHs. rCGTase of 0.384 μg was incubated at various temperatures for 30 min at temperature and pH as described below. The β-CD concentration was measured based on the decrease in color intensity of phenolphtalein at 550 nm.

Optimum Temperature and Thermostability. The optimum temperature of purified rCGtase was determined by incubating 0.02 unit activity (UA) rCGtase with 1% (w/v) gelatinized sagoo starch in 50 mM Tris-Cl pH 6.0 at different temperatures, ranging from 26-80 °C for 30 min and the reaction was stopped using 1.2 M HCl. The gelatinized sagoo starch was prepared by heating sagoo starch suspension at 80 ˚C for 15 min. Before the β-CD was measured, the reaction was neutralized using 1.2 M NaOH. The reactions were carried out using the rCGtase assay procedure mentioned above (Goel and Nene 1995). The temperature stability of the enzyme was measured by incubating 0.02 UA rCGtase with an equal volume of 50 mM Tris-Cl buffer (pH 7.0) for 30 min, followed by incubation with 1% (w/v) gelatinized sagoo starch for 30 min. Residual activities were measured with the standard assay as mentioned above (Goel and Nene 1995).

Optimum pH and pH Stability. Three buffers were used to characterize optimum pH and pH stability. As the enzyme activity can be different depending on the buffer system, normalization process is necessarily required. The optimum pH of the purified rCGTase was determined by reacting 0.029 UA enzyme with 1% (w/v) gelatinized sagoo starch in various pHs, pH 4 and 5 using 50 mM sodium acetate buffer, phosphate buffer (pH 6-8) and glycine-NaOH buffer (9-10) at optimum temperature for 30 min. The reactions were stopped by boiling for 5 min then neutralized by 75 mM NaOH for acid condition and 75 mM HCl for base condition. Then, the subsequent steps were done according to the rCGTase assay described above (Goel and Nene 1995). The pH stability of the rCGtase was measured by incubating 0.029 UA enzyme at various pHs (4-10) in buffers mentioned above without substrate for 30 min. Then the enzyme was reacted with 1% (w/v) gelatinized sagoo starch in optimum temperature and pH. The remaining activity of the enzyme was assayed by the standard assay method (Goel and Nene 1995).

Time Course β-CD Production. After the optimum pH and temperature were determined, 0.001 UA enzyme was reacted with 1% (w/v) gelatinized and raw sagoo starch in optimum pH and temperature for 0.5-72 h. The β-cyclization activities were measured with the standard assay as mentioned above.

Product Specificity. 0.029 UA enzyme was reacted with 1% (w/v) gelatinized sagoo starch in optimum pH, temperature and incubation time. The cyclization ratio of different CDs produced was analyzed using HPLC with refractive index detector and NH_2 column (Waters, Sperisorb). The flow was set at 1 mL/min with 70 : 30 acetonitrile-water as mobile phase (Kinalekar *et al.* 2000).

Small Scale β-CD Production. The β-CD was produced in 100 mL reaction containing 0.15 UA rCGtase and 1% (w/v) gelatinized sagoo starch in optimum pH, temperature and incubation time. The β-CD was measured by phenolphtalein method (Goel and Nene 1995).

RESULTS

In order to find the best growth condition for rCGTase production, optimization was done using three growth media (LB, TB, and SOB) and two temperatures (16 and 25 °C) were used for induction. In all media and both temperatures, rCGTase demonstrated as a 76.39 kDa protein was produced but at different level. The highest level was obtained when LB medium was used and induction was carried out at 25 °C for 6 h (Fig 1A). For further experiment, the overproduction of rCGtase was carried out using LB medium and induction was done at 25 °C for 6 h.

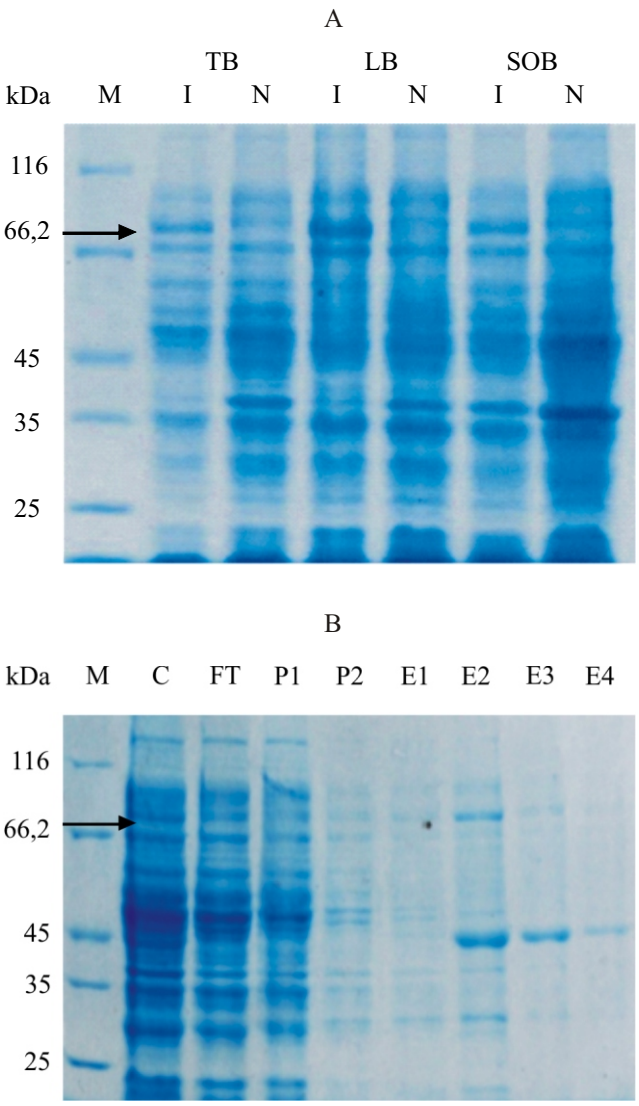

Fig 1 10% SDS-PAGE analysis of rCGTase in overproduction and purification. (A) rCGtase overproduction using LB medium at 25 °C for 6 h. (B) Purified rCGTase. M; protein marker; N: no IPTG induction; I: IPTG induction; TB: Terrific Broth; LB: Luria Bertani; SOB: Super Optimum Broth; C: crude extract; FT: flow-through; W: wash; E: elution. The arrow showed rCGTase protein band.

For enzyme characterization, rCGTase was required in the purified form. The rCGTase was copurified with a 45 kDa protein (Fig 1B), identified previously as LacI protein. 8 mL of partially purified rCGTase in concentration 0.038 μg mL^{-1} was produced from 4 x 250 mL culture. The 76.39 kDa protein displayed both starch hydrolysis and β-cyclization activities as determined by zymography method (Fig 2). This demonstrated that the rCGTase produced in this

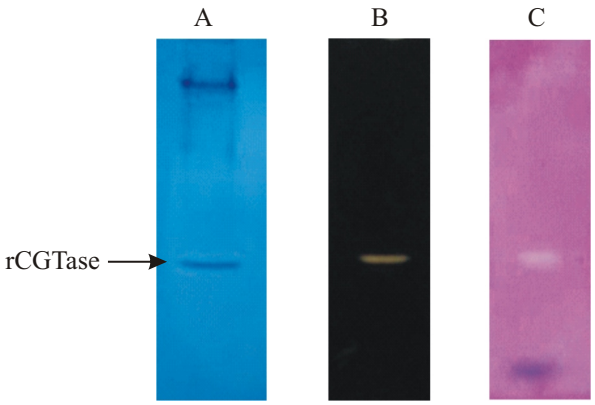

Fig 2 Result of zymography assay. (A) Coomassie blue staining. (B) KI/I_2 staining. (C) Phenolphtalein staining.

work was active. Although the purified rCGtase was still contaminated with LacI, there was only rCGtase band that showed hydrolysis and β-activity.

Our previous work showed that sagoo starch was the best substrate for CGTase of *Bacillus* sp A2-5a. The characteristic of this CGTase using sagoo starch as substrate has not been reported previously. Therefore, this current work focused on the partial characterization the enzyme using sagoo starch. The first character to be studied was its optimum temperature and its thermostability. The purified rCGtase exhibited the highest β-cyclization activity at 55 °C (Fig 3A). The activity increased from 26 °C to 55 °C, decreased above 50 °C and was almost no activity (10%) at 80 °C. However, it displayed 90% activity at 50-60 °C (Fig 3A). In terms of its thermostability, the temperature half life (T_{50}) was shown to be 56 °C for 30 min incubation (Fig 3B). rCGTase exhibited biphasic phenomenon of its activity with two optimum pHs, pH 6 and pH 9 (Fig 3C). It displayed no activity at pH 4 but then its activity increased and reached the first optimum pH, at pH 6. From pH 6 to pH 8, its activity declined and reached the lowest activity (about 60%) in the range at pH 8. With regards to its pH stability, rCGTase retained its activity (>80%) at wide range of pHs (5-10) (Fig 3D).

The production of β-CD in the gelatinized starch was maximum at 24 h, rCGtase did not show β-cyclization activity for raw sagoo starch (Fig 4A). But using gelatinized sagoo starch, the β-CD produced per min was highest at 30 min incubation and reduced considerably until 72 h incubation (Fig 4B). The ratio of α, β, and γ-CD using 1% w/v gelatinized sagoo starch was 4 : 81 : 15, respectively, while using 10% w/v raw sagoo starch was 5.3 : 76.4 : 18.3. In terms of β-CD production, 542 mg was produced in 100 mL reaction using 1% w/v gelatinized sagoo starch and 0.15 UA rCGtase at optimum pH, temperature and incubation time.

DISCUSSION

In present work, we reported better rCGTase overproduction condition and some characters of the rCGTase that are central to its use in the β-CD production using sagoo starch. Induction at 25 °C for 6 h in LB medium was the best condition for rCGTase overproduction using the starch. A number of characteristic of rCGTase of *Bacillus* sp. A5-2a including its optimal temperature and pH, its thermostability and pH stability demonstrated that this enzyme has good thermostability and works at quite wide range of pHs. Using gelatinized sagoo starch as substrate, the enzyme maintains its product specificity with β-CD as a predominant product and in 100 mL of culture, the amount of β-CD produced in optimal condition was 524 mg. The enzyme is unable to act on raw starch and then gelatinized sagoo starch should be used for the β-CD production.

rCGTase was formed in the cytoplasm of *E. coli* in the form inclusion body. In this present work, induction was performed at 16 °C in attempt to obtain higher amount of soluble rCGtase. Our current result showed that the yield of soluble rCGTase was the same as that of previous work at 25 °C and the yield of the total rCGtase, in the form of soluble and inclusion body was the highest using LB as growth medium.

Optimum temperature and thermostability. The optimum temperature of rCGtase using sagoo starch was the same as that of native CGtase A2-5a (Komentani *et al.* 1994) and rCGTase A2-5a in *B. subtilis* ANA-1 (Ohdan *et al.* 2000), ranging of 50-55 °C. The T_{50} of rCGtase using sagoo starch (56 °C) was lower than native CGTase A2-5a (64.4 °C) (Kelly *et al.* 2009). The difference was probably due to the presence of $CaCl_2$ in the assay activity of native CGTase A2-5a (Kelly *et al.* 2009). Moreover, in this research the reaction was done at pH 7.0, while the pH stability assay of native CGTase A2-5a was carried out at pH 5.5 which was close to optimum pH of rCGtase at pH 6.

Optimum pH and pH Stability. The pH optimum of rCGtase using sagoo starch (pH 6.0) as substrate was slightly different from native CGTase A2-5a (pH 5.5) (Ohdan *et al.* 2000). The difference of optimum pH was probably caused by the difference of incubation temperature. In this research, the incubation temperature was at 55 °C while native CGTase A2-5a was at 40 °C. The CGTase which has optimum activity

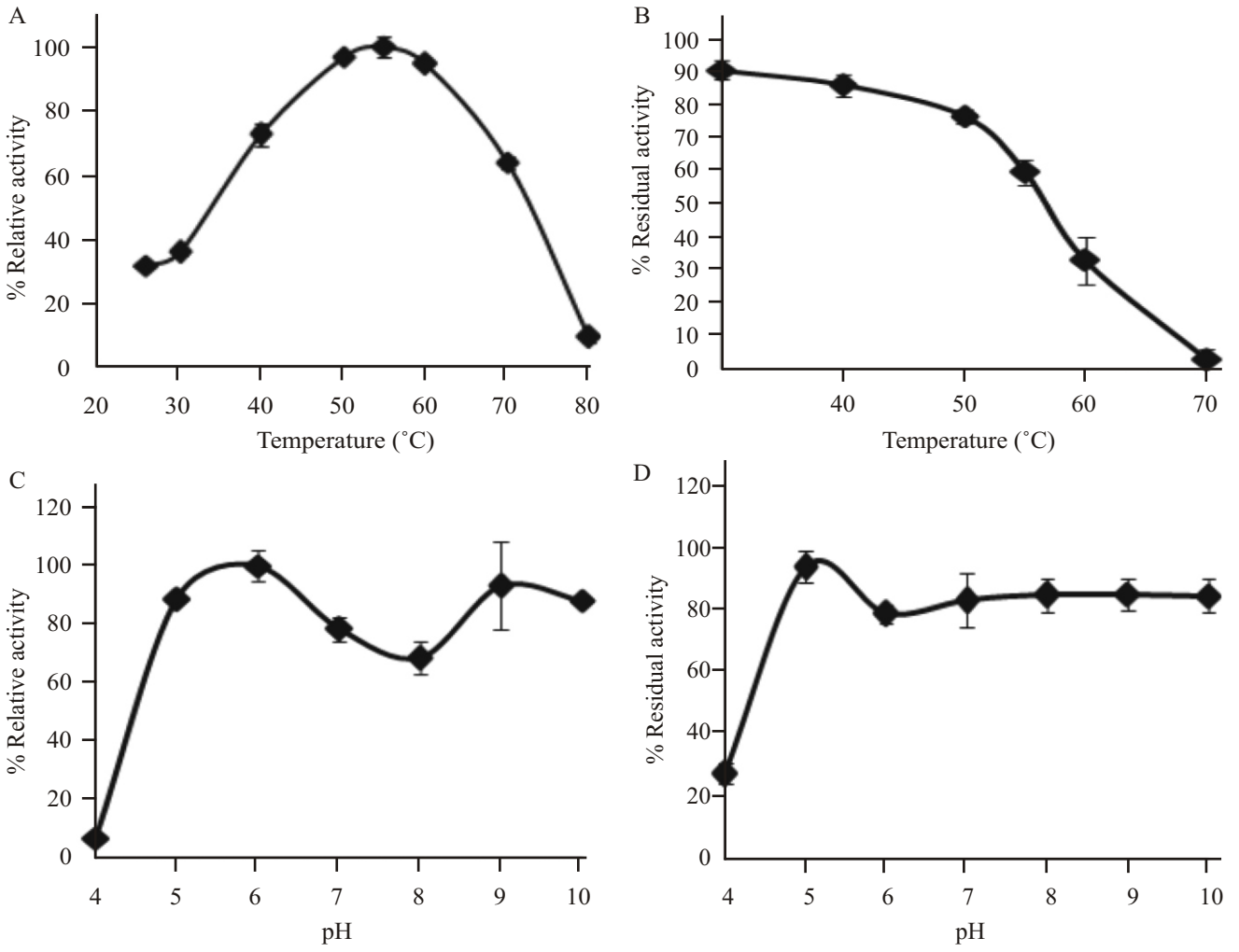

Fig 3 Properties of the purified rCGTase A2-5a using sagoo starch as substrate. (A) Temperature profile of purified rCGTase. For this temperature profile, enzymatic activity was measured in Tris-Cl pH 7.0. (B) Thermal stability of purified rCGTase. Thermostability was determined by preincubating the enzyme in Tris-Cl pH 7.0 at designed temperatures for 30 min. (C) pH profile purified rCGTase. The reaction pHs were adjusted to 4-10 with the following buffers: Na-acetat (pH 4.0-5.0), Na-phosphate (pH 6.0-8.0), and Glycine-NaOH (pH 9.0-10.0). (D) pH stability purified rCGTase. pH stability was determined by preincubating enzyme at following buffer Na-acetat (pH 4.0-5.0), Na-phosphate (pH 6.0-8.0), and Glycine-NaOH (pH 9.0-10.0) for 30 min. The given values in all activity assays are the means of triplicates, and the error bars indicate the standard deviation of these triplicates of independent experiment

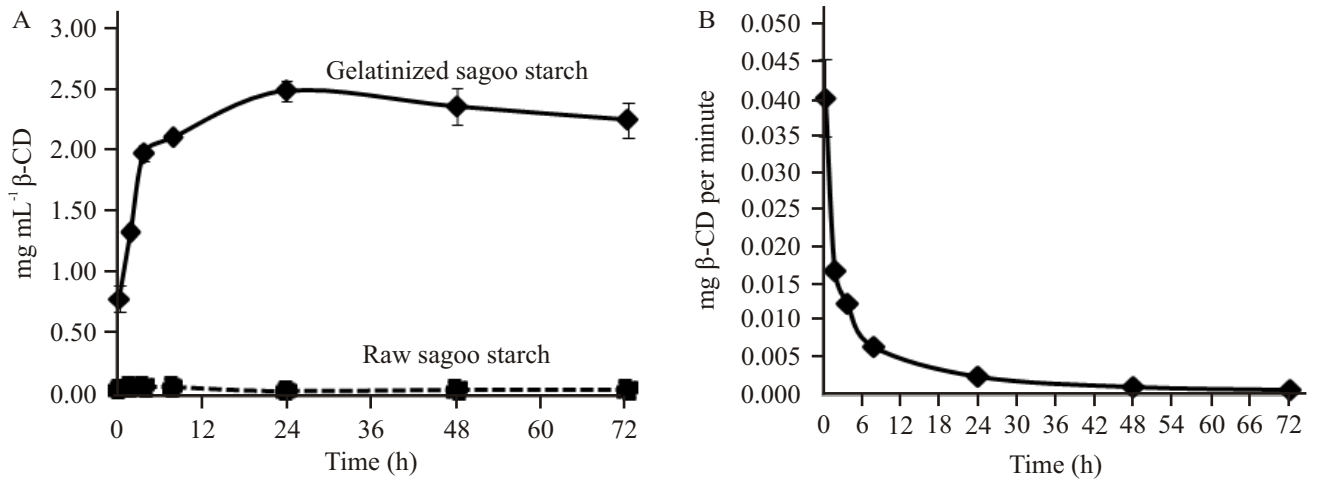

Fig 4 Time course of β-CD production using purified rCGTase *Bacillus* sp. A2-5a using 1% (w/v) gelatinized sagoo starch as substrate. The reaction was carried out at 55 °C in 100 mL of 50 mM phosphate buffer pH 6.0. (A) amount β-CD produced during 72 h. (B) mg β-CD produced per min during 72 h.

at pH 6 is from *Bacillus* sp. TS1-1, G1 and 17-1 (Rahman *et al*. 2006; Ong *et al*. 2008; Kaneko *et al*. 1989). rCGTase retained >50% of its activity at pHs 5.0-10.0, while native CGTase A2-5a retains 50% of its activity at pHs 9.0-10.0 (Komentani *et al*. 1994).

Time course experiment of β-CD production. rCGtase using sagoo starch produced maximum β-CD at 24 h reaction time, in contrast, the β-CD produced per minute was highest at 30 min incubation time. It showed that the activity of the rCGtase decreased in longer period of reaction. In CDs ratio assay using 1% (w/v) gelatinized sagoo starch, the α, β, and γ-CD ratio was 4 : 81 : 15, respectively. The α, β, and γ-CD ratio from native rCGTase is 5 : 77 : 18 (Kelly *et al*. 2009), respectively and β-CD was still the predominant product. In other research using CGTase from *Bacillus circulans* and sagoo starch as substrate, β-CD is 65% from total CDs produced (Charoenlap *et al*. 2004). The 5.42 ± 0.75 g L^{-1} β-CD produced from 100 mL reaction at optimum condition had not purified yet.

In conclusion, the difference of substrate did not influence of some characteristics of rCGTase A2-5a such as optimum temperature and pH but the β-CD yield was higher when using sagoo starch as substrate than using soluble starch. Further study of the rCGTase and β-CD production followed by β-CD purification should be done in the near future to obtain purified rCGTase and β-CD in larger scale using sagoo starch; therefore their use in industrial scale can be applied.

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