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Isolation of β-Cyclodextrin Gycosyl Transferase (β-CGTase) Producing Bacteria from Potato Plantation Soil

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Abstract

Cyclodextrin glycosyltransferase (CGTase) is an extracellular enzyme produced by several microorganisms, particularly bacteria, that converts starch into cyclodextrin (CD). CD has various applications in fields such as food, textiles, pharmaceuticals, chemistry, and biotechnology. The objective of this study was to isolate β-CGTase-producing bacteria from soil in a potato plantation located in Kerinci, and to conduct their characterization. Screening was conducted using Horikoshi agar media with phenolphthalein and methyl orange color indicators. The results indicated that one isolate, coded CK-2, produced the optimum β-CGTase enzyme when incubated for 30 hours at 37 ºC and pH 10. The CGTase enzyme converted starch to CD when incubated for 60 minutes at 37 ºC and pH 7. The CK-2 isolate was identified as monobacilli Gram-positive bacteria, having spores, positive in the catalase test, and motile. The study concluded that the bacteria producing β-CGTase enzyme are found in the soil of potato plantations in Kerinci, which is believed to originate from the genus *Bacillus sp*.

Keywords: CK-2 isolate; cyclodextrin (CD); cyclodextrin glycosyltransferase (CGTase); soil.

Abbreviations: CD: cyclodextrin; CGTase: cyclodextrin glycosyltransferase.

INTRODUCTION

Cyclodextrin glycosyl transferase, which is classified with CGTase number E.C 2.4.1.19, is a bacterial enzyme belonging to the R-amylase family that has the ability to convert starch into cyclodextrin (Jeang et al., 2005). The CGTase enzyme can synthesize starch to form three main cyclodextrin compounds, namely α-CD, β-CD, and γ-CD (Kesaulya, 2015). This enzyme converts starch to cyclodextrin (CD) through the transglycosylation and starch hydrolysis pathway. The resulting CD is a glucose residue compound bound by α-1,4 glycosidic to form cyclic oligosaccharides (Kamble & Gupte, 2015).

Cyclodextrin is a cup-shaped molecule with a cavity in the middle that is hydrophobic on the inside and hydrophilic on the outside. The specialty of cyclodextrins lies in their ring structure, which has the ability to enclose guest molecules in the cyclodextrin cavity. This can be applied in several ways, including modifying the physicochemical properties of molecules such as stability, solubility, bioavailability, conjugate preparation, and the bonding of several polymers (Duchene, 2011; Jansook et al, 2018; Poulson et al. 2022).

The formation of cyclodextrins must be in accordance with the environmental conditions and reactions needed for growth and reproduction so that some CGTase-producing bacteria can form three cyclodextrin molecules, namely α-CD with 6 glucose molecules, β-CD with 7 glucose molecules, and γ-CD with 8 glucose molecules (Kesaulya, 2015). Due to the difference in the number of molecules that make up each cyclodextrin, the main difference between them is the size of the nonpolar cavity and their solubility in water (Koo et al., 2017; Braga, 2019).

Research conducted by Higuti et al. (2003) concluded that there are 75 strains of CGTase-producing bacteria that exist in various types of soil. Based on the summary of Qi & Zimmermann (2005), CGTase-producing bacterial isolates are currently known to produce the most β-CD. Miftahurrohmah & Moordiani (2014) found that the CGTase-producing JS-1 isolate from soil in Jatinangor was a Gram-positive bacterial isolate that was bacillus-shaped, motile, facultative anaerobic, and had a positive catalase test, in line with research by Rostinawati & Lestari (2017) showing that CGTase was produced by the bacteria Bacillus sp. The aim of this research is to obtain isolates of bacteria producing the enzyme β-CGTase from potato plantation soil in Kerinci

and identify isolates producing β-CGTase based on biochemical characteristics.

MATERIALS AND METHODS

This research was conducted at the Pharmaceutical Microbiology Laboratory, Harapan Ibu Jambi College of Health Sciences from April 2019 to July 2019. The samples examined in this research were soil samples taken from the potato plantations, specifically the soil closest to the potato tubers. A 1-gram sample was taken at one point and diluted into 9 mL of sterile distilled water to create a dilution of 10-8. Then, 100 µl of suspension from dilutions 10-2 to 10-8 was poured into solid Horikoshi medium and incubated for 24 hours at 37 ºC.

The presence of a yellow hollow zone around the growing colony indicated the ability of the bacteria to produce the enzyme β-CGTase. The colonies were then identified using macroscopic, microscopic, and biochemical tests, and inoculated into liquid Horikoshi media to measure CGTase activity. CGTase activity was measured using spectrophotometry, which involved observing the absorbance of the color indicator bound by cyclodextrin to create a curve of cyclodextrin concentration against the incubation time of the CGTase enzyme.

Absorbance was measured by inserting a color indicator compound into the cyclodextrin to form an inclusion. The measurement is carried out on the color indicator captured by the cyclodextrin, which was produced from the CGTase enzyme. The more cyclodextrin present, the more compound was captured, resulting in a smaller absorbance value of the color indicator. The percent decrease in absorbance of the color indicator was then correlated to the CGTase incubation time using the following formula:

Percent reduction in phenolphthalein (%)

$$U/mL = \frac{R0-R}{R} \times 100\%$$

R_0 : Control absorbance (Time 0)
R : Sample absorbance

RESULTS AND DISCUSSION

Result

Screening of Isolates Producing the β-CGTase Enzyme

The isolate successfully obtained from potato plantation soil, which exhibited β-CGTase activity, was derived from a soil dilution of 10-2. A yellow hollow zone was observed on the β-Horikoshi media, and this isolate was designated as CK-2. The CGTase activity secreted by the CK-2 isolate in the β-Horikoshi medium is illustrated in Figure 1.

Figure 1. Horikoshi agar as control (A), Horikoshi agar after CK-2 inoculation (B)

Isolate Identification

Macroscopically, the colonies of the CGTase-producing isolate that grew on β-Horikoshi agar had a round shape with a whitish-yellow color, flat edges, and a flat elevation. Microscopically, identification and characterization were carried out through several tests:

Biochemical Test

Biochemical tests were conducted using IMVIC media, including SIM media, Veges paskeuer, methyl red, Simon citrate & Triple Sugar Iron Agar, and sugar media. The results of the isolate identification can be seen in Table 1.

Table 1. Results of identification of CK-2 isolates.

Identification	Results
Sulfur Indol Motility	(-),(-),(+)
Methyl Red	(+)
Veges Paskeuer	(-)
Triple Sugar Iron Agar	K/K
Simon Citrate	(-)
Glukosa	(-)
Dextrosa	(-)
Mannitol	(-)
Sucrosa	(-)
Lactosa	(-)
Gram	(+)
Spora	(+)
Katalase	(+)

Gram Staining and Endospores

In Gram staining, rod-shaped bacteria are purple in color, indicating that they were Gram-positive. The bacteria had subterminal spores because they had a green dot on 1/4 of the cell. The bacteria also produce the enzyme catalase.

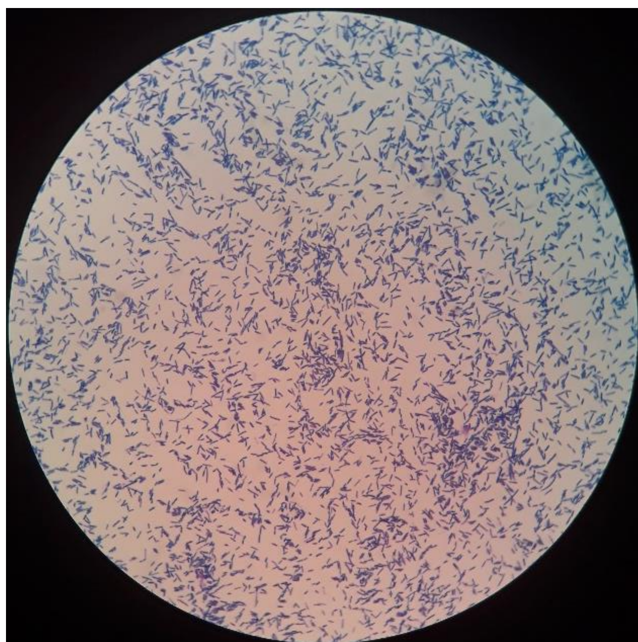

Figure 2. Result of Gram staining under light microscope with magnification 1000x.

Characterization of the incubation time of enzymes on CGTase activity

The characterization of enzyme incubation time was conducted to determine the optimal time for the enzyme to catalyze starch and form CD. According to the results of the characterization, as shown in Figure 3 (A), the 60-minute incubation time exhibited the lowest phenolphthalein absorbance, indicating that this is the optimal time for CGTase activity in producing CD.

Preparation of a Growth Curve for Isolate CK-2

In the growth curve, the results of the OD600 measurement shown in Figure 3 (B) indicate that the isolate experienced a log phase starting from the 12th hour, followed by a stationary phase at the 24th hour. Subsequently, the death phase was observed from the 30th to the 72nd hour of incubation.

Characterization of Isolates in β-CD

Production The best time for an isolate to produce the β-CGTase enzyme was seen based on incubation of the CK-2 isolate at a temperature of 37 ºC. Isolate CK-2 secreted the CGTase enzyme maximally after 30 hours of incubation. In Figure 3 (C), it can be seen that the phenolphthalein absorbance was the smallest at 30 hours of incubation, followed by the 36th hour of incubation. Figure 3 (D) illustrates that isolate CK-2 secreted the maximum amount of CGTase at the 30-hour mark, as indicated by a percent decrease in phenolphthalein absorbance reaching 98.39%.

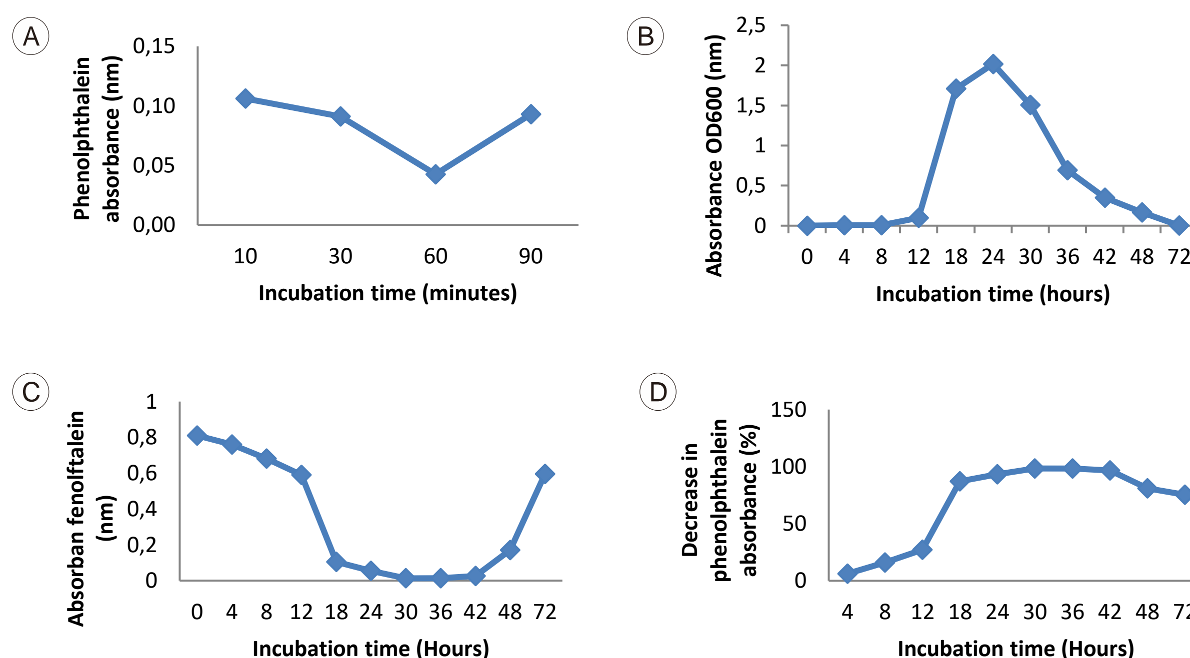

Figure 3. (A) Decreased phenolphthalein absorbance corresponds to CD formation. (B) Growth curve of isolate CK-2. (C) Phenolphthalein absorbance over incubation time. (D) The percentage decrease in phenolphthalein absorbance is directly proportional to β-CD formation.

Discussion

Screening and identification of isolates

An isolate of bacteria producing the CGTase enzyme has been successfully isolated from potato plantation soil in the Kerinci area. This isolate exhibits a yellow hollow zone on Horikoshi media. The formation of this yellow hollow zone is attributed to the isolate's capability to produce the enzyme β-CGTase, which converts starch into the compound β-CD. Consequently, this compound binds to phenolphthalein, causing the disappearance of the reddish-purple color of phenolphthalein around the colony.

Based on the results of biochemical identification of bacteria, it is suspected that the CK-2 isolate comes from the genus Bacillus (Kiranmayee Rao & M. Lakshmi, 2007). This identification has several advantages, namely that it is easy to carry out and costs less. However, it has the disadvantage of a lower level of accuracy due to several types of species that exhibit nearly identical biochemical metabolic results. Therefore, further molecular identification of the CK-2 isolate is necessary. This aligns with several local studies that have identified Bacillus as CGTase-producing bacteria, including Bacillus sp BK-1 (Yunianto et al., 2000), Bacillus sp BGG-1 (Perbadi, 2001), Bacillus sp LT1B and PT2B (Sagita, 2011), as well as Bacillus sp BV-3 (Vionita, 2017).

Characterization of enzyme incubation time on CGTase activity

This characterization was carried out in order to obtain the optimum incubation time for the enzyme that reacts with starch to produce β-CD. Figure 3 (A) shows that the best time for the enzyme to react with starch to produce CD is at 60 minutes of incubation. In the test carried out by Rostinawati et al (2016), the incubation time for CGTase with starch was 30 minutes but with the starch chain breaking first by gelatinization. This research also did not carry out enzyme purification so that a mixture of substances originating from the media still remained. It is possible that the incubation time was longer in this study due to the need for additional time for the reaction to break the starch structure and the disruption of the bond between the enzyme and starch by residual substances from the media.

- *Making an Isolate Growth Curve*

In the growth curve of the CK-2 isolate, it is known that from the initial time of incubation to the 12th incubation hour there is a lag phase, namely the time when the isolate adapts and is then able to reproduce. This is different from research conducted by Kamble & Gupte (2014) which is known to be the lag phase of Bacillus. producing CGTase at 0 hours to 8 hours of incubation, this difference is probably caused because the CK-2 isolate takes longer to adapt to the pH 10 liquid Horikoshi medium, while the isolate used by Kamble & Gupte (2014) is an isolate that has been proper pH optimization for growth.

Next, the CK-2 isolate experienced a log phase starting from the 12th hour of incubation to the 24th hour of incubation, indicating that the CK-2 isolate reproduced maximally during that incubation hour. In line with the difference in the lag phase with the research of Kamble & Gupte (2014), there was also a difference in the log phase. In this study, the log phase was obtained for 22 hours of incubation, namely from the 8th hour to the 30th hour of incubation. This can be caused by adjustments to the nutrient concentration that they make, such as optimizing the concentration of peptone, yeast extract and starch so that the possibility of bacterial growth can last longer. Meanwhile, the stationary phase in this study started at the 24th incubation hour until before the 30th incubation hour, this shows a state of balance between the growth rate and death rate of bacteria at that time and is in line with the reduction in nutrients in the media, so the number of deaths continues to increase and the growth rate continues to decrease, this occurs at the 30th to 72nd incubation hours. This phase is also different from the research of Kamble & Gupte (2014) which concluded that the stationary phase from their growth curve measurements started from the 30th incubation hour to the 176th incubation hour, while the death phase is known to occur later, this difference is probably caused by the addition of nutrients. In their media, the starch used was tapioca starch, the addition of 0.2% Ammonium dihydrogen Phosphate and a difference in Magnesium Sulfate concentration of 0.12%.

- *Characterization of Isolates in β-CD Production*

The characterization of isolates in the production of β-CD, as depicted in Figure 3 (C), reveals that the 30th hour of incubation exhibits the lowest phenolphthalein absorbance value. This observation suggests that this specific incubation time is optimal for bacteria to produce β-CD, resulting in a significant 98.39% increase in β-CD formation, illustrated in Figure 3 (D). Furthermore, the 30th hour of incubation marks the conclusion of the stationary phase, indicating a correlation between β-CGTase production and this phase. Specifically, the formation of β-CGTase escalates concomitantly with the conclusion of the stationary phase.

CONCLUSIONS

Bacteria producing β-CGTase, named isolate CK-2, have been found. Based on biochemical identification, the CK-2 isolate is a gram positive bacterium in the form of a bacillus, facultative anaerobe, motile and positive in the catalase test. This isolate is thought to be a bacterium from the genus Bacillus. The CGTase enzyme secreted by the CK-2 isolate has optimum cyclization activity in

incubation time 60 minutes at 37 ℃. This bacterium is one of the CGTase-producing bacteria which can produce the maximum CGTase enzyme at the 30th hour of incubation at a temperature of 37 ℃.

Authors' Contributions: Helman Kurniadi, Desi Sagita, and Barmi Hartesi designed the research, carried out laboratory work, and analyzed the data. Soil sampling was carried out by Helman Kurniadi.

Competing Interests: Regarding competing interests, it is important to note that there was no conflict of interest observed in this study.

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