Inulinase Activity of Thermophilic Bacteria isolated from Hot Springs of Penen Village, North Sumatera, Indonesia

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Abstract

Inulinase is an enzyme that catalyzes inulin to produce D-fructose as the end product which is considered as safe alternative sweetener to sucrose. This enzyme can be extracted from plants and vegetables but it is difficult to obtain it in high quantity and the production cost also high. The main problem in utilizing this enzyme in industrial is the instability against fluctuation of temperature that usually occurs in industrial process. Therefore thermophilic microbial inulinase has great potential for these industrial needs. The aim of this study was to isolate and characterize the thermophilic bacteria that possess the inulinase activity from hot springs in Penen Village, North Sumatera. To screening the inulinase producing bacteria, the modified Czapex Dox agar supplemented with 1,5% inulin powder was used. The temperature used for incubation is 48°C. The clear zone surrounds the bacteria after Lugol's iodine treatment on medium indicated the nucleolytic activity. As 4 isolates of thermophilic bacteria namely UTMP 11, UTMP 14, UTMP 15 and UTMP 17 showed the inulolytic activity. The biochemical characterization of these isolates showed negative in catalase test, negative on citrate test, non-motil, negative Gram staining, and rod-shaped morphology. Molecularly, this bacteria identified as Bacillus subtilis strain JCM 1465 and Bacillus tequilensis strain 10b. From the result, these isolates have potential as source of thermophilic inulinase production in food industry for the production of fructose.

Keywords: Thermophilic bacteria; inulinase; enzyme; hot springs

Introduction

Inulin consisting of linear -2,1-linked polyfructose chains with terminal glucose units and also a good source for producing high fructose through hydrolysis ([1]). Inulin is found in many plant families including dahlia tubers (Cichorium endivia) and Jerusalem artichoke (Helianthus tuberosus) and acts as reserve carbohydrates (2). To produce fructose, acid hydrolysis was carried out on inulin at high temperature, 80-100°C, however, the fructose easily degrades in this condition, producing difructose anhydrides difructose, a coloring product of fructose (3). The alternative approach to produce fructose-free from by-product can be done with enzymatic hydrolysis using inulinase. Inulinase is an enzyme that is able to produce fructose or fructooligosaccharides molecules through hydrolysis of inulin. Recently,
Inulinase has increased applications in industrial sector, especially in producing high fructose syrup. Inulinase has been reported from a variety of plants, and animals but they are present in very minute quantities. The main problem in the use of enzymes in industrial is instability of enzymes due to exposure of high temperature, changes in physical and chemical factors (4). Therefore, the best sources of inulinase are microorganism, because easy in cultivation, genetic manipulation, high production yield and usually inducible and extracellular in nature. Genetic changes using recombinant DNA technology can be easily done on microbial cells to increase enzyme production. Another advantage of microbial enzymes is the stability performance of the enzymes, so it can work in varied chemical and physical conditions that often occur during industrial processes (5). It is known that an organism’s enzymes are conditioned to work optimally at its growth condition, including pH, salinity and temperature. One source of thermostable enzyme is thermophilic bacteria. Extremophilic microorganisms include members of all three domains of life, archaea, bacteria, and eukarya. Thermophilic microorganisms are growing optimally at temperature more than 45°C. The most enzyme produced by groups of thermophilic microorganisms is widely used because thermostability and resistance to change in physical and chemical factors (6). Based on this, the study on thermostable enzymes has attracted attention in recent years. Study regarding isolation and production of thermostable enzyme from microorganism have got wider attention. North Sumatera is one of the province in Indonesia which has many natural hot springs. Some of these hot springs have been commercialized as tourist attractions. The hot spring in Penen village is one of the spring which has not yet been explored. The studies on the exploration of thermophilic microbes and thermostable enzymes from hot springs in North Sumatera are still limited. The aim of this study is to isolate and identify inulinase producing thermophilic microorganisms, from hot springs in North Sumatera.

Material and Methods

Samples collection

The hot spring chosen in this study is the hot spring in Penen village, the regency of Deli Serdang, North Sumatera. This hot spring located at 3°18’5”N and 98°38’41”E. The location of sampling sites was shown in Figure 1.
A sampling of water and sediment was conducted at three points, namely at the upstream, middle and downstream of location. During the sampling pH and temperature are measured. 10 mL of water from each sampling points was collected using well sampler, and stored in a sterile falcon tube. Sediment samples were collected using Ekman grab and stored in sterile sample bottles. All samples were stored in an icebox and analyzed further on the same day in the laboratory.
Enrichment and Isolation of bacteria

1 mL of each water sample from each sampling point is mixed into a test tube and homogenized with the vortex. For sediment samples, each 1 g of sediment from each sampling point was mixed into a test tube containing 9 mL sterile distilled water and homogenized with the vortex. As 1 mL of mixed water sample and 1 g of mixed sediment sample were mixed in 9 mL of Tryptic Soy Broth, incubated for 24 hours at 49°C. The serial dilution was conducted up to 10⁻³ dilution followed by inoculation on the surface of Tryptic Soy Agar. The Petri dish was incubated for 48 hours at 49°C. The growing colonies were characterized morphologically and biochemically.

Biochemical and morphological characterization

The growing bacteria were characterized biochemically. The biochemical characteristic of isolates was determined by gelatin assay, catalase test, citrate test, motility, and Gram staining.

Screening of inulinase activity
The inulinase activity of isolates was determined by inoculating on Czapek-Dox agar plates enriched with inulin (1% w/v) and incubated at 48°C for 48 hours. The plates were treated with Lugol’s iodine for 3 to 5 minutes and washed with distilled H2O to remove excess Lugol’s iodine. The clear zone around the colonies indicating inulin hydrolysis. Four bacterial with the largest clear zones if inulin hydrolysis were subcultured and kept for further studies.

Isolation, 16SrRNA gene amplification and sequencing

DNA isolation is carried out based on the protocol established by company (TianGen). The PCR solution consisted of 1µL forward Primer 16S, 1µL reverse primer 16S, 12.5µL GoTaq Green Master Mix 2X, 1 µL DNA template, 0.5 µL DMSO and 9 µL Nuclease free water. The program of PCR used in this study was : pre denaturation at 95° for 90 seconds, continued with 30 cycles consists of denaturation at 95°C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 90 seconds, and final extension at 72 °C for 3 minutes. The primers used in this study were Primer 27 F: 5`- AGA GTT TGA TCC TGG CTC AG - 3` dan Primer 1492 R: 5`- GGT TAC CTT GTT ACG ACT T – 3. The sequence result then trimmed and assembled using BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequence result then submitted into BLAST (Basic Local Alignment Search Tool) with genome data that has been registered at NCBI (National Center for Biotechnology Information) at http://www.ncbi.nlm.nih.gov/BLAST, to determine species that have closest similarity molecularly.

Result and Discussion

Biochemical and morphological characterization

From 11 bacterial isolates that successfully isolated from sediment and water from hot springs in Penen Village, 4 isolates showed good inulinas activity based on the formation of the colourless zone around the colonies. These bacteria were isolated from the sediment, namely UTMP 11, UTMP 14, UTMP 15 and UTMP 17. These isolates showed good growth on Saboraud Dextrose Agar media. The growth of the isolates was showed and Figure 3.
Figure 3. The growth of isolates UTMP 11, UTMP 14, UTMP 15 and UTMP 17 on Saboraud Dextrose Agar after 48 hours at 48° C

The biochemical characterization of the isolates was shown in Table 1.

| Isolates code | Citrate test | Catalase test | Starch hydrolysis | Gelatin hydrolysis | motility |
|---------------|--------------|---------------|-------------------|-------------------|---------|
| UTMP 11       | +            | +             | +                 | -                 | -       |
| UTMP 14       | +            | -             | +                 | -                 | -       |
| UTMP 15       | +            | +             | +                 | -                 | -       |
| UTMP 17       | +            | +             | +                 | -                 | -       |

From Table 1, it is shown that all the isolates showed starch hydrolysis activity, indicating that the isolates also produced the amylase. Lugol’s iodine solution can be used to detect the presence of starch. Iodine solution will form a blue to brown color. Hydrolyzed starch does not produce change of color (7). A similar result reported by Abejo et al (2018), which succeed to isolate amylase producing bacteria from hot springs in Southern Ethiopia (8). Out of 72 isolates, 10 amylases positive were screened on the basis of starch hydrolysis, indicated by the formation of clear zone around the colony on the growth medium supplemented by starch. The isolates also showed positive in citrate test. A citrate test was used to select the bacteria that have the ability to consume citrate as its sole source of carbon and ammonium as the sole nitrogen source (Ayitto). This is in accordance with Tariq et al (2018) which reported Bacillus species from sediment and swept clean flora, showed positive results in citrate utilization. 3 of 4 isolates resulted in positive in catalase test (9). Catalase is an oxidoreductase enzyme. This enzyme works as detoxification system in living cells against reactive oxygen species that are formed as a by-product of metabolic reactions (10). The Gram staining of these isolates showed that all the isolates are Gram-positive with rod-shaped bacteria. The result of Gram staining was showed in Figure 4.
Figure 4. Result of Gram staining under light microscope with magnification 1000×. (a) UTMP 11; (b) UTMP; (c) UTMP 14; UTMP 15.

The aim of Gram staining is to divide the group of bacteria into positive and negative results based on their cell wall and cell wall permeability. Decolorization will cause significant damage to the surface of Gram-negative cells and only a small amount of damage to Gram-positive cells. Gram-negative bacteria are thought to have thin-walled cells and rich in lipid, causing cells to lose the first dye (crystal violet) and bind the second dye (safranin). Gram-positive cells are thick-walled cells and lipid-poor, appear blue from retaining the crystal violet.

Inulinase activity

Inulinase activity of bacteria is indicated by the formation of a clear zone on Czapex-Dox media supplemented with 1% inulin. The inulinase activity of isolates UTMP 11, UTMP 14, UTMP 15 and UTMP 17 showed in Figure 5.
Figure 5. The inulinase activity of isolates UTMP 11, UTMP 14, UTMP 15 and UTMP 17. The formation of the clear zone due to inulin hydrolysis showed by the white arrow.

Studies on isolation of thermophilic bacteria producing inulinase are still very few reported. Most studies of inulinase-producing bacteria are mesophilic bacteria and fungal. Microbial inulinase has great potential in industrial to produce fructose from inulin. Inulinase produced by microorganism consist of exo-inulinase and endo-inulinase. Exo-inulinase splits off the terminal fructose units from non-reducing end of the inulin, and also hydrolyzed sucrose and raffinose. Endo-inulinase targets the β-2,1 linkage of inulin and hydrolyzes it into fructose (11). Therefore, inulinase has a wide usage in industrial to produce fructose syrup by exo-enzymatic hydrolysis of inulin with D-fructose content over 95% or for the production of oligofructose syrups by endo-enzymatic hydrolysis (12). The plate screening method is generally used to detect extracellular hydrolytic enzymes produced by bacteria. The presence of inulinase on agar plates can be detected by formation of clear zone surrounding the bacterial colony after incubation. (5). The previous study reported bacterial inulinase isolated from the soil of sugarcane field. 4 microbes including Bacillus subtilis, Lactobacillus casei, Pseudomonas aeruginosa and Achromobacter sp were identified as inulinase producers. The optimum parameters of these bacteria were found at 40°C and pH 5 (2). Another study reported that inulinase producing bacteria obtained from rhizosphere of Taro, Dahlia, Garlic, and Onion. Among all the isolates, Bacillus sp considered as good inulinase produce and showed good enzyme production at 37°C and pH 6 using Dahlia as raw inulin source (13).

Conclusions
The study reported that thermophilic bacteria isolated from hot springs show inulinase activity. There are 4 isolates that show good inulinase activity at temperature 48°C, namely UTMP 11, UTMP 14, UTMP 15 and UTMP 17. All isolates are Gram-positive and rod-shaped bacteria. This revealed that thermophilic bacteria have potential as a source of enzyme inulinase. For further study, it is necessary to identify the inulinase producing thermophilic bacteria also production and characterization of crude inulinase produced by these bacteria.

Molecular Identification

The result of molecular identification of UTMP 11, UTMP 14, UTMP 15 and UTMP 17 was shown in table 2.

| Sequence code | The similarity result of homology-based on Basic Local Alignment Search Tool (BLAST) |
|---------------|-----------------------------------------------------------------------------------|
| UTMP 11       | Bacillus subtilis strain JCM 1465 |
|               | Accession no: [NR_113265.1](https://www.ncbi.nlm.nih.gov/nuccore/NR_113265.1) |
|               | Homology: 99.86% |
|               | Query coverage: 100% |
| UTMP 12       | Bacillus subtilis strain JCM 1465 |
|               | Accession no: [NR_113265.1](https://www.ncbi.nlm.nih.gov/nuccore/NR_113265.1) |
|               | Homology: 99.72% |
|               | Query coverage: 100% |
| UTMP 14       | Bacillus tequilensis strain 10b |
|               | Accession no: [NR_104919.1](https://www.ncbi.nlm.nih.gov/nuccore/NR_104919.1) |
|               | Homology: 99.79% |
|               | Query coverage: 100% |
| UTMP 15       | Bacillus subtilis strain JCM 1465 |
|               | Accession no: [NR_113265.1](https://www.ncbi.nlm.nih.gov/nuccore/NR_113265.1) |
|               | Homology: 99.64% |
|               | Query coverage: 100% |
| UTMP 17       | Bacillus tequilensis strain 10b |
|               | Accession no: [NR_104919.1](https://www.ncbi.nlm.nih.gov/nuccore/NR_104919.1) |
|               | Homology: 99.57% |
|               | Query coverage: 100% |

Based on the identification result, the isolates identified as Bacillus subtilis strain JCM 1465 and Bacillus tequilensis strain 10b. This is the first study to report that Bacillus subtilis strain JCM 1465 and Bacillus tequilensis strain 10b showed inulinase activity. Inulinase is produced by different types of bacteria including Bacillus sp, Lactobacillus, Clostridium, and Pseudomonas. From previous study, a total of ten isolates including Bacillus subtilis, isolated from sugarcane soil, showed inulinase activity. The molecular
weight of this enzyme was estimated at 45 KDa and optimum conditions were found to be 40°C and pH 5 (2). The previous report for *Bacillus tequilensis* which isolated from coastal mud describes that this strain showed the amylolytic enzyme (14).

Conclusion

based on the result, this is the first study that reported that thermophilic Bacillus subtilis strain JCM 1465 and Bacillus tequilensis strain 10b showed the inulinase activity. these strains might be a source of industrially enzymes mainly inulinase

Acknowledgment

The author would say thanks to the Ministry of Research, Technology and Higher Education which funded this research through collaborative research between Universitas Prima Indonesia and Universitas Riau (contract number: T/61/L1.3.1/PT.01.03/2019)

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