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RAW STARCH-DEGRADING AMYLASE FROM BACTERIA IN KARANG MUMUS RIVER, SAMARINDA

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Abstract

Raw starch-degrading amylase (RSDA) is an enzyme that has the ability to degrade starch granules into glucose without undergoing gelatinization. This research was conducted to explore and characterize extracellular RSDA-producing bacteria from body water of the Karang Mumus River, Samarinda. RSDA activity was analyzed qualitatively in nutrient agar containing 1% starch granules, bacterial colonies with RSDA activity are clear halos around bacterial colonies after the plates were flooded with iodine solution. Five of the 14 bacterial colonies secreted an RSDA extracellularly. The RSDA enzyme from the 5 bacteria was tested for amylase activity using the Dinitrosalicylic Acid (DNS) method. Bacteria with colony code KM 5 had the highest RSDA activity of 0.332 U/mL.The optimum working conditions for RSDA are at pH 5 and a temperature of 40°C. Identification of bacterial genotypes using the 16S rRNA gene showed that KM5 was *Klebsiella* sp, referred to as *Klebsiella* KM5.

INTRODUCTION

Starch is the main ingredient in various industries, especially the food industry. Starch is also the main ingredient in making bioethanol. Native starch exists naturally as insoluble semi-crystalline granules of different particle sizes and shapes which are assembled by amylose and amylopectin[1]. The important step of starch processing is gelatinization, which requires high energy. Eliminating the gelatinization step will reduce costs in the starch industry. The way to eliminate the gelatinization steps is to use an amylase that is capable of degrading raw starch, known as Raw Starch Degrading Amylase (RSDA)[2].

Amylase is produced from plants, animals, and microorganisms, but the RSDA that has been studied comes from bacteria. RSDA from bacteria has many advantages such as the amylase production capacity of bacteria is more economical, can be done in large quantities, and can be manipulated for desired characteristics [3].

The crude starch-degrading amylases that have been studied come from various bacteria. RSDA-producing bacteria include *Pontibacillus sp. ZY* [4], *Bacillus megaterium* NL3 comes from seawater [5], *Panninobacter phragmatetus* comes from Natar hot springs [6], *Bacillus mojavonsis* SO-10 [7], *Roseateles terrae* HL11 comes from soil [8].

The unique characteristics of enzymes are required for industrial processes involving low temperatures and a wide pH range. Side reactions in industry may occur at high temperatures which must be avoided [9]. Furthermore, in addition to temperature, many industrial processes are also carried out under extremes of pH, pressure, salinity, or in the presence of detergents, non-aqueous solvents, among others[10]. Unique enzymes can be sourced from microorganisms in various environments, including those with high salinity and varying pH levels in rivers.

The Karang Mumus River in Samarinda is a river whose watershed is used for residential areas, places for various types of employment such as tofu and tempe processing, hotels, markets, livestock farms, and agricultural areas, and some of them are still empty land. The type of land used can have a major impact on the level of microbial pollution in rivers. The content of *E. coli* at seven sampling points in the Karang Mumus River, Samarinda did not meet the *E. coli* parameter requirements [11]. In this research, bacteria have been isolated from

Karang Mumus River water that is capable of producing RSDA and characterizing the RSDA.

EXPERIMENT

Material

The equipment used was an incubator (Memmert), water bath (Memmert), autoclave SA-232X (Tomy, Japan), Genesys 10S UV-Vis spectrophotometer (Thermo Scientific), laminar air flow and micropipettes (Eppendorf, Germany). Meanwhile, the materials used were nutrient agar (Merck), tryptone (Himedia), yeast extract (Criterion), NaCl (Merck), Bacto agar (Himedia), soluble starch (Merck), I_2 (Merck), and dinitro salicylic acid/DNS (Merck). Commercial-grade rice starch granules were purchased from a local market in Samarinda, Indonesia.

Procedure

Bacteria Isolate Screening

Bacteria isolate was inoculated on nutrient agar plates (2% w/v nutrient agar).

Qualitative and Quantitative RSDA Activity

In the qualitative selection of bacteria that produce RSDA enzymes, the media, namely Nutrient, contains sterile raw starch (1% w/v). Each single colony of bacteria was streaked onto the agar surface. After that, the bacterial culture was incubated at room temperature for 24 hours. The bacteria that had been incubated were added to the iodine solution. The clear zone formed around the bacteria indicated the presence of RSDA-producing bacteria.

Quantitative determination of RSDA activity was determined by using the 3,5-dinitrosalicylic acid (DNS) method [12]. The starch-hydrolyzing activity of RSDA was determined by measuring the release of reducing sugar from soluble starch. The reaction mixture contained 25 µL of the enzyme and 25 µL of 1% (w/v) of soluble starch in 50 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4) pH 6.0. After incubation at room temperature for 30 min, 50 µL of DNS was added to the mixture, and the reaction was stopped by boiling for 10 min. The mixture was diluted with ddH_2O to a final volume of 1 mL. Absorption was measured at 540 nm and the activity was calculated using soluble glucose as the standard. One unit of RSDA activity was defined as the amount of enzyme releasing 1 µmol glucose per minute under the assay condition.

Bacteria Identification

The selected bacteria had grown on nutrient agar at a temperature of 37°C for 24 hours and then sent to Genetika Science Laboratorium, PT. Genetika Science Indonesia, Jakarta for bacterial species barcoding. Bacteria were identified based on the identification of 16S rRNA.

Genomic DNA was extracted by using the quick-DNA Bacterial Miniprep Kit (Zymo Research, D6005). PCR amplification with MyTaq HS Red Mix (Bioline, BIO-25048). The primer used were 27F (5'–AGAGTTTGATCMTGGCTCAG–3') and 1492R (5'– TACGGYTACCTTGTTACGACTT– 3'). The conditions for the PCR are as follows: DNA denaturation at 95°C for 10 sec, primer annealing at 52°C for 15 sec, and elongation at 72°C for 15 sec. The initial and final cycles included denaturation at 95°C for 1 min. PCR lasts for 35 cycles.

Effect of pH and temperature on α-amylase activity

The optimal pH of α-amylase activity was determined using by DNS method by evaluating the hydrolysis reaction in the pH 4.0–9.0 range using 50 mM succinic buffers and phosphate buffer at room temperature.

The optimal temperature of α-amylase activity was determined by evaluating the hydrolysis reaction in the 30–80◦C at optimum pH.

Scanning electron microscopy

Amylolytic activity on raw starch granules from rice was checked by mixing 10% of the respective raw starch suspension in a volume ratio 1:1 with the enzyme solution. Reaction mixtures were incubated at room temperature for 2 days, then centrifuged and the pellet was dried in an oven at 40°C. The dry pellets were sent to Lembaga Ilmu Hayati, Teknik dan Rekayasa Universitas Airlangga for photographing by using a scanning electron microscope.

RESULT AND DISCUSSION

RSDA Activity

The results of bacterial isolation from Karang Mumus River water samples selected 14 single colonies of bacteria. Qualitative selection of RSDA-producing bacteria showed that five of the 14 single colonies of bacteria had RSDA activity (**Figure 1**).

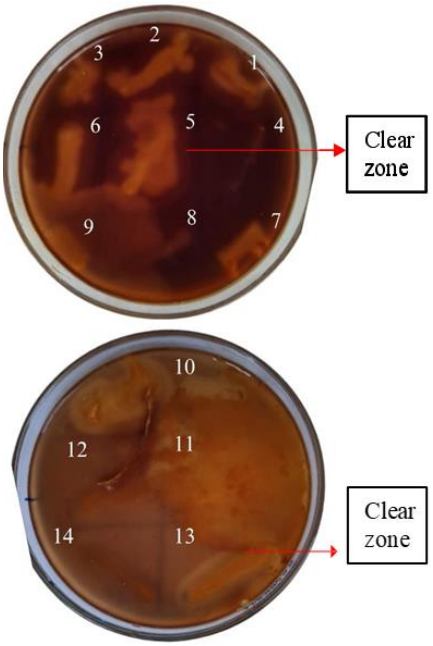

Figure 1. Qualitative screening of bacterial RSDA activity.

Five bacteria that showed qualitative RSDA activity were then determined to have RSDA activity quantitatively using the DNS method [8]. The bacterial isolate code KM5 had the highest activity of 0.332 U/mL. RSDA from KM5 isolate was characterized and used for further tests.

Table 1. RSDA activity.

Bacteria	RSDA Activity (U/mL)
KM 2	0,104
KM 5	0,332
KM 6	0,116
KM 11	0,096
KM 13	0,072

Bacteria Identification

The KM5 bacteria, which was the RSDA-producing bacteria with the highest activity, was then identified by its species. The PCR results of the 16S rRNA gene obtained a DNA fragment with a size of around 1400 bp. The gene fragment resulting from the PCR was sequenced to determine the nucleotide sequence.

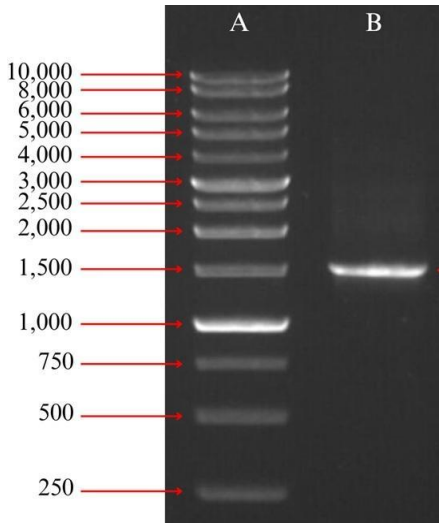

Figure 2. Electropherogram of 16S rRNA gene; A, molecular DNA marker; B, 16S rRNA gene of KM5.

The 16S rRNA gene sequence of isolate KM 5 was then aligned using the Basic Local Alignment Search Tool (BLAST) on the ncbi.nlm.nih.gov website. BLAST uses the neighbor-joining method to compare with worldwide DNA sequencing data deposited in gene bank databases. BLAST results are shown in **Figure 3**. BLAST and phylogenetic tree results show that isolate KM5 has the closest similarity to *Klebsiella* sp. strain LM1. Based on this, isolate KM5 is regarded to be a representative of *Klebsiella* sp..

Several studies state that *Klebsiella* sp is capable of producing strach-degrading amylase. Halophilic *Klebsiella pneumonie* can produce amylase [13] and *Klebsiella* sp. from industrial soil can produce α-amylase [14]. These two studies have not stated that the amylase produced by *Klebbsiella* is capable of degrading raw starch.

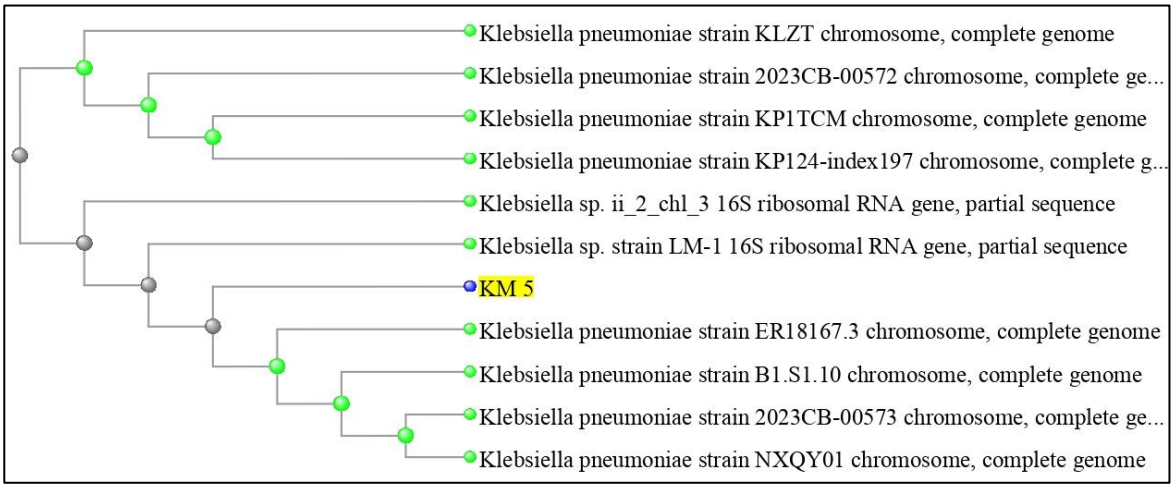

Figure 3. The phylogenetic tree of KM5.

pH Optimum on Amylase Activity

The optimum pH was determined to determine the effect of pH on amylase activity. The chosen pH range is 4-9 considering that most amylases work at that pH. Amylase of *Klebsiella* KM5 activity was relatively low at pH 5 (**Figure 4**). This enzyme is relatively stable in acidic conditions at pH 4-7. At alkaline pHs, 8 and 9, the activity is greatly reduced compared to the optimum pH, but at least RSDA still has activity. These characteristics may become important for industrial processes that require a wide pH range. This wide pH range is beneficial in industry because the pH in industrial processes does not need to be managed tightly [15], [16].

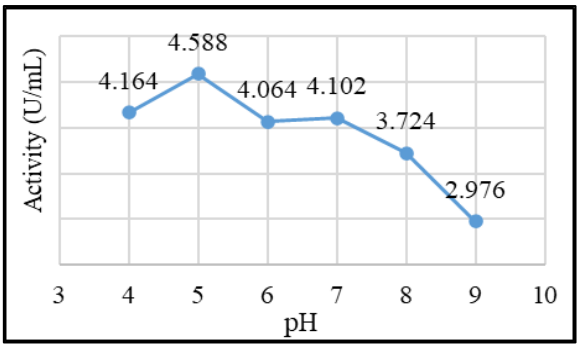

Figure 4. The effect of pH on RSDA activity.

Most amylases work optimally at pH 5-6. Amylase from *Citrobacter portucalensis* showed that a high amylase activity of 4.96 U/mL was obtained at pH 5.0 [17]. Amylase from *Pontibacillus* sp. ZY showed high activity at pH 6.0–7.5 [18]. RSDA from *Bacillus aquimaris* MKSC 6.2 showed high activity at pH 6 [19].

Temperature Optimum on Amylase Activity

The amylase of *Klebsiella* KM5 was measured at variation temperatures ranging from 30 to 80°C (**Figure 5**).

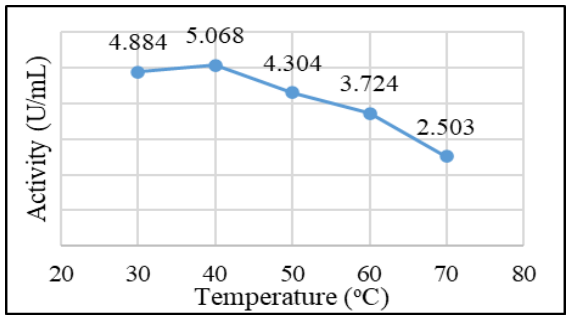

Figure 5. The effect of temperature on RSDA activity.

The optimum temperature of amylase from KM5 is 40°C. KM5 amylase catalyzed optimal at moderate temperatures. This moderate temperature is beneficial for industrial processes because it will reduce energy use in industry. This is also in line with the benefits of using RSDA in industry, which can also reduce energy consumption [9], [16].

Raw Starch Pattern Degradation

The ability of amylase to hydrolyze raw starch was investigated by incubating a suspension of rice starch granules in a neutral phosphate buffer (pH 7.0) for 48 hours at room temperature. This approach was used to ensure that the starch granules were degraded by raw starch-degrading amylase (RSDA) rather than by an acidic or basic buffer. The hydrolysis patterns were produced by the α-amylase's distinct attack on rice starch

granules as seen by scanning electron microphotographs (**Figure 6**).

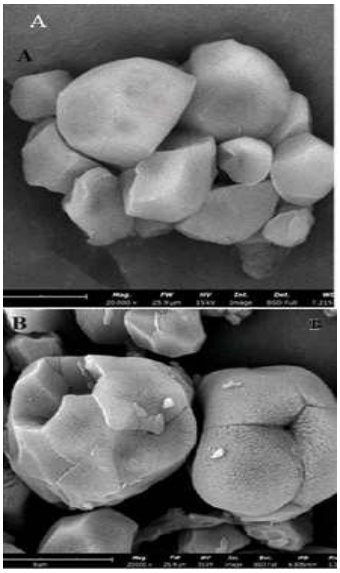

Figure 6. Scanning electron microscopy of rice raw starch untreated (A) and treated (B) rice raw starch granules by amylase of *Klebsiella* sp. KM5.

The action of the enzyme produced big deep holes on the surface of rice starch granules. The amylase from different bacterial sources forms different hydrolysis patterns in the same type of raw starch [20]. The amylase *Bacillus* sp. ALSHL3 action resulted in large and deep holes on the surface of rice starch granules [21]. Rice granules were hydrolyzed by the amylase of *B. amyloliquefaciens* strain ABBD which were dispersed across the surface granules [22]. Raw starch degrading α-amylase from Jeotgalibacillus malaysiensis D5 can degrade raw rice granules only around 16%, this is thought to be because rice starch contains a lot of amylose, thereby less prone enzyme activity [23].

CONCLUSION

KM5 isolates obtained from the body water of Karang Mumus River in Samarinda is *Klebsiella* sp. This bacteria has the potential to produce raw starch-degrading amylase. This RSDA bacteria works optimally at pH 5 and temperature 40^{o}C.

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