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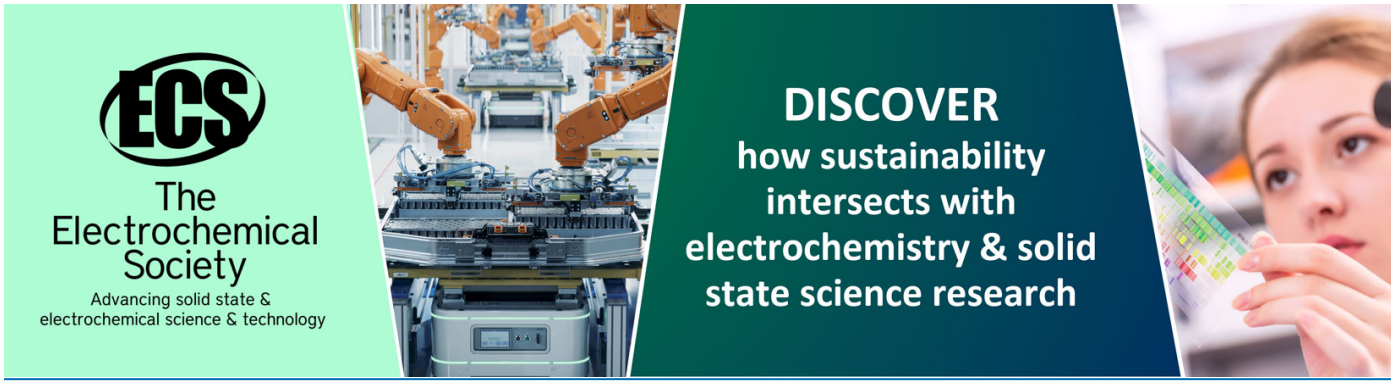

Identification of α-amylase gene by PCR and activity of thermostable α-amylase from thermophilic *Anoxybacillus thermarum* isolated from Remboken hot spring in Minahasa, Indonesia

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Abstract. Information of thermophilic bacteria in Indonesian hot springs is limited, especially in the province of North Sulawesi. The study aims to isolate and identify thermophilic bacteria from Remboken hot springs at Minahasa Indonesia, identify the α-amylase gene and its enzyme activity. To identify the thermophilic bacteria, it was conducted the morphological analysis and biochemistry tests. Th microbial species was identified using PCR and sequencing of 16S rRNA followed by BLAST search on the EZBioCloud database, whereas the α-amylase gene was identified by Polymerase chain reaction (PCR). Starch hydrolysis test was used to analyze α-amylase activity qualitatively. It was done using iodine method (Fuwa method). The results showed that thermophilic bacteria isolated belong to the species *Ureibacillus suwonensis*, *Anoxybacillus thermarum*, and *Anoxybacillus mongoliensis*. PCR analysis showed that *Anoxybacillus thermarum* FRM-RBK02 possesses an α-amylase gene. The strain was able to hydrolyze amylose, indicated by a clear zone around the growth; hence exhibiting α-amylase activity. The the crude amylase of *Anoxybacillus thermarum* FRM-RBK02 exhibited optimum activity at 80°C and pH 7.0. Overall, *Anoxybacillus thermarum* FRM-RBK02 has α-amylase gene and could produce thermostable α-amylase with amylolytic capability.

Keywords: α-amylase, 16S rRNA gene, thermophilic bacteria

1. Introduction

Thermophiles are the microorganisms which are adapted to live at high temperatures. They are stable to such high temperatures because they have several modifications in their structural components and biomolecules such as proteins, lipids, enzymes, ribosome, RNA and DNA [1]. Thermophilic microorganisms have gained a great deal of attention in the last two decades [2-4]. This is due to the fact that enzymes from thermophilic microbes are capable of functioning at high temperatures, where most mesophilic proteins are denatured, and are even active at elevated temperatures [6-9].

Amylases, starch degrading enzymes, is a class of important enzymes used in industry and accounts for high proportion of the enzyme market [10]. Amylases can be divided into three types, *viz*. α-amylase that hydrolyzes α-1,4 bonds and bypasses branched linkages, β-amylase that breaks down α-1,4 and cannot bypass α-1,6 branch linkages and produces maltose as a product, and γ-amylase that hydrolyzes α-1,4 and α-1,6 linkages and breaks down the substrate from the non-reducing end, and in turn releasing monosaccharides as the end product.

Thermostable α-amylase has gained importance in starch processing, brewing and sugar production, pharmaceutical industries, textile industries and in detergent manufacturing [11, 12]. α-Amylases produced by thermophilic bacteria are more favorable than those produced by mesophiles because stability at higher temperatures give benefits such as increased substrate solubility, decreased viscosity of the medium and lowered risk of microbial contamination or higher rates of concurrent non-enzymatic reactions [13]. In light of this fact, more studies are needed to find new thermophilic bacteria and thermostable amylases.

This paper reports an effort to isolate, identify, and characterize thermophilic bacteria from two hot springs in Remboken, North Sulawesi Indonesia with particular reference to a strain, designated *Anoxybacillus thermarum* FRM-RBK02. Furthermore, the presence of α-amylase gene in its genome,

the potential of this bacterial strain to produce thermostable α-amylase, and the activity of this enzyme were also investigated.

2. Experimental Method

2.1. Sample Collection and Characterization

A total of four water samples were collected from hot springs using 50ml sterile Falcon tubes. Two hot springs in Remboken, Minahasa, North Sulawesi Indonesia were chosen to collect the samples. Muddy water samples were used immediately for enrichment in nutrient broth at 50°C. One-day enrichment culture was streaked on nutrient agar to obtain separate colonies. Pure cultures of the bacterial isolates were determined for their thermophilic characteristics. Isolates in test tubes containing nutrient broth were incubated at initial temperature of 50°C for 12 h. After a 48 h incubation period each broth culture of bacteria were streaked onto freshly prepared nutrient agar. Bacterial isolates growing in the plates were selected and again tested for their tolerance at elevated temperature. Finally, isolates that could tolerate temperature of 80°C was selected for further study. Characterization of each isolate was done by examination of colony color, size, elevation, margin, texture, and pigmentation, in addition to Gram staining. Various biochemical tests like indole, motility, citrate, methyl red, catalase and oxidase tests were performed.

2.2. PCR Amplification and Sequencing of 16S rDNA.

Genomic DNA from pure strains is extracted using a DNA extraction kit (Plant Genomic Mini Kit, Geneaid). Universal bacterial primer 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3') and the domain bacteria-specific primer 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') were used for 16S rDNA amplification. Amplification of DNA was carried out under the following conditions: denaturation at 94°C for 5min followed by 30 cycles of 94°C for 45 s, 48°C for 45 s, 72° C for 90 s, and final extension at 72° C for 5 min. Amplified PCR products of bacterial isolates were analyzed by electrophoresis with 1% agarose gel. The purified PCR products were sequenced by 1st BASE, Malaysia, using Genetic Analyzer (Applied Biosystems 3130 XL, Switzerland). The deduced sequences were subjected to BLAST algorithm all available databases to retrieve for homologous sequences. For species identification, BLAST search was conducted on EZBioCloud database [14].

2.3. PCR amplification of α-amylase gene

Primers used to amplify α-amylase gene were amy3-F (5'- ACGAACGGCGAGGGTGCAGC -3') and ldh2-R (5'- GCCGCTGCCGATGACGCG -3'). Amplification of DNA was carried out under the following conditions: denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 65°C for 45 s, 72°C for 90 s, and final extension at 72°C for 5 min. Amplified PCR products of bacterial isolates were analyzed by electrophoresis with 1% agarose gel.

2.4. Starch Hydrolysis Test

The starch hydrolysis test determines the ability of microorganisms to degrade starch in the media by producing hydrolytic extra cellular enzymes. Bacteria were propagated on basal media containing 1% (w/v) soluble starch. Plates were inoculated with the isolated bacteria and incubated at 65°C for 24 hours. Colonies were exposed to 2% iodine solution and observed for the zone of hydrolysis around each colony. Changes in the plate was monitored. The starch in the plate was changed to blue-brown by the iodine reagent. Zones where starch has been processed by bacterial growth display clear halos in the midst of the dark plate

2.5. Quantitative amylase activity test

The activity of α-amylase was assay using modified method of Fuwa [15]. In a typical run, 50 µL of soluble starch in citrate-phosphate buffer, pH 7.0 was mixed with 50 µL of enzyme solution. After incubation at 70°C for 10 min, the enzymatic reaction was terminated by the addition of 50 µL 1 M HCl. To the reaction mixture, 50 µL iodine solution containing 2% KI and 0.2% I2 and 800 µL citrate-phosphate buffer, pH 7.0 was added. Absorption was measured at 600 nm and the activity was calculated using soluble starch as the standard. Hydrolysis of 1 mg mL−1 soluble starch in 1 min was defined as 1 U mL−1 of enzyme activity. The optimal pH of *α*-amylase activity was determined by evaluating the hydrolysis reaction in the range of pH 4.0–8.0 using citrate-phosphate buffer at 70°C. The optimal temperature of *α*-amylase activity was determined by evaluating the hydrolysis reaction in the temperature range of 30–90°C at pH 7.0.

3. Results and Discussion

In this study, bacterial isolates were obtained from two hot springs in Remboken Minahasa, North Sulawesi Indonesia. The temperatures of the hot springs were 57 - 66°C and pH 7-8. Based on morphological characteristics and various identification tests conducted, such as indole, motility, citrate, methyl red, catalase, oxidase and Gram staining that the bacteria belonged to Gram-positive bacilli (data not shown).

To identify the isolates to species level, molecular characterization was conducted by sequencing of 16S rRNA gene. PCR amplification was done to amplify the 16S rRNA gene. **Figure 1** shows the results of PCR analysis of four isolates. All isolates yielded a single band about 1.5 kb, which corresponded with the expected size of the 16S rRNA gene fragment.

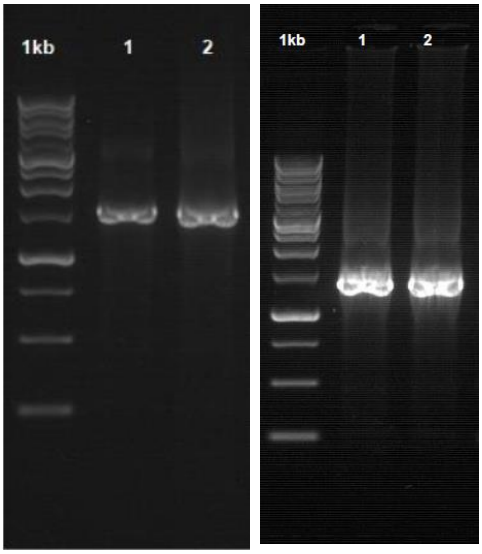

Figure 1. PCR products of four isolates obtained, designated FRM-RBK01, FRM-RBK02, FRM-RBK03, and FRM-RBK04, from left to right.

The final identification of the isolates was assessed by the 16S rDNA sequencing. The 16S rDNA sequences from the four haplotypes were aligned with their closely related reference bacterial sequences obtained from the GenBank by Basic Local Alignment Search Tool (BLAST) program. Sequence analysis showed high similarity with those of the reference strains available in the GenBank databases. Based on BLAST search conducted on EZBioCloud database [14], the four haplotypes shared highest similarity with *Anoxybacillus thermarum* for FRM-RBK01 and FRM-RBK02*, Ureibacillus suwonensis* for FRM-RBK03 and *Anoxybacillus mongoliensis* for FRM-RBK04.

To investigate the presence of α-amylase gene, PCR amplification was conducted using amy3- and ldh2-R pair of primers [16]. All isolates yielded a single band about 1,1 kb, which corresponded with the expected size of the α-amylase gene fragment.

Based on the abovementioned findings, one strain namely *Anoxybacillus thermarum* FRM-RBK02 was chosen to investigate the amylolytic ability and the quantitative activity of α-amylase. *Anoxybacillus thermarum* was chosen because previous studies reported that α-amylase from this species exhibits relatively high stability against several surfactants and has a potential to be used for commercial liquid and solid detergents at 55°C [17]. *Anoxybacillus thermarum* is a relatively new aerobic thermophilic bacterium which was first isolated from the mud of hot spring in Euganean, Abano Terme, Padova Italia [18]. This species is a rod shape bacterium, Gram-positive, motile, and can grow at temperatures of 55 - 67°C.

Starch hydrolysis test conducted to investigate the amylolytic ability of *Anoxybacillus thermarum* FRM-RBK02 yielded a positive result, which was indicated by the formation of a clear zone around the growth **(Figure 2**). This finding confirms the result of α-amylase gene amplification that this strain of

bacterium possesses an α-amylase gene and the product of gene, *viz*. α-amylase enzyme, is secreted to the agar medium and is capable of degrading starch in its surroundings.

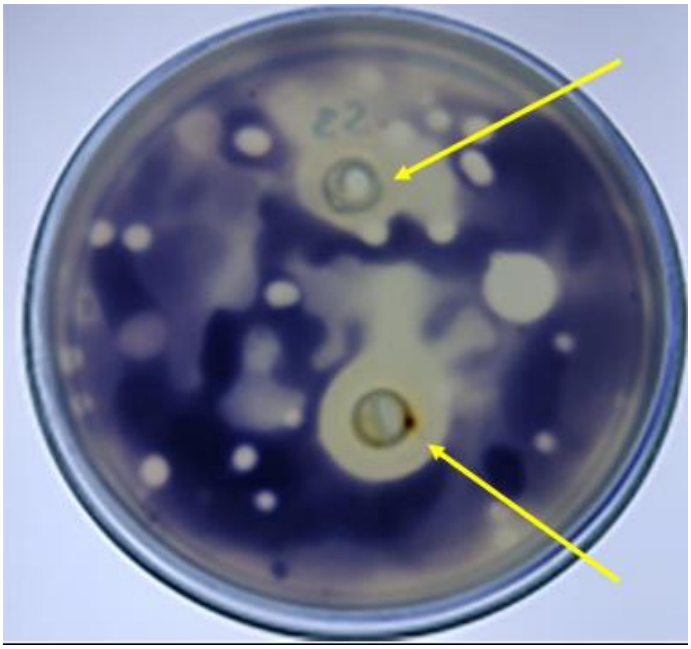

Figure 2. Result of starch hydrolysis test on *Anoxybacillus thermarum* FRM-RBK02. Note the formation of clear zone around the growth (yellow arrows).

To investigate the activity of α-amylase quantitatively, an assay employing the modified method of Fuwa [15] was conducted. Results showed that *Anoxybacillus thermarum* FRM-RBK02 has an α-amylase activity of 0.76 U/mL at 70°C incubation temperature and pH 7. Since stability of enzyme is very sensitive to pH and temperature, a series of experiments were done to study the effects of varying temperatures and pH.

Effects of pH on amylase activity were observed by conducting amylase assay with citrate-phosphate buffer of different pH (4.0–8.0) in which 1% soluble starch solutions were prepared. The pH stability of amylase was incubated at 70°C, as previously described. It was found that the α-amylase produced by *Anoxybacillus thermarum* FRM-RBK02 is stable at a broad range of pH, but optimum enzyme activity was observed at pH 7.0 (**Figure** 3). At pH 8.0, the enzyme still retains approximately 72% of its initial activity. This finding corroborates the optimum pH of α-amylase as previously reported by other workers [19-21].

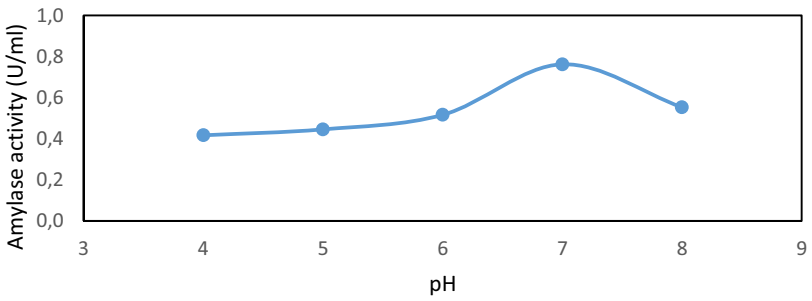

Figure 3. Effects of reaction pH on amylase activity

To investigate the effects of temperature on amylase activity, amylase assay was done at temperature 30–90°C in an incubator. The thermostability of amylase was studied by incubating the crude amylase at different temperatures as indicated for 10 minutes. It was found that α-amylase produced by *Anoxybacillus thermarum* FRM-RBK02 showed higher activity at high temperatures, with optimum activity was observed at 80ºC (**Figure 4**). At 90°C the enzyme still retained approximately 87% of its initial activity. Activity of amylase at high temperatures revealed in this study is comparable with that of previously reported by other workers. Rekadwad [22] found that *Geobacillus amylase* was stable at 90ºC temperature and retained its 85% initial activity. Similarly, Ivanova *et al*. [23] reported that *Bacillus licheniformis* showed optimum activity at 90ºC.

This high-temperature activity characteristic of the α-amylase lends the strain *Anoxybacillus thermarum* FRM-RBK02 a potential microbe to produce α-amylase required for high temperature applications. Due to the elevated temperatures used during some industrial applications (such as sugar manufacturing processes and/or raw material pretreatments in bioethanol production), stability at high temperatures is an important feature for the utilization of α-amylase [24]. In addition, other reasons to choose thermostable enzymes in industries is of course the intrinsic thermostability, which implies possibilities for prolonged storage (at room temperature), increased tolerance to organic solvents, reduced risk of contamination, as well as low activity losses during processing even at the elevated temperatures often used in raw material pre-treatment [25].

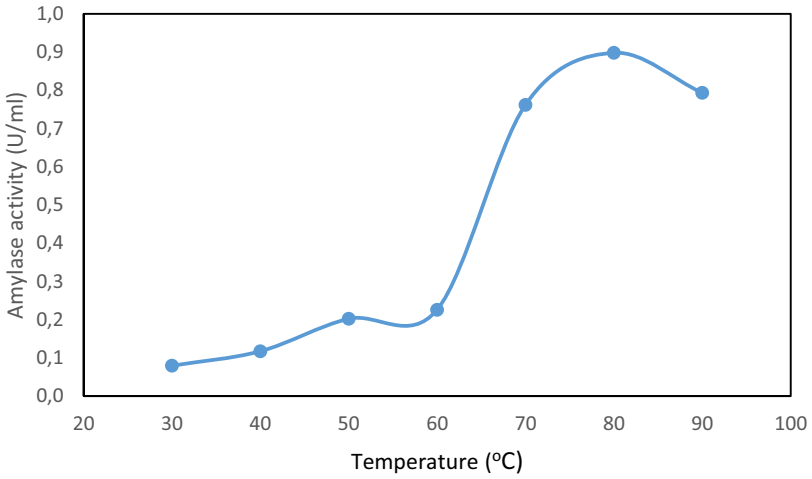

Figure 4. Effects of temperature of reaction on amylase activity

4. Conclusions

In this work, we report the isolation, identification, and characterization of an isolate of thermophilic bacterium isolated from a hot spring in Remboken, Minahasa, North Sulawesi, Indonesia. Based on morphological, biochemical and molecular identification, this isolate was designated *Anoxybacillus thermarum* FRM-RBK02. This bacterial strain has an α-amylase gene in its genome and is able to produce extracellular α-amylase with amylolytic ability. The optimum activity of α-amylase produced by this strain was observed at 80ºC and pH 7.0. Based on the findings of this work we propose that *Anoxybacillus thermarum* FRM-RBK02 may be commercialized after optimizing conditions for enzyme production

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