ISSN: 1410-8917
Jurnal Kimia Sains & Aplikasi
e-ISSN: 2597-9914

Jurnal Kimia Sains dan Aplikasi 23 (7) (2020): 238–243

Jurnal Kimia Sains dan Aplikasi
Journal of Scientific and Applied Chemistry

Journal homepage: http://ejournal.undip.ac.id/index.php/ksa

Characteristics of Raw-Starch Degrading Amylase Bacteria from Natar Hot Spring Lampung

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https://doi.org/10.14710/jksa.23.7.238-243

Article Info

Article history:

Received: 28th March 2020
Revised: 28th June 2020
Accepted: 30th June 2020
Online: 31st July 2020

Keywords:
α-amylase; raw starch; *Panninobacter*

Abstract

Indonesia has a diversity of hot spring as a habitat of bacteria. One of the hot springs is Natar hot spring, Lampung. This study is to report the characteristics of a bacterium called Nat1 isolate that produces amylase to degrade raw starch from Natar hot spring. Water samples were taken from hot springs with a temperature of 45°C and a pH of 7.0. Nat1 was isolated by screening on the medium of Starch-Luria Bertani at 37°C. Its amylase-producing bacteria showed an optimum amylolytic activity of a crude enzyme of Nat1 isolate in soluble starch was 267.2774 U/mL at 60°C. Genotypic identification results using the 16S rRNA gene showed that the Nat1 isolate is identified as *Panninobacter phragmatetus*. A crude enzyme of Nat1 isolate showed a novel amylase ability and could degrade the raw starch substrates, such as corn and sago, with the amount of reducing sugar for each raw starch, 37.0688 µmol/mg, and 24.2697 µmol/mg. In conclusion, Nat1 amylase is potentially used in industry for its ability to degrade raw starch directly.

1. Introduction

Acid hydrolysis of starch is considered a simple method, as the acid is cheap and quickly obtained [1]. The result of starch hydrolysis by acid are glucose, xylose, arabinose as the main products. On the other hand, furans (furfural and hydroxymethylfurfural) as undesirable byproducts are also obtained [2]. Acid hydrolysis of the starch industry, especially the food and beverage industry, is considered unsafe if consumed [3]. Therefore, enzymatic hydrolysis was developed using α-amylase as an alternative to starch hydrolysis.

Starch hydrolysis using α-amylase does less production of unwanted byproduct, has specific and higher yield, approximately obtained 95% more of glucose [4]. Starch hydrolysis by amylase is also used in the detergent industry for stain removal applications [5]. Based on Business Communication Company (BCC) Research, the global enzyme market for the detergent industry is projected to increase to 1.3 billion in 2021 [6]. Amylase production in the world is 30% of the total production of all enzymes [7].

Amylase bacteria are used predominantly in industrial applications because their production is more accessible, cheaper, and faster than other amylase microbes [8]. Furthermore, bacterial amylase is easily performed for genetic engineering studies. A large number of amylase bacteria species have been isolated, mostly are *Bacillus species* [9]. Amylase, which is involved in the thawing step in the starch processing industry, requires thermostable amylase in a high-temperature process [7]. The study of thermostable amylase-producing-thermophilic bacteria from hot springs is one of the trends of screening thermophilic bacteria that produce thermostable amylase. Some of the thermophilic bacteria produce thermostable amylase have been reported [10, 11, 12, 13, 14, 15].

Furthermore, α-amylase is widely known for degrading various substrates [16], which could be a soluble substrate and a raw substrate. Based on a recent

study of amylase, there are some raw starch degrading α-amylases; α-amylase from *Bacillus aquimaris* MKSC 6.2 (BaqA) [17], *Geobacillus thermoleovorans* (GTA) [18], and *Geobacillus thermoleovorans* (Gt-amy II) [19] have been reported. The ability of some α-amylases, as mentioned above, that can directly hydrolyze a raw starch is considered useful in the starch-processing industry. The initial step causes gelatinization is to introduce amylose and amylopectin to be easily degraded by amylase. This step requires high temperature and can be neglected if using raw starch degrading α-amylases to reduce the industrial cost.

Indonesia has a diversity of natural hot spring as a habitat for thermophilic bacteria. One of the hot springs is Natar hot spring in Lampung. Limited information about the identification of thermophilic bacteria that can produce α-amylase from Natar is the basis of research on the screening of thermophilic bacteria that produce amylase in Natar. Based on the previous study, one bacterium (Nat1 isolate) with amylolytic activity has been screened from Natar hot spring in Lampung. The purpose of this study is to determine the characteristics of Nat1 isolate.

2. Methodology

This research was divided into three steps to determine the characteristics of Nat1 isolates. Bacterial characteristics were determined in the form of (i) morphology and physiology of Nat1 isolates, (ii) identification of Nat1 isolate genotypes based on 16S ribosomal RNA analysis, and (iii) determination of amylolytic activity in soluble and raw starches and (iv) scanning electron microscopy of raw starch granules treated by Nat1 isolates.

2.1. Equipment and Materials

In this research, the tools used were incubator (Memmert), water bath (Memmert), autoclave SA-232X (Tomy, Japan), Genesys 10S UV-Vis spectrophotometer (Thermo Scientific), and micropipettes (Eppendorf, Germany). While the materials used were tryptone (Himedia), yeast extract (Criterion), NaCl (Merck), bacto agar (Himedia), soluble starch (Merck), I_2 (Merck), and dinitrosalicylic acid/DNS (Himedia). Commercial grade corn and sago starch granule were purchased from a local market in Bandar Lampung, Indonesia.

2.2. Bacterial Identification

Nat1 isolate was inoculated on Luria Bertani agar plates (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 2% w/v agar, 1% w/v soluble starch). Nat1 isolate was grown at a temperature of 37°C for 24 hours and then sent into Genetika Science Laboratorium, PT. Genetika Science Indonesia, Jakarta for bacterial species barcoding. Bacteria were identified based on the identification of full-length 16S rRNA. The bacterial identification included extraction of genomic DNA with quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005), PCR amplification with *MyTaq* HS Red Mix (BIO-25047) and purification, then bidirectional PCR product sequencing [20]. The resulting fragment of 1.3 kb was compared to other 16S rRNAs in the GenBank database using the NCBI BLAST tool. A phylogenetic tree was constructed using neighbor-joining by NCBI BLAST Tree Method with 1000 bostraps. The morphology and physiology properties of bacteria were determined at the Laboratory of Bacterial Diagnostic, Balai Veteriner Lampung, Bandar Lampung, Indonesia.

2.3. Determination of Amylolytic Activity on Soluble Starch

Nat1 isolate was grown in 50 mL Luria Bertani (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) on shaker at temperature of 37°C and 150 rpm for 24 hours. The amylolytic activity was determined by measuring the amount of reducing sugar using the DNS method [21]. Amylase assay was performed from a mixture of 25 µL α-amylase bacterial crude and 25 µL 1% w/v of soluble starch. A 50 µL DNS solution stopped the activity. The mixture was incubated in the water bath at 50°C for 10 minutes, and then the reaction stopped in the boiling water for 10 minutes. As a control reaction, DNS reagent was added before adding amylase. All reaction was performed triplicates. The absorbance was measured at 500 nm. The protein concentration was determined using the Bradford method with the absorbance measured at 595 nm [22]. One unit of amylase activity is defined as the amount of amylase needed to produce 1 µmol reducing sugar under specified conditions.

2.4. Determination of Amylolytic Activity on Raw Starch

The amylolytic activity of Nat1 isolate on corn and sago raw starch granule was determined by incubating 1% w/v of each raw starch with 1 mL of crude in a shaker at a temperature of 37°C and 150 rpm for 24 hours. Followed by 5000 rpm centrifugation for 10 min, the supernatant's amylolytic activity was measured by the DNS method as it was in line with the amylolytic activity's determination of soluble starch [21]. The activity was performed triplicates. Meanwhile, the crude pellets were sent into Laboratorium Terpadu dan Sentra Inovasi Teknologi, Universitas Lampung, Bandar Lampung, Indonesia, for scanning electron microscopy of hydrolysis amylase of the raw starch granule.

3. Results and Discussion

Based on the previous study, one Nat1 isolate was screened for their amylase activity when growing in selective media containing soluble starch. The appearance of the clear zone after staining with KI/I_2 solution around the Nat1 colony indicating the ability to hydrolyze soluble starch. Nat1 isolate has a crude amylolytic activity of 240.7267 U/mL in a 1% soluble starch substrate using DNS reagents at a temperature of 50°C. Hot springs are a common source of bacteria that produce enzymes, one of which is amylase. Several hot springs in Indonesia can be the source of bacterial growth and have potential applications in the starch-processing industry. *Bacillus megaterium* has been isolated from Hatuasa hot spring in Tuhelu Village Ambon, which excreted amylase after being screened on a starch agar plate [10]. *Bacillus licheniformis* BT5.9 has been identified as a potential bacterium producing a thermostable α-amylase from Changar hot springs in Malang [11]. Two

isolates (BR 002 and BR 015) of thermostable α-amylase were isolated from Bora Hot Springs, Central Sulawesi [12]. *Anoxybacillus flavithermus* AE3 was isolated as an α-amylase-producing bacterium from Bukit Kili hot spring in Solok, West Sumatera [13]. Amylase producing bacteria have been isolated from Dondang hot spring in Muara Jawa Sub District, Kalimantan [14]. One colony was identified as *Thermoactinomyces saachari* based on morphology and physiology identification as α-amylase bacteria from Singgahan hot spring, Tuban, East Java [15].

3.1. Morphology and Physiology Characteristics of Nat1 Isolate

The morphological analysis results showed that the Nat1 isolate produced circular, flat, light yellow colonies on agar plates. It has pink-red rods, coccobacilli, Gram-negative bacteria, positive for catalase and oxidase production, and γ-hemolytic on blood agar plates. The physiological tests for Nat1 isolate were carried out with Microbact GNB 12A/B/E, 24 E., The results of the Nat1 isolate physiological test, are shown in Table 1.

Table 1. The result of physiology characteristic of Nat1 isolate

Test	Result	Test	Result
Lysine	Positive	Gelatine	Negative
Ornithine	Negative	Malonate	Negative
H_2S	Negative	Inositol	Negative
Glucose	Positive	Sorbitol	Negative
Mannitol	Negative	Rhamnose	Negative
Xylose	Positive	Sucrose	Positive
ONPG	Positive	Lactose	Positive
Indole	Negative	Arabinose	Positive
Urease	Positive	Adonitol	Negative
V-P	Negative	Raffinose	Negative
Citrate	Positive	Salicin	Positive
TDA	Negative	Arginine	Negative

3.2. Identification of Nat1 Isolate

Fragments of 1322 base pairs of Nat1 isolate DNA were amplified and determined (Figure 1). The nucleotide sequence of 16S rRNA gene fragment Nat1 isolates (Figure 2) was aligned and compared to the 16S rRNA of various bacteria from GenBank data. The phylogenetic tree was constructed and showed that Nat1 isolate belongs to *Panninobacter phragmatetus* (Figure 3). It is suggested that amylase bacterial from Natar hot spring in Lampung, be identified as *Panninobacter phragmatetus* with strain name Nat1.

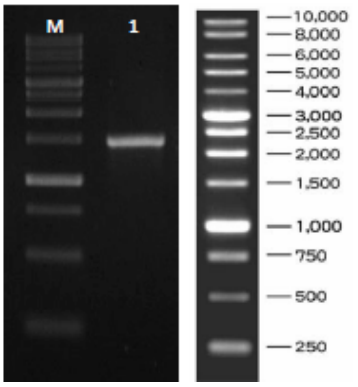

Figure 1. Electropherogram of 16S rRNA gene fragment Nat1 isolate. Line M, molecular marker; line 1, 16S rRNA gene fragment Nat1 isolate.

CGGTTAGCGCACCACCTTCGGGTAACCCAACTCCCATGGT
GTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC
GCGTCATGCTGTTACGCGATTACTAGCGATTCCAACTTCA
TGCTCTCGAGTTGCAGAGAACAATCCGAACTGAGACGGCT
TTTGGAGATTAGCTCCGGGTCGCCCCTTCGCTGCCCACTG
TCACCGCCATTGTAGCACGTGTGTAGCCCAGCCCGTAAGG
GCCATGAGGACTTGACGTCATCCCCACCTTCCTCTCGGCTT
ATCACCGGCAGTCCCCCTAGAGTGCCCAACTCAATGCTGG
CAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACC
CAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACC
TGTCCTGGCGTCCCCGAAGGGAACCCACGGTCTCCCGTGG
TAGCACCAAATGTCAAGGGCTGGTAAGGTTCTGCGCGTTG
CTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCC
CGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCC
AGGCGGGAAGCTTAATGCGTTAACTGCGCCACCAAGATGC
ATGCACCCTGACGGCTAGCTTCCATCGTTTACGGCGTGGA
CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGC
ACCTCAGCGTCAGTACCGGGCCAGTGAGCCGCCTTCGCCA
CTGGTGTTCTTCCGAATATCTACGAATTTCACCTCTACACT
CGGAGTTCCACTCACCTCTCCCGGACTCCAGACTCCCAGTA
TCAAAGGCAGTTCCGAGGTTGAGCCCCGGGATTTCACCCC
TGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTGA
TTCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCT
GGCACGAAGTTAGCCGGGGCTTCTTCTGCGAGTAACGTCA
TTATCCTCCTCGCTGAAAGAGCTTTACAACCCTAGGGCCT
TCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCA
TTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTG
GGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAG
ACCAGCTACTGATCGTCGCCTTGGTGAGCCATTACCCCACC
AACTAGCTAATCAGACGCGGGCCAATCCTTCGGCGATAAA
TCTTTCCCCCGTAGGGCGCATGCGGTATTAGCAGCCGTTT
CCAGCTGTTGTTCCGCACCAAAGGGTATGTTCCCACGTGT
TACTCACCCGTCTGCCACTCCCCGTTACCAGGG

Figure 2. The nucleotide sequence of the 16S rRNA gene from Nat1 isolate

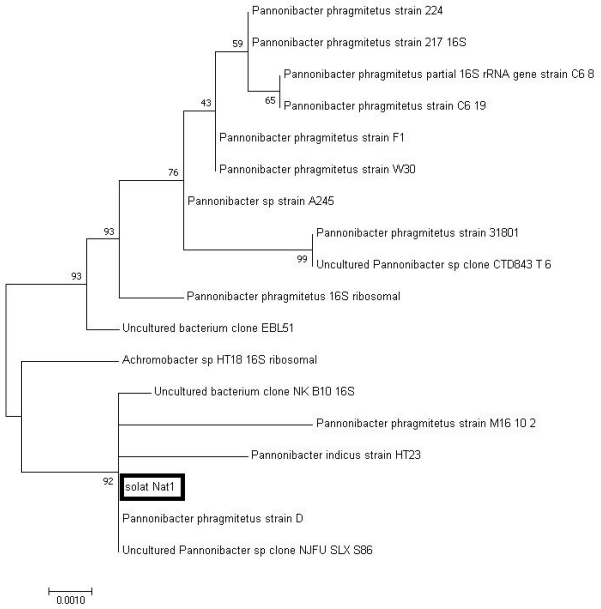

Figure 3. The phylogenetic tree of Nat1 isolate

3.3. Amylolytic Activity on Soluble Starch

The crude enzyme of *Panninobacter phragmatetus* strain Nat1 shows amylase activity in soluble starch and raw starch. The crude enzyme of Nat1 isolate can hydrolyze soluble starch at a temperature range 40–80°C (Figure 4). Nat1 isolate displayed an optimum amylase activity in soluble starch at a temperature of 60°C, with the amylolytic activity of 267.2774 U/mL determined by the DNS method [21]. Nat1 shows the amylolytic activities in soluble starch at a temperature of 40°C, and 50°C is 131.435 U/mL and 240.7267 U/mL, respectively. Nat1 can still actively hydrolyze soluble starch at temperature 70°C and 80°C, in which the amylase activities are 114.0236 U/mL and 23.5817 U/mL, respectively. Previous studies reported that several hot spring bacteria produce amylase, each of which had optimum amylolytic activity at 70°C [10], 50°C [11], 50°C and 70°C [12].

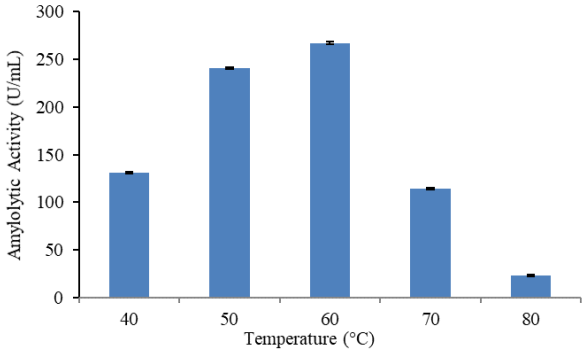

Figure 4. Amylolytic activities of Nat1 isolate on 1% soluble starch

Temperature is one of the important factors in the starch-processing industry. The starch industry needs a high-temperature process. Starch is gelatinized, then liquefied with amylase up to a temperature of 105°C. Followed by the saccharification step on hydrolyzed starch by enzyme needed glucoamylase, which can optimally be hydrolyzed at a temperature of 50°C and 60°C [23]. The specific characteristic of moderate thermostable microorganism used in industrial has optimal amylase activity at 50°C and above [24]. Nat1 has the potential to be used in the starch industry as a moderate thermostable bacterium, which has optimal amylolytic activity at a temperature of 60°C and still has amylolytic activity around 8% at a temperature of 80°C than the optimal activity.

3.4. Amylolytic Activity on Raw Starch

The determination of Nat1 amylase activity in raw starch has been carried out by measuring the supernatant adsorption after incubating a mixture of the Nat1 crude enzyme with corn and sago starch for 24 hours at 37°C. The amylolytic activities of Nat1 on corn and sago raw starch granule are 37.0688 µmol/mg and 24.2697 µmol/mg, respectively, determined by the DNS method [21]. The results of scanning electron microscopy of amylase treated corn granules by the Nat1 crude enzyme show that corn granules have small pores on the surface (Figure 5A), compared to corn granules starch, which had not been applied to amylase hydrolysis (Figure 5B).

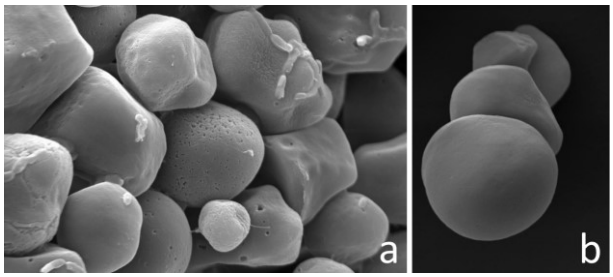

Figure 5. Result of scanning electron microscopy of corn granule. A. amylase treated corn granule by the crude enzyme of Nat1. B. amylase untreated corn granule.

Amylase treated corn granules were incubated with Nat1 crude enzyme. Small pores on the surface of amylase treated corn granules by Nat1 show that Nat1 crude enzyme can hydrolyze a corn raw starch granule. The results compared to the untreated corn granules which have no small pores on the surface. This indicates that Nat1 crude amylase can directly hydrolyze raw corn starch. Previous studies reported that α-Amylase from *Bacillus aquimaris* MKSC 6.2 and *B. amyloliquifaciens* ABBD showed the same hydrolyze pattern to raw corn starch, that holes were found on the surface of corn raw starch [17, 25].

The crude enzyme of *Panninobacter phragmatetus* strain Nat1 can be a novel amylase because of its ability to hydrolyze raw starch directly along with other strains that had been reported previously [26]. The study reported that *Bacillus aquimaris* MKSC 6.2 could degrade various raw starch granules, such as corn, cassava, sago, potato, and rice [17]. *Geobacillus thermoleovorans* (Gt-amy II) had the optimum ability to directly hydrolyze a corn raw starch granule [19]. Amylase from Anoxybacillus strains SK3-4 (ASKA) and Anoxybacillus strains DT3-1 could degrade sago and potato granule starch [27]. The gelatinization step in the starch industry needs high temperature and could be abolished if using α-amylases to directly degrade raw starch [17][17][17][17][17]. The

advantage of using raw starch degrading α-amylases can save the cost. Nat1 isolate has the ability as a raw starch degrading α-amylases so that it becomes promising and potentially used as an enzyme in the starch industry.

4. Conclusion

The result shows that Nat1 isolate as a moderate thermostable bacterium from Natar hot springs in Lampung Province produces amylase that degrades soluble starch and raw starch granules, such as corn and sago. A novel amylase from Nat1 isolate has the potential to be use in the starch-processing industry.

Acknowledgment

This research was funded by the Research grant program on Institut Teknologi Sumatera SMART 2019 with a contract number of B/299/IT9.C1/PT.01.03/2019.

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