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IDENTIFICATION OF THERMOPHILIC BACTERIA *BACILLUS BADIUS* W.IIISRNA_2.1 IS A POTENTIALLY NOVEL STRAIN FROM MATAUMPANA HOT SPRINGS, BUTON, INDONESIA, AS PRODUCERS OF URICASE ENZYME

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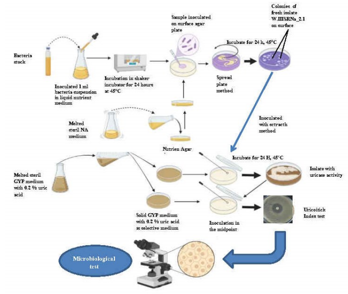
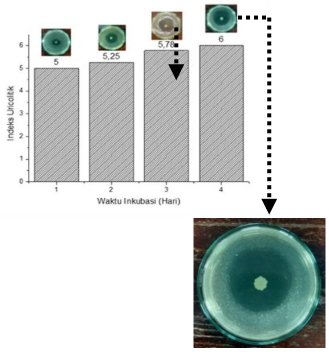
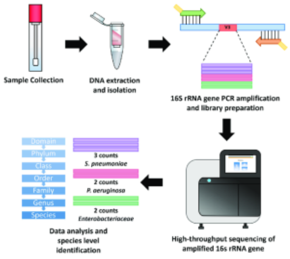
Graphical abstract







Abstract

Thermophilic bacteria are unique microorganisms because they can survive and thrive in high temperatures and extreme environments such as hot springs. Microorganisms that survive in environment like this contain active compounds, one of which is the enzyme uricase. Uricase is an enzyme that plays an important role in the nitrogen metabolism pathway to catalyze oxidation of uric acid into allantoin, CO_2 and H_2O_2 which are water soluble easily. This study aims to identify thermophilic bacterial species producers of uricase enzyme isolated from Mataumpana hot spring water samples and determine the effect of incubation time on bacterial growth and uricase enzyme production. Bacterial identification includes morphological and biochemical tests as well as species-level molecular tests using 16S rRNA, then determining the effect of incubation time on bacterial growth and uricase enzyme production. The results of the analysis showed that the uricase-producing thermophilic bacterial isolate W.IIISRNa_2.1 strain was identified as coming from the gram-positive Bacillus genus and had a very close relationship *with the Bacillus badius species with a similarity of 98.97%, so the isolate was named Bacillus badius* W.IIISRNa_2.1. This bacterium has an optimal optical density (OD) at 42 hours of incubation of 0.962, which is different from the incubation time for production of uricase, was 12 hours with an enzyme activity of 0.4928 U/ml and a protein concentration of 4.7715 mg/ml. *Bacillus badius* W.IIISRNa_2.1 is a potentially novel strain from hot springs and new natural sources which is reported to produce the uricase which is very promising in the medical field.

Keywords: Thermophilic bacteria, uricase, uric acid, Bacillus badius, mataumpana hot springs

Abstrak

Bakteria termofilik adalah mikroorganisma unik kerana mampu bertahan hidup dan berkembang dalam suhu tinggi serta persekitaran ekstrem seperti mata air panas. Mikroorganisma yang bertahan dalam persekitaran seperti ini mengandungi sebatian aktif, salah satunya ialah enzim urikase. Urikase adalah enzim yang memainkan peranan penting dalam laluan metabolisme nitrogen dengan mengkatalis oksidasi asid urik kepada allantoin, CO_2, dan H_2O_2 yang mudah larut dalam air. Kajian ini bertujuan untuk mengenal pasti spesies bakteria termofilik penghasil enzim urikase yang diasingkan daripada sampel air panas Mataumpana dan menentukan kesan masa inkubasi terhadap pertumbuhan bakteria dan pengeluaran enzim urikase. Pengenalpastian bakteria merangkumi ujian

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morfologi dan biokimia serta ujian molekul pada peringkat spesies menggunakan 16S rRNA, kemudian menentukan kesan masa inkubasi terhadap pertumbuhan bakteria dan pengeluaran enzim urikase. Hasil analisis menunjukkan bahawa isolat bakteria termofilik penghasil urikase, strain W.IIISRNa_2.1, dikenal pasti berasal daripada genus gram-positif Bacillus dan mempunyai hubungan yang sangat rapat dengan spesies Bacillus badius dengan kesamaan 98.97%, maka isolat tersebut dinamakan *Bacillus badius* W.IIISRNa_2.1. Bakteria ini mempunyai ketumpatan optik (OD) optimum pada masa inkubasi 42 jam sebanyak 0.962, berbeza dengan masa inkubasi untuk pengeluaran urikase, iaitu 12 jam dengan aktiviti enzim sebanyak 0.4928 U/ml dan kepekatan protein sebanyak 4.7715 mg/ml. *Bacillus badius* W.IIISRNa_2.1 merupakan strain berpotensi baharu dari mata air panas dan sumber semula jadi baharu yang dilaporkan menghasilkan urikase yang sangat berpotensi dalam bidang perubatan.

Kata kunci: *bakteria termofilik, urikase, asid urik, Bacillus badius, mata air panas mataumpana*

1.0 INTRODUCTION

Thermophilic bacteria are unique microorganisms that have received special attention because they can survive and develop at high temperatures, even in extreme environments like hot springs [1]. Microbes that can survive in an environment like this contain active compounds, so that they can survive. One contains thermostable extracellular enzymes that are resistant to high temperatures, chemical reagents, and extreme pH compared to their homologous mesophilic microorganisms. Therefore, research on thermophilic bacteria is very important because they produce various enzymes that have industrial and medical applications. Although the catalytic potential of enzymes has been recognized in recent decades, recent advances in biotechnology have led to an expansion of their use as biocatalysts. One enzyme attracting attention is uricase, which has wide application potential in health and industry [2]. Uricase is a good potential enzyme in the medical field. Because it is used as a reagent in clinical biochemistry, as an immobilized-uric acid receptor biosensor, and as a protein medicine Rasburicase for treating hyperuricemia. In cases of gout associated with renal complications, direct uricase injection is preferrable.

Uricase or urate oxidase is an enzyme that plays an important role in the nitrogen metabolism pathway to catalyze the enzymatic oxidation of uric acid into allantoin, CO_2, and H_2O_2, which are more soluble in water [1, 3, 4, 5, 6]. Uric acid is the final result of the metabolic process of purine nucleic acid, the presence of which in the body is needed as a natural antioxidant; however, if uric acid production exceeds normal limits, it will cause various dangerous diseases. This is known as hyperuricemia; this condition occurs because the gene that expresses the uricase enzyme is inactive during evolution in the human body [7, 4, 8, 9, 10]. Uricase is very promising in the medical field [11]; for example, it can be used as a medicinal protein to treat diseases caused by high uric acid levels or hyperuricemia [12]. Also, uricase is used as a reagent in laboratory diagnosis to measure uric acid levels in blood or other biological fluids [13, 14, 12]. Several studies have used uricase as an immobilized electrode matrix in uric acid biosensors [4, 15, 16].

Various living creatures, except humans, can produce the uricase enzyme, such as bacteria, fungi, animals, and plants [5, 11, 17]. However, enzyme production from animal and vegetable sources is still limited to exploration due to the low efficiency of the production process, ethical issues, and environmental reasons[18]. Uricase from microorganism sources, especially bacteria, is preferred. Enzymes from microbes are also simpler to produce, purify, and optimize processes [5]. Several sources of the uricase enzyme from bacteria have been found, for example, *Bacillus altitudinis W.IIISRNs_1.1*[1], *Alcaligenes faecalis* GH3[19], *Bacillus cereus*[5], *Ochrobacterium anthropi*[7], *Streptomyces rochei* NEAE-25[21], *Pseudomonas putida*[22]. Considering that the uricase enzyme is increasingly important in treatment and diagnosis, new enzyme sources,especially from microorganisms, are continuously sought to obtain better-quality enzymes[23].

The discovery of new thermophilic bacteria could open the door to more efficient and stable enzyme production at high temperatures. Recent research shows that hot springs have great potential in producing bacteria, especially Bacillus species, which produce uricase enzymes with high activity and better thermal stability compared to other sources, as done by [1] and [24]. Mataumpana hot springs in Buton, Indonesia, is an environment rich in thermophilic microbes due to the water of high temperature. This location provides an ideal habitat for uricase-producing bacteria that can survive and thrive in extreme conditions. Therefore, in this study, In this study, we focused on separating thermophilic bacteria that produce uricase, identifying them morphologically, physiologically and molecularly.

The aim of this study was to identify one of the thermophilic bacteria producing the uricase enzyme that has been isolated from the Mataumpana hot springs and to determine the effect of incubation time on bacterial growth and enzyme production. *Bacillus badius* W.IIISRNa_2.1 is a new type of thermophilic bacteria that produces uricase in Indonesia, especially in Southeast Sulawesi, which has not been reported previously.

2.0 METHODOLOGY

The materials used in this study include isolate of thermophilic bacteria W.IIISRNa_2.1 from water of the hot spring of Mataumpana, agar powder (Sigma), Peptone (Sigma), $Na_2B_4O_5(OH)_4.8H_2O$ (Merck), BH_3O_3 (Merck), $NaH_2PO_4.H_2O$ (Merck), K_2HPO_4, $MgSO_4.7H_2O$, NaCl (Merck), NaOH (Merck), uric acid (Sigma), beef extract (Sigma), Ethanol 75%, Aquadest, folin (Merck), yeast extract (Sigma), Bovine serum albumin (Sigma), Glucose (Merck).

Isolation of Uricase Enzyme-Producing Bacteria from Hot Springs and qualitative test of uricase activity

Thermophilic Bacteria were isolated from Hot Water samples. Isolation began with Water samples (1 mL) suspended in 25 mL of enrichment medium containing 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, and 1.0% (w/v) NaCl. Then incubated at 45ºC for 48 hours in a 150 rpm with shaker incubator. Then the incubated sample was spread in solid NA medium by the spread plating method to be grown for 24 hours [7]. The best isolate that grew was selected, and then each isolate with different colonies was separated in solid enrichment medium by the quadrant streak method. Furthermore, the pure isolate was transferred in solid selection media containing uric acid as a specific substrate for the uricase enzyme, incubated at 45ºC, for 1 - 7 days. Isolates grown in Glucose Yeast Peptone (GYS) selection medium with the composition of 1.0% Glucose (w/v); 0.5% yeast extract (w/v); 0.5% peptone (w/v); 0.2% beef extract (w/v); 0.5% NaCl (w/v); 0.01% $MgSO_47H_2O$ (w/v); 0.01% K_2HPO_4 (w/v); 0.2% uric acid (w/v); and 2.0% Bacto agar (w/v)[1];[24];[6], if they produce a clear zone around the colony, they are indicated as uricase-producing isolates because they can decompose the substrate around the colony. Next, the isolate was grown again in selective medium under optimal conditions for bacterial growth to determine the uricolytic index (UI) as a qualitative test of uric acid degrading activity. Then an analysis of the physiological and morphological properties was carried out as well as molecular identification of the bacteria [25]; [1].

$$UI = (Clear\ zone\ Diameter) / (Colony\ Diameter)$$

Identification of Morphological and Physiological of Bacteria

The rejuvenated isolate of the thermophilic bacterial strain W.IIISRNa_2.1 was cultured for 18-24 hours at 45°C on slant agar media without uric acid. Next, morphological identification and biochemical tests are carried out. Morphological characterization was carried out according to the method of Bergey's Manual of Bacteriological Determinants and by Cappuccino and Sherman. Macroscopic identification is carried out by comparing colonies in terms of shape, colony edges, surface texture, and colony color, and microscopically observed with a microscope at 1000 times magnification. Cell observations include gram staining, cell shape, and spore staining. The physiological observations include the reactions of catalase, indole, oxidase, citrate, Voges – Proskauer, 6% NaCl, and carbohydrate tests (glucose, arabinose, sucrose, and mannitol) [7].

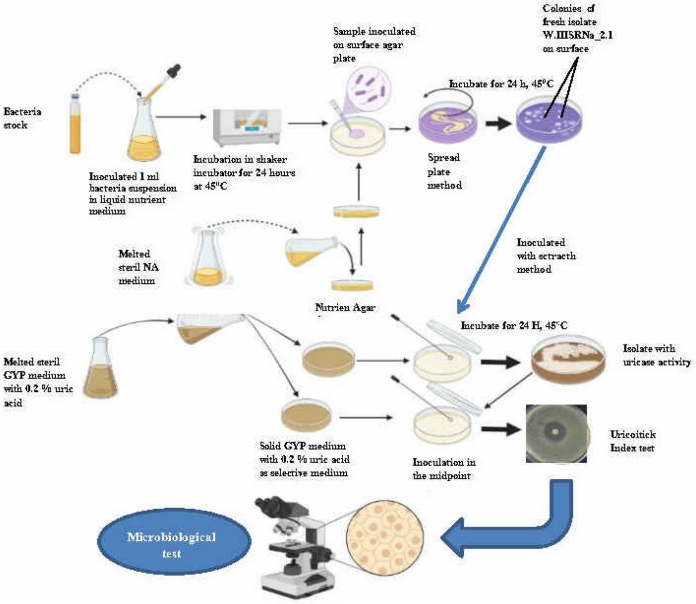

Figure 1 Illustration scheme of microbial rejuvenation, uricolytic index test, and microorganism analysis

Molecular identification of uricase-producing bacteria

This stage begins with isolating of total chromosomal DNA from the thermophilic bacteria strain W.IIISRNa_2.1. Chromosomal DNA isolation is carried out using the organic extraction method. DNA extraction kit A from Geneaid was used for genomic DNA extraction. Next, DNA amplification and 16S rRNA gene sequence analysis use the Polymerization Chain Reaction (PCR) method, which uses chromosomal DNA templates. Purified template DNA in the amount of 0.4-0.5 µL (± 200 ng) was amplified with a Ready-to-Use PCR Bead Kit (Pharma, Biotechnology) using universal primers specific for bacteria front primer 63f (5'-CGA GCC TAA CAC ATG

CAA GTC) and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC). Mixture 18.5 µL ddH2O; 2.5 µL buffer; 1.0 µL primer 63f; 1.0 µL 1387r; 0.5 µL dNTPs mix; 1.0 µL of DNA polymerase and 0.5 µL of template were put into an Eppendorf tube, then put into PCR (Gene Amp® PCR System) under the following conditions: pre-PCR (94^0C, 2 minutes), denaturation (95^0C, 30 seconds), annealing or primer installation (55^0C, 30 seconds), primer extension (72^0C, 1 minute) for 30 cycles, and post-PCR (75^0C, 5 minutes). The purified PCR product was sent to the Indonesian Science Genetics Laboratory for sequencing. Sequences obtained from sequencing were analyzed using the Basic Local Alignment Search Tool (BLAST) from NCBI (National Centre for Biotechnology Information) to identify species [7]; [24]. A phylogenetic tree of bacterial strain W.IIISRNa_2.1 was then built and established using the Treecon program to determine relationships in its phylogenetics [7]; [24]; [1].

Effect of Fermentation Time on Bacterial Growth and Uricase Enzyme Production from the Thermophilic Bacillus badius W.IIISRNa_2.1

This stage begins with making inoculum media as a starter. Isolate W.IIISRNa_2.1 is cultured in 20 mL of medium containing uric acid with a modified composition: uric acid 0.2% (w/v), beef extract 0.2% (w/v), meatball peptone 0.5% (w/v), NaCl 0.5% (w/v), yeast extract 0.5% (w/v), K_2HPO_4 0.01% (w/v), $MgSO_4{\cdot}7H_2O$ 0.01% (w/v), Glucose 1.0% (w/v) [7]; [13]; [26]; [1] and incubated in a shaker water bath WB5-18 incubator at 45^0C for 24 hours at a speed of 180 rpm. It is used as an inoculum for fermentation media. The composition of the fermentation media is the same as the inoculum fermentation media. Next, 10 mL of inoculum or starter that has been incubated is inoculated into 100 mL of production media and incubated with shaking at 45^0C for 72 hours in an incubator shaker. Samples were taken every 12 hours to measure microbial growth known as Optical Density (OD) using a UV-Vis Spectrophotometer at a wavelength of 600 nm and centrifuged at 3500 rpm in centrifuge (HERMLE Z 366 K), temperature 4°C for 30 minutes. Apart from that, enzyme activity and protein content were also measured [1].

Uricase enzyme assay

A total of 75 µL of 3.57 mM uric acid was added to 3 mL of 20 mM sodium borate buffer pH 8, followed by 30 µL of crude enzyme and incubated for 10 minutes. For the blank solution, 30 µL of enzyme was replaced with borate buffer, which was also allowed to stand for 10 minutes. The reaction was stopped by heating the reaction mixture in boiling water for 5 minutes and then cooling to room temperature. The reaction mixture and blank were measured for the absorbance of the decrease in uric acid content using a UV-Visible Spectrophotometer (T60 PG Instruments) at a wavelength of 293 nm with the unit of uricase activity being Unit/mL. One unit (U) of enzyme activity represents the amount of uricase required to convert 1 mole of uric acid per minute under optimal conditions [5]; [6].

$$\text{Uricase Activity} = \frac{\Delta A \text{ x Vtotal}}{12.6 \; x \; Ve \; x \; t}$$

Note:
ΔA = Blank Absorbance-Sample Absorbance
Vt = Total Volume in measurement (mL)
Ve = Enzyme Volume
12.6 = uric acid extinction coefficient at λ293nm (Cm^2 μmol^{-1})
t = Incubation time (minutes)

Content Protein assay

Determination of protein content was carried out using the Lowry method. Two solutions are used in this method, namely: Lowry A and Lowry B. The first consists of 2% Na_2CO_3 in a mixture of 0.1 N NaOH, 1% $CuSO_4$, and sodium potassium tartrate in a ratio of 100:1:1, and the other is a phospho-tungstic-phospho-acid solution molybdate (Foline) in distilled water (1:1). Protein content was measured using a spectrophotometer at the maximum wavelength (670 nm) using BSA (Bovine Serum Albumin) as a standard [1].

3.0 RESULTS AND DISCUSSION

Isolation of Uricase Enzyme-Producing Bacteria from Hot Springs and qualitative test of uricase activity

The bacterial isolate W.IIISRNa_2.1 is one of 50 isolates of thermophilic bacteria producing the uricase enzyme, which have been isolated from the water and sediment of the Mataumpana hot spring in Buton Regency, Southeast Sulawesi [14]. However, this isolate is sourced from water in hot springs. As an indicator that these bacteria produce uricase, it can be seen from the bacteria that grow optimally on solid GYP (glucose yeast peptone) medium containing 0.2% uric acid as a substrate for the uricase enzyme, which is incubated for 7 days at pH 7.0 and a temperature of 45^0C (optimum conditions) and produces a clear zone around the bacterial colony during incubation (Figure 2). The presence of a clear zone indicates that the uricase enzyme in microbes has been induced and can degrade uric acid in the growth medium [1]. Furthermore, a qualitative test was carried out on the level of uricase activity on isolate W.IIISRNa_2.1 by observing the clear zone produced in solid medium containing 0.2% uric acid every day (24 hours) for 4 days of incubation at optimum conditions and measuring the uricolytic index (UI), which is the diameter of the clear zone formed divided by the diameter of the bacterial colony.

In this study, on the last day of incubation, isolate W.IIISRNa_2.1 had a colony diameter of 1.0 cm and a clear zone diameter of 6 cm, so the resulting uricolytic index was 6 cm. The development of the colony size and clear zone produced by the bacteria during the qualitative test of the uricase enzyme activity of the bacteria during 96 hours of incubation can be seen in Figure 3. Every day of incubation shows the development of bacterial growth, both in colony size and clear zone size, increasing over time, but the size of the clear zone develops much larger than the bacterial colony itself. This can be seen in the 24-hour incubation of the clear zone produced by isolate W.IIISRNa_2.1, which is quite significantly large and continues to increase at the end of incubation while the colony size does not show a significant increase, resulting in a large uricolytic index (UI). This illustrates the strength of the activity of the uricase enzyme produced by the bacterial isolate W.IIISRNa_2.1 in hydrolyzing uric acid, which is insoluble in the medium, into allantoin, which is easily soluble in water.

Table 1 Morphological characteristics of thermophilic bacteria

Test		Result
SIM	H_2S	Negative
	Indole	Positive
	Motility	Positive
MRVP	Methyl Red	Positive
	Voges-Proskauer	Negative
	Citric	Negative
	Urea	Negative
	Lactose	Positive
	Sucrose	Positive
	Glucose	Positive
	Mannitol	Positive
	Catalase	Positive
	NaCl 60%	Negative

Identification of Morphology and Physiology of Bacteria

Morphological identification was carried out to observe the bacteria macroscopically and microscopically. In this study, the results of the isolate W.IIISRNa_2.1 morphology test can be seen in Table 1. From the table, it can be seen that microscopically, this thermophilic bacterium is a gram-positive type of bacillus bacteria because its cell wall absorbs a bluish-purple color when stained with crystal violet and safranin [9]. It has non-spore bacilli-shaped cells, as seen in Figure 4. This is in line with the uricase enzyme-producing thermophilic bacteria *Bacillus altitudinis* strain W.IIISRNs_1.1, which is also a gram-positive bacillus genus isolated from the sediment of the Mataumpana hot spring [1]. Gram staining is a useful tool for characterizing the type of bacteria based on the composition of their cell walls; Gram-positive bacteria have a thick peptidoglycan layer, while Gram-negative bacteria have a thick lipid layer [2]. Macroscopically, isolate W.IIISRNa_2.1 shows that the bacteria are small, round like sand grains, milky white in color, with flat edges and smooth, convex surfaces.

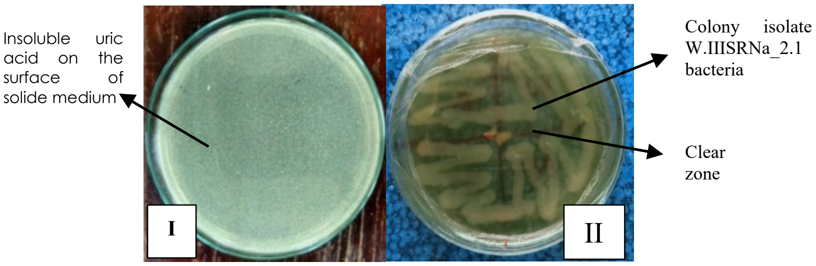

Figure 2 (I). Solid GYP medium conditions with insoluble uric acid before incubation (II). Colony of Thermophilic bacteria-producing uricase strain W.IIISRNa_2.1 in solid GYP medium with 0.2% uric acid at 45°C, pH 7

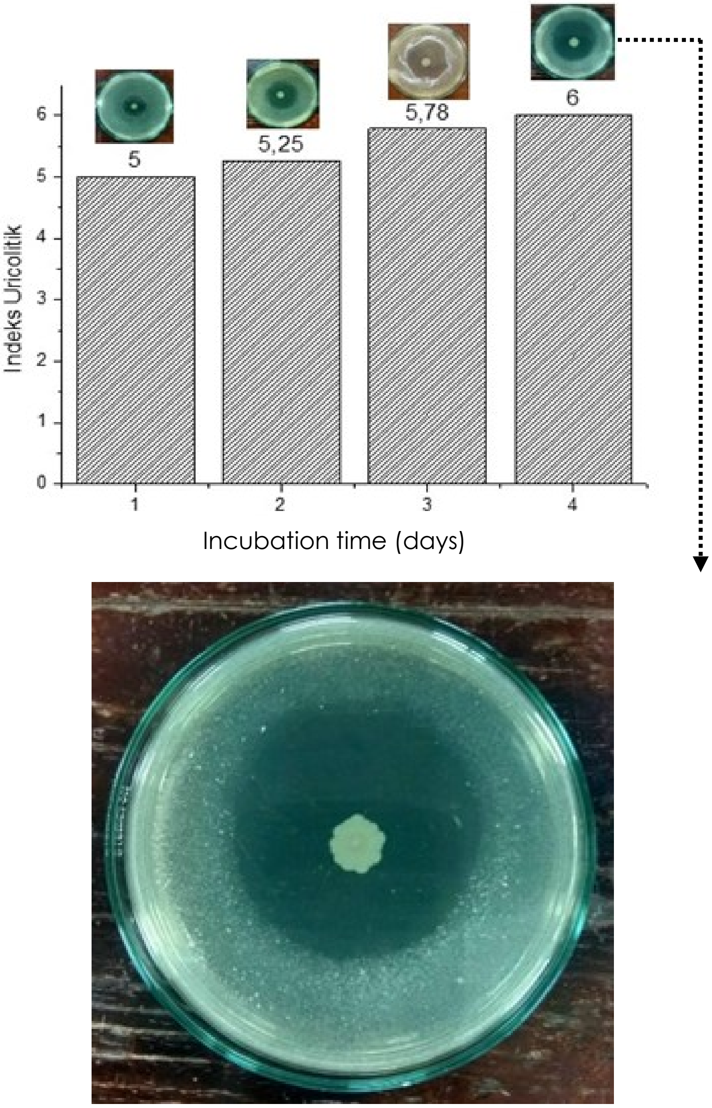

Figure 3 Result of Uricolytic Index test with clear zone around colony isolate W.IIISRNa_2.1 at each incubation time

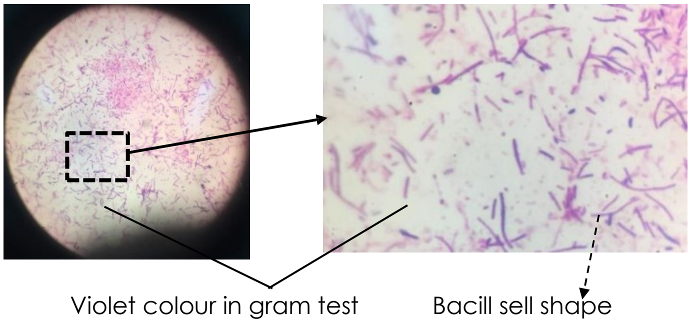

Figure 4 Observation results of gram staining of thermophilic bacteria producing uricase IsolateW.IIISRNa_2.1 under a microscope with 1000x magnification

The IMViC test is a series of tests consisting of indole, Methyl Red, Voges-Proskauer, and Simon's Citrate tests. The indole test aims to see the ability of organisms to degrade the amino acid tryptophan and produce indole. The MR test was carried out to determine the ability of bacteria to produce mixed acids by adding methyl red reagent. If the test result is red (+), the bacteria can ferment the mixed acids, lowering the pH to ≤ 4.4. If the test result is yellow (-), the bacteria ferment other substances, resulting in a pH of ≥ 6 [27]. The Simon's Citrate test was conducted to see the ability of microorganisms to utilize citrate as a source of carbon, indicating whether the bacteria produced the enzyme citrate permease. This test uses the bromothymol blue indicator; if citrate acid is metabolized, carbon dioxide will be formed, which reacts with water and sodium to form sodium bicarbonate, an alkaline product. The bromothymol blue indicator will then change from green to blue [14].

Table 2 Result Physiological and Biochemical Identification of uricase-producing thermophilic bacteria strain W.IIISRNa_2.1

Morfological Identification		**Result**
Macroscopic	Form colonies	Round
	Colony color	Milk white
	Edge of colony	Flat
	Colony surface	Smoth & convex
Microscopic	Gram color	purple
	Gram Stain	Positive (+)
	Spores	Negative (-)
	Sell shape	Bacilli

The Sulphide Indole Motility Test (SIM) is a multitest medium that can be used to detect indole production by microorganisms and observe the characteristics of bacteria based on their ability to move. Triple Sugar Iron Agar (TSIA) is a medium used to distinguish between Gram-negative bacteria based on their ability to ferment sugars, namely glucose, lactose, and sucrose, as well as its ability to produce H_2S. If the butt part is yellow, the bacteria can ferment glucose; if the bacteria can ferment lactose and sucrose, the slant part will be yellow. If the bacteria produce H_2S, the butt part will be black, and the agar will break. The results of the physiological and biochemical identification of thermophilic bacteria producing uricase, isolate W.IIISRNa_2.1, are seen in Table 2.

Based on the results of DNA chromosome isolation from thermophilic bacteria isolate W.IIISRNa_2.1, confirmed by agarose gel electrophoresis, a single fragment was observed on the electroferogram. This proves that the isolation of DNA chromosomes has been done quite well and can be used as a template in the PCR amplification process. Amplification of the 16S-rRNA gene by PCR was carried out for 30 cycles with an annealing temperature of 55°C and elongation at 72°C using universal primer pairs for bacteria [1].

An electroferogram of the 16S-rRNA amplicons of the uricase-producing thermophilic bacteria isolate W.IIISRNa_2.1 is shown in Figure 5. Based on the image, it can be seen that the resulting amplicon has a fragment size of 1300 bp, which is a common size for the length of 16S rDNA amplified with a universal primer. These amplicons were then sequenced using the Dideoxy Sanger method with a reverse primer (1387r) and a forward primer (63f). The nucleotide base sequences were blasted to identify the genus and species of bacteria. The results of aligning the nucleotide sequences of bacterial isolates with the nucleotides of several bacterial species that have been registered in the Gene Bank database allow for the identification of common and specific bacterial species. Based on the analysis results, it shows that the DNA sequence identity of isolate W.IIISRNa_2.1 is 98.97% with the *Bacillus badius* gene (Table 3), indicating a fairly high validation for the identity of the genus and species of bacteria obtained. Identification will be considered ambiguous or biased when the homologous identity of the 16S-rRNA sequence is below 70% [28].

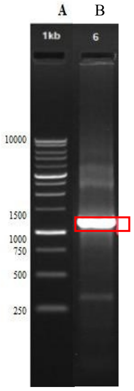

Figure 5 Electropherogram of amplicon 16S rDNA, A. 1 kb DNA Marker Ladder (Gene Ruler 1 kb DNA Ladder), B. 6= DNA of strain W.IIISRNa_2.1

Based on the nucleotide sequence of the 16S-rRNA gene, a phylogenetic tree can be constructed to study the relationship of bacteria with other bacteria registered in the GenBank database. DNA sequence alignment was carried out using the ClustalW program integrated with Bioedit, and phylogenetic analysis of kinship relationships was conducted using the MEGA 6 program. The phylogenetic tree or dendrogram that was compiled provided nodes and branches showing the close relationship between groups of organisms [1]. The phylogenetic tree of the thermophilic uricase-producing bacterial isolate W.IIISRNa_2.1 strain, as seen in Figure 6, indicates that the W.IIISRNa_2.1 isolate in this study is related to the Bacillus genus group. The results of phylogenetic analysis show that the uricase-producing thermophilic bacterial isolate from the Mataumpana hot springs is closely related to *Bacillus badius* strain NBPM-293. Based on these results and the relationship to the isolation location, namely Watumotobe Village (W) and the author (SRN), the isolation point came from point 3 at the time of sampling (III) and from the water sample (a), thus the isolate was named *Bacillus badius W.IIISRNa_2.1*.

Table 3. Sequences Producing Significant Alignment of thermophilic bacteria strain W.IIISRNa_2.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus wudalianchiensis strain CIRRC/R1 16S ribosomal RNA gene, partial sequence	Bacillus wudalianchiensis	2246	2246	100%	0.0	98.97%	1529	MK937834.1
Bacillus badius strain SB-7 16S ribosomal RNA gene, partial sequence	Bacillus badius	2246	2246	100%	0.0	98.97%	1489	MF321853.1
Bacillus badius strain JU13 16S ribosomal RNA gene, partial sequence	Bacillus badius	2246	2246	100%	0.0	98.97%	1391	KX230141.1
Bacillus badius partial 16S rRNA gene, strain DSM 30822	Bacillus badius	2246	2246	100%	0.0	98.97%	1544	LT549008.1
Bacillus badius strain ACCC60106 16S ribosomal RNA gene, partial sequence	Bacillus badius	2246	2246	100%	0.0	98.97%	1517	MZ067936.1
Bacillus badius strain F 16S ribosomal RNA gene, partial sequence	Bacillus badius	2246	2246	100%	0.0	98.97%	1444	KJ000879.1
Bacillus badius strain NBPM-293 chromosome, complete genome	Bacillus badius	2246	22416	100%	0.0	98.97%	3868812	CP082363.1
Bacillus badius strain SCTB117 16S ribosomal RNA gene, partial sequence	Bacillus badius	2246	2246	100%	0.0	98.97%	1478	JN650282.1
Bacillus badius strain KO_CM52 16S ribosomal RNA gene, partial sequence	Bacillus badius	2246	2246	100%	0.0	98.97%	1331	GQ497939.1
Bacillus sp. ES-SL-2 16S ribosomal RNA gene, partial sequence	Bacillus sp. ES-SL-2	2246	2246	100%	0.0	98.97%	1517	FJ529039.1
Bacillus sp. CC 3 16S ribosomal RNA gene, partial sequence	Bacillus sp. CC 3	2246	2246	100%	0.0	98.97%	1432	EU716646.1
Bacillus badius strain 12ELE1 16S ribosomal RNA gene, partial sequence	Bacillus badius	2242	2242	100%	0.0	98.89%	1520	KJ722449.1
Bacillus badius strain SY29 16S ribosomal RNA gene, partial sequence	Bacillus badius	2242	2242	100%	0.0	98.89%	1446	FJ494699.1
Bacillus badius strain NBRC 15713 16S ribosomal RNA, partial sequence	Bacillus badius	2242	2242	100%	0.0	98.89%	1481	NR_112633.1
Bacillus sp. (in: Bacteria) strain D1P012 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	2241	2241	100%	0.0	98.89%	1462	MN989044.1
Bacillus badius strain SCSB-17 16S ribosomal RNA gene, partial sequence	Bacillus badius	2241	2241	100%	0.0	98.89%	1481	MK995595.1
Bacillus badius strain B19 16S ribosomal RNA gene, partial sequence	Bacillus badius	2241	2241	100%	0.0	98.89%	1465	MG552833.1
Bacillus badius strain 2017G 16S ribosomal RNA gene, partial sequence	Bacillus badius	2241	2241	100%	0.0	98.89%	1393	MK355611.1
Bacillus sp. (in: Bacteria) strain LQ65 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	2241	2241	100%	0.0	98.89%	1451	MG025799.1
Bacillus badius strain FJAT-47859 16S ribosomal RNA gene, partial sequence	Bacillus badius	2241	2241	100%	0.0	98.89%	1433	MG651261.1

Effect of Fermentation Time on Bacterial Growth and Uricase Enzyme Production from the thermophilic *Bacillus Badius* W.IIISRNa_2.1

The uricase enzyme, present in organism cells, is isolated or removed from its host microorganism using a fermentation method with conditions highly dependent on the optimum requirements of the source microorganism. Therefore, to obtain enzymes in maximum and potential quantities, it is necessary to identify the optimal production time and conditions. In this study, the fermentation process began by growing *Bacillus badius* W.IIISRNa_2.1 bacteria in modified glucose yeast peptone (GYP) broth containing 0.2% uric acid as a substrate, incubated in a shaker incubator at 45°C for 72 hours, 180 rpm, with variations in fermentation time. This process was carried out to determine the effect of fermentation time on bacterial growth and to establish the optimum conditions for producing the uricase enzyme, thereby yielding the enzyme with the highest activity in large quantities.

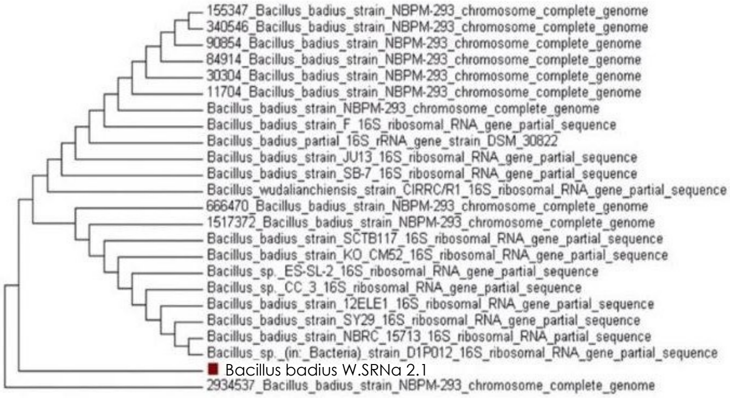

Figure 6 Phylogenetic position of strain W.IIISRNa_2.1 based on 16s rRNA sequence

Fermentation time significantly influences the bacterial growth or optical density (OD) of *Bacillus badius* W.IIISRNa_2.1, as shown in Figure 7. From the growth curve, it can be observed that *Bacillus badius* W.IIISRNa_2.1 bacteria reached optimal cell growth at the end of the stationary phase, 42 hours into incubation, with an OD value of 0.962 measured at a wavelength of 600 nm. Following this, biomass production declined at 48 hours of incubation, marking the onset of the death phase. This decline is due to the accumulation of toxic materials and very limited nutrients, leading to the death of many cells. The number of dead cells increases exponentially, opposite to the logarithmic growth phase. Additionally, bacterial cells begin to break down due to the influence of the enzyme itself (autolysis), leading to complete bacterial death; however, at the 60th hour, the bacteria remain alive and active. This indicates that *Bacillus badius* W.IIISRNa_2.1 has a prolonged logarithmic phase.

Along with determining the level of bacterial growth or optical density, enzyme activity values are also measured to determine the optimum time for uricase production. The uricase enzyme activity of *Bacillus badius* W.IIISRNa_2.1 from 0 hours of incubation continued to increase gradually and reached a maximum at 12 hours of incubation with an activity of 0.4928 U/mL. This is due to the increasing growth of bacteria, leading to increased production of enzymes used to hydrolyze substrates. However, the optimum activity of the uricase enzyme from *Bacillus badius* did not occur at the same time as the highest optical density was achieved, namely at 42 hours of incubation and reflected the dynamics of microorganism growth and enzyme expression that differed in time, in this case the production of uricase from the isolate *Bacillus badius* W.IIISRNa_2.1 is optimal at the beginning of the exponential phase, not at the peak of biomass production. This indicates that these bacteria are easily adaptable to its growth environment for uricase production. Because the enzyme is produced in response to certain environmental conditions, while microbial growth requires broader conditions, which means that environmental conditions that respond to uricase production do not support optimal growth of *Bacillus badius* bacteria so that enzyme production and the optimum time for microbial growth can be different. The production of enzymes such as uricase is often secondary, meaning it does not always parallel maximal cell growth. In the early exponential phase, microbial metabolism is at its peak and the *Bacillus badius* W.IIISRNa_2.1 gene regulation system triggers the expression of certain enzymes, such as uricase. After the exponential phase, although the cell number increases (OD increases), enzyme activity can decrease due to negative feedback regulation, nutrient depletion, or accumulation of metabolites that inhibit enzyme expression.

Therefore, it has an impact on the application of uricase enzyme production, where the optimal harvest time for uricase production is not at maximum OD, but at the highest enzyme activity, which is 12 hours. If fermentation is continued for up to 42 hours (to achieve high OD), it is likely that the enzyme produced has been degraded or decreased in activity, so that production efficiency decreases. This has a direct impact on production costs and process effectiveness, because there are fewer active enzymes so that the results are lower and energy and nutrients are wasted, resulting in increased production costs. Thus, the optimal time to produce uricase enzyme from *Bacillus badius* W.IIISRNa_2.1 in modified GYP medium containing 0.2% uric acid at a temperature of 45 °C is 12 hours, which is used to produce uricase for research purposes.

The production time for the uricase enzyme varies depending on the source microorganism; for example, uricase from M. luteus has a production time of 24 hours with an activity of 3.75 U/mL [6], *Sphingobacterium thalpophilum* (VITPCB5) has a production time of 24 hours with an activity of 3.75 U/mL [29], *Bacillus cereus* has a production time of 18 hours with an activity of 37.9 U/mL [5], *Bacillus cereus* SKIII has a production time of 3 days with an activity of 0.73 U/mL [12], and *Bacillus altitudinis* W.SRNs_1.1 has a production time of 36 hours with an activity of 0.5198 U/mL.

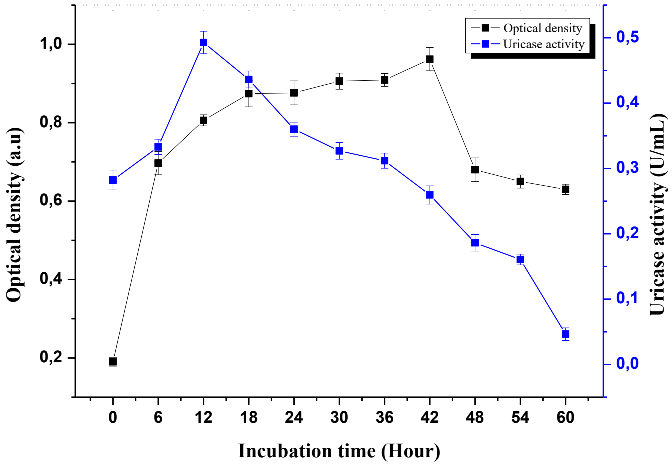

Figure 7 The curve of uricase enzyme production and growth of *Bacillus badius* W.IIISRNa_2.1 at λ 600 nm against incubation time at substrate concentration (uric acid) 0.2%, pH 7.0; 45°C

Next, measurements of the protein concentration are carried out to determine the specific activity value of an enzyme. In this study, the protein content of the uricase enzyme was measured using the Lowry method with Bovine Serum Albumin (BSA) as the standard. The reaction results between Lowry's reagent molecules and protein molecules at each incubation time form a blue solution. Protein content is determined based on the absorbance value, which is proportional to the protein content at the maximum wavelength (670 nm) against the BSA standard curve. The crude protein content of the uricase enzyme from *Bacillus badius* W.IIISRNa_2.1 at optimum activity was 4.7715 mg/mL, resulting in a specific enzyme activity (enzyme activity divided by enzyme protein concentration) of 0.1033 U/mg.

4.0 CONCLUSION

Results of morphological and physiological analysis. The uricase-producing thermophilic bacterial isolate W.IIISRNa_2.1 from the Mataumpana hot spring water sample was identified as coming from the gram-positive Bacillus genus, and molecular analysis showed a very close relationship with the *Bacillus badius* species with a similarity of 98.97%. So, this isolate was named *Bacillus badius* W.IIISRNa_2.1. This new species of bacteria produces uricase from hot spring water. This bacterium has an optimal optical density (OD) at 42 hours of incubation of 0.962, which is different from the incubation time for producing the uricase enzyme, namely 12 hours with an enzyme activity of 0.4928 U/ml and a protein concentration of 4.7715 mg/ml.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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