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Research Article

Phenotypic Characterization and Identification of Potential L-Asparaginase-Producing Thermohalophilic Bacteria from Wawolesea Hot Spring, North Konawe, Southeast Sulawesi, Indonesia

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Abstract

Background and Objective: L-asparaginase-producing thermohalophilic bacteria have the potential of producing an enzyme tolerant to high heat and salt levels. This enzyme, L-asparaginase, can be used as a biological agent for the cancer therapy of acute lymphoblastic leukemia and melanoma as it has a specific ability to inhibit the formation of nutrients for cancer cells. This enzyme is also used effectively in food industries operating at high temperatures due to its ability to reduce acrylamide, a trigger of cancer cells. This study sought to figure out the phenotypic characters of and identify potential L-asparaginase-producing thermohalophilic bacteria from Wawolesea Hot Spring, North Konawe, Southeast Sulawesi. **Materials and Methods:** The characterization conducted on potential L-asparaginase-producing thermohalophilic bacterial isolates consisted of the following: Colony morphological characterization, covering the shapes, edges, internal structures, elevations and colours of the colonies, cell morphological characterization, covering gram staining, endospore formation and motility, biochemical characterization, covering catalase, Methyl Red and Voges Proskauer (MR-VP), gelatin hydrolysis, citrate, indole and carbohydrate fermentation tests and physiological characterization, covering pH effect, salinity, oxygen demand and temperature effect tests. Bacterial isolate identification was carried out in two stages, namely phenetic identification based on the phenotypic characterization data determine through a preliminary identification and numeric-phenetic identification. **Results:** The characterization results showed that the bacterial isolates AAT 1.4, AAT 3.2 and CAT 3.4 were *Bacillus*-shaped, Gram-positive, motile, catalase-positive and aerobic. Based on the numeric-phenetic analysis results, the isolates AAT 1.4 and CAT 3.4 had a 92.9% similarity to *Bacillus subtilis*, while isolate AAT 3.2 had a 92.9% similarity to *Brevibacillus limnophilus*. **Conclusion:** According to the numeric-phenetic analysis results, the isolates AAT 1.4 and CAT 3.4 belong to the species *Bacillus subtilis*, while isolate AAT 3.2 belongs to the species *Brevibacillus limnophilus*.

Key words: Thermohalophilic bacteria, phenotypic characterization, L-asparaginase, numeric-phenetic analysis, Wawolesea Hot Spring, *Bacillus subtilis*, *Brevibacillus limnophilus*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer disease control is now commonly performed through surgery, chemotherapeutic and radiation methods. However, the treatment mechanism is still considered less than effective. One of the alternative methods in cancer treatment is enzyme-based treatment and one of the enzymes that are currently in use for cancer cell treatment is L-asparaginase.

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) is an enzyme that hydrolyzes L-asparagine into aspartic acid and ammonia¹. The L-asparaginase administered to cancer cells can break down L-asparagine, so it is expected that L-asparaginase may hinder the cancer cells' growth². L-asparaginase is widespread in nature, commonly found in bacteria, plants and the serum of mice, but it is absent in humans^{3,4}. Pradhan *et al.*⁵ reported that, L-asparaginase can be produced in considerable amounts from microorganisms of the genera *Escherichia*, *Aerobacter*, *Erwinia*, *Serratia*, *Xanthomonas*, *Pectobacterium*, *Photobacterium* and *Streptomyces*. However, in today's days, enzyme-based industries are demanded the capability and stability of the enzyme.

Among the microorganisms that are able to produce the enzyme with high levels of capability and stability are thermohalophilic bacteria. These bacteria are a group of bacteria that are capable of living in high-heat and high-salinity environments. According to Ruginescu *et al.*⁶, thermohalophilic bacteria can grow in conditions with temperatures of 50°C and above and with salinity in the range of 2-30% and it was recorded that they grow best at 3-15% salinity. Thermohalophilic bacteria can be found in sea sediments, soil and deserts with high salt levels or in high-salt hot springs.

Jamaluddin *et al.*⁷ reported 14 potential L-asparaginase-producing thermohalophilic bacterial isolates that were successfully isolated from Wawolesea Hot Spring, with the highest enzyme activities recorded in AAT 3.2, AAT 1.4 and CAT 3.4, each at 86.61, 84.711 and 81.88 $\mu\text{mol mL}^{-1}$. However, these bacterial isolates are unknown as to their identities. It is necessary to identify the potential L-asparaginase-producing bacteria so they can be used more widely for industrial purposes.

Prior to being identified, these L-asparaginase-producing bacteria were characterized phenotypically by morphological (colony and cell), biochemical and physiological characterization. By characteristic, the bacteria were identified

in numeric-phenetic terms by comparing the characters of the L-asparaginase-producing bacterial isolates to those of a reference isolate.

MATERIALS AND METHODS

Place and time: This study was conducted from May to September, 2022 at the Microbiology Laboratory and the Genetics Laboratory of the Biology Department of Universitas Halu Oleo, Southeast Sulawesi, Indonesia.

Selected bacterial isolates culture: The selected bacterial isolates were grown on slant media (NA medium: 8 g of nutrient broth and 20 g of agar in 1000 mL of sterile distilled water) and liquid media (NB medium: 5 g of peptone and 3 g of beef extract in 1000 mL of sterile distilled water), then they were incubated at 32°C for 24 hrs.

Characterization of potential L-asparaginase-producing bacteria: Bacterial characterization was conducted by observations of the morphological (colony and cell), biochemical and physiological characters.

Colony morphological characterization: A colony morphological observation was carried out by growing a selected bacterial isolate in an NA medium for 24 hrs. The morphological characters observed were the shapes, edges, internal structures, elevations and colours of colonies in reference to a characterization book⁸.

Cell morphological characterization: The cell morphological observation covered Gram staining, endospore formation and motility. The Gram observation was performed by extracting an inoculum loopful of the 24 hrs old bacteria cultured in a slant NA medium and making a smear of the bacteria on a slide, which was heat-fixed by passing it over a Bunsen burner several times. The smear was then added with a few drops of crystal violet for 1 min and rinsed with distilled water. Then, the smear was added 95% ethanol was drop by drop until the smear appeared clear from the crystal violet stain before it was then rinsed with distilled water. Several drops of safranin were added to the smear for 45 sec and then the smear was rinsed with distilled water and dried before being observed under a microscope. If the bacteria were stained red, then they were categorized as Gram-negative, but if they were stained bluish-green, then they were categorized as Gram-positive⁸.

An endospore observation was carried out by extracting an inoculum loopful of bacteria from a pure culture, which was then suspended in sterile distilled water on a slide. The slide was then heat-fixed by passing it over a Bunsen burner several times. After being left drying and cooling, the smear was added with a few drops of Malachite Green and was left for ten minutes. The smear was then rinsed with flowing water and air-dried. After being added several safranin drops were for five seconds, the smear was then rinsed with flowing water and dried. Then, it was observed under a microscope with high-power magnification. Vegetative cells should be red, endospores should be transparent and exospores should be green⁸.

A motility test was performed by inoculation of a bacterial isolate by stabbing an inoculum needle perpendicularly to half the height of the semisolid NA medium in a test tube. The tube was then incubated for 48 hrs and the bacterial movement trace was observed. An occurrence of foggy turbidity marked that the bacteria were motile⁸.

Biochemical characterization: The biochemical characterization covered catalase, Methyl Red and Voges Proskauer (MR-VP) (7 g of peptone, 5 g of glucose and 5 g of KH_2PO_4 solved in 1,000 mL of sterile distilled water), citrate, indole and carbohydrate fermentation tests. A catalase test was conducted by extracting a 24 hrs old isolate from a pure culture and setting it on a slide, which was then added with several drops of 3% H_2O_2 . The test result was positive if gas bubbles were formed around the bacterial colonies⁹.

The MR-VP tests were carried out by growing a bacterial isolate from a pure culture in an MR-VP broth medium and incubating them for 24-48 hrs. For the Methyl Red (MR) Test, five drops of methyl red were added into a tube. A positive test result was marked by a turn of the medium into red and a negative test result was by a turn into yellow. Meanwhile, the Voges Proskauer (VP) Test was conducted by growing a bacterial isolate from the pure culture in an MR-VP broth medium and incubating them for 24-48 hrs. Then, the tube was added with ten drops of a Barritt's A reagent and shaken. It was added further with ten drops of a Barritt's B reagent and shaken for 20-30 sec. A positive test result was marked by a red colour⁹.

A citrate test was conducted by preparing a slant Simmons citrate agar medium (20 g of agar, 5 g of NaCl, 2 g of sodium acetate, 1 g of $(\text{NH}_4)_2\text{H}_2\text{PO}_4$, 1 g of K_2HPO_4 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.08 g of BTB solved in 1000 mL of sterile distilled water) in a test tube. One inoculum loopful of a bacterial isolate from pure culture was inoculated into the medium and incubated for 1-2 days. A positive result was

marked by a turn in the medium colour from green to blue, which indicated that the bacteria were able to use citrate as a source of energy⁹.

An indole test was conducted by pouring a 1% tryptone liquid medium into two test tubes, each at 10 mL. The first tube was filled with a microbial isolate from pure culture, while the second tube was used as a control. The tubes were incubated for 48 hrs at 37°C. An observation was performed for indole by adding 0.2-0.3 mL of Erlich's or Kovac's reagent into each tube. The tubes were shaken gently and left upright for the reagent solution to be concentrated on the surface of the medium. The presence of indole could be observed from the occurrence of red colour on the upper layer of the medium, in contrast to the result obtained from the control tube. This procedure was also applied to each of the other isolates.

A glucose fermentation test was carried out by preparing a glucose fermentation medium (5 g of test carbohydrate, 13 g of NB and 0.08 g of Bromothymol Blue solved in 1000 mL of distilled water) in a test tube, then in a Durham tube. Then, an inoculum loopful of pure culture was taken and inoculated into each tube filled with the glucose that was to be tested in the fermentation test, then it was incubated for 1-2 days. A positive reaction could be observed with a turn in the medium colour from green to yellow or with the occurrence of gas bubbles trapped in the Durham tube. If there was only a change in medium colour, then the fermentation that took place was categorized as acidic fermentation. If only trapped gas bubbles were observed, then the fermentation type was an alcoholic. If both occurred, then the fermentation was categorized as mixed fermentation⁹.

Physiological characterization: The physiological characters observation covered the pH effect, osmotic pressure (tolerance to salinity), oxygen demand and thermophilic temperature. A pH effect test was conducted by growing a bacterial isolate in several test tubes that were filled with NA media and added with NaOH and HCl based on the tolerance appropriate at pH 5, 7 and 8. A salinity tolerance test was conducted by growing a bacterial isolate in several test tubes that were filled with NA media and added with NaCl to each tube at varying concentrations, namely, 2, 5, 7 and 10%.

An oxygen (O_2) demand test was conducted by extracting one inoculum loopful of a bacterial isolate from pure culture and inoculating it in several NB-filled test tubes. The tubes were incubated for 24 hrs, after which the growth types were observed: Aerobic bacteria would grow on the surface, anaerobic bacteria would grow below the surface or at the bottom of the test tube and facultative anaerobic bacteria

would cause turbidity throughout the test tube. A test of tolerance to temperature effect was conducted by extracting an inoculum loopful of a bacterial isolate from pure culture and inoculating it into several NB-filled test tubes. The tubes were then incubated at 5, 30, 45, 50, 55, 65, 70 and 80°C for 24 hrs. An observation of the growth in the media was then conducted.

Bacterial identification: An identification of potential L-asparaginase-producing thermohalophilic bacterial isolates was conducted in two stages, namely, phenetic identification based on the phenotypic characters determined through a preliminary identification and numeric-phenetic identification.

Preliminary identification: A preliminary identification was conducted based on the key characteristics of a genus, including the Gram-staining character, cellular shape, motility, endospore formation and aerobic/anaerobic/facultative anaerobic growth condition¹⁰. All characters of a selected bacterial strain were verified against the key characters of a certain genus according to Bergey's Manual of Determinative Bacteriology¹¹ and *Brevibacillus* as a Biological Tool: A Short Review¹².

Numeric-phenetic identification: The phenotypic properties obtained from the test on each character were coded or converted as follows: Score 1 for positive properties, score 2 for negative properties. The data were then analyzed using the MVSP (MultiVariate Statistical Package) program, version 3.1. To figure out one species' similarity with another species, the simple matching coefficient (SSM) method was employed.

The classification was conducted using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithms. The analysis results were then presented in a dendrogram. The dendrogram generated was used as a basis to find out the similarity between the test bacterial isolates and the reference bacterial genera.

RESULTS AND DISCUSSION

Morphological characters of the bacterial isolates: The morphological characters of potential L-asparaginase-producing thermohalophilic bacteria were identified from colony and cell morphological characterization results. The morphological characters of the bacterial isolate colonies observed included colours, shapes, edges, elevations and internal structures in Table 1 and Fig. 1.

Table 1 shows that, in terms of colony morphological characters, the three potential L-asparaginase-producing bacterial isolates were colored differently, that is, the isolates AAT 3.2 and CAT 3.4 were cream, while the isolate AAT 1.4 was white. According to Fig. 1, the isolates AAT 1.4 and CAT 3.4 shared the same circular shape with an entire colony edge, while the isolate AAT 3.2 had an irregular colony shape with a lobate colony edge (ear-like shape). The elevation characters of the isolates AAT 1.4, AAT 3.2 and CAT 3.4 were effuse (thin and even), raised (with clearly observed elevation) and low convex, respectively. As for internal structures, the three isolated colonies were all opaque.

The cell morphological characters of the potential L-asparaginase-producing thermohalophilic bacterial isolates based on the observation in the NB medium are provided in Table 2.

Table 1: Colony morphological characters of the potential L-asparaginase-producing thermohalophilic bacterial isolates

No.	Isolate code	Colony shape	Elevation	Edge	Internal structure	Colour
1	AAT 1.4	Circular	Effuse	Entire	Opaque	White
2	AAT 3.2	Irregular	Low convex	Lobate	Opaque	Cream
3	CAT 3.4	Circular	Raised	Entire	Opaque	Cream

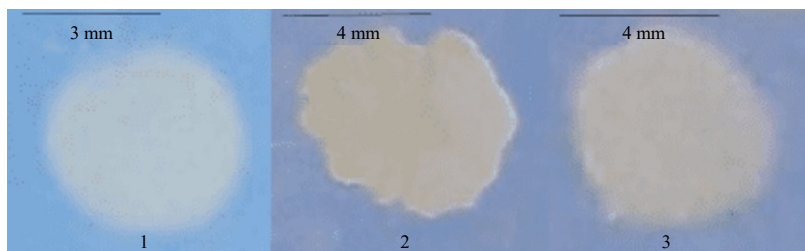


Fig. 1: Colony morphological characters of the bacterial isolates in NA medium

1: AAT 1.4 isolate, 2: AAT 3.2 isolate and 3: CAT 3.4 isolate

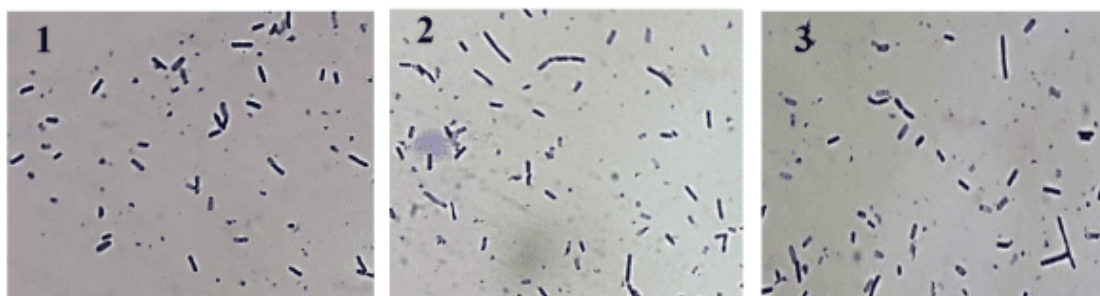


Fig. 2: Shapes and the cell morphological characters of the bacterial isolates at 400X magnification

1: AAT 1.4 isolate, 2: AAT 3.2 isolate and 3: CAT 3.4 isolate

Table 2: Cell morphological characters of the potential L-asparaginase-producing thermohalophilic bacterial isolates

No.	Isolate code	Cell shape	Gram character	Endospore	Motility
1	AAT 3.2	Bacilli	Positive	Positive	Motile
2	AAT 1.4	Bacilli	Positive	Positive	Motile
3	CAT 3.4	Bacilli	Positive	Positive	Motile

Based on the results of the cellular observation (Table 2), the three potential L-asparaginase-producing thermohalophilic bacterial isolates were similar in cellular characters, that is, the three isolates were rod-shaped (bacil), Gram-positive, capable of forming endospores and motile. The results of the cell morphological observation of the potential L-asparaginase-producing thermohalophilic bacterial isolates were depicted in Fig. 2.

Figure 2 shows that, the three bacterial isolates were *bacillus* in shape. Bacilli are predominantly found in water as they have a better ability to absorb nutrients than cocci, hence, their better survivability in low-nutrient environment conditions. Their easy availability in water is attributable to the materials, nutrients and shape of the molecules or ions transported from the environment to their membrane by a number of specific mechanisms¹³.

The three potential L-asparaginase-producing thermohalophilic bacterial isolates were Gram-positive. This was known from the three bacterial isolates' ability to maintain the methyl purple colour during Gram-staining (Fig. 2). The high prevalence of Gram-positive bacteria is attributed to the difference in the cell wall component. The cell wall structure of Gram-positive bacteria contains thicker peptidoglycan than that of Gram-negative bacteria. It is for this reason that Gram-positive bacteria are better at surviving high-heat and high-salinity environmental conditions. As found by various research works^{14,15}, Gram-positive bacteria are more abundant in extreme environments, such as those with high heat and salinity.

As for endospores, the observation results of the three bacterial isolates showed that there were spores removed from the cells and there were some that were still intact in the cells. Spores that came off of vegetative cells (exospores) would be stained by Malachite Green into a green colour, while the vegetative cells would be stained red. The spores that were still intact inside the cells (endospores) would have a transparent look, hence difficult to be stained by Malachite Green (Fig. 3). As stated by McKenney *et al.*¹⁶, in endospore staining, vegetative cells would be red in colour, while exospores would be green. The results of the observation of the potential L-asparaginase-producing thermohalophilic bacterial isolates are depicted in Fig. 3.

Based on the endospore observation results, endospores were formed at the edge of the cell in an oval shape. The spores formed in the three bacterial isolates showed a form of defensive response to the environmental factor of high temperature. Bacterial spores generally function as a defensive mechanism in extreme environments such as environments with chemical substances, heat, a lack of water and radiation^{17,18}.

A motility test is a test aimed to figure out a bacterium's movement in a growing medium. Based on the motility test results in this study, the three bacterial isolates were motile, given that bacterial movements in the medium were captured under a microscope. Besides, bacterial motility is also marked by isolate movement trace in a SIM (sulfide indole motility) medium (Fig. 4). As stated by Kearns¹⁹, positive motility is shown by isolated growth that is spread throughout the medium.

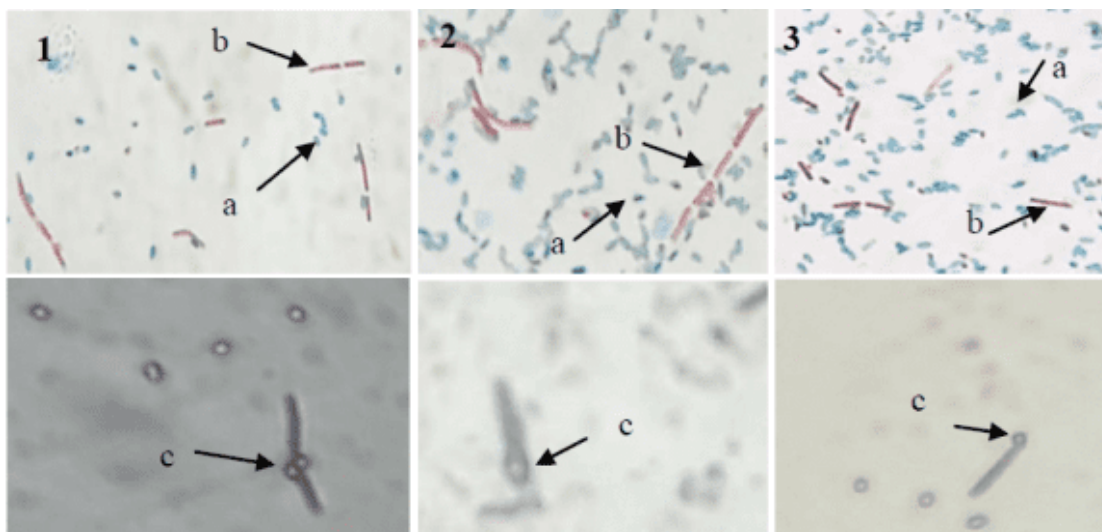


Fig. 3: Results of endospore staining at 1000X magnification

a: Exospores, b: Vegetative cells and c: Endospores, 1: AAT 1.4 isolate, 2: AAT 3.2 isolate and 3: CAT 3.4 isolate

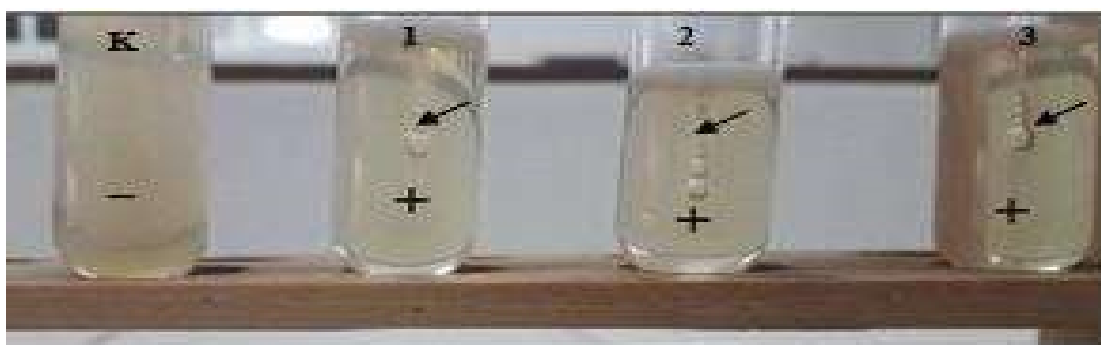


Fig. 4: Results of the motility test on the bacterial isolates

K: Negative control, 1: AAT 1.4 isolate, 2: AAT 3.2 isolate and 3: CAT 3.4 isolate

Table 3: Biochemical characters of the potential L-asparaginase-producing thermohalophilic bacteria

No.	Isolate code	Catalase test	Methyl Red test	Voges Proskauer test	Gelatin hydrolysis	Citrate test	Indole test	Sugar fermentation test (gas/colour change)				
								Lactose	Glucose	Sucrose	Mannitol	Xilosa
1	AAT 1.4	+	-	+	+	+	-	-/+	-/+	-/+	-/+	-/+
2	AAT 3.2	+	-	+	+	-	-	-/+	-/+	-/+	-/+	-/-
3	CAT 3.4	+	-	+	+	+	-	-/+	-/+	-/+	-/+	-/+

+: Ability existed, -: Ability did not exist, +/+ : Gas occurred/a colour change occurred, +/- : Gas occurred/a colour changed did not occur, -/+ : Gas did not occur/a colour change occurred and -/- : Gas did not occur/a colour change did not occur

Motility is presumed to be associated with the presence of a flagellum in the cell structure. Bacilli are typically motile, while cocci are non-motile. Haiko and Westerlund-Wikström²⁰ stated that, bacilli that live in water have flagella that they use as a means of mobility, allowing them to move towards a more advantageous environmental condition or away from an environment that is harmful to their lives.

Biochemical characters of the bacterial isolates: The potential L-asparaginase-producing thermohalophilic bacterial isolates were biochemically characterized through catalase, indole, citrate, Methyl Red and Voges Proskauer (MR-VP) and glucose fermentation tests. The biochemical characters of the bacterial isolates were presented in Table 3.

The catalase test results of the three bacterial isolates showed positive reactions. A positive reaction was marked by the formation of gas bubbles around an isolated colony that was added with several drops of 3% H_2O_2 solution. The gas bubbles formed were Oxygen (O_2) that was produced from the breaking down of Hydrogen Peroxide (H_2O_2) by the catalase enzyme produced by the microorganism⁹. According to Mahaseth and Kuzminov²¹, hydrogen peroxide is toxic to cells as it is able to deactivate the enzymes in the cells. Thus, microorganisms that are anaerobic in nature will break down hydrogen peroxide into Oxygen (O_2) and Water (H_2O). Hydrogen peroxide is formed during aerobic metabolism, so bacteria that live in an aerobic environmental condition will produce catalase to break down this compound to eliminate its toxicity to the cells.

A Methyl Red Test is intended to detect a bacterium's ability to produce a stable acid and keep it from glucose fermentation⁹. An addition of a Methyl Red reagent can show a pH indicator, which remains red at pH 4.5 or below⁹. The Methyl Red Test results of the three bacterial isolates were negative (-), which was indicated by a yellow colour of the solution. This showed that the three isolates were unable to ferment carbohydrate, hence, failing to lower the medium's pH.

The Voges Proskauer Test results of the bacterial isolates, on the other hand, showed positive reactions. This was shown by a change in the medium's colour after the addition of 5% α -naftol and 40% KOH. Certain bacteria are capable of fermenting glucose to acetone through the butanediol fermentation pathway. In an alkaline atmosphere, acetone and butanediol will be oxidized into diacetyl, which will react with α -naftol to give a red colour²².

A gelatin hydrolysis test aims to know whether there is a gelatinase-catalyzed gelatin hydrolysis process. Gelatinase will break down gelatin into amino acids, which will be used in the growing process. Hydrolyzed gelatin cannot form gel and turns liquid once taken out from a refrigerator²³. The gelatin hydrolysis test results of the three bacterial isolates were positive. This was indicated by the gelatin medium's melting even after 30 min of storage in a refrigerator. As stated by Zheng *et al.*²⁴, a microorganism's ability to hydrolyze gelatin is characterized by the melted state of the gelatin medium even after refrigeration.

The citrate test was conducted to figure out the three bacterial isolates' ability to use citrate as a source of carbon for cell metabolism. The test results of the isolates AAT 1.4 and CAT 3.4 were positive, while the test result of the isolate AAT 3.2 was negative. A positive result is marked by a change in the medium colour from green to blue after 24 hrs of incubation²⁵. These results showed that the isolates AAT 1.4 and CAT 3.4 possessed citrate permease (citrase) that made it

easy to transport citrate into cells, while the isolate AAT 3.2 did not. The test medium used was Simmons citrate agar, which used citrate as the only source of carbon. In the case of bacteria that could use citrate for that purpose, acids would be removed from the medium, causing an increase in pH and a change in medium colour from green to blue²⁶.

An indole test, meanwhile, is aimed to detect a certain bacterium's ability to produce tryptophanase to break down tryptophan amino acid into pyruvate acid and indole. The indole test results of the three bacterial isolates were negative, as marked by the formation of yellow rings on the surface of the media after the addition of several drops of Kovac's reagent. This showed that the three bacterial isolates were unable to break down tryptophan amino acid in the tryptone broth medium, hence, no red ring formed on the surface of the medium. A positive result is marked by a red ring formed on the surface of the medium after an addition of Kovac's reagent that contains para-dimethylaminobenzaldehyde. A red ring is formed when indole reacts to the aldehyde from Kovac's reagent²⁷.

From the carbohydrate fermentation test, positive results were obtained in several sugar types tested, namely, glucose, sucrose, mannitol, lactose and xylose, but the isolate AAT 3.2 could not ferment carbohydrate of xylose type. This is based on the indicator observed, that is, when there was a change in the medium colour from green, which indicated a neutral state, to yellow, which indicated acidity. This change in colour was observable due to the addition of a solution named BTB (Bromthymol Blue) into the carbohydrate fermentation medium. According to Mamlouk and Gullo²⁸, carbohydrate fermentation may occur either aerobically on the medium surface or anaerobically at the bottom of the medium. On the surface of a medium, glucose is catabolized into pyruvate and later perfectly degraded in a citrate acid cycle to produce CO_2 , H_2O and energy. Meanwhile, at the bottom, glucose catabolism produces organic acids and energy.

Physiological characters of the bacterial isolates: The physiological characters of the potential L-asparaginase-producing thermohalophilic bacterial isolates were provided in Table 4.

The test results of the three bacterial isolates grown on the NB medium as to the oxygen demand showed that the three isolates were aerobic. This character is marked by growth on the surface of the liquid medium after 24 hrs of incubation (Table 4). This is as stated by Mamlouk and Gullo²⁸, that aerobic bacteria will grow on the surface of a medium, anaerobic bacteria will grow in colonies at the bottom of a medium, facultatively anaerobic bacteria will grow in dispersion throughout a medium and microaerophilic bacteria will grow in colonies slightly under the surface of a medium.

Table 4: Physiological characters of the potential L-asparaginase-producing thermohalophilic bacterial isolates

No.	Code	Oxygen (O ₂) demand	NaCl concentration (%)				pH tolerance			Heat tolerance (°C)							
			2	5	7	10	5	7	8	5	30	45	50	55	65	70	80
1	AAT 1.4	Aerobic	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-
2	AAT 3.2	Aerobic	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-
3	CAT 3.4	Aerobic	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-

+: Growth and -: No growth

A NaCl tolerance test of the three bacterial isolates was performed at varied NaCl concentrations, namely, 2, 5, 7 and 10%. The tolerance level of a bacterial isolate can be seen from the presence of growth in a NaCl-containing medium. The NaCl concentration in the medium is inextricably linked to Na⁺ and Cl⁻ ion needs. Certain bacteria have particular Na⁺ and Cl⁻ ion needs according to their physiological demands. The test results showed that the three isolates could live well in the medium with various NaCl concentrations (Table 4). It was found that the three bacterial isolates had wide-ranging tolerance to NaCl. It is thus reasonable to categorize these three bacterial isolates as facultative halophilic bacteria. As stated by Kearns¹⁹, microorganisms that need 3.5-4% NaCl in their growths are called obligate halophilic microorganisms, while those that do not require NaCl, either in high concentrations or low, are called facultative halophilic microorganisms.

Bacteria may grow optimally in high-salinity environments by maintaining their osmotic pressure equilibrium. A high osmotic pressure outside the cell causes the bacterial cell to absorb a high amount of liquid²⁹. On the contrary, a low osmotic pressure outside the cell will deprive the cell of liquid as the liquid inside the cell will be drained out to keep the osmotic pressure equilibrium in and out of the cell, leading to the cell having a plasmolysis³⁰.

The pH tolerance test results showed that the three bacterial isolates could tolerate the three interventions at pH 5, 7 and 8. This was marked by the presence of clear growths on the medium at those pH levels (Table 4). The pH levels would influence the cell's enzymatic activity. In general, bacteria grow in the pH range 4-9, but they will grow optimally in the neutral pH range 7-8³¹. Sub-optimal pH levels of the medium will disrupt the enzymatic functioning and growth of bacteria. Increases and decreases of the hydrogen ion concentration will slow down chemical reactions due to the inhibited enzyme reaction, which will influence the growth rate and survivability of bacteria^{31,32}. According to Baker-Austin and Dopson³², acidophilic microorganisms grow optimally in the pH range 1.0-5.5, neutrophilic microorganisms do in the pH range 5.5-8.0, alkaliphilic microorganisms do in the pH range 8.5-11.5 and extremely alkaliphilic microorganisms do at pH>10.

A heat tolerance test was conducted at temperatures of 5, 30, 45, 50, 55, 65, 70 and 80°C. Whether an isolate is tolerant to heat is observable through its ability to grow in a medium with an intervention corresponding to the temperature. Based on the test results, as provided in Table 4, the three isolates were able to grow at 30, 45, 50, 55 and 65°C but were not at 5, 70 and 80°C. This shows that the three bacterial isolates were categorized as facultative thermophiles as they could grow at 30°C rather than obligate thermophiles as they could not grow at 70 and 80°C^{33,34}. The isolates' ability to tolerate heat at 45, 50, 55 and 65°C was caused by the spores formed in the bacteria's cells (Fig. 3). Bacterial spores are structures that are formed by bacteria to survive extreme environmental conditions, including high temperatures^{17,18}.

Identities of the potential L-asparaginase-producing thermohalophilic bacteria: The potential L-asparaginase-producing thermohalophilic bacterial isolates were identified based on phenetic identification and phylogenetic identification.

Phenetic identification

Preliminary identification: Based on the preliminary identification that was performed using the Profile Matching method, the potential L-asparaginase-producing thermohalophilic bacterial isolates had characters assumed to be matched with the genera *Bacillus* and *Brevibacillus*. This was supported by the similarities of the key characters of the selected test isolates with the a forementioned genera, as can be seen in Table 5.

Numeric-phenetic identification: The phenotypic characters of the potential L-asparaginase-producing bacterial isolates were analyzed based on numeric systematics and there were 30 of them. The results of the characterization of the test bacteria were provided in Table 6.

A numeric systematics analysis was conducted using the MVSP software, version 3.1. The results for the three isolates based on the phenotypic characters are visualized in the form of a dendrogram as can be seen in Fig. 5.

Table 5: Profile matching results revealing the similarities in key characters of the genera *Bacillus* and *Brevibacillus* with the bacterial isolates

Character	<i>Bacillus</i> ¹	<i>Brevibacillus</i> ²	AAT 1.4	AAT 3.2	CAT 3.4
Cell shape	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>
Gram reaction	Positive	Positive	Positive	Positive	Positive
Catalase	Positive	Positive	Positive	Positive	Positive
Motility	Positive	Positive	Positive	Positive	Positive
Endospore formation	Positive	Positive	Positive	Positive	Positive
O ₂ demand	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Xylose fermentation	Positive	Negative	Positive	Negative	Positive

¹Key genus characters based on Bergey's Manual Determinative of Bacteriology¹¹ and ²Key genus characters based on *Brevibacillus* as a Biological Tool: A Short Review¹²

Table 6: Phenotypic characters of the potential L-asparaginase-producing bacterial isolates and the reference species

	Codes of isolates and reference species*								
Character	AAT 1.4	AAT 3.2	CAT 3.4	<i>Bacillus subtilis</i>	<i>Bacillus megaterium</i>	<i>Bacillus pumilus</i>	<i>Brevibacillus limnophilus</i>	<i>Brevibacillus laterosporus</i>	<i>Brevibacillus halotolerans</i>
Morphology									
Circular colony shape	+	-	+	+	-	+	-	-	+
<i>Bacillus</i> cell shape	+	+	+	+	+	+	+	+	+
Gram reaction: Positive	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Endospore formation	+	+	+	+	+	+	+	+	+
Aerobic growth	+	+	+	+	-	+	+	-	+
Facultative anaerobic growth	-	-	-	-	+	-	-	+	-
Biochemical test									
Catalase	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	-	+	-	+	+	+
Indole test	-	-	-	-	-	-	-	-	-
Citrate test	+	-	+	+	+	-	+	-	-
Voges Proskauer	+	+	+	+	-	+	+	-	-
Fermentation									
Glucose	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	-	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+
Xylose	+	-	+	+	+	+	-	-	-
Physiological test									
2% NaCl	+	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+	+	+
7% NaCl	+	+	+	+	-	+	+	+	+
10% NaCl	+	+	+	+	-	-	+	-	+
Temperature 5 °C	-	-	-	+	-	+	-	-	-
Temperature 30 °C	+	+	+	+	+	+	+	+	+
Temperature 45 °C	+	+	+	+	+	+	+	+	+
Temperature 50 °C	+	+	+	+	+	-	+	+	+
Temperature 65 °C	+	+	+	-	-	-	-	-	-
pH 5	+	+	+	+	+	+			
pH 7	+	+	+	+	+	+			
pH 8	+	+	+	+	+	-			

*Characters of the reference species were based on Bergey's Manual Determinative of Bacteriology¹¹ and *Brevibacillus* as a Biological Tool: A Short Review¹², +: There was a reaction/growth and -: There was no reaction/growth

The analysis result dendrogram (Fig. 5) based on the levels of similarities of the three potential L-asparaginase-producing bacterial isolates with six reference species from the genera *Bacillus* and *Brevibacillus* shows that there were four clusters formed. The isolate AAT 3.2 joined *Brevibacillus limnophilus* in the third cluster with a similarity level of 92.9%.

Meanwhile, the isolates AAT 1.4 and CAT 3.4 had a similarity level of 100%. The latter two isolates were then assigned to join *Bacillus subtilis* in the fourth cluster with a similarity level of 89.3%. The separation of the isolates AAT 1.4 and CAT 3.4 from the isolate AAT 3.2 was due to distinguishing characters from the existing phenotypic characters.

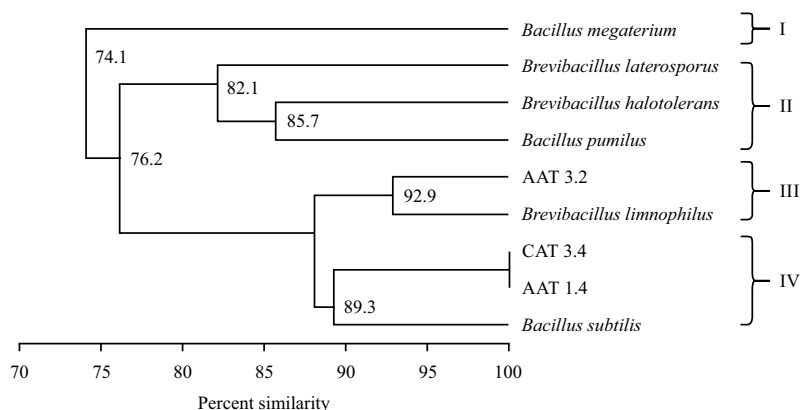


Fig. 5: A dendrogram showing the similarities of the three potential L-asparaginase-producing bacterial isolates⁶ strains based on the simple matching coefficient (SSM) analysis and unweighted pair-group method with arithmetic averages (UPGMA) algorithms following the phenotypic characters

The first distinguishing character was the bacteria's ability to use citrate, where the isolates AAT 1.4 and CAT 3.4 were able to use citrate, while the isolate AAT 3.2 could not use citrate as its source of carbon (Table 3 and 6). Another distinguishing character was the ability to ferment xylose, where the isolates AAT 1.4 and CAT 3.4 were able to ferment xylose, while the isolate AAT 3.2 was not.

CONCLUSION

Based on the results and discussion in this research, it can be concluded that the phenotypic characters of the isolates AAT 1.4 and CAT 3.4 were as follows: Being *Bacillus* in shape, Gram-positive, endospore-forming, catalase-positive, capable of xylose fermentation and motile. These characters were the key characters of the genus *Bacillus*. Meanwhile, the phenotypic characters of isolate AAT 3.2 were as follows: Being *Bacillus* in shape, Gram-positive, endospore-forming, catalase-positive, incapable of xylose fermentation and motile. These characters were the key characters of *Brevibacillus*. The distinguishing character between the genus *Bacillus* and the genus *Brevibacillus* was the xylose fermentation ability. The results of the numeric-phenetic analysis of the isolates AAT 1.4 and CAT 3.4 were identical to the species *Bacillus subtilis* subsp., *Subtilis* BS-2 and those of isolate AAT 3.2 were identical to the species *Brevibacillus limnophilus* DSM 6472.

SIGNIFICANCE STATEMENT

This study discovered the L-asparaginase-producing thermohalophilic bacteria which has the potential to produce

enzymes that are heat and salt resistant. This enzyme can be used for the cancer therapy of acute lymphoblastic leukemia and melanoma as it has a specific ability to inhibit the formation of nutrients for cancer cells. Therefore, this study will help researchers to find the enzymes involved in the treatment of leukemia cancer. In this study, 3 bacterial isolates were found that have the potential to produce L-asparaginase enzymes, namely AAT 1.4 and CAT 3.4 isolates as *Bacillus subtilis* and AAT 3.2 isolates as *Brevibacillus limnophilus*.

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