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Isolation and Phenotypic Identification of Amylolytic Bacteria from *Oryctes rhinoceros* L. Larvae Decomposing Empty Palm Oil Fruit Bunches

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ABSTRACT. *Oryctes rhinoceros* L. has the ability to produce enzymes since its digestive system contains 65% usable symbiotic bacteria. The aims of the research were to isolate and phenotypic identify the amylolytic bacteria that produce amylase enzymes from horn beetle larvae. The methods involve using isolated and screened bacteria. The morphology and biochemical of the isolates were described. The detected isolate's genomic DNA was isolated and 16S rRNA sequencing was used to examine it. The results of screening and bacterial isolation obtained five isolates that produced amylase. Five selected bacterial isolates had amylolytic index ranging from 0.135-1.370 mm at 37 °C. The five bacterial isolates were rod-shaped gram-negative bacteria. Based on the 16S rRNA gene sequence and phylogenetic tree analysis showed that EA1 is closely related to *Ochrobactrum* sp with a similarity level of 97.71% and EA2 is related to *Pseudomonas mendocina* with a similarity level of 98.52%.

Keywords: Amylolytic Bacteria, Identification, Isolation, 16S rRNA sequencing

INTRODUCTION

The amylase enzyme is one of the many enzymes that are widely utilized in a variety of activities and play a significant role in the industrial sectors (Biswas et al., 2020). Amylase enzyme is a biocatalyst that catalyzes the formation of maltose, dextrin, D-glucose, and oligosaccharides by breaking α-D-(1,4)-glycosidic bonds in starch, glycogen, and oligosaccharides. Until recently, Indonesia still imported amylase enzymes from several countries. This is due to the lack of local industries that produce these enzymes (Nisa et al., 2021). Even though Indonesia has a high microbial biodiversity and the potential to produce amylase enzymes.

Amylase enzymes can generally be found in various living things, such as microorganisms. Several types of microorganisms, such as fungi that produce amylase enzymes such as *Aspergillus* sp. and *Pennicilium* sp. (Silaban et al., 2020), also produce amylase. It can also be produced by *Bacillus stearothermophilus, Bacillus licheniformis*, and *Bacillus amyloliquefaciens* (Putri, 2021). Microorganisms have been widely used as a source of enzymes because they produce enzymes that can be utilized in very varied amounts and types. In addition, microorganisms can be cultured to obtain the enzymes they produce. Amylolytic bacteria are one of the types of microorganisms that are often used to obtain amylase enzymes. The ability of the amylolytic bacteria can be seen from the bacterial isolates capable of hydrolysing starch (Arfah et al., 2018). This is indicated by the presence of an amylolytic index value, which can be measured by comparing the diameter of the clear zone with the diameter of the colony that has been incubated at a certain temperature. This clear zone describes the ability of extracellular amylase to hydrolyse starch. One source of microorganisms that can be extracted to produce amylolytic bacteria is the larvae of the horn beetle (*Oryctes rhinoceros* L.).

The horn beetle is a major pest on various plantation crops, especially in the *Arecaceae* family, such as oil palm plants. One of the waste products from the production of oil palm is empty fruit bunches. Fresh fruit bunches (FFB) yield 23-25% oil palm empty fruit bunches, 6.5% shell, 13-15% fiber, 5.5-6% seeds, and 16-20% crude palm oil for every ton of FFB processed (CPO) (Rahma, 2018). Horn beetle larvae are often found on dead or weathered tree trunks, which can be used as nests as well as a source of food for the larvae. Oil palm trunks are very popular with horn beetle larvae because they contain the required organic matter (Fauzana & Ustadi, 2020). The larvae have non-pathogenic microorganisms in their intestines that are capable of harboring 105–109 prokaryotic cells. In the intestines of larvae, about 65% of symbiotic bacteria are used in the process of food degradation in their digestive system (Saha et al., 2019). Recent studies have shown that the gut of horn

beetle larvae can be extracted to obtain various types of enzymes. However, there are still very few studies that identify amylolytic bacteria found in the gut of horn beetle larvae.

Identification is the process of using established criteria for nomenclature and classification by comparing the characteristics of an unknown individual with a known individual. Identification can be done by genomics or phenotypic. Genomic identification includes identification that utilizes genomic analysis, which is an analysis that studies the relationship of a species to its relatives based on the sequence of molecular markers. The molecular marker used in this study was the 16S rDNA sequence (Wullur et al., 2020). The 16S rDNA sequence is a marker because it has a region with a conserved and diverse nitrogen base sequence. Sustained nitrogen base sequences can be used to construct phylogenetic trees, while diverse base sequences can be used to track diversity and locate lines within species. Several studies related to the identification of 16S rRNA in larvae have been carried out, such as in longhorned beetle larvae which is closely related and is in the same group as C. *rubripes* and P. *semipunctata* (Setyolaksono et al., 2017). Other studies, namely from termite (*Coptotermes* sp.) gut, which has similarities with *Brevibacillus parabrevis*, *Brevibacillus* sp. and *Pseudomonas alcaligenes* (Mulyani et al., 2018). Research related to the isolation of amylolytic bacteria from horn beetle larvae has not been much reported. These larvae include nuisance pests that can cause damage and death to oil palm fruit bunches (OPEFB) plants. This research was done to isolate and identify isolates and analyzed using 16S rRNA sequencing.

EXPERIMENTAL SECTION

Materials

The analytical grade materials required for this study were all purchased from Merck (Indonesia). Horn beetle larvae were obtained from the decay of oil palm in Bulu Taba District, North Mammuju Regency, West Sulawesi, Indonesia.

Instrument

Morphological observations of each isolate were carried out using a microscope (Microscope Binocular/Olympus CX23LED). DNA amplification was carried out using a polymerase chain reaction (PCR) machine. The PCR results were determined by the DNA base sequence (sequencing) and then analyzed using Applied Biosystem model 3730XL automatic DNA sequencing system (Applied Biosystem). The phylogenetic tree was developed by neighbor-joining in MEGA 11.

Culture and Bacteria Isolation

One larvae sample was taken. The intestine was taken with sterile tweezers. Then 1 g was weighed and dissolved in 9 mL of 0.9% physiological NaCl and homogenized. A total of 1 mL was diluted to a dilution of 10^{-6}. A total of 1 mL was diluted to a dilution of 10^{-6}. Additionally, 0.1 mL was grown on a selective medium (A mixture of 0.2% (b/v) bacto-yeast extract, 0.05% (b/v) $MgSO_4$, 0.05% (b/v) NaCl, 0.015% (b/v) $CaCl_2$, 2% (b/v) nutrient agar, 1% (b/v) dissolved starch added with distilled water) using the scatter technique, and the results were cultured in an incubator at 45 °C for 24 hours. The single colony and had different colony morphological characteristics (e.g., color, colony, growth shape, and edges) were individually isolated, scratched into an amylolytic selective medium and incubated for 24 hours at 37 °C. The growing colonies were scratched by quadrants to obtain pure isolates on an amylolytic selective medium and incubated for 24 hours at 37 °C (Ulfat et al., 2020).

Screening of Amylase-Producing Symbiont Bacteria

The pure isolate was isolated, spotted on an amylolytic selective medium, and incubated for 48 hours at 37 °C. Bacterial isolates that grew dripped with iodine solution (2% I_2 and 0,2% KI). It was determined that isolates of amylolytic bacteria producing amylase enzyme were present if there was a clean zone around the colony because they could hydrolyze starch. Then, to calculate their amylolytic index, pure bacterial isolates with a clear zone were regrown on an amylolytic selective medium. A caliper was used to measure the clean zone's diameter. The culture stock was prepared on an inclined medium before evaluating each isolate. This is necessary because the iodine test, which uses a disinfectant-like iodine solution, kills bacterial isolates. Additional characteristics of bacterial isolates with high clear zone diameters and steady growth patterns are described (Arfah et al., 2015).

Phenotypic Identification of Amylase-Producing Bacterial Isolates

Initial identification of bacterial isolates was carried out based on morphology and physio-biochemical characteristics. Physical identification was carried out by observing morphology using a microscope (Microscope Binocular/Olympus CX23LED) at 1,000x magnification, including: shape of the colony; surface of the colony as seen from the side; colony edge as seen from above; and colony color (Oktiarni et al., 2021).

Microscopic characterization of amylase producing bacteria isolates with gram staining

The culture of bacterial isolates aged 24 hours was created by smearing physiological NaCl on glass objects, drying in the air, and fixing on a Bunsen burner. A crystal violet (Gram-A solution) was drip-applied to the preparations, which were then washed under running water after 3 minutes. Then it was dripped with Gram B solution (iodine lugol) and left for 1 minute, then washed with running water. It was then cleaned once more under running water after

being drizzled with Gram C (alcohol) until the last of the color had been removed. The last stage of preparation was dripped with Gram D solution (safranin) and left to dry, after which it was dripped with immersion oil and viewed under a 1,000x magnification microscope. Observations were made by examining the morphology and color of the cells. Gram-negative bacteria were red, whereas Gram-positive bacteria were purplish blue (Arfah et al., 2015).

Biochemical analysis of a selected bacterial isolate

According to Bergey's Manual of Determinative Bacteriology, biochemical experiments (Febriyanti et al., 2021) to determine the physiological characteristics of bacteria, such as the Simon Citrate Agar (SCA), Catalase, Triple Sugar Iron Agar (TSIA), motility, Methyl Red-Vogue Proskauer (MR-VP), and carbohydrate fermentation tests have been performed.

16S rRNA molecular identification and phylogeny

DNA extraction of potential endophytic bacteria was carried out by centrifugation of colonies of potential isolates that had been suspended in a sterile saline solution. The pellets were resuspended by adding 0.5 mL of InstaGene Matrix (Bio Rad, USA). After that, it was incubated at 56 ºC for 30 minutes, then praised at 100 ºC for 10 minutes. The supernatant containing concrete DNA was ready to be used for the amplification process. DNA amplification was carried out using a polymerase chain reaction (PCR) machine. The results of the PCR can be known qualitatively through electrophoresis using 1% agarose gel, which is then visualized with the help of a UV transilluminator. The cycles that occur in PCR are about 35 cycles. The primers used were 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3'). 1 of 20 of the overall reactions volume was added as DNA concrete. 35 cycles of DNA amplification were performed, with the initial denaturation settings being 94 °C for 45 seconds, 55 °C for 60 seconds, and 72 °C for 60 seconds. PCR product purification using the Montage PCR Clean up kit (Milipore). Furthermore, the PCR results were determined by the DNA base sequence (sequencing) using primers 518F (5'- CCAGCAGCCGCGTA ATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') [24], as well as big dye terminator cycle sequencing kit (Applied Biosystem) and then analyzed using Applied Biosystem model 3730XL automatic DNA sequencing system (Applied Biosystem). Sequence results were aligned with GenBank data using the BLAST program from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) (Tamura et al., 2011). Selected sequences from the NCBI database were aligned using CLUSTX (http://www.clustal.org/). Gaps and the 5′ and 3′ ends of the alignment were edited using BioEdit sequence alignment editor software version 7.2.5 (http: //www.mbio.ncsu.edu/bioedit/page2.html). The phylogenetic tree was developed by neighbor-joining in MEGA 11.

RESULTS AND DISCUSSION

Results of Screening and Isolation of Amylase-Producing Amylolytic Bacteria from the Intestines of Horn Beetle Larvae

Based on the results of screening and isolation of bacteria found in the intestines of horn beetle larvae, 162 bacterial isolates were obtained. There were 5 amylolytic bacteria that produced amylase. The findings demonstrated that the five bacterial isolates that were taken from the intestines of the horn beetle larvae produced amylase; this is shown by the amylolytic index obtained in each isolate. The amylolytic index can be determined by dropping the growing bacterial colonies with an iodine solution, which aims to determine the ability of bacteria to hydrolyse starch into maltose or glucose. Amylolytic activity can be characterized by the formation of a clear zone on the growth bacterial medium. The extent of the clear zone produced was due to the ability of each isolate to produce amylase enzymes (Saha et al., 2019). **Figure 1** shows that isolate EA3 has the largest amylolytic index, namely 1.370 mm, and the smallest amylolytic index is shown in isolate EA5, which is 0.145 mm with a clear zone around the colony containing 2% starch. The amylolytic index is obtained by comparing the diameter of the bacterial colony and the clear zone; the amylolytic index can be calculated.

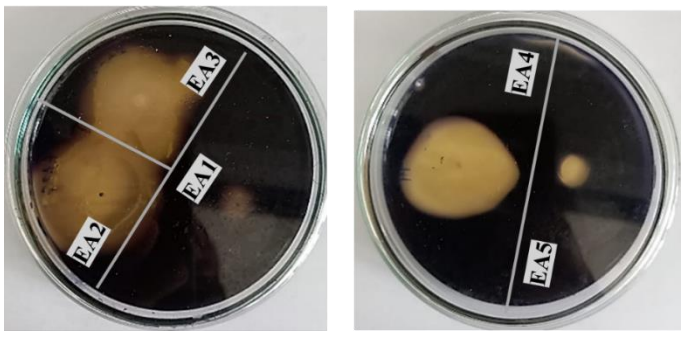

Figure 1. Bacterial isolates were spotted on selective containing 2% amylum. The clear zone around the bacteria indicated the amylum degradation by amylase that produced the bacteria.

Table 1. The Results of observations of bacterial colony morphology and bacterial biochemical test results from horn beetle larvae

Characteristic	Result				
	EA1	EA2	EA3	EA4	EA5
Colony color	Milky white	Beige	Beige	Milky white	Milky white
Colony shape	Round	Fibrous	Fibrous	Fibrous	Fibrous
Colony edge	round-regular	Irregular	Irregular	round-regular	round- regular
Gram colony	Red	Red	Red	Red	Red
Gram	Negative	Negative	Negative	Negative	Negative
Elevation	Arise	Flat	Flat	Arise	Arise
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Sucrose	-	-	-	-	-
Maltose	+	-	+	+	+
Catalase	+	-	+	+	+
Motile	-	-	-	+	-
MR	+	-	+	+	+
VP	-	-	-	-	-
SCA	-	-	+	+	-
Indole	-	-	-	-	-
TSIA	+	-	-	-	-

EA : Bacterial Isolates; (-) : It does not change color; (+) : Change color; MR : Methyl red test; VP : Voger Proskauer test; TSIA : Triple sugar ion test.

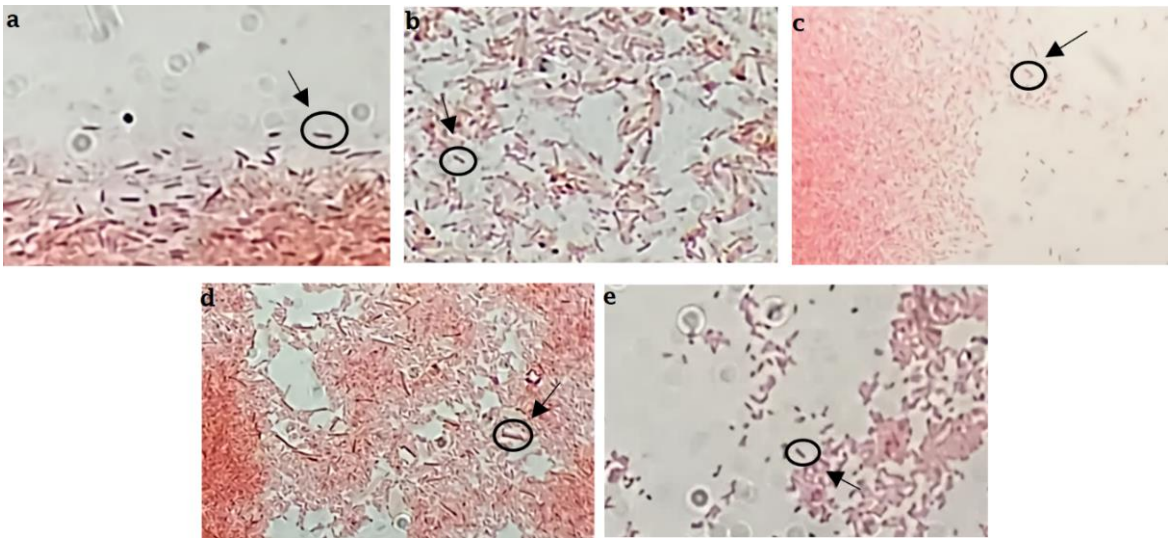

Figure 2. Gram staining of horn beetle larvae using a microscope at 1000x magnification. The five bacteria include gram-negative bacteria in the form of bacilli (a) Isolate Code EA1 (b) Isolate Code EA2 (c) Isolate Code EA3 (d) Isolate Code EA4 (e) Isolate Code EA5

Morphological and Biochemical Characteristics of Selected Bacterial Isolates

Based on macroscopic observations, characteristics of bacterial isolates can be known. Several bacterial isolates had almost the same morphological characteristics such as color, shape, edge, elevation, and gram color as bacterial colonies. Simon Citrate Agar (SCA), Catalase, Triple Sugar Iron Agar (TSIA), motility, Methyl Red-Voges Proskauer (MR-VP), and carbohydrate fermentation assays are among the biochemical tests used to determine the characteristics of the bacterial isolate from the horn beetle. Identification of bacteria by approach Biochemistry is done by looking at the behavior of bacteria against sugar fermentation and seeing the enzyme activity they have (Rahma., 2018).

The results of the observation of colony morphology obtained beige and milky white bacterial colors. The shape of the colonies is round and fibrous. The edges of the bacterial colonies are round-regular and irregular. The elevation is raised and flat. Observation of bacterial isolates using a microscope after staining is shown in **Figure 2.**Gram staining was used to identify the isolate's characteristics based on variations in the gram-positive and gram-negative bacteria's cell wall structures (Ulfat et al., 2020). When

viewed under a microscope, gram-positive bacteria will continue to display a crystal violet purple color. Gram-negative bacteria are unable to maintain a crystal purple color, but safranin dye is absorbed on the cell wall and causes it to appear red under a microscope (Hayat., 2022). Based on **Figure 2**, it is known that the five bacterial isolates were rod-shaped gram-negative bacteria. According to (Biswas et al., 2020) observations of colonies of chosen bacterial isolates, rounded colonies predominate, and there are two isolates that are Gram-positive and seven that are Gram-negative from the digestive tract of O. *rhinoceros* L. larvae.

The biochemical character of the bacterial isolates of horn beetle larvae (**Table 1**) showed that, in the molality test, there was one citrate-positive isolates out of five bacterial isolates. Citrate test serves to determine the carbon source of bacteria using or not using citrate, the test indicates that of the five bacterial isolates, only one isolate used citrate as a carbon source (Dawodu & Akanbi, 2021). The Methyl Red-Voges Proskauer (MR-VP) test revealed that all of the bacterial isolates were negative for Voges Proskauer (VP) and four of the isolates were positive for Methyl Red (MR). To find out whether bacteria can oxidize glucose by creating acid as the end product and in large concentrations, the Methyl Red-Voges Proskauer (MR-VP) test was used (Woo et al., 2008). The catalase test was carried out by adding a 3% H_2O_2 solution to the medium. Positive catalase was indicated by the formation of O_2. This was because the bacteria hydrolysed hydrogen peroxide (H_2O_2) using the catalase enzyme. The results showed that there were 4 catalase-positive bacterial isolates. In the TSIA test, there were 2 isolates that showed positive reactions marked by a change in the color of the media to yellow, which indicated the ability of bacteria to produce acid and gas from the fermentation of glucose, lactose, and sucrose. Tests for the fermentation of carbohydrates include those using glucose, sucrose, maltose, and lactose. The test results obtained for all the bacterial isolates were positive for glucose and positive for lactose. In the maltose test, there was one bacterial isolate that was negative for maltose and all bacterial isolates were negative for sucrose (Harahap et al., 2018).

Molecular Analysis of The Selected Bacterial Isolates

16S rRNA gene from two amylolytic bacteria (EA1 and EA2) was successfully amplified and showed DNA band of around 1000–1300 bp (**Figure 2**). The products obtained from the PCR results were then sequenced to obtain the nucleotide sequence of the selected isolates. Results of the Basic Local Alignment Search Tool (BLAST) approach for sequencing dialysis samples can be found at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Molecular analysis using BLAST was selected based on the sequence from the type of material. Sequence from type is an important part of the taxonomy because it has a very high level of confidence (Ginting et al., 2021). The results of the molecular identification of amylolytic bacteria isolates EA1 and EA2, respectively, can be seen in **Table 2** and **Table 3.**

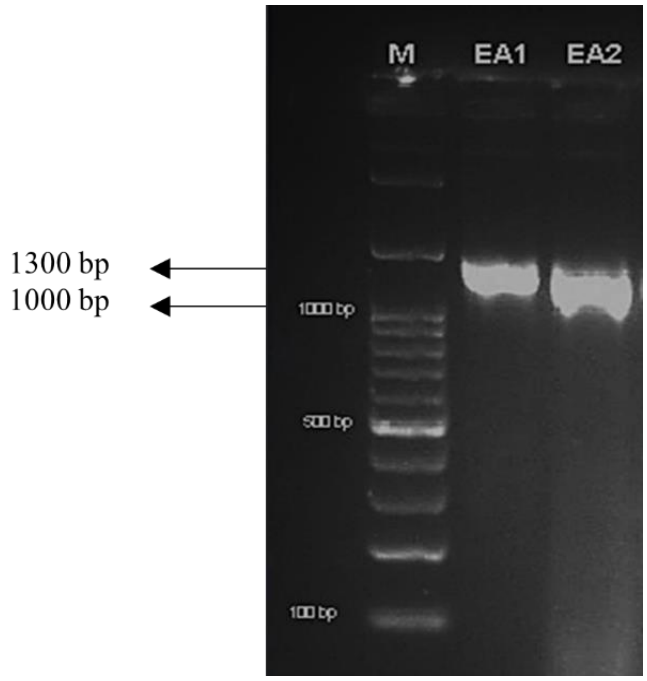

Figure 3. DNA amplification results for EA1 and EA2 using a polymerase chain reaction (PCR) machine. The PCR results were known qualitatively through electrophoresis using 1% agarose gel, which were then visualized with the help of a UV transilluminator. Isolates Information: M : Marker, EA1 : Selected isolate code EA1, EA2 : Selected isolate code isolate EA2

Table 2. The best homology analysis 16S rRNA gene sequence of amylolytic bacteria from EA1 isolate using the Basic Local Alignment Search Tool (BLAST) NCBI

Description	Max Score	Total Score	E value	Ident
Ochrobactrum sp. strain A7 16S ribosomal RNA gene, partial sequence	2100	2100	0.0	97.71%
Ochrobactrum sp. KT48 16S ribosomal RNA gene, partial sequence	2097	2097	0.0	97.63%
Ochrobactrum sp. K38 16S ribosomal RNA gene, partial sequence	2097	2097	0.0	97.63%
Brucella intermedia strain CCI5 16S ribosomal RNA gene, partial sequence	2095	2097	0.0	97.63%

Table 3. The best homology analysis 16S rRNA gene sequence of amylolytic bacteria from EA2 isolate using the Basic Local Alignment Search Tool (BLAST) NCBI

Description	Max Score	Total Score	E value	Ident
Pseudomonas mendocina strain MAE1-K chromosome, complete genome	2148	8229	0.0	98.52%
P. guguanensis strain A52 16S ribosomal RNA gene, partial sequence	2143	2143	0.0	98.44%
P. guguanensis 16S ribosomal RNA gene, partial sequence	2143	2143	0.0	98.44%
P. guguanensis strain Iraqi ZG.K.M 16S ribosomal RNA gene, partial sequence	2143	2143	0.0	98.44%

Figure 3. Phylogenetic tree EA1 from analysis of 16S rRNA gene sequence of bacterial strain showing the evolutionary relationship of the bacterial isolates within previously characterized species.

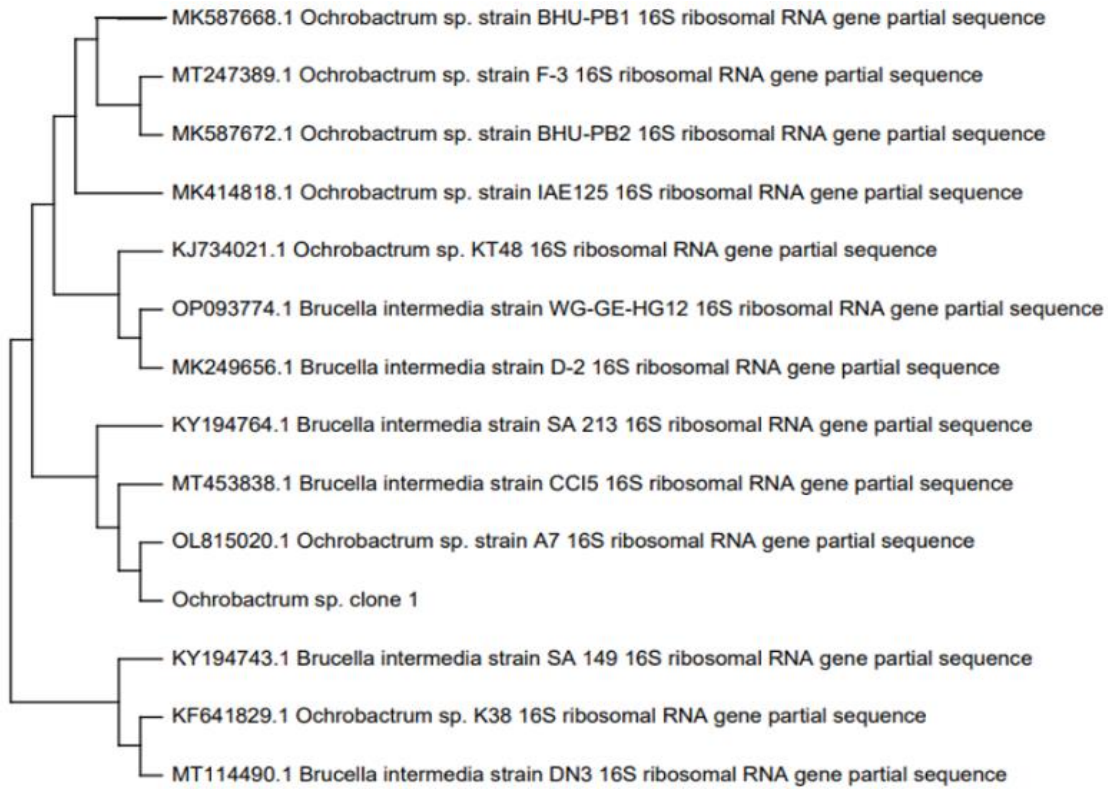

Figure 4. Phylogenetic tree EA2 from analysis of 16S rRNA gene sequence of bacterial strain showing the evolutionary relationship of the bacterial isolates within previously characterized species.

Based on the homology analysis obtained from BLAST, it can be seen that the percentage of target bacteria is similar to other bacteria. The nucleotide sequence of the 16S rRNA gene was analyzed using the BLAST program from NCBI and several bacterial species were obtained that had affinity with the bacterial isolates EA1 (**Table 2**) and EA2 (**Table 3**). This phylogenetic analysis is expressed in a phylogenetic tree. Phylogenetic trees are created to determine the evolutionary relationships between different species. The results of phylogenetic tree analysis based on the 16S rRNA gene showed that the EA1 sample had the closest relationship with *Ochrobactrum* sp. (**Figure 3**) with a similarity rate of 97.71% (**Table 2**). According to Odumosu et al. (2022), *Ochrobactrum* sp. is a gram-negative and rod-shaped bacterium and is motile; this bacterium has a conformity with the characteristics of the EA1 bacterial isolate.

Analysis of the 16S rRNA gene in the bacterial isolate EA2 showed that the isolate is related to *P. mendocina* (Figure 4) with a similarity of 98.52% (**Table 3**). Based on research conducted by Gani et al. (2019), bacteria that have amylolytic, preteolytic and lipolytic properties of *P. pseudomallei* bacteria index 97.81%, *P. stutzeri* index 61.21% and *P. stutzeri* index 97.81% with gram-negative characters, rod-shaped and aerobic bacteria. These characteristics are compatible with the EA2 isolate.

CONCLUSIONS

The conclusion of this study was based on the results of screening and bacterial isolation obtained five isolates that produced amylase. Five selected bacterial isolates had amylolytic index ranging from 0.135-1.370 mm at 37 °C. The five bacterial isolates were rod-shaped Gram-negative bacteria. Based on the 16S rRNA gene sequence and phylogenetic tree analysis showed that EA1 is closely related to *Ochrobactrum* sp with a similarity level of 97.71% and EA2 is related to *P. mendocina* with a similarity level of 98.52%.

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