Identification of Sago-Pulp Amylolytic Bacteria and Its Utilization for Granulated Fertilizer

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Abstract

Previous research stated that bacteria isolated from sago waste from the traditional sago industry in Palopo had the potency to produce amylase. This study aims to confirm the ability of bacteria isolated from sago pulp waste to produce amylase, evaluate the ability of these amylolytic bacteria to produce IAA fix nitrogen, identify selected bacteria, and apply selected bacteria into granules biofertilizers. Bacteria were isolated from sago pulp waste and grown on a 1% starch agar medium. The amylolytic activity was analyzed qualitatively using iodine and quantitatively using the 3,5-Dinitrosalicylic Acid (DNS) method at pH 6. Amylolytic bacteria were analyzed for IAA production using Salkowsky reagent and nitrogen fixation ability through the Serra Kit method. Potential bacteria were identified based on the similarity of the 16S rDNA sequence. The selected bacteria were grown in a 10mL NB medium, and then bacteria were inoculated and air-dried to obtain the granules. The selected bacteria were put into granules and re-isolated using the total plate count (TPC) method. Eight amylolytic bacteria were obtained from sago pulp waste. L1E isolate had the highest amylolytic activity of 1.228 $U.mL^{-1}$, and L1D isolate had the highest IAA production of 69.8 $g.mL^{-1}$. Isolate L1E was identified as *Alcaligenes faecalis* with a 99.45% similarity index, and L1D was identified as *Serratia surfactantfaciens* with a 99.09% similarity index. Isolate L2G was identified as *Alcaligenes aquatilis* with a similarity index of 99.8% of the 16S rDNA sequence. The viability of bacteria from granules was 1.41 x 10^8 $CFU.g^{-1}$.

Keywords: Amylase enzyme, amylolytic bacteria, granulated fertilizer.

INTRODUCTION

Indonesia has approximately 1.2 million ha of sago plantation producing around 8.4-13.6 million tons of sago annually. Sago becomes the staple food for some areas in Indonesia as it contains high nutrient and calories sources. In South Sulawesi, especially Palopo City, sago becomes the main source of food and income for the local community. The processing of sago remains limited to produce sago flour for basic food. Sago flour can be produced from the extraction of sago stalks. The cycle of sago processing produces solid and wastewater, including the bark of sago, fibrous sago residue (pulp), and wastewater. It is about 26% of sago bark and 14% of sago pith count of the total weight of the sago block. Sago waste remains to have about 65.7% of dissolved starch [1].

The sago waste produced annually ranges between 1,838 – 2,100 $tons.year^{-1}$ [2]. Sago pulp waste in the sago industry is not handled properly, allowing the accumulation of solid waste and causing acidity in the soil, then polluting the environment. Sago waste can be used as a substrate for bacterial growth and to be used to produce various commercially useful products. The starch content in sago pulp ranges from 41.7 to 65% [2]. Sago waste can be well hydrolyzed into reducing sugars by bacteria with a degrading activity such as amylolytic bacteria to degrade the starch components. Therefore, the use of potential bacteria to degrade starch is needed to maximize the bioconversion of sago pulp [3].

Biofertilizer is a material containing certain live microbes that function as a plant growth-promoting (PGP) producer, a nitrogen fixer, and a decomposer. One type of bacteria that can be used is amylolytic bacteria. Amylolytic bacteria such as the genus Bacillus, Enterobacter, and Klebsiella are known to have the ability to fix nitrogen [4]. Therefore, this study focused on obtaining amylolytic bacteria from sago pulp in Palopo, South Sulawesi, that can be further used for amylase, IAA production, and nitrogen-fixing, so it will be a biofertilizer agent to solve the sago pulp waste problem.

MATERIAL AND METHOD

A Sampling of Sago Pulp Waste Collection

The sago pulp waste was collected from the traditional industry of sago in Palopo, South Sulawesi, at two locations, location 1 (L1) in Salubulo and location 2 (L2) in Songka (Fig. 1). Both locations were chosen based on the different activities of traditional sago industries,

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which led to the assumption the sago pulp waste differs in its storage time. Sample of sago pith waste was taken from a pile of sago pulp waste at the bottom and near the ground, then stored in a plastic bag in an isotherm box. Measured environmental parameters, such as C/N ratio, organic compound, and pH, were measured to complete the information of physicochemical conditions in both locations. The organic compound and C/N ratio were analyzed in Soil Laboratory, Faculty of Agriculture, University of Brawijaya.

Figure 1. Locations of sago waste sampling (location 1: Salubulo, location 2: Songka)

Isolation and Screening of Amylolytic Bacteria

Isolation of amylolytic bacteria was carried out using starch media (1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.05% MgSO4, 0.05% NaCl, 0.015% $CaCl_2$, and 2% bacteriological agar) with method plate [2,5]. All plates were incubated at 30°C for 48 h. The isolates were screened used iodin 1% by flooding the isolates inoculated in another petri dish, and a clear zone indicated a positive value. The isolated colonies were photographed, then grouped.

Crude Enzyme Production of Selected Bacteria and Screening of Amylolytic Bacteria

Each selected bacteria were inoculated into 25 mL starch broth media then incubated in a shaker incubator at 30°C 120 rpm for 48 h. Five ml of the bacterial culture was taken to observe the optical density at 54 nm, equalize the sample density, taken 1 mL of each, then transferred to 50 mL of new starch broth media, incubated at 37°C 120 rpm. Five mL of the samples were taken at 24, 48, 72, and 92 hours. The optical density (OD) was measured at a wavelength of 540 nm [6]. The production of crude enzyme was obtained by centrifugation of culture medium at 4°C, 10000 rpm for 10 minutes. The supernatant was defined as the crude amylase enzyme then taken to analyze the enzyme activity [7].

Data Collection

Amylolytic Activity Assay

Amylase activity was tested using the DNS method [8]. One mL of crude amylase filtrate from centrifugation (supernatant) was put into a test tube, then added liquid starch media with 1% sago as a source of starch and then incubated at 37°C for 15 min. One g.100mL^{-1} of starch solution was added to the blank tube (as blank) and then incubated at 15 min, 37°C without added the enzyme. One mL of the DNS reagent was added to each test tube to stop the reaction

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after 15 min. The tube was heated to boiling for 5 min, added 1 mL of K-Nartrate solution, cooled with running water for 15 min and 1 mL of distilled water added. Each solution in the test tube was then determined by the color intensity using a spectrophotometer at 540 nm. The absorbance value was plotted with a glucose standard curve. Each test sample was repeated three times for enzyme activity [8,9].

Indole-3-Acetic acid Assay

The amount of IAA produced by amylolytic bacteria was determined by a colorimetric technique using Van Urk Salkowski reagent and Salkowski's method [10]. The isolates were grown in Triptyc soy broth and incubated at 28°C for four days at a shaker incubator. Data were obtained from the sampling of 24, 48, 72, and 96 hours. The broth was centrifuged after incubation. The supernatant was reserved, and 1mL was mixed with 2 mL of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HCLO_4$ solution) and kept in the dark. The optical density (OD) was recorded at 530 nm after 30 min [11].

Nitrogen Fixation Assay

Cultures of each isolate were equalized for age, culture volume, and cell density. Each isolate culture in the logarithmic growth phase was inoculated as much as 5 mL in 50 mL of liquid Nfb without the addition of Bromothymol Blue. The control (media without the addition of inoculum) was also prepared. Controls and test cultures were incubated in a shaker incubator at 120 rpm at 30°C for seven days. The culture was taken 2 mL and centrifuged at 10,000 rpm at 28°C for 15 minutes. The supernatant was taken 1 mL and put into a test tube. The ammonia content of the supernatant was determined based on the Sera Ammonia Test Kit. A change in color indicated the nitrogen-fixing activity of each isolate from clear to green.

Bacteria Identification Based on 16S rDNA

Bacterial chromosomal DNA was extracted using a Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZYMO RESEARCH, USA). The 16S rDNA sequence was amplified by Polymerase Chain Reaction (PCR) using universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG3') and 1492r (5'-GGTTACCTTGTTAACGACTT-3') with PCR program: pre-denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 min, annealing at 55°C for 30 s, and extension at 72°C for 5 min. The amplicon of 16S rDNA was confirmed by 1.5% agarose gel electrophoresis and visualized using a UV transilluminator. The amplicon of 16S rDNA was purified and sequenced in First BASE, Malaysia. The 16S rDNA sequences of bacteria were aligned with reference strains from the GenBank database using the MEGA V.6 program, and the phylogeny tree was constructed and inferred according to the Neighbor-joining algorithm and Tamura-Nei model [12].

Hemolytic Activity Assay

Hemolytic activity was assayed to determine phenotypically non-pathogenic bacteria. Bacterial isolates were tested by growing on 5% blood agar media, then incubated at 30°C for 24 hours. Bacterial isolates were observed for alpha hemolysis, beta hemolysis, and gamma hemolysis activities. Isolates with gamma-hemolytic activity (no clear zone was formed around the colony) indicated that the bacterial isolates were not pathogenic [13].

Granulated Fertilizer Production

One oose of pure bacterial isolate was selected into a culture bottle containing 10 mL of sterile NB media and then incubated in an incubator shaker at 150 rpm, temperature 28 C for 24 hours. Bacterial density was measured using a spectrophotometer (OD=0.5, wavelength 470nm) then 2.5 mL (5% v/v) of starter culture was inoculated into a 50 mL sterile NB medium as a production medium. This medium was then incubated in a shaker incubator at a speed of 150 rpm at 28°C for 48 hours.

Sago waste as much as 300 grams (part of the crude fiber removed) then dried at a temperature of 40-45°C until the water content is about 15%. The sago pulps were mixed with bacterial starter culture and molasses (ratio 1:1) until compact and could be clenched, then passed through a 14 mesh sieve and air-dried for 24 hours. The evaluation of the obtained biofertilizer granules was then tested for bacterial viability.

Cell density in compost media was calculated using the TPC (Total Plate Count) method. Cell density was calculated after the granules were successfully formed. A total of 1 g of the granule product was diluted in 9 mL of sterile NaCl, then homogenized and diluted to a dilution level of 10-10, then 100 L was taken and poured into sterile Petri dishes. The cup was then poured with sterile NA medium and moved to form a figure of eight and incubated at 28°C for 24 hours, and the Colony Forming Unit was calculated. Count colonies between 30-300.

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RESULT AND DISCUSSION

Amylolytic Bacteria Isolates

The bacteria isolation of amylolytic bacteria from sago pulps waste obtained eight isolates consisting of five isolates from Salubulo (L1A, L1B, L1C, L1D, and L1E) and three isolates Songka (L2F, L2G, and L2H). The amylolytic bacteria in Salubulo had a density of 59 x 10^5 CFU.g^{-1} more than17.35 x 10^5 CFU.g^{-1}, and there was a significant difference. The difference in bacterial density was influenced by the content of organic matter, especially C-organic and the C/N ratio was significantly higher in the sago pulp samples in Salubulo than in Songka (Table 1).

Table 1. Comparison of the physico-chemical and biological parameters of sago pulp waste in Sabulo and Songka based on T-test

Parameter	Sabulo (Location 1)	Songka (Location 2)
C-organic (%)	38.9±1.22^b	31.5±3.25^a
N-total (%)	0.2±0.06^a	1.2±0.69^a
C/N ratio	174±38.19^b	26.8±28.69^a
Organic compound (%)	67.3±2.10^b	54.5±5.62^a
pH	5.9±0.66^a	4.7±0.35^a
Total plate count (10^5 CFU.g^{-1})	59±7.46^a	17.35±7.98^b

Note: The same notation after the values shows that the parameters do not differ between locations (p>0.05)

High organic C content in a substrate causes microorganisms to overgrow, and at a high C/N ratio, nutrients for microorganisms are well met so that the density of microorganisms is higher. In addition, carbon is a source of energy for the growth of microorganisms, while nitrogen plays an important role in the preparation of amino acids [1].

The physico-chemical conditions that most influence the growth of bacteria are the available carbon and nitrogen sources. The low organic C content in sago pulp samples taken from Songka was due to the activity of carbohydrate fermentation by microorganisms to form organic acids such as acetic acid, pyruvic acid, and lactic acid [14,15].

Amylase Activity in Bacteria

L1E isolate had the highest ability to produce amylase enzyme, as much 1,407 U.mL^{-1} at 48 and hours incubation, followed by L1B isolate 1.228 U.mL^{-1}, and L2H isolate 1.066 U.mL^{-1} at 72 hours incubation (Table 2). Amylase is an extracellular enzyme that can hydrolyze α-1,4-glycidic bonds into dextrins in starch and other small molecules that make up glucose. Amylase enzymes can be produced by different species of B*acteria, Archae,* and *Actinomycetes* [16]. Strain bacteria isolated from sago pulp had a good ability to produce amylase enzyme. The best amylolytic bacteria has produced the highest enzyme concentration at 72 hour incubation time. In case, L1E isolate has high amylase acitvity in 42 hours incubation. It could be caused by the environment such as pH and temperature were capable for the production activity [9].

Identification of Amylolytic Bacterial Species

The phylogenetic identification based on the similarity of the 16S rDNA sequence showed that the isolate L1E was identified as Alcaligenes faecalis NC260419C. The 16S rDNA gene sequence is the gold standard marker in the identification of bacteria. The gene with a length of 1500 bp has alternating conserved and variable regions that permit to design universal primers on the conserved regions and to use the variable for taxonomic classification, therefore the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG3') and 1492r (5'-GGTTACCTTGTTAACGACTT-3') were used in this experiment. The technique is used to accurately determine the phylogenic relationship between bacteria with over 100,000 sequences available at an online database [17].

Table 2. The activity of amylolytic bacteria in different incubation time

Isolates	Enzyme activity in different incubation times (U.mL^{-1})			
	24 hour	48 hour	72 hour	96hour
L1A	0.19 ± 0.14^{Aa}	1.01 ± 1.45^{Ab}	0.39±0.03^{Aab}	0.23± 0.06^{Aab}
L1B	0.81 ± 0.49^{Ba}	0.88 ± 0.93^{Bb}	1.23±0.44^{Bab}	0.23± 0.14^{Bab}
L1C	0.19 ± 0.07^{Aa}	0.51 ± 0.08^{Ab}	0.26±0.06^{Aab}	0.31 ± 0.29^{Aab}
L1D	0.35 ±0.14^{ABa}	0.96±0.04^{ABb}	0.54±0.25^{ABab}	0.49 ± 0.14^{ABab}
L1E	0.56± 0.52^{ABa}	1.41±0.876^{ABb}	1.3±0.025^{ABab}	0.65 ± 0.55^{ABab}
L2F	0.23±0.06^{ABa}	0.456±0.30^{ABb}	0.79±0.18^{ABab}	0.53 ± 0.05^{ABab}
L2G	0.26 ± 0.14^{Aa}	0.67 ± 0.54^{Ab}	0.35±0.02^{Aab}	0.23 ± 0.07^{Aab}
L2H	0.37± 0.07^{ABa}	0.80 ± 0.08^{ABb}	1.07±0.81^{ABab}	0.74 ± 0.07^{ABab}

Note: Amylase enzyme activity using Tukey's test. The same notation after the values (the capital letters for the same isolate at different times, lowercase letters for different isolates at the same time) shows that the parameters do not differ between locations (p>0.05).

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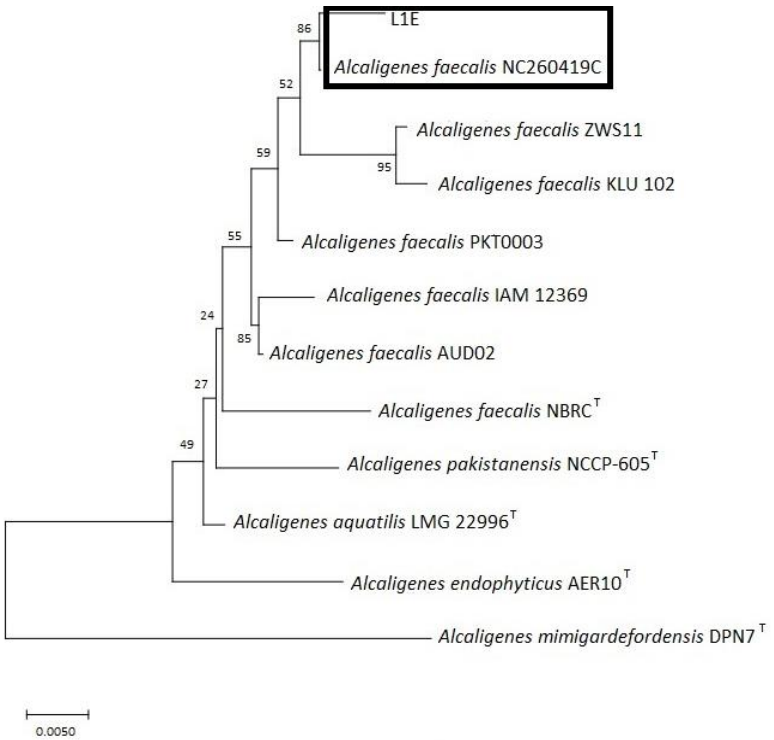

Figure 2. Phylogeny tree showing the relationship between L1E isolates and comparison bacterial strains based on 16S rDNA sequences.

These isolated bacteria had the highest ability to produce amylase enzymes. The results of the 16S rDNA sequence analysis showed that the relationship between L1E isolates and *Alcaligenes faecalis* NC260419C was 99.85% (Fig. 2).

Research on the ability of *Alcaligenes faecalis* bacteria to produce amylase enzymes is still very limited. The results of previous studies showed that the bacterial species *Alcaligenes faecalis* isolated from Taptani hot springs, Odisha, had the ability to produce an amylase enzyme of 4.125U.mL^{-1} at 84 hours of incubation [18]. The isolate of *Alcaligenes faecalis* bacteria isolated from soil had a maximum amylase enzyme activity at 48 hours of incubation, which was 663.67 U.mL^{-1} [19].

IAA Hormone Production

L1D isolate had the highest ability to produce IAA hormone, which was 69.83 g.mL^{-1} at 24 hours incubation, followed by L1E isolate58.91 g.mL^{-1} at the same time and L2F isolate 54.45 g.mL^{-1} at 72 hours incubation (Table 3). Indole-3-Acetic Acid (IAA) is a crucial phytohormone produced by plant growth-inducing bacterial strains. The IAA hormone can increase nutrient absorption activity and better root growth so that it can trigger plant growth [20,21]. The bacteria that produce IAA are *Aeromonas, Bacillus, Azotobacter, Burkholderia, Enterobacter, Pseudomonas, Microbacterium, Sphinogomonas, Mycobacterium,* and *Rhizobium* [22].

The L1D isolate with the highest ability to produce IAA was *Serratia surfactantfaciens* XY1011, with a similarity index of 99.8% (Fig. 3). *Serratia* sp. is a candidate for the growth-promoting agent in plants because it can produce IAA and break down phosphate. The results of previous studies stated that the isolate could produce 123.2 g.mL^{-1} IAA after 144 hours of incubation [23]. *Serratia surfactantfaciens* is a group of gram-negative bacteria and members of the Enterobacteriaceae family. These bacteria are often found in water, soil, insects, and plant surfaces [24].

Serratia is associated with plants as endophytic bacteria and free-living species in the rhizosphere. Many *Serratia* species have the ability to increase plant growth, one of which is because it has the ability to produce IAA and also as a biological control agent in pathogenic fungi that spread in soil that infects various plants such as *Erwinia tracheiphila, Pseudomonas syrigae* pv. *Lachrymans,* and *Fusarium oxysporum* [25]. The bacterial isolates that trigger plant growth are *Serratia* sp. ZoB14 It also protects ginger plants from the pathogenic fungus *Pythium myriotylum* [26].

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Table 3 The IAA production in different incubation times

Isolates	IAA production in different incubation times (g.mL^{-1})			
	24 hours	48 hours	72 hours	96 hours
L1A	34.08 ± 3.54^{hijk}	36.97 ± 10.42^{ijkl}	47.04 ± 0.47^{lmn}	34.18 ± 1.59^{hijk}
L1B	19.15 ± 0.98^{cdef}	24.83 ± 0.67^{efgh}	22.00 ± 1.35^{efg}	17.89 ± 0.37^{bcdef}
L1C	8.16 ± 0.99^{abcd}	14.08 ± 5.19^{abcde}	19.66 ± 0.77^{def}	5.47 ± 1.08^{a}
L1D	69.83 ± 9.68^{p}	44.18 ± 1.37^{klmn}	24.08 ± 1.41^{efgh}	17.14 ± 1.70^{bcdef}
L1E	58.91 ± 3.46^{op}	51.15 ± 5.78^{mno}	51.77 ± 6.69^{mno}	28.13 ± 1.89^{fghij}
L2F	32.68 ± 4.95^{ghijk}	54.45 ± 4.92^{no}	42.28 ± 2.18^{klm}	22.11 ± 2.32^{efg}
L2G	35.03 ± 0.67^{jkl}	38.02 ± 1.49^{hijk}	35.54 ± 0.82^{bcdef}	27.11 ± 2.26^{fghij}
L2H	41.02 ± 2.56^{klm}	33.09 ± 2.41^{ghijk}	25.51 ± 7.23^{efghi}	9.55 ± 0.56^{abcd}

Note: The different notation after the values indicates a significant difference between isolates and between incubation times ($p<0.05$)

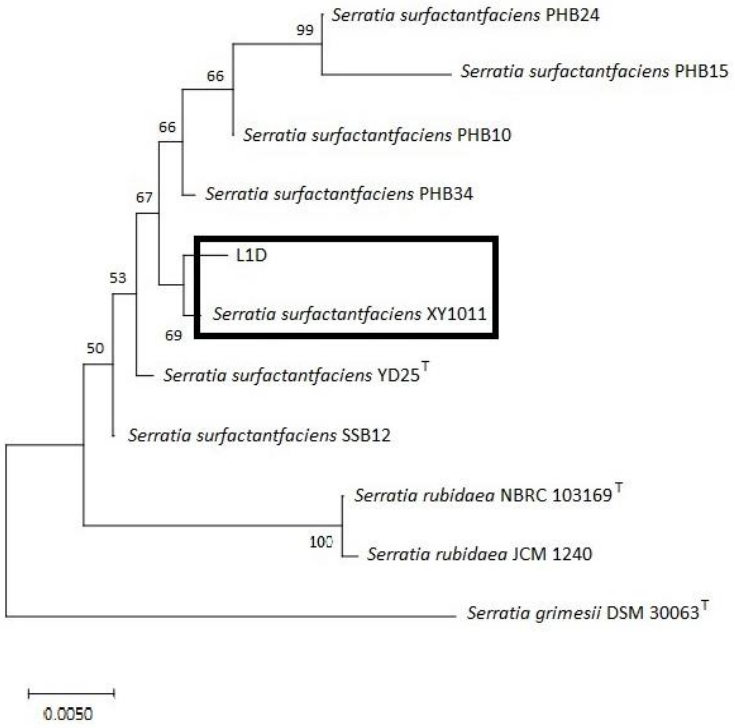

Figure 3. Phylogeny tree showing the relationship between L1D isolates and comparison bacterial strains based on 16S rDNA sequences

Isolates of *Serratia* sp. 5D and RTL100 isolated from root nodules showed the ability to increase yields by 25.55% and 30.85% on fertile soils in irrigated areas and nutrient-deficient soils in rainfed areas when compared to negative controls. These results indicate that the isolates of *Serratia* sp. 5D and RTL100 can function as effective inoculants in increasing nutrient-deficient soil fertility [27].

Nitrogen Fixing Assay

The semi-quantitative N_2 fixation potential test results using the Sera Ammonium Test Kit are presented in Table 4. There were six isolates from a total of eight isolates that could produce ammonia with a concentration of 0.1-1 mg.L^{-1}. The test results showed that isolates L1A, L1B, L1C, L1D, and L1E could fix N of 0.25mg.L^{-1}. Meanwhile, isolates L2F and L2H could not fix N. Nitrogen-fixing bacteria convert N_2 in the air into ammonia using nitrogenase. The nitrogenase is very sensitive to oxygen; the oxygen concentration as present in the atmosphere (21%) will inhibit the work of enzymes in reducing N_2 becomes NH_3^+. On the other hand, nitrogen fixation requires the energy of ATP to be synthesized. Through the process of oxidation, especially phosphorylation, which means it requires oxygen. To overcome the opposing needs, leghemoglobin plays a role in helping by binding, transferring, and providing oxygen in the respiration process [28]. Based on the criteria for the highest ammonia production capability, L2G was selected for identification.

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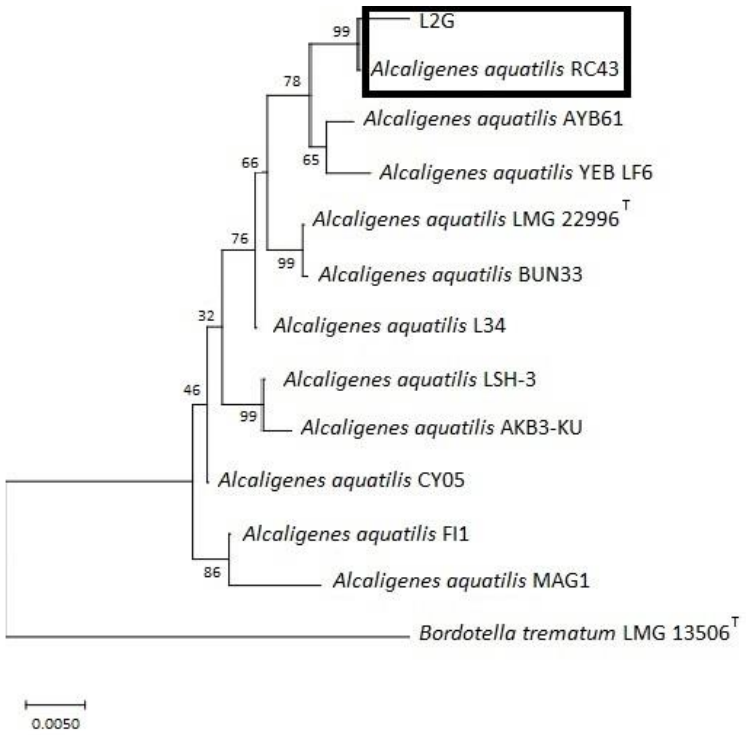

Figure 4. Phylogeny tree showing the relationship between L2G isolates and comparison bacterial strains based on 16S rDNA sequences

Table 4. Semi-quantitative potential assay for nitrogen fixation of amylolytic bacterial

Isolate	Ammonia Production
L1A	++
L1B	++
L1C	++
L1D	++
L1E	++
L2F	+
L2G	+++
L2H	+

Note: ++++ = > 1 mg.L^{-1} dark green color, +++ = 0.5 – 1 mg.L^{-1} green color, ++ = 0.1 – 0.4 mg.L^{-1} light green color, + = < 0.1 mg.L^{-1} yellow color; accumulation of ammonia in bacteria culture

Identification of Nitrogen-fixing Bacteria

The isolate that could fix nitrogen (L2G isolate) was known as the bacterium *Alcaligenes aquatilis* RC43 with a similarity index of 99,8% (Fig. 4.) *Alcaligenes aquatilis* bacteria can live and grow in extreme environments. In addition, these bacteria can grow in an environment polluted by gasoline and its derivatives because it has the ability to fix nitrogen [20].

Hemolysis Assay

The result of the hemolytic activity test of the three potential isolates (*Alcaligenes faecalis, Serratia surfactantfaciens,* and *A. aquatilis*), there was one non-pathogenic isolate, namely *Alcaligenes aquatilis* isolate with no hemolysis and no changes or no reaction on blood agar after 24 hours of incubation. *Alcaligenes faecalis* and *Serratia surfactantfaciens* isolates were indicated to have beta hemolysis activity with changes in red blood cells to become apparent. Based on this, one isolate was suspected to be non-pathogenic, and two isolates were pathogenic (Table 5).

Table 5. Pathogen confirmation test on selected isolates

Isolates	Hemolytic activity		
	β-hemolytic	α - hemolytic	γ - hemolytic
L1D	+	-	-
L1E	+	-	-
L2G	-	-	+

Note: the value (+) indicates the isolate has the activity, the value (-) indicates the isolate does not have the activity. L2G isolate was choosed as non-pathogenic bacteria

Granulation

The sago pulps were inoculated with L2G isolate, and then the granulation process was carried out. The granules formed were then seen for their bacterial density using the Total Plate Count (TPC) method. TPC results from granulation samples obtained a bacterial density of 1.41 x 10^8 CFU.g^{-1}. According to the regulation of the minister of agriculture no. 28/Ministry of

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Agriculture/SR.103/S/2009 about biofertilizers and soil improvers, the microorganism formula to be used as the starter of biofertilizer were 10^5 CFU.mL^{-1} [29].

Figure 5. Granules from sago pulp inoculated with L2G isolates

CONCLUSION

Identification using 27f and 1492r primers showed that L1E isolate was identified as *Alcaligenes faecalis*, and L1D isolate was identified as *Serratia surfactantfaciens*. Both L1E and L1D, respectively, have high amylolytic activity and produce high IAA hormones. The L2G isolate was identified as *Alcaligenes aquatilis* RC43 with an ability to fix nitrogen. *Alcaligenes aquatilis* bacteria showed indications of gamma hemolysis, so it assumed that it is safe to use as fertilizer. The results of TPC of granulated fertilizer showed living bacteria numbers of 1.41 x 10^8 CFU.g^{-1}, where this number met the standard requirements for biological fertilizers.

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