Isolation and Characterization of Nitrogen - Fixing

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Research Article

Isolation and Characterization of Nitrogen-Fixing Bacteria and Producing IAA (Indole Acetic Acid) From Rice Rhizosphere from The Soppeng Regency

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ABSTRACT
Rhizosphere microorganisms in the plant root area can increase the ability of plants to utilize nutrients, tether free nitrogen in the air, and can produce Indole Acetic Acid compounds. This study aims to obtain rice rhizosphere bacteria as nitrogen-fixing and producing Indole Acetic Acid which has the potential as a booster for plant growth. Rhizosphere bacteria are isolated from the soil around the roots of rice plants through serial dilution. There were thirty isolates of rhizosphere bacteria that could grow on agar nutrient medium. Morphological and biochemical characterization results obtained seventeen isolates that had different morphological, gram reaction, and catalase enzyme content. Ten isolates of rhizosphere bacteria that can grow on Burk's nitrogen medium and produce growth hormone Indole Acetic Acid. The highest concentration of Indole Acetic Acid was found in bacteria with HR2P isolate code, followed by HR15P isolate and HR29P isolate. The rhizosphere bacterial isolate is an isolate that has the potential as a bacterium for plant growth.

Keywords: Rizosfer, Indole Acetic Acid, isolates

INTRODUCTION

Soppeng Regency is one of the regencies in South Sulawesi that is experiencing rapid development in agriculture, on the other hand, the rate of population growth has also increased. Along with population growth, the need for rice is increasing. In meeting nutritional needs, around 90% of the population consumes rice because it contains about 40-80% of calories and 45-55% of protein[1]. Efforts are made to meet the needs of the population, namely to increase rice production by cultivating plants that utilize microorganisms that are safe, environmentally friendly, and sustainable.

Rhizosphere microorganisms are soil microorganisms originating from plant roots that can form mantles in the root area and increase the ability of plants to utilize nutrients. Rhizosphere microbes play a role in spurring plant growth, controlling plant pests and diseases and making efficient use of nitrogen fertilizer[2][3].

In agriculture, the use of nitrogen fertilizer as plant nutrients is increasingly being used. It is estimated that around 100 million tons per year of global consumption of nitrogen fertilizer[4][5][6]. The negative impact on the environment from the excessive use of synthetic nitrogen fertilizers, there is an accumulation of organic matter in the soil and groundwater pollution. Rhizosphere microorganisms as nitrogen fixers play an important role in the availability of nitrogen for plant growth because of the potential to convert free nitrogen from the air into ammonia that plants need[7][8]. Nitrogen-fixing bacteria are found in almost every niche ecological soil capable of binding nitrogen from the air, both symbiotic (root-nodulating bacteria) and non-symbiotic (free-living nitrogen-fixing rhizobacteria)[9][10].

Some rhizosphere microorganisms produce the product compound Indole Acetic Acid (IAA) which functions as a molecule carrying signals of microbial communication with plants and can stimulate plant growth[11][12][13]. IAA is a

phytohormone with auxin which helps the process of plant cell development and plays a role in the growth of xylem and phloem[14]. This study aims to obtain rice rhizosphere bacteria as nitrogen-fixing and IAA-producing potential as a booster for plant growth.

MATERIALS AND METHODS

Isolation and morphological and biochemical characterization of rhizosphere bacteria

Bacterial isolation was obtained from the soil in the rooting area of healthy rice plants from the Soppeng district. Done with serial dilutions[15]. The soil is weighed as much as 1 gram and mixed with 10 ml of sterile water. A total of 1 ml was put into a test tube containing 9 ml of sterile water and homogenized until it is diluted 10^{-6}. A 0.1 ml bacterial suspension was poured and spread onto a petri dish containing the Nutrient Agar (NA) medium. Incubated at a temperature of 28^0 C. Characterization of bacteria was carried out based on the shape, size, edges, elevation, and color of the colony on the NA medium[16].

Gram Stain Test

A 3% KOH (Potassium Hydroxide) solution was dropped on a glass object and added 1 loop of the bacterial isolate from the NA medium using an inoculation needle. The suspension is mixed for ± 60 seconds and rotated repeatedly in a clockwise direction and slowly raised upward[17]. If the suspension is slimy, sticky and lifted together with the inoculation needle indicates a positive reaction that indicates bacteria are gram-negative, and vice versa, if the suspension is not slimy and not sticky, indicates a negative reaction that indicates bacteria are gram-positive[18].

Catalese Test

The catalase test is to determine the ability of microbes to degrade Hydrogen peroxide (H_2O_2). H_2O_2 3% solution that has been dropped into glass object is mixed with bacterial isolates as much as one loop was taken from the NA media. A positive reaction is characterized by the formation of air bubbles that show bacteria produce catalase enzymes that can break down H_2O_2 into water (H_2O) and oxygen (O_2)[19].

The ability of rhizosphere bacteria to tether nitrogen

Bacterial isolates were grown on Burk's media and incubated at 28^0 C for 7 days. The composition of Burk media is $MgSO_4$ 20 g, K_2HPO_4 80 g, KH_2PO_4 20 g, $CaSO_4$ 13 g, $FeCl_3$ 1.45 g, Na_2MoO_4 0.253 g and sucrose 20.0 g dissolved in 1,000 ml distilled water and sterilized with an autoclave at 121^0 C for 15 minutes[20].

The ability of rhizosphere bacteria to produce IAA growth hormones

The rhizosphere bacteria's capability to produce IAA growth hormones is carried out by growing bacteria on NA media containing 200 ppm L-tryptophan. Incubated at 28^0 C for seven days. A 10 ml bacterial suspension was centrifuged at 8000 rpm for 10 minutes. 2 ml supernatant was added with two drops of orthophosphoric acid and 4 ml Salkowski reagent. Incubated in a dark room for 24 hours. Changes in suspension to a pink color show the ability of bacteria to produce IAA growth hormone[21][22]. Optical density is measured at a wavelength of 535 nm in mg units. L^{-1} [23][24].

RESULTS AND DISCUSSION

Isolation and morphological and biochemical characterization of rhizosphere bacteria

The results from the rhizosphere bacteria isolations found 30 isolates that could grow and colonize the NA medium. The bacterial colony that was found was cultured back on the NA medium (Figure 1). NA medium is a common medium for microbial growth and has nutrients for microorganisms that are not selective or heterotrophic. Nutrients contained in culture media will affect bacterial growth[25][26].

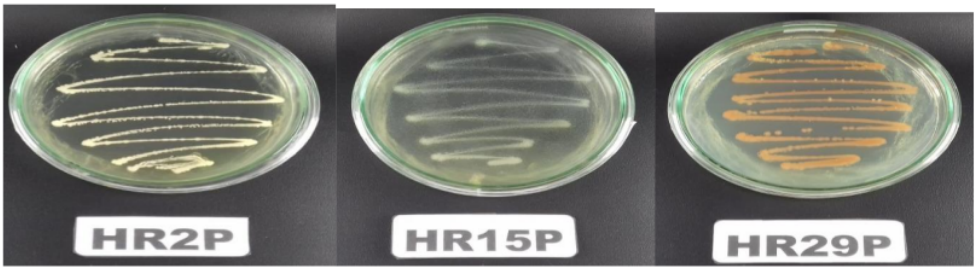


Fig.1: Purification of bacterial colonies

Morphological characterization results of bacterial isolates found 17 isolates that have different sizes, shapes, edges, elevations, and colors. Bacterial isolate colonies were grouped according to sizes, such as two tiny, two small, nine moderate sizes, and four large colonies. Grouping based on shape is dominated by the circular shape consisting of 13 colonies, the remaining four colonies were irregular in shape. The edges of the colony were generally entire, ie 10 isolates, four undulate, and three lobate colonies. Colony elevation varies from raised, convex, and flat. The color of the colony is predominantly white (Table 1). Intracellular pigments produced by bacteria cause differences in colony color[27][28][29]. Microbial pigments can play a role in the pathogenesis of diseases with cytotoxic properties [30]. Variable microbial characters indicate the different types of bacteria present in the plant's rhizosphere[31][32].

The gram reaction test found 12 isolates of gram-negative bacteria and five isolates of gram-positive bacteria (Table 1). Gram-positive bacteria have thin fat and thick cell walls, while gram-negative bacteria have thick fat and thin-walled cells that are in the periplasm chamber. KOH solution attacks fat (lipid bilayer) and makes gram-negative bacterial cells rupture, whereas gram-positive is not affected[17][33]. Gram-negative bacteria produce a positive KOH reaction because the cell wall has a thin layer of peptidoglycan that is very easily destroyed when exposed to alkaline materials. When the bacterial cell wall is destroyed in the 3% KOH, the suspension becomes thick within 5-60 seconds and the thread release is caused by the release of unfragmented DNA strands (Figure 2A). Gram-positive bacterial cell walls are not affected by a 3% KOH solution, this is due to the positive gram having a thick layer of peptidoglycan and a large amount of teichoic acid[34][35][36][37]. Gram-negative and gram-positive nonpathogenic bacteria have the potential to be biocontrols in controlling plant diseases[38][37].

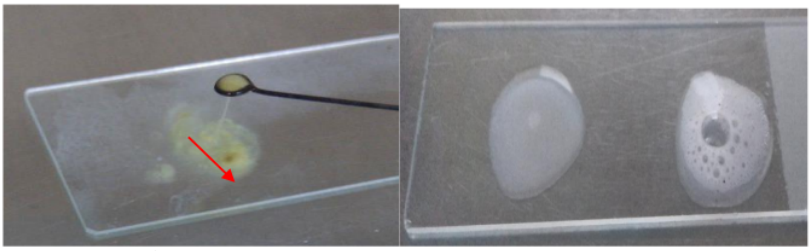
The catalase test results obtained 13 bacterial isolates that can produce air bubbles that indicate the bacteria can break down H_2O_2 into water and oxygen which means the bacteria have the enzyme catalase (Figure 2B). Catalase is a hemoprotein that can react with peroxide compounds. Hydrogen peroxide can be produced by bacteria under certain conditions and is a poison that can damage the bacterial metabolic system. The breakdown of hydrogen peroxide into other compounds, that are not dangerous, only occurs if there is a catalase enzyme and causes bacteria to survive[39], [40]. Hydrogen peroxide compounds are formed during aerobic metabolism[41]. Bacteria that can break down H_2O_2 with the enzyme catalase can form a defense system from the toxic H_2O_2 it produces[42].

Bacterial isolates that did not produce as many air bubbles as the four isolates could be expressed as negative catalase (Table 1). This means that H_2O_2 given is not broken down by the bacteria so it does not produce oxygen. The negative catalase bacterium does not have the catalase enzyme that breaks down H_2O_2[39].

Table 1: Morphological characterization of nitrogen-fixing rhizosphere bacteria and IAA production

Isolate	Colony Morphology					Reaction	
	Size	Shape	Edge	Elevation	Color	Gram	Catalase
HR1P	large	sirkuler	entire	raised	white	+	+
HR2P	moderate	sirkuler	entire	convex	cream	-	+
HR5P	moderate	irregular	entire	raised	white	-	+
HR6P	moderate	irregular	entire	raised	white	-	+
HR7P	pinpoint	sirkuler	undulate	raised	white	-	+
HR8P	moderate	sirkuler	undulate	raised	cream	-	-
HR9P	moderate	sirkuler	entire	raised	white	-	+
HR10P	small	irregular	entire	raised	white	-	+
HR15P	pinpoint	sirkuler	entire	convex	white	-	+
HR18P	large	sirkuler	entire	raised	white	+	-
HR19P	large	irregular	undulate	flat	white	+	+
HR20P	moderate	sirkuler	lobate	raised	white	-	-
HR22P	moderate	sirkuler	undulate	raised	cream	+	-

HR23P	small	sirkuler	entire	flat	white	-	+
HR28P	large	sirkuler	lobate	flat	white	+	+
HR29P	moderate	sirkuler	lobate	convex	yellow	-	+
HR30P	moderate	sirkuler	entire	flat	white	-	+



A B

Fig.2: Characterization of rhizosphere bacteria, A. Gram reaction using 3% KOH, B. Catalase enzyme reaction using 3% H_2O_2 solution

Rhizosphere bacterial ability to fix nitrogen

Analysis of the ability of rhizosphere bacteria to fix nitrogen found 10 isolates that could grow on Burk's medium (Figure 3). This medium is selective because it does not contain nitrogen, so only microorganisms that can bind free nitrogen can grow on this medium[43]. The form of bacteria that can grow on Burk's medium is a bacterial isolate that has a round shape, flat edge, convex elevation, and is a type of gram-negative. In general, the characters found in nitrogen-fixing cells are round and gram-negative. N sources contained in various organic compounds and from the air can meet the needs of bacteria for the element N. There are two classes of nitrogen-fixing bacteria, namely symbiotic nitrogen-fixing bacteria that are symbiotic with legume and non-symbiotic plants that live freely in the rhizosphere[44][45].

Rhizosphere bacteria contained in the soil can carry out the process of nitrification and denitrification so that N is available off in the form of NO_2 into the atmosphere[46]. Microorganisms that are in the soil contribute to plant productivity, especially in nitrogen-fixing and produce growth promoters[45][47]. If enough N elements are available for plants, the chlorophyll content in the leaves will increase and the photosynthesis process will also increase so that the more assimilates are produced, resulting in better plant growth [48],[49].

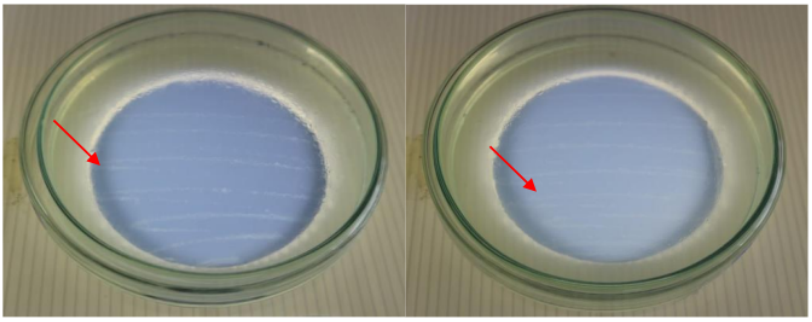


Fig.3: Analysis of nitrogen-fixing bacteria in Burk's media

The ability of rhizosphere bacteria to produce IAA growth hormones

The ability of rhizosphere bacteria to produce IAA growth hormones varies based on the change in color to pink after adding Salkowski's reagents. A total of 10 isolates showed color changes compared to controls (Figure 4). This indicates that the bacteria can produce IAA growth hormone. The addition of L-tryptophan to the medium is very important because it is a major precursor in the IAA biosynthetic pathway so that it can increase the concentration of IAA. IAA biosynthesis by rhizosphere bacteria can utilize tryptophan originating from root exudates or

dead cells [50], [51], [52]. The availability of suitable precursors is a primary factor in the microbial secretion of secondary metabolites[53], [54].

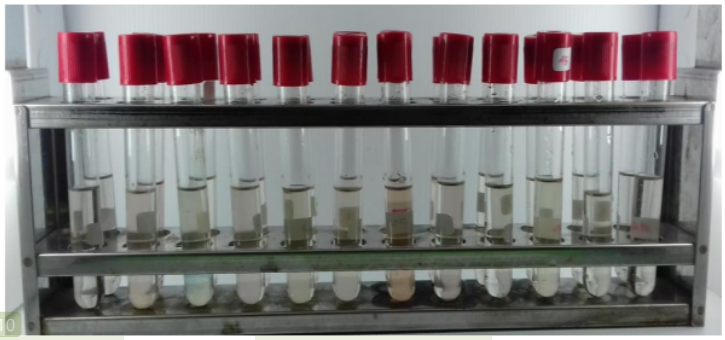


Fig.4: The ability of rhizosphere bacteria to produce IAA with a pinkish color change

Analysis of the highest IAA production capability produced by HR2P isolates was 3.359 mg.L^{-1}, followed by isolates HR15P at 3.297 mg.L^{-1} and HR29P isolates with IAA concentrations of 3.109 mg.L^{-1}. In contrast, the lowest IAA concentration produced by HR10P isolate was 0.875 mg.L^{-1} (Figure 5). The concentration of IAA produced by bacterial isolates varies based on the type of isolate and its ability to convert tryptophan contained in the media to IAA hormones[55]. Besides that, it can also be influenced by environmental factors, amino acid availability, growth rate, and other N sources[56], [11].

Rhizosphere bacteria that produce IAA-growing hormones have a metabolic pathway through L-tryptophan synthesis [57]. And play a role in the process of division, elongation, and enlargement of plant cells[58][59]. IAA compounds produced by rhizosphere bacteria can be reused by plants because rhizosphere bacteria can interact directly with plants by producing phytohormones, vitamins, or organic molecules that are easily absorbed by plant roots[60],[61]. The IAA-producing rhizosphere bacterium has the potential to spur plant growth which can benefit plant development.

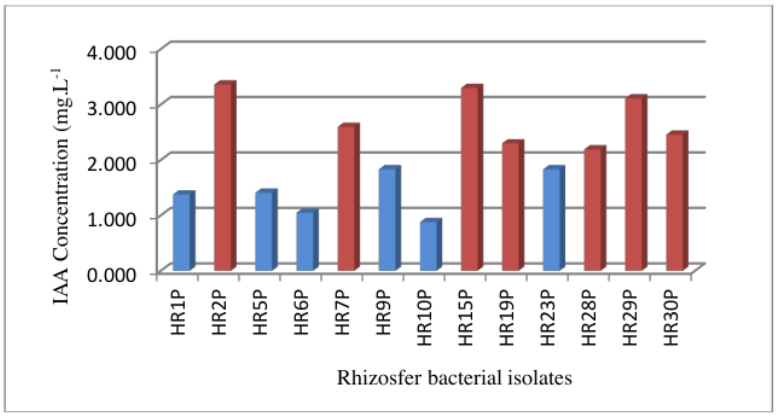


Fig.5: Analysis of IAA concentrations using a 535 nm UV-VIS spectrophotometer

CONCLUSION

Thirty isolates from rice rhizosphere bacteria were successfully grown on the nutrient agar medium. Morphological and biochemical characterization results obtained seventeen isolates that had different morphological, gram reaction, and catalase enzyme content. Ten isolates of rhizosphere bacteria that can grow on Burk's nitrogen medium and produce growth hormone Indole Acetic Acid. The highest concentration of Indole Acetic Acid was found in bacteria with the isolate code HR2P with a concentration of 3.359 mg.L$_{-1}$, followed by isolate HR15P 3.297 mg.L$_{-1}$ and isolate HR29P with a concentration of Indole Acetic Acid of 3.109 mg.L$_{-1}$. The bacterial isolates HR2P, HR15P, and HR29P have the potential as bacterial stimulants for plant growth.

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Competing Interests

The authors declare no competing interests.

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