The ability of bacteria from legume plant roots grown on former coal mining soil to produce Indole-3-Acetic Acid (IAA)

Yuni Sri Rahayu1*, Yuliani1, and Mahanani Tri Asri1

1 Undergraduate Program of Biology, Universitas Negeri Surabaya, Surabaya, Indonesia

Abstract. In general, coal mining is carried out openly using heavy equipment to take and move soil in the topsoil area until coal mining is possible to be conducted. As a consequence, the nutrient level is low because there is physical, chemical, and biological soil damage. Bioremediation is one of the alternatives to improve former coal mining land by utilizing soil microorganisms that have a role in soil plant hormone levels, such as auxin-produced root rhizosphere bacteria. This study aimed to isolate and characterize rhizosphere bacteria of legume plant roots grown on former coal mining soil, and to determine qualitatively and quantitatively its ability to produce IAA hormones. The characterizations include gram properties, colony morphology, arrangement of isolate, and cell shape. Then, the bacterial ability to produce IAA qualitatively and quantitatively respectively using the Salkowski method and spectrophotometry were tested. The results revealed that there were eleven isolates of legume plant root rhizosphere bacteria grown on the former coal mining soil that were able to produce IAA hormones with an average concentration of 15.949 ppm (2IA4); 10.762 ppm (4IE3); 9.700 ppm (ID3); 9.422 ppm (3IB4); 7.970 ppm (2IA3); 7.847 ppm (6IIB3); 7.268 ppm (8IIIB4); 6.804 ppm (IIDD5); 6.459 ppm (IE5); 5.379 ppm (7IIIB3); and 5.086 ppm (5IB3). Isolates of rhizosphere bacteria with the highest concentration have the potential to be chosen as a growth booster for legume plants grown on former coal mining soil to increase legume crop productivity.

1 Introduction

Large-scale former coal mining lands can be found abundant in the East Kalimantan region. However, not all coal mining companies carry out land processing before the area is abandoned. Meanwhile, this marginal land has the potential to be used as productive land, although efforts are needed to minimize the limiting factors. An effort that can be made to overcome the damage to former coal mining land is bioremediation. Bioremediation is an effort that can be made to restore a polluted environment using biological processes, namely by utilizing organisms to carry out the detoxification process of polluted environments [1]. Bioremediation can be carried out by utilizing multi-symbiotic interactions between plants and soil microorganisms which play an important role in the dynamics of soil nutrients. This

* Corresponding author: yunirahayu@unesa.ac.id

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).
includes exploring the potential of bacteria from former coal mining lands which can produce phytohormones that play a role in increasing plant growth and production. The study of the utilization of multi-symbiotic microorganism interactions concerning plant growth and production on marginal soils such as former coal mines is important if former coal mining land is made productive for planting media, especially when it is supported by the increasing demand for chemical-free agricultural products.

Reducing the use of chemical fertilizers in agriculture brings many benefits to improve soil biochemical properties. Chemical fertilizers used above the normal threshold can become pollutants that are harmful to the environment and to plants. The use of chemical fertilizers can have a negative impact on the soil where the soil structure hardens and loses its porosity [2]. Organic matter enriched with root-fertilizing microbes in agricultural soil can increase soil respiration and enzymatic activity [3]. Therefore, the efforts to find alternative bacteria that have the potential to increase soil nutrients and produce phytohormones, especially auxin (Indole-3-acetic acid, or IAA) are an important part of the marginal land of former coal mining land. Auxin is known to play an important role in various plant growth mechanisms, such as the formation of lateral roots, cell differentiation [4], cell division, and shoot formation and development [5]. On the other hand, IAA hormones originating from the rhizosphere have an important role, especially in the cycle of plant organic matter and the interactions between soil microbes and plant roots [6].

In general, the hormone auxin (IAA) can be produced by plants. However, IAA can also be produced by microorganisms and fungi [7]. The auxin hormone produced by plants is called endogenous IAA, while produced by organisms other than plants such as bacteria is called exogenous IAA. Endogenous auxins are produced in plant meristems and play a role in cell elongation. Meanwhile, exogenous IAA at low concentrations can be utilized by plants in the process of growth and development of shoots and root hairs [8]. Microorganisms capable of producing IAA are microorganisms that have a symbiosis with plants, such as rhizosphere microbes (bacteria around plant roots) or endophytic microbes [6]. This is also supported by research conducted in a previous study which found that plant growth hormone can be synthesized independently by plants or from endophytic bacteria [9]. Rhizosphere microbes act as part of PGPR (Plant Growth-promoting Rhizobacteria), or Rhizobacter affect triggering plant growth [10]. A number of bacteria are able to produce IAA such as Bacillus, Pseudomonas, Enterobacter, Agrobacterium, Azospirillum, and Rhizobium [11].

The results of previous studies showed that the rhizosphere bacterial isolates of the tomato plant had activity in producing IAA hormones even without the presence of a tryptophan precursor [12]. Similar research was also conducted previously by isolating bacteria from various soils which showed that Bacillus spp. and Serratia spp., R1 line was able to produce the highest IAA of 121.1 ppm on day 6 and was able to increase the number of adventitious roots in maize by 14.4 ± 1.476. Bacillus spp. able to produce IAA around 53.1-71.1 ppm, while Serratia spp. around 3.14 ppm and the optimum on day 8 was 20.05 ppm [13]. Rhizosphere bacteria secluded from the root and stem nodules of various leguminous plants show the ability to produce IAA, NH3, siderophore, HCN, and ACC deaminase [14]. The rhizosphere bacteria also have the ability to produce hydrolytic enzymes.

The purpose of this study was to describe the potential of isolates of IAA hormone-producing bacteria from the rhizosphere soil of leguminous plants which grow in abundance in former-coal mining land in East Kalimantan. This research hopefully can act as part of a solution or alternative to biological fertilizers to repair land damage, to increase plant growth and productivity by utilizing the multisymbiotic interaction of microorganisms with plants on marginal lands including former coal mining lands.
2 Material and Method

This research was a descriptive study conducted from November 2022 to March 2023, with research stages in the form of isolation, characterization, and testing of the ability of bacterial isolates to produce IAA hormones. The tool used to test the production of IAA by bacteria was a UV-Vis spectrophotometer. The materials used in this study included sterile distilled water, Nutrient Agar (NA) for bacterial isolation media; Nutrient Broth (NB), L-tryptophan for IAA-tested culture media; and Salkowski reagent to test IAA production ability by bacterial isolates, as well as legume plant rhizosphere soil samples.

In this study, samples of the rhizosphere of legume plants were taken from former coal mining areas in North Penajam Paser Regency, East Kalimantan Province. Soil samples were taken from 15 location points, and 11 dominant isolates were obtained. Each type of rhizosphere soil was qualitatively tested first to detect the presence of the IAA hormone, which was done by taking 1 gram of soil and dissolving it in 9 ml of distilled water, then diluting it up to $10^{-9}$. Then 1 ml was taken to be suspended in nutrient broth (NB) + L-tryptophan 0.1 mg/l. After incubation for 48 hours at 25-30 °C, the bacterial culture was centrifuged at 6000 rpm for 15 minutes to obtain the supernatant. The supernatant obtained was then taken 2 ml and reacted with 1 ml of Salkowski reagent. A positive test result as an IAA producer was indicated by a change in the color of the supernatant from yellow to pink to red after 30 minutes of incubation in a dark room [15].

Soil samples with positive test results were taken 1 ml from the results of dilutions $10^{-8}$ and $10^{-9}$ for pour plate on 0.1 mg/L NA + L-tryptophan medium, then incubated for 24 hours at 25-30 °C. From each type of soil sample, the most dominant isolate was taken for purification. Purification was carried out using the streak plate method with an incubation time of 24 hours. Furthermore, isolates of rhizosphere bacteria that produce IAA hormones were characterized based on colony morphology (shape, edges, elevation, surface, and color) and gram staining under a 1000x magnification light microscope.

One swab of each isolate of IAA-producing rhizosphere bacteria was cultured on NB + L-tryptophan 0.1 mg/l and then incubated for 48 hours to test its ability to produce IAA qualitatively with the same method. The development of the pink color is considered positive for IAA production [16]. Next, a quantitative test was carried out by preparing a graded concentration of IAA solution (0 ppm; 1 ppm; 5 ppm; 10 ppm; 15 ppm; 20 ppm; 50 ppm) which was reacted with Salkowski's reagent, then the absorbance was measured at 520 nm using a UV-Vis spectrophotometer. So that the IAA standard curve can be obtained. Each absorbance value obtained will produce a regression equation of the form: $y = mx + n$. Where $y$ = absorbance (A); $m$ = regression coefficient; $x$ = IAA (ppm) and $n$ = interval. This standard curve can be used to calculate the concentration of IAA that can be produced by isolates of legume plant rhizosphere bacteria by comparing their absorbance at the same wavelength. Furthermore, data analysis was carried out descriptively regarding isolation, characterization, and the ability of each isolate to produce IAA hormone qualitatively and quantitatively.

3 Result and Discussion

There were 11 bacterial isolates found as dominant isolates from 15 soil samples with colony morphological characteristics as shown in Table 1. It can be seen that legume plant rhizosphere bacterial isolates have a diameter of 3-8 mm, with shapes: round, irregular; elevation: raised, convex, flat, umbonate; edge: entire, undulate, lobate; smooth surface; and yellow, white. Meanwhile, the results of the gram staining test showed that all isolates were gram-positive (+) bacteria. Gram-positive bacteria retain crystal violet dye during the
staining process so that it will be blue or purple under a microscope because the primary dyes are difficult to wash [17].

Table 1. Morphological characteristics of legume plant rhizosphere bacterial colonies

<table>
<thead>
<tr>
<th>No</th>
<th>Isolate Code</th>
<th>Diameter</th>
<th>Form</th>
<th>Elevation</th>
<th>Edge</th>
<th>Surface</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5IB3</td>
<td>5 mm</td>
<td>Round</td>
<td>Raised</td>
<td>Entire</td>
<td>Smooth</td>
<td>White</td>
</tr>
<tr>
<td>2</td>
<td>2IA4</td>
<td>4 mm</td>
<td>Round</td>
<td>Raised</td>
<td>Entire</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
<tr>
<td>3</td>
<td>7IB3</td>
<td>5 mm</td>
<td>Round</td>
<td>Raised</td>
<td>Entire</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
<tr>
<td>4</td>
<td>IE5</td>
<td>7 mm</td>
<td>Irregular</td>
<td>Raised</td>
<td>Undulate</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
<tr>
<td>5</td>
<td>6IB3</td>
<td>8 mm</td>
<td>Irregular</td>
<td>Raised</td>
<td>Lobate</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
<tr>
<td>6</td>
<td>4IE3</td>
<td>2 mm</td>
<td>Round</td>
<td>Flat</td>
<td>Entire</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
<tr>
<td>7</td>
<td>ID3</td>
<td>3 mm</td>
<td>Round</td>
<td>Convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>White</td>
</tr>
<tr>
<td>8</td>
<td>IID5</td>
<td>2 mm</td>
<td>Round</td>
<td>Convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
<tr>
<td>9</td>
<td>8IB4</td>
<td>3 mm</td>
<td>Round</td>
<td>Raised</td>
<td>Entire</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
<tr>
<td>10</td>
<td>3IB4</td>
<td>4 mm</td>
<td>Round</td>
<td>Umbonate</td>
<td>Entire</td>
<td>Smooth</td>
<td>White</td>
</tr>
<tr>
<td>11</td>
<td>2IA3</td>
<td>3 mm</td>
<td>Round</td>
<td>Convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

Based on the qualitative tests and observations made, it showed that all isolates were positive as IAA-producing rhizosphere bacteria with quite diverse abilities. Isolate 2IA4 had the deepest color, while isolate 5IB3 had the faded color (Figure 1). Based on this, it can be seen that in the qualitative test, if there is a pink color change in the supernatant after the bacterial culture is reacted with the Salkowski reagent, then the bacteria are IAA-producing bacteria [15]. Bacteria with the capability of producing IAA will turn red when Salkowski drops because there is an interaction between IAA and Fe to form a complex compound $[\text{Fe}_2(\text{OH})_2(\text{IA})_4]$. The darker the pink color, the higher the concentration of IAA produced by bacteria [18]. IAA growth hormone functions as an important molecular signal in the regulation of plant development, promotes the development of host plant roots, increases resistance to pathogens, and promotes plant growth. In addition, IAA assists in the production of longer roots with an increased number of root laterals and root hairs which are involved in nutrient uptake [19]. In addition, exogenous IAA also improved the drought tolerance of plants due to endogenous plant hormone concentration changes and modulation of genes involved in drought stress response and leaf senescence [20].

In testing the ability of bacteria to produce IAA, Salkowski’s reagent was used to detect the presence of the IAA hormone which was marked by the change in color to red indicating that oxidation of the indole ring (group in IAA compounds) had occurred [18]. Other research states that the red color formed in positive test results comes from the $[\text{Fe}_2(\text{OH})_2(\text{IA})_4]$ complex, where Fe comes from Salkowski and IAA is indole-3-acetic acid in bacterial supernatants [21]. Salkowski reagent is a mixture of 0.5 M ferric chloride ($\text{FeCl}_3$) and 35% perchloric acid ($\text{HClO}_4$) [22]. Salkowski’s test has since been widely used for detecting IAA from microorganisms.

The supernatant may contain active compounds or secondary metabolites produced by bacteria, one of which is the IAA hormone [15, 23]. The results of the qualitative test showed that isolate 2IA4 showed the densest color, while the faded color showed isolate 5IB3 (Figure 1). Quantitative test results using the spectrophotometric method showed that isolate 2IA4 was an isolate of the rhizosphere bacteria of legume plant roots with the best ability to produce IAA hormone, based on the average concentration of 15.949 ppm. Meanwhile, the lowest ability was produced by isolate 5IB3 with a concentration of 5.379 ppm (Table 2). These results are supported by previous research that a deeper red color indicates a higher concentration of IAA contained in the test sample, and vice versa [24].
The difference in the ability to produce IAA hormones is influenced by the bacterial metabolic activity which is determined by the genome sequence it has, which is related to its role in encoding certain enzymes that are able to catalyze reactions to bacterial metabolic activity [25]. If bacteria do not have certain genes capable of encoding enzymes that play an important role in a metabolic process, the metabolic process will not occur [26]. This means that the difference in the ability of isolates of rhizosphere bacteria from legume plant roots to produce IAA hormones is probably influenced by differences in the genome sequence of each bacterial isolate, so there are differences in the availability of enzymes for IAA biosynthesis which causes the concentrations of IAA produced to also be different. The genes involved in IAA biosynthesis are the IAAH gene which encodes the IAM hydrolase enzyme and iaaM which encodes the tryptophan monooxygenase enzyme in the IAM (indole-3-acetamide) pathway, the ipdC gene which encodes the indole-3-pyruvate decarboxylase enzyme in the IPyA (indole-3-pyruvic acid), as well as unknown genes in other IAA biosynthetic pathways [27]. Another researcher also stated that these genome sequences affect variation in L-Trp utilization in making IAA that impacts on the amount of IAA produced from bacterial isolates [28].

In this study, growth media for legume plant root rhizosphere bacteria was added with 0.1 mg/l L-tryptophan to provide precursor material for the formation of IAA so that the resulting concentration of IAA is more optimal [29] because it is known that most bacteria are capable of producing IAA from tryptophan [30]. L-tryptophan was a precursor that affected IAA production. Thus, the increase of L-tryptophan concentration influenced to IAA production of the strains [31]. Even without the addition of L-tryptophan, actually, the bacterial isolates are still able to produce IAA hormones, namely through the independent tryptophan pathway [32]. However, the concentration of IAA that can be produced by endophytic bacteria tends to be higher when exogenous tryptophan is administered [33]. Even so, each type of bacteria can have different optimum L-tryptophan concentration limits to be able to produce IAA hormones [29].

It is known that bacteria in the root environment are found to use the tryptophan-dependent pathway with the main pathways IAM and TAM (Tryptamine) [29]. The IAM pathway is said to be the best pathway in symbiotic bacteria because it can synthesize IAA from tryptophan through two steps, while the other pathways take at least three steps [34]. In the IAM pathway, tryptophan will be converted by tryptophan monooxygenase into IAM, then hydrolyzed by IAM hydrolase to become IAA [7]. The main genes driving the IAM pathway are iaam and iaaH, which encode tryptophan monooxygenase and indole-3-acetamide hydrolase, respectively [35]. Meanwhile, in the Tryptamine (TAM) pathway, which begins with tryptophan carboxylation to tryptamine, then oxidized by amine oxidase to become IAAl, then oxidized to IAA [36]. Apart from these two pathways, several studies have stated that the main pathway of IAA biosynthesis found in bacteria is the IPyA (Indole-3-pyruvic acid) pathway, namely Arthrobacter pascens [26], Acinetobacter baumanii [13].
and *Enterobacter* sp. [29]. Other tryptophan dependent pathways found in bacteria are the Tryptophan Side-chain Oxidase (TSO) pathway and the Indole-3-Acetonitrile (IAN) pathway [37].

### Table 2. Morphological characteristics of legume plant rhizosphere bacterial colonies

<table>
<thead>
<tr>
<th>No</th>
<th>Isolate Code</th>
<th>Absorbance Value (A)</th>
<th>IAA Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5IB3</td>
<td>0.141</td>
<td>5.379</td>
</tr>
<tr>
<td>2</td>
<td>2IA4</td>
<td>0.341</td>
<td>15.949</td>
</tr>
<tr>
<td>3</td>
<td>7IIIB3</td>
<td>0.153</td>
<td>6.030</td>
</tr>
<tr>
<td>4</td>
<td>IE5</td>
<td>0.161</td>
<td>6.459</td>
</tr>
<tr>
<td>5</td>
<td>6IIB3</td>
<td>0.187</td>
<td>7.847</td>
</tr>
<tr>
<td>6</td>
<td>4IE3</td>
<td>0.243</td>
<td>10.762</td>
</tr>
<tr>
<td>7</td>
<td>ID3</td>
<td>0.223</td>
<td>9.700</td>
</tr>
<tr>
<td>8</td>
<td>IIID5</td>
<td>0.168</td>
<td>6.804</td>
</tr>
<tr>
<td>9</td>
<td>8IIIB4</td>
<td>0.176</td>
<td>7.268</td>
</tr>
<tr>
<td>10</td>
<td>3IB4</td>
<td>0.217</td>
<td>9.422</td>
</tr>
<tr>
<td>11</td>
<td>2IA3</td>
<td>0.190</td>
<td>7.970</td>
</tr>
</tbody>
</table>

A quantitative test of legume plant rhizosphere bacteria as a producer of IAA used the IAA standard curve which had been made with the regression equation $y = 0.0189x + 0.0086$ ($R = 0.9899$). The absorbance of the pink color formed on each bacterial isolate was measured, so that the concentration of IAA that can be produced during the 48-hour incubation period of bacteria can be known based on the standard IAA curve as shown in Table 2.

Based on Table 2, it can be seen that the concentration of IAA produced by legume plant rhizosphere bacterial isolates ranged from 5.379 ppm-15.949 ppm. The isolates that were able to produce IAA from highest to lowest were isolates of 15.949 ppm (2IA4); 10.762 ppm (4IIIE3); 9.700 ppm (ID3); 9.422 ppm (3IB4); 7.970 ppm (2IA3); 7.847 ppm (6IIB3); 7.268 ppm (8IIIB4); 6.804 ppm (IIID5); 6.459 ppm (IE5); 5.379 ppm (7IIIB3); and 5.086 ppm (5IB3).

These results confirm that the genetic factors of bacterial isolates have a very strong influence in expressing their ability to produce IAA, considering that the environmental conditions in the implementation of this study were the same. Other researchers said that co-expression patA, ilvB3, and fusE, increased IAA yield by 60% in strain bacteria that isolated [38]. When viewed from the value of the ability to produce IAA, then when compared with research by Dewi et al. [39] is relatively small because with the same concentration of tryptophan the highest IAA produced reached 158, 651 ppm. Another study indicated that all were isolated strains from tomatoes were able to produce indole acetic acid even without the presence of a tryptophan precursor [12].

### 4 Conclusion

The results showed that there were 11 isolates of legume plant root rhizosphere bacteria grown on the former coal mining soil that were able to produce IAA hormones with an average concentration of 15.949 ppm (2IA4); 10.762 ppm (4IIIE3); 9.700 ppm (ID3); 9.422 ppm (3IB4); 7.970 ppm (2IA3); 7.847 ppm (6IIB3); 7.268 ppm (8IIIB4); 6.804 ppm (IIID5); 6.459 ppm (IE5); 5.379 ppm (7IIIB3); and 5.086 ppm (5IB3). Isolates of rhizosphere bacteria with the highest concentration have the potential to be used as a growth booster for legume plants grown on former coal mining soil to increase legume crop productivity.
Acknowledgement

The authors thank the Directorate of Research, Technology and Community Service, Directorate General of Higher Education, Research, and Technology of the Ministry of Education and Culture for funding the present study in fiscal 2023 through Rector’s Decree Number 948/UN38/HK/PP/2023 on April 12, 2023. Thanks to DAPT-EQUITY Program, Lembaga Pengelola Dana Pendidikan (LPDP), Ministry of Finance, Indonesia for supporting this publication. This study was carried out with the help of students, laboratories, and other facilitators at the Department of Biology, Universitas Negeri Surabaya.

References

1. A. D. Wulandari, and V. I. Meitiniarti, Journal of Science and Science Education 5, 1 (2021)
5. U. Patma, A. P. P. Lollie, A. M. S. Luthfi, Jurnal Online Agroektoreknologi 1, 2 (2013)
6. R. B. Sukmadi, Jurnal Sains dan Teknologi Indonesia, 14, 3 (2012)
23. T. Kaeoket, V. Thongbaiyai, N. Ngamwongsatit, K. Kaeoket, BMC Veterinary Research 18, 60 (2022)
33. A. M. Tangapo, Jurnal Bios Logos, 10, 1 (2020)
37. Y. Zhao, Molecular Plant, 5 (2012)
38. H. Sun, J. Zhang, W. Liu, Biotechnology Biofuels, 15, 81 (2022)