pISSN 2302-1616, eISSN 2580-2909
Vol 10, No. 1, June 2022, pp. 112-120
Available online http://journal.uin-alauddin.ac.id/index.php/biogenesis
DOI https://doi.org/10.24252/bio.v10i1.28451

The potency of bacteria isolated from the hydroponic rockwool of field mustard (*Brassica rapa* L.) for nitrogen fixation and indole acetic acid (IAA) production

Anindita Riesti Retno Arimurti[1*], Vella Rohmayani[1], Nurhidayatullah Romadhon[2], Tara Puri Ducha Rahmani[3], Lee James Watson[4], Kenari Sri Wahyuni[1], Nova Ulumiya[1]
[1]Study Program of DIII Medical Laboratory Technology, Faculty of Health Sciences,
Universitas Muhammadiyah Surabaya
*Email: aninriesti@gmail.com
Jl. Raya Sutorejo No. 59, Sutorejo, Mulyorejo, Surabaya, East Java, Indonesia. 60113
[2]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Muhammadiyah Surabaya
Jl. Raya Sutorejo No. 59, Sutorejo, Mulyorejo, Surabaya, East Java, Indonesia. 60113
[3]Department of Biology, Faculty of Science & Technology, Universitas Islam Negeri Walisongo Semarang
Jl. Walisongo No. 3-5, Tambakaji, Ngaliyan, Semarang, Central Java, Indonesia. 50185
[4]Chemtech Environmental Limited
Unit 6 Parkhead, Greencroft Industrial Park, Stanley, County Durham, DH9 7YB. United Kingdom

ABSTRACT. Rockwool is a growing media widely used by hydroponic farmers. The hydroponic system contains high amounts of nutrients in order to provide an ideal environment for microorganisms to grow. Microorganisms can help spur plant growth through many mechanisms such as N_2 fixation, plant stress control, nutrient extraction from soil, anti-pathogenic functionality, production of various types of plant hormones, and biological control. This study aimed to determine the potential of bacteria in hydroponic rockwool of field mustard (*Brassica rapa* L.) for nitrogen fixation and IAA (Indole Acetic Acid) production. The study began with isolating Hydroponic Rockwool Bacteria from *Brassica rapa* L., followed by identifying and growing isolates on NA, NB, and LB media for 48 h to determine their spesies. After 48 h, counting the number of bacteria and characteristics of the colonies. Then the bacterial colonies found were purified on NA media. After knowing their spesies, the potential Nitrogen fixation was tested using NFBB media for six days. Then the potential for IAA production was tested using LB medium for one week. The results showed that 11 (eleven) bacterial isolates could not fixation Nitrogen but had the ability to produce IAA hormone, with IAA concentrations ranging from 4.09 to 21.99 ppm.

Keywords: DNA extraction method; fecal DNA; molecular analysis; PCR amplification; spotted deer

Article History: Received 11 February 2022; Received in revised form 14 April 2022; Accepted 19 May 2022; Available online 30 June 2022. Ver: Pre-Press
How to Cite This Article: Arimurti ARR, Rohmayani V, Romadhon N, Rahmani TPD, Watson LJ, Wahyuni KS, Ulumiya N. 2022. The potency of bacteria isolated from the hydroponic rockwool of field mustard (*Brassica rapa* L.) for nitrogen fixation and indole acetic acid (IAA) production. *Biogenesis: Jurnal Ilmiah Biologi*. vol 10(1): 112–120. doi: https://doi.org/10.24252/bio.v10i1.28451.

INTRODUCTION

Hydroponic systems involve agricultural cultivation without using soil media, where the provision of water and nutrients can be conducted simultaneously (Rizal *et al.*, 2020). Several advantages of hydroponic cultivation systems are saving land use, controlled quality of crop yields through controlling nutrients given to plants, independence in planting and harvesting time (not reliant on the weather or season) so that it can be regulated according to the market needs. Types of plants often grown by utilizing hydroponic systems include vegetables such as tomatoes, spinach, mustard, kale etc. The availability of air and water can be optimized in the root zone with the presence of a solid matrix such as Rockwool.

The hydroponic system contains high amounts of nutrients and provides an ideal environment for microorganisms to grow. The pH level and temperature of the water can be optimally conditioned for the growth and survival of microorganisms. Although some bacteria, such as *Agrobacterium tumefaciens, Xylella fastidiosa*, and *Pseudomonas syringae* can be pathogenic to plants, most bacteria in the hydroponic system are beneficial to plants (Roumiantseva *et al.*, 2018). Many of these bacteria are able to suppress the growth of particular plant pathogenic bacteria to protect plants (Triwidodo, 2021). In addition, other studies have suggested that microorganisms in hydroponic systems play a role in increasing the nitrification process by the addition of nitrifying bacteria (Deng *et al.*, 2015) or

using Plant Growth Promoters (PGPR) such as *Azospirillum brasilense* and *Bacillus* spp. to improve plant performance (Bartelme *et al.*, 2018).

Plant growth-driving microbes in a hydroponic system are needed to support plant growth since they can maintain the efficiency of fertilizer usage. Those microbes also act as natural biological agents in fighting pathogens infecting many hydroponic plants; hence the vegetable crop yields can ultimately be increased. Microbes can promote plant growth and yield through several mechanisms, which are: nitrogen fixation, facilitation of access to nutrients, direct stimulation of plant growth, and production of organic compounds (Calvo *et al*., 2014). This ability can reduce the number of nutrients needed to maintain optimal crop yields (Dasgan *et al*., 2012). Many studies state that microbes capable of spurring plant growth can increase nutrient absorption under certain conditions, demonstrating their effectiveness in reducing fertilizer use without negatively affecting greenhouse crop yields (Ruzzi & Aroca, 2015). Furthermore, microbes associated with plants in the hydroponic system can protect from plant pathogens through their ecological niche mechanisms. Some microbes that have been tested for their ability to prevent plant pathogens are *Pseudomonas* spp., *Bacillus* spp., *Enterobacter* spp., *Streptomyces* spp., *Gliocladium* spp., and *Trichoderma* spp. (Lee and Lee, 2015). Nevertheless, the benefits provided by such microbes depend on their abilities in colonizing plant roots, surviving, and multiplying for extended durations against other microbes (Olanrewaju *et al*., 2017).

Nitrogen is one of the most important macronutrients for plant growth. The presence of Nitrogen in the atmosphere in the form of N_2 cannot be utilized directly because plants do not have enzymes that can help convert that nitrogen into biological forms that benefit plants. Proteins are required for all key functions in plants, and nitrogen is one of the most important components of protein. Because nitrogen is a component of chlorophyll, it aids in the photosynthesis process, which determines the plants' total yields. To support their growth and production, plants require a combination of nitrogen forms such as ammonia or nitrate as the primary nutrients (Zhang *et al*., 2015 in Rahmani *et al*., 2020). The primary source of Nitrogen in plants mainly originates from the Nitrogen fixation process that occurs due to symbiosis between the plants and bacteria contained in the planting media. On the other hand, the presence of IAA (Indole Acetic Acid) hormone-producing microbes will generate phytohormones that can accelerate plant growth. Therefore, it is crucial to conduct research to test the potency of Bacterial Nitrogen fixation and IAA production.

MATERIALS AND METHODS

Isolation with multilevel dilution method. The research was conducted in Laboratory of Microbiology, Faculty of Health Science, Universitas Muhammadiyah Surabaya. A sample of hydroponic rockwool of field mustard (*Brassica rapa* L.) was taken from the garden of the Greenhouse Office of Department of Food Security, Universitas Muhammadiyah Surabaya. The Rockwool was separated from the plants by cutting. Rockwool derived from the plants aged five weeks then weighed with digital scales of 24 and 25 grams. The weighted mixture was then dissolved in 225 ml of 0.85% NaCl or physiological salt in 500 mL sized Erlenmeyer. Then the mixture was shaken with a shaker for 15 minutes to dissolve and release the microorganism attached to the surface of the Rockwool and plant roots into the salt solution. A physiological saline solution containing Rockwool was then shaken and diluted using a serial dilution method until 10-6. The dilution was conducted in a tube containing 9 mL of 0.85% physiological salt as much as 1 mL in each dilution. Before proceeding to the subsequent dilution, the dilution tube was put in a vortex to homogenize the mixture. After obtaining six dilution tubes, 0.1 mL was taken and put in a petri dish (this procedure was repeated 3 times for each dilution). The three Petri dishes in each dilution were poured with NA (Nutrient Agar) media, NB (Nutrient Broth) media, LB media (Luria Bertani) agar ± 12 mL, then the dish was shifted to form a figure 8 to flatten the media. The hardened media was incubated at 30°C for 48 h (Putri *et al*., 2020).

Bacterial enumeration and bacterial characterization (Macroscopic). After incubating the bacteria for 48 h, the number of bacterial colonies found was counted using the Colony Counter (to enumerate the most dominant bacterial colony from all of Petri dishes dilutions). The selected petri dish must meet the TPC (Total Plate Count) requirements; that is, the number of colonies that can be counted from each type of colony found must range from 30 – 300 colonies. Colonies are not scattered or located not more than half of the Petri dish. Suppose in one series of dilutions there are two dishes that meet these requirements at two different dilutions. In that case, the TPC value of the number of colonies in the more dilute dilution is divided by the TPC of the number of colonies in the more concentrated dilution. If the quotient is ≤, then the TPC value for the type of bacteria or mold is then averaged. On the other hand, if the quotient is > 2, then the TPC value of the bacteria or mold is taken from the TPC value in the most concentrated dilution. The bacteria found were labeled according to the type of media and to what species. The colonies found were characterized based on their macroscopic appearance. The variables observed were the shape, color, edge, elevation, size, texture, appearance, optical property of each colony to which the isolate code was assigned (Mathialagan *et al*., 2018).

Bacterial purification. Petri dishes containing NA media were prepared and divided into four parts with a permanent marker. The bacterial colonies found and already coded based on the dilution were respectively sampled with an ose needle aseptically and quickly near a Bunsen lamp. The bacterial isolate on the tip of the ose needle was scratched on NA media which had been divided into four parts by the four-quadrant method. After being streaked, the petri dish is closed and rotated near the bunsen lamp. The same procedure was conducted on other bacterial isolates. Streaked bacterial isolates were then incubated at 30℃ for 24 h. Then, bacterial colonies growing in the fourth quadrant, which was isolates of one pure bacterial species, were inoculated using the streak method on slanted NA media as culture stock (Axler-DiPerte, 2017).

Biochemical reaction test. Reaction biochemical test, including oxidase test, catalase test, MR-VP test, simmons citrate test, sugar fermentation test, H2S and gas production, motility test. Oxidase test on each isolate was prepared on NA slant media aged 24 h and tested using a kit. Furthermore, the catalase test on each isolate was prepared on 24 h old NA slant media, then one eye of ose was taken and placed on a glass object and given 1-2 drops of H2O2. Next, one eye of ose of bacterial isolate was taken from NA slant media aged 24 h to be tested for MR-VP Broth, for the Methyl-Red test, 5 drops of Methyl-Red reagent were added, for the Voges-Paskuer test, 1 mL of MR-VP media that had been prepared Incubated transferred to another empty tube and added 0.6 mL of 5% alphanapthol + 0.2 mL of 40% KOH and waited for about 15 minutes. Next, a sample of bacterial isolate was taken from the NA slant media aged 24 h, put in a test tube containing Simmons citrate medium, inoculated with a needle with a sharp tip and inserted into the media (perpendicularly) and then pulled by scratching the surface of the media aseptically. Then the sugar fermentation, H2S production, and gas tests were carried out, each bacterial isolate was prepared in a 24-hour-old NA slant, a test tube containing TSIA (Triple Sugar Iron Agar) medium. Inoculated with a needle with a pointed tip and inserted into each medium (perpendicularly) and then withdrawn by scratching the surface of the media aseptically. Then the motility test was carried out, one eye of ose was taken and put into a test tube containing SIM (Sulfide Indole Motility) medium. Furthermore, the identification of the bacterial isolate genus was carried out using a dichotomous key based on the biochemical properties, microscopic appearance and macroscopic appearance of the bacteria according to Bergey's Manual of Determinative Bacteriology (Syahri *et al*., 2019).

Test the ability of bacteria in nitrogen fixation. Each bacterial isolate was prepared on a 24-hour-old NA slant media. Each isolate was taken as much as two oses (ose from ose needle), inoculated on Nitrogen Free Bromothymol Blue Semi-Solid (NFBB) media, and incubated at 30° for six days. Positive results were indicated by a change in the color of the media to blue and the formation of a white ring-like floc on the surface of the media (Fig.1a) (Singh, 2013).

Test of bacterial ability in IAA production. Each bacterial isolate was prepared on NB media aged 24 h, 5 mL of culture was taken from each isolate to be inoculated on 45 mL of Luria Bertani Broth media (so the total volume after inoculation was 50 mL). The cultures were incubated for six days on a rotary shaker. A total of 10 mL of each isolate was put in a propylene tube for centrifugation at 6000 rpm for 15 minutes. A total of 2 ml of the supernatant from each isolate was transferred to another empty test tube, then 2 mL of Salkowsky's reagent was added and then incubated in a dark room for 30 minutes. The color changes that occurred were observed. Positive results of IAA production are indicated by a change in color to pink (pink) (Fig.1b). Quantitatively, the content of IAA in the media can be measured using a spectrophotometer at a wavelength of 535 nm (blank: LB media + Salkowsky reagent). The absorbance value of each isolate was recorded, then converted into IAA content (ppm) using the IAA standard curve (Singh, 2013).

RESULTS AND DISCUSSION

After biochemical tests (Table 1) and bacterial identification were carried out, the identified bacterial isolates were then rejuvenated. Then, the 11 bacterial isolates were tested for their nitrogen fixation potential by growing them on NFB medium for one week. The nitrogen fixation potential test results of 11 isolates are listed in Table 2.

Table 1. The biochemical reaction of isolate from hydroponic rockwool of field mustard plant (*Brassica rapa* L.)

No.	Isolate code	Reaction biochemical test								Bacteria identification
		SS	MR	VP	SIM	TSIA Gas	H_2S	Kat	O_2	
1	NA1	+	+	+	+	+	-	-	+	*Mycobacterium* sp.
2	NA2	-	+	+	+	-	-	-	+	*Bacillus* sp.
3	NA4	-	-	+	+	-	-	+	+	*Micrococcus* sp. *Staphylococcus* sp.
4	NA6	+	+	+	+	+	-	-	+	*Mycobacterium* sp.
5	PDA2	+	+	+	+	-	-	+	+	*Vibrio* sp. *Aeromonas* sp.
6	PDA3	-	+	+	+	-	-	-	+	*Mycobacterium* sp.
7	PDA4	+	+	+	+	-	-	+	+	*Bacillus* sp.
8	LB1	+	+	+	+	-	-	-	+	*Streptococcus* sp. *Enterococcus* sp.
9	LB2	+	-	-	+	+	-	-	+	*Lactobacillus fermenti*
10	LB3	+	+	+	+	-	-	+	+	*Mycobacterium* sp.
11	LB4	-	+	+	+	-	-	+	+	*Neisseria* sp. *Veillonella* sp.

About 11 rockwool bacterial were isolated from field mustard, including *Mycobacterium* sp., *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Vibrio* sp., *Aeromonas* sp., *Streptococcus* sp., *Enterococcus* sp., *Lactobacillus fermenti, Neisseria* sp., and *Veillonella* sp. All samples of these bacterial isolates did not have the ability to fix nitrogen, but had the ability to produce the IAA hormone (Table 2; Fig. 1). This is because each bacterium, when stimulating plant growth, can go through many mechanisms such as N_2 fixation, plant stress control, nutrient extraction from the soil, anti-pathogens, production of various types of plant hormones, and biological control (Olanrewaju *et al.*, 2017).

In a hydroponic system, adequate nutrition has been provided by a nutrient solution added to the circulation of the growing media so that the presence of microbes in the root system plays a role in protecting plants from pathogens and the production of plant growth hormones. In general, microbes can thrive after growing plants in a hydroponic system that contains exudates, compounds in a nutrient solution, and dead plant material (Hosseinzadeh *et al.*, 2017).The composition of microbes in the hydroponic system can be influenced by environmental factors and sources of nutrients (Lahkar

et al., 2018). Some microbes can be pathogenic to plants, but the population of pathogenic microbes is generally less than the population of non-pathogenic organisms (Grunert *et al.*, 2020).

Table 2. Test results of potential nitrogen fixation ability of isolate from hydroponic rockwool of field mustard plant (*Brassica rapa* L.)

No.	Isolate code	Nitrogen fixation potential test results
1	NA1	-
2	NA2	-
3	NA4	-
4	NA6	-
5	PDA2	-
6	PDA3	-
7	PDA4	-
8	LB1	-
9	LB2	-
10	LB3	-
11	LB4	-

Some bacterial groups that have been found in the hydroponic system include those of the genus *Bacillus amyloliquefaciens, Bacillus cereus, Bacillus subtilis*, and *Bacillus thuringiensis*, which are found in the hydroponic system of cucumber, carrot, lettuce, and tomato plants (Chinta *et al*., 2014), Enterobacter Aerogenes which was found in the hydroponic system of cucumber plants (Liu *et al.*, 2018) and *Streptomyces griseoviridis* which was found in the hydroponic system of cucumber and tomato plants (Lahkar *et al*., 2018). The results of this study found the Genus *Bacillus*. The mechanism of the Genus *Bacillus* spp. in stimulating plant growth and producing antibiotics against phytopathogenic organisms is still not widely known (Nihorimbere *et al*., 2012). *Bacillus subtilis* is a Gram-positive bacterium and is able to stimulate plant growth with its ability to increase the concentration of water or nutrient solutions with high salinity concentrations. Other species, *Bacillus amyloliquefaciens*, can increase the efficiency of water use in tomatoes and is also able to increase crop yields, both in quality (higher vitamin C than the control group) and quantity (8-9% higher yields) (Souto *et al*., 2017). *Bacillus licheniformis* had increased the diameter and weight of tomatoes and peppers and increased the higher yield of each plant (Bjerrum-Bohr *et al*., 2016). In addition to the beneficial genera of bacteria found, several genera are suspected of not providing benefits or even being pathogenic in the hydroponic systems. Those bacterial genera include the suspected genus of *Vibrio* sp. and *Aeromonas* sp., several strains of *Vibrio* spp. are widely distributed in the aquatic environment and have a toxic effect on aquatic organisms, and some *Vibrio* spp. also found to be pathogenic against plants (Herschy, 2012). *Aeromonas* spp. can usually be found in soil and water environments.

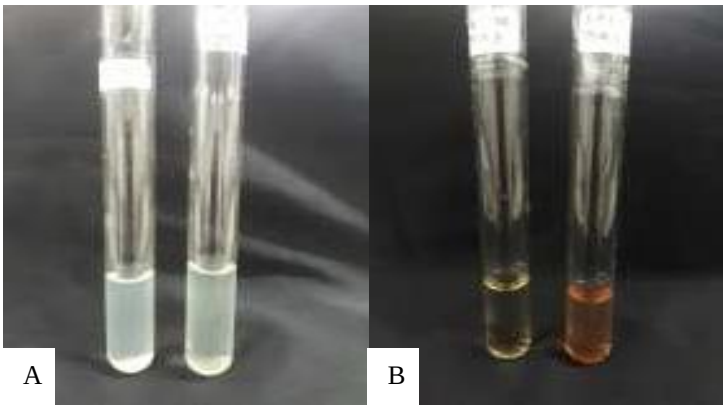

Fig. 1. The ability of 11 isolates: A. Bacterial ability in nitrogen fixation; B. Bacterial ability in IAA production.

After testing the potential for nitrogen fixation, the potential for IAA production was tested using LB medium for one week. The results of the qualitative IAA production potential test of 11 isolates are listed in Table 3.

Table 3. Qualitative results of IAA isolate production potential test from hydroponic rockwool of field mustard plants *(Brassica rapa* L.)

No.	Isolate code	IAA production	Absorbance (535 nm)	IAA concentration (ppm)
	Blank		0.00	0.00
1.	NA1	+	0.35	18.3
2.	NA2	+	0.09	4.62
3.	NA4	+	0.09	4.62
4.	NA6	+	0.36	18.6
5.	PDA2	+	0.37	14.62
6.	PDA3	+	0.34	17.78
7.	PDA4	+	0.28	19.36
8.	LB1	+	0.08	4.09
9.	LB2	+	0.42	21.99
10.	LB3	+	0.30	15.67
11.	LB4	+	0.08	4.09

After obtaining the results of the IAA production potential test qualitatively, then proceed with the quantitative results described by the IAA standard curve. IAA standard curves of 11 isolates are shown in Figure 1. Based on the R2 value (figure 2), it showed that there is a close relationship between the absorbance value and the IAA concentrate.

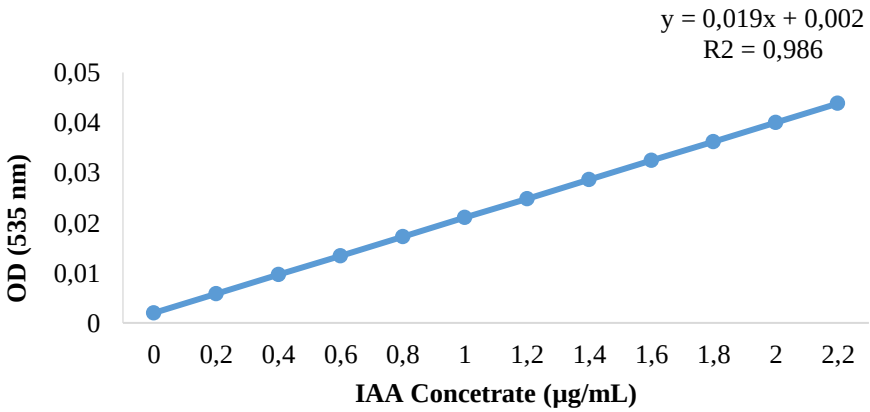

Fig. 2. IAA standard curve isolate from hydroponic rockwool of field mustard plant (*Brassica rapa* L.)

After being converted to the IAA standard curve equation formula as above, it was found that isolate LB2 produced the highest concentration of IAA with 21.99 ppm. IAA production of all isolates can be seen in Fig. 3.

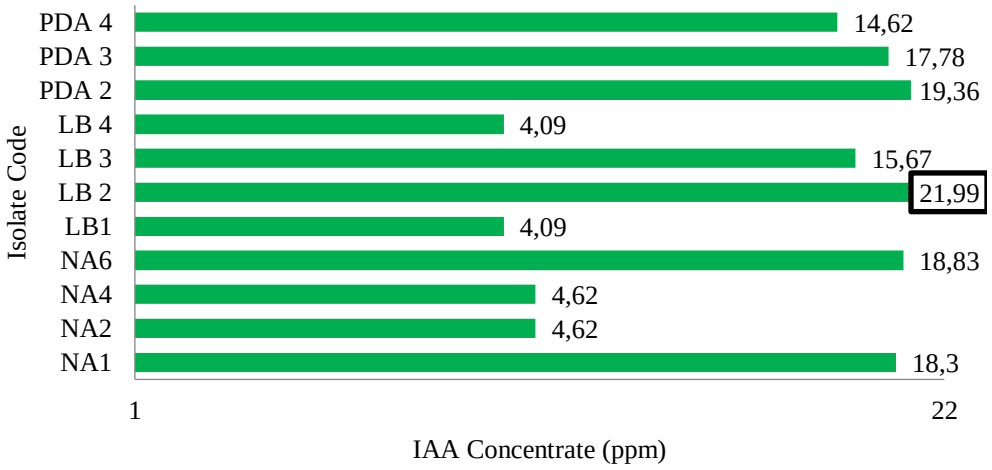

Fig. 3. The concentration of IAA isolates from hydroponic rockwool of field mustard plants (*Brassica rapa* L.)

Based on the Fig. 3, 11 isolates were found to be able to produce IAA with concentrations ranging from 4.09 to 21.99 ppm. Isolate LB2, which has similarities with the species of *Lactobacillus fermenti*, can produce IAA with the highest concentration of 21.99 ppm. In several previous studies, it has been reported that the group of lactic acid bacteria had the ability to do activities in stimulating plant growth. *Lactobacillus casei* was able to produce up to 2 ppm IAA on LB media with the addition of tryptophan. In contrast, on YMD media, without the addition of tryptophan, it can produce up to 40 ppm of IAA (Mohite, 2013). IAA-producing bacteria have been reported in several studies. These include the Genera *Agrobacterium*, *Burkholderia*, *Bacillus*, *Erwinia*, *Flavobacterium*, *Pantoea*, *Pseudomonas* (Novotná and Suárez, 2018), *Microbacterium*, *Mycobacterium*, *Rhizobium*, and *Sphingomonas* (Pan *et al*., 2020), including *Enterobacter* (Kumar Ghosh *et al*., 2015). *Vibrio* spp. Strains isolated from estuarine grassroots produce phytohormone indole-3-acetic acid (IAA). Some species of the Genus Aeromonas isolated from rice plants were able to produce IAA up to 30 ppm (Susilowati *et al*., 2015). Bacterial strains of *Bacillus cereus* (So3II) and *B. subtilis* (Mt3b) have the potential to produce IAA bacteria under different growth and environmental conditions (Wagi and Ahmed, 2019). It is known that several genera such as *Bacillus, Mycobacterium*, and *Lactobacillus* can be potential candidates for bacteria that can stimulate plant growth through the ability to produce IAA and the ability to colonize in a hydroponic environment (as microflora). The role of these bacteria, especially those related to the ability to fight pathogens (which is often an obstacle in hydroponic planting systems), needs to be investigated further so that eminent bacteria can be obtained to stimulate plant growth in hydroponic systems in the future.

CONCLUSION

It can be concluded that there were 11 bacterial isolates found from the hydroponic Rockwool of the Field Mustard plant. Out of the 11 isolates, none of the bacteria found had the ability to fix nitrogen. However, all of the 11 isolates had the ability to produce IAA hormone, with the highest concentration of IAA produced by isolate LB2, which was 21.99 ppm.

ACKNOWLEDGEMENTS

The authors would like to thank Muhammadiyah Central Leadership Higher Education and Development Assembly for funding this research and guiding researchers to complete this research and this publication. This research received a Basic Research grant with contract number: 0842.362/PD/I.3/C/2021.

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