

CHARACTERISATION AND BIOASSAY OF BIOFILMED HALOTOLERANT N-FIXER BIOFERTILIZERS FOR IMPROVING THE RICE SEEDLING GROWTH ON SALINIZED GROWING MEDIA

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ABSTRACT

Salinity affects the water potential in plants and causes a devastating effect on rice growth and lead to a significant rice yield loss. The research was done to screen the halotolerant of N-fixer bacteria from saline ecosystem which capable to form the biofilm attached to roots system. Five of composite soil samples were taken from plant rhizospheres of saline ecosystems and resulted 15 bacterial isolates. Seven isolates which capable to form a biofilm were selected for bioassay using the rice seedling on salinized growing media. The experiments was arranged as randomized block design, consisted 8 treatments and provide with 3 replications. Rice seedlings were grown on salinized Fahraeus Gmedia (4 dS m⁻¹) for 21 days on green house. The observed responds were included plant height, root length and dry weight. The experimental results showed that the bacterial isolates gave a significant effect on plant height, dry weight roots and total biomass. The highest plant height and total biomass were by the Isolates of C33 and E52 were 7.93 cm and 7.56 mg. The root was increased by 38, 20, and 20 %, respectively and total biomass was increased by 41, 39, and 64 %, respectively This finding concludes these promising bacterial isolate of salt tolerant biofilmed N-fixers could develop as bioagent to alleviate the salinity stress to improve the rice growth on saline lowland rice fields.

Keywords: Salinity stress, Lowland rice, Rhizobacteria, rice growth, biofilm.

1. INTRODUCTION

Rice is an important commodity and staple food for Indonesia. [1] Indonesian people consume rice as the main staple food. Most Indonesian people consume rice for their carbohydrate supply. This causes a very high dependence on rice. So, rice had an important role for the Indonesian economy. The agricultural practices is forced to intensify to produce more food to meet the requirement of rapidly growing population in Indonesia. The rice consumption is about 94,9 kg capita- 1 year-1 or the total need of rice is about 19,99 million tons year-1 , while the currently rice production is about 31,33 million tons [2]. In addition, the areal of lowland rice ("known as sawah") is shrinking due the land conversion to non agricultural uses. [3] Shows that the conversion rate of paddy fields to non-rice fields is 96.512 ha year-1. Moreover, most of the rice field along the coastal areal are subjected to salinization due to the rise of sea levels and global climate change [4]. Consequently, the extent of rice cultivation on saline soils is required to increase the rice production and to secure the food security.

The potential of saline for rice cultivation is about 0,44 millions ha and mostly situate along the coastal area [5]. High sodium content or salinity is the main constraint for rice growth and development. There are five classications of saline soils based on electric conductivity including very low (<1 dS m⁻¹), low (1–2 dS m⁻¹), medium (2–3 dS m⁻¹), high (3–4 dS m⁻¹), and very high (>4 dS m⁻¹) [6]. The effect of salinity on plants is very complicated. Salinity cause ion toxicity, osmotic and secondary stress. Excessive Na⁺ ion on the surface the plant roots inhibit the absorption of K⁺ by root due to lower osmotic potential [7]. Salinity impairs the crop productivity and quality seedling establishment and stunted plant growth [8].

Effort to alleviate the salinity effect on rice growth can be done by engineering rhizomicrobiome or rhizosphere through introducing or application of beneficial halotolerant N-fixers as biofertilizers or bioagent. Biofertilizers is a efficient microbial fertilizers that give an important role to increase the rice yield productivity by biological nitrogen fixation, producing growth hormones, vitamins and other growth promoters required for plant growth [9]. Application N-fixers biofertilizers such as Azotobacter and Azospirillum inoculant contribute to the improvement of plant growth and fertilizers efficiency [10].

The latest study revealed that attached bacterial to roots hairs to thin layer of microbe, called as biofilm gain more attention for improving the ability of rice to adapt on saline ecosystem. These microbes following to biotic or abiotic surfaces that is protected from environmental stresses by a self-produced extracellular matrix [11]. The growth and yield of rice was increased by the biofilm biofertilizers inoculant [12].

This study is focused to screen the potential halotolerant rhizobacterial of biofilm N-fixer isolates through characterization and bioassay that could be developed as microbial fertilizers (biofertilizers) or bioagent for improving rice growth and productivity under saline ecosystem in sustainable ways.

2. MATERIALS AND METHODS

2.1. Materials

This research was held from July 2021 to November 2021. About 1 kg of composite soils samples (five subsamples) were taken from 0-20 cm depth using an auger soil probe from each ecosystem using. composite soil samples were taken from different ecosystems from Cimrutu Village in Patimuan District of Cilacap Regency. The location of soils sampling is shown in Table 1.

Table 1. Sampling site and vegetation

Sample Code	Coordinate	Ecosystem	Vegetation
A	07°37'29.3"S 108°49'00.3"E	Saline	Oryza sativa
B	07°37'29.6"S 108°49'00.1"E	Saline	Eleocharis dulcis
C	07°37'29.1"S 108°48'56.3"E	Saline	Oryza sativa with Nymphee sp.
D	07°37'29.1"S	Saline	Monochoria

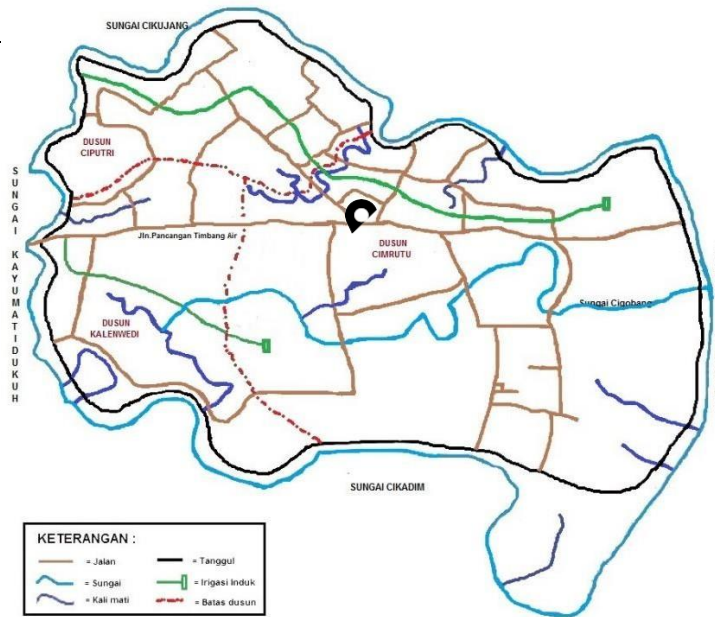


Figure 1. Acces and location of sampling area in Cimrutu village of Cilacap

The materials to be used in this research are isolates from 5 soil samples from different ecosystems, Inpari 34 rice seeds, Ashby media, Fahraeus media saline at 4 dS m⁻¹, aquadest, parchment paper, aluminum foil, spiritus, crystal violet, NaCl pro analysis, plastic wrap, cotton.

2.2. Screening of N-fixers and Purification Isolates

Screening of halotolerant N-fixer rhizobacteria (HNR) using salinized selective media of Ashby's Mannitol Nitrogen Free (Mannitol, K₂HPO₄, MgSO₄, NaCl, K₂SO₄, CaCO₃) were conducted at the Soil Biology Laboratory, Greenhouse, Department of Soil Science and Land Resources, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor District, Sumedang Regency, West Java. The dilution method was used for the isolation of HNR. About 10 g of rhizosphere soil in 90 ml of Ashby's Mannitol liquid medium and then incubated for 3 x 24 hours. At the end of incubation about 0.5 cm from the surface of the medium pellicle was visible. These bacteria configurate a pellicle 0.5 cm from the media, which will then move to the surface when nitrogen in the cell accumulates [13]. The pellicle formed on the surface of the liquid Ashby was then grown in a Petridish containing solid Ashby media by streaking to obtain pure isolate. Purification was done by aseptic scraping in a laminar space and was incubated for 1-2 days until new pure colonies were formed. Replanting was carried out after the pure colonies grew on the media, which was done by taking separate colonies and streaking them on the Ashby media so that pure bacteria were obtained.

2.3. Characterization and Biofilm Test of Nitrogen-Fixing Bacteria Isolates

The morphological observations of isolates were carried out microscopically which included the shape of the bacteria under a microscope with 1000 times magnification and the types of bacteria using the Gram staining method. According to Gram staining method, bacteria divided into 2 groups, namely Gram Positive Bacteria and Gram Negative Bacteria. The staining index is that gram-positive bacteria will be violet and gram-negative bacteria will be red.

Biofilm test was carried out qualitatively with Microtiter Plate Assay (MPA). Preparation was carried out by inoculation of one agar isolate into the Nutrient Agar (NA) medium and biofilm analysis using liquid NA media. Biofilm staining was carried out by removing planktonic cells from the microtiter plate. Plate microtiter which giving a violet color on the walls indicates the presence of bacteria that can form biofilms. [14].

2.4. Biological assay (Bioassay)

Bioassay for halotolerant nitrogen-fixing rhizobacteria (HNR) using rice variety of Inpari 34 were carried out with selective media Fahraeus free nitrogen (CaCl₂, MgSO₄, KH₂PO₄, Na₂HPO₄.2H₂O, C₆H₅FeO₇, Micro nutrient) [15]. This research method uses a randomized block design, consisted 8 treatments (control and seven isolates) and provide with 3 replications. The rice seeds were coated with sterile parchment paper and given sterile distilled water to be germinated in a petri dish for five days at a temperature of 30oC. Previously, the rice seeds were sterilized using HgCl₂ 0.2% for ± 2 minutes and given 70% alcohol for ± 1-2 minutes, then rinsed three times using distilled water. In this test, 90 ml of salinated Fahraeus media was used which was planted in a test tube with a size of 100 ml. At the top of the tube is propped up using plastic and a pipe mouth. Observations were made including root length (cm) and shoot length (cm) which were carried out every 4 days. Meanwhile, the root weight and Plant Dry Weight was observed on the last day of observation.

3. RESULTS AND DISCUSSION

3.1 Morphology of Nitrogen-Fixing Bacteria

The morphological and microscopically observations of fifteen isolates showed that bacterial shape belong to bacilli or cocci and the colony color is red or violet (Table 2 and Figure 2), and the qualitative capability to form the biofilm is shown in Figure 3.

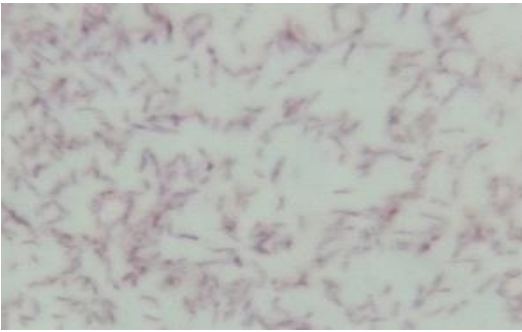
Table 2. Morphological Gram staining results and biofilm test of HNR isolates

Isolat Code	Bacterial Shape	Bacteria Cells Color	Gram Test	Biofilm Test
A11	Bacil	Violet	+	-
A12	Cocci	Violet	+	-
A13	Bacil	Red	-	+
B21	Bacil	Red	-	+
B22	Bacil	Red	-	+
B23	Cocci	Violet	+	-
C31	Cocci	Violet	+	-
C32	Cocci	Violet	+	-

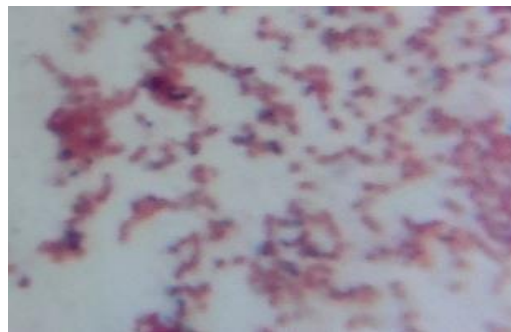
C33	Bacil	Red	-	+
D41	Bacil	Violet	+	-
D42	Cocci	Violet	+	-
D43	Bacil	Red	-	+
E51	Cocci	Violet	+	-
E52	Bacil	Red	-	+
E53	Bacil	Red	-	+

Table 1 shows that the seven selected isolates (A13, B21, B22, C33, D43, E52, and E53) were characterized as *Azotobacter* sp. The genus of *Azotobacter* can be characterized by gram-negative pleomorphic nature, which means that it does not have an irregular shape starting from bacil to cocci [16].

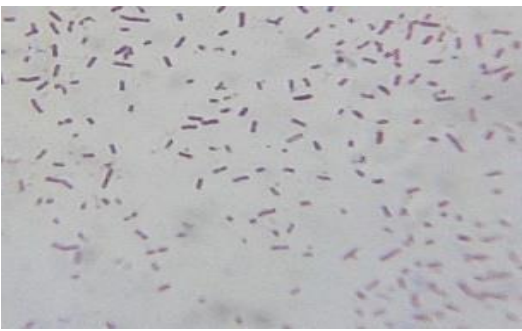
Results of Gram staining are seven isolates selected on Figure 2.



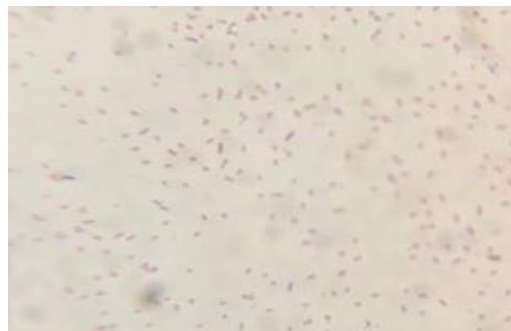
A13



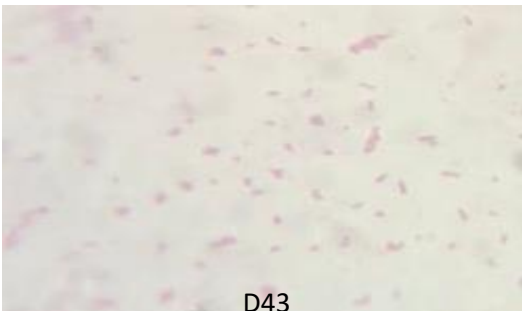
B21



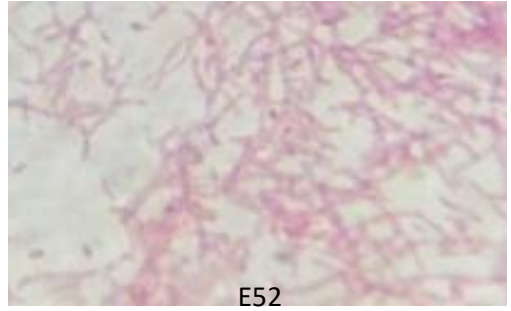
B22



C33

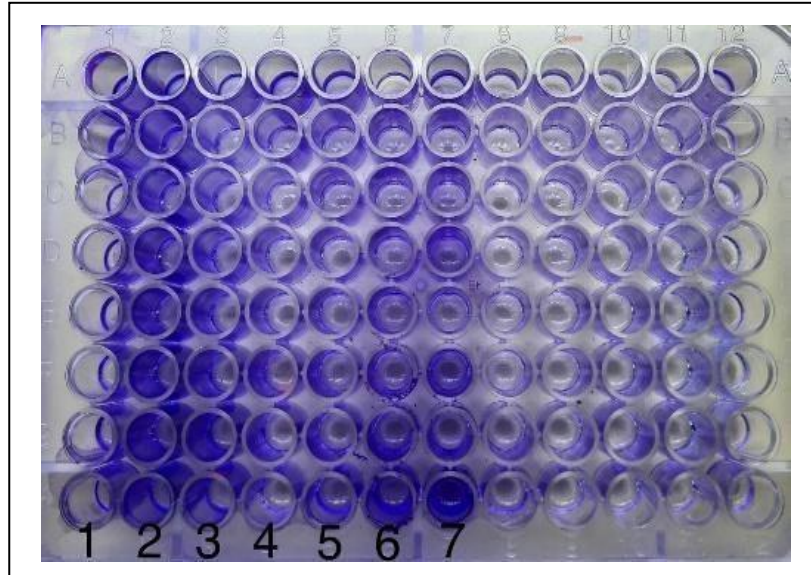


D43



E52



Figure 2. The Appear of bacterial isolates according gram stain**Figure 3.** The qualitative capability to form the biofilm of isolate (1 = A13, 2 = B21, 3 = B22, 4 = C33, 5 = D43, 6 = E52, and 7 = E53)

In Figure 3 revealed that seven bacterial isolates were able to produce biofilms which were indicated by the presence of purple dye on the walls of the microtiter plate after being stained with 0.1% crystal violet. Number 1 is isolate A13, number 2 is isolate B21, number 3 is isolate B21, number 4 is isolate C33, number 5 is isolate D43, number 6 is isolate E52, and number 7 is isolate E53. If an unwashed purple color is formed, the amount of bound dye is assumed to be equal to the amount of microbial biofilm in the well [14]. Biofilm formation can also be enhanced if added by amino acids [17]. Amino acids obtained from root exudates, research showed that the chemotactic effect of root exudates in the form of amino acids was able to help *Azotobacter chroococcum* in increasing the biofilm on the root surface [18].

3.2 Growth Character of Rice Seedling

The growth roots length, shoot-roots dry weigh and total biomass were significantly affected by the inoculation of HNR, while the plant height was not affected (Table 3 and Table 4). The highest roots length and root increment was given by the isolate of C33 and follow by the isolate D43 and E52 (figure 4). Moreover, the roots length increased by 14.16 cm, 12.36 cm and 12.33 cm. Which stated that with the addition treatment of nitrogen-fixing rhizobacteria inoculants produced rice plants with thicker root conditions and higher rice plant stems than controls [19]. Nutrient-providing rhizobacteria that live in the root rhizosphere (rhizobacteria) have a role, one of which is N₂ fixing and produce growth hormones such as IAA. The root was increased by 38, 20, and 20 %, respectively.

Table 3. Effect isolate inoculation on plant height and roots length of rice seedling after 21 days on Salinized Media (4 dS m⁻¹)

Selected isolate Code	21 Days After Planting		
	Plant Height (cm)	Root Height (cm)	Root Increment (%)
Control	6.33 a	10.23 a	-
A13	7.50 a	11.00 ab	7
B21	4.63 a	11.33 abc	10
B22	5.56 a	11.43 abc	11
C33	7.93 a	14.16 d	38
D43	6.60 a	12.36 c	20
E52	5.86 a	12.33 c	20
E53	6.33 a	11.60 bc	13

Note: The average value followed by the same letter in the same column shows no significant difference at the 5% level according to Duncan's Test.

The activity of HNR on root length is shown visually in Figure 4.



Figure 4. Shoot and roots performance of rice seedling affected by HNR isolate

Addition, Figure 4. This shows a significant difference between control treatment and C33,D43 and E52 isolates under saline conditions which had the highest root length among all treatments. C33, D43 and E52 isolates able to independently increase root length in saline conditions. It is suspected that N- fixing bacteria activity is more likely to work in secreting growth hormone IAA which has a function in root elongation. Which states that HNR is a group of bacteria that live in the rhizosphere area and act as a plant growth promoter. HNR bacteria also produce nitrogenase enzymes which function to fix nitrogen in the atmosphere and then transfer it to plants for use in metabolic processes [20].

It is clear that rice plants in saline conditions will show an impact in the form of salt stress in the form of root symptoms being short and not dense, then plant height are short and pale leaf color but the addition of N-fixing bacteria isolates in saline media can help rice plants grow more well then the control treatment. In the control of rice plants, the results of the non-saline biological test had quite good growth, but due to the absence of the addition of N-fixing bacteria isolates, the growth of rice was not because plant growth was influenced by the availability of nutrients in the growing media. The increase in plant growth is the influence of bacteria that can change elements that are not available to be available to plants so that the control has lower growth than all treatments given HNR isolates. Plant have different various respons to salinity stress (salt tolerance) [21]. There are plants that could survive in high saline soil conditions and while others cannot [22].

3.3 Shoots Dry Weight, Roots Dry Weight, and Biomass Total

Furthermore, Table 4 show that shoot and roots dry weight and as were affected by the inoculation of HNR significantly compared to control the highest shoot dry weight was resulted by the isolate E52, while the root dry weight and total biomass were given by the isolates of C33, D43 and E53. The total biomass was increased by 41, 39, and 64 %, respectively.

Tabel 4. Effect of bacterial isolate inoculation on shoot-root dry weight and biomass of rice seedling 21 days on salinized media

Isolate Code	Shoot Dry Weight (mg)	Root Dry Weight (mg)	Biomass Total (mg)	Increment (%)
Control	3.13 ab	1.46 a	4.6 ab	-
A13	3.03 ab	1.36 a	4.4 a	- 4
B21	4.00 bc	1.46 a	5.46 bc	18

B22	2.10 a	1.50 a	3.60 a	- 21
C33	4.00 bc	2.53 b	6.53 c	41
D43	4.00 bc	2.40 b	6.40 cd	39
E52	5.00 d	2.56 b	7.56 d	64
E53	4.00 bc	1.70 a	5.70 cd	23

Note: The average value followed by the same letter in the same column shows no significant difference at the 5% level according to Duncan's Test.

Based on Table 4 it can be seen that the addition of N-Fixing bacteria isolates increased the dry weight of the dead and roots along biomass total which gave the highest results, which was found in treatment G with isolate E52 which was 5.00 mg, 2.56 mg and 7.56 mg in saline compared to the control. The concentration of inoculant would have an effect on nitrogen fixation applied to both saline and non saline media [23].

The soaking of the seeds with N-fixing bacteria will produce one of them with high dry weight [24]. Isolates of N-fixing bacteria were able to increase plant biomass because they could produce phytohormones IAA which are growth- promoting hormones in plants [25].

The results of the root dry weight and total biomass given by the isolates of C33 by 2.53 mg and 6.53 mg, D43 by 2.40 mg and 6.40 mg and E53 by 1.70 mg and 5.70 mg compared to control which is 1.46 mg and 4.6 mg (Table 4). Total biomass represent the overall development of plants towards the shoot or roots. The highest total biomass in isolates that was because these bacteria produce growth hormones, this is in line with the statement of The formation of plant biomass was influenced by the hormones auxin, cytokinin, and abscisic acid (ABA) [26]. Salinity in rice plants results in reduced root length, plant height, crown dry weight, and root weight [27].

3. CONCLUSION

Seven of selected isolates (A13, B21, B21, C33, D43, E52, E53) has the ability to form biofilm. The selected of HNR isolates gave the significant effect on roots length, shoot dry weight, roots dry weight and total biomass. Compared to control, the highest roots and total biomass were resulted by the isolates E33, D43 and E53. The total biomass was increased by 41, 39, and 64 %, respectively. This results concludes that HNR isolates could use to develops bioagent as biofertilizers to alleviate the salinity stress and to improve the rice growth and productivity of rice on saline soils.

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