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Plant growth-promoting characterization of zinc-solubilizing bacteria from rice cultivation in Malaysia as a potential biofertilizer

Irsyad S. Ramly[1], Nur Maizatul I. Othman[1, 2,*], Kee Z.A.Tan[3] and Siti A.S. Nordin[1]

[1]Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA Melaka, Malacca, Malaysia
[2]Soil Conservation and Management Research Interest Group (RIG), Universiti Teknologi MARA (UiTM), Selangor, Malaysia
[3]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia
[*]Corresponding author: nurmaizatul@uitm.edu.my

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Abstract

This laboratory experiment was designed to isolate zinc-solubilizing bacteria (ZSB) and evaluate their potential use as a plant growth-promoter. A mineral salts medium (MSM) with the addition of insoluble zinc was used in plate observation of zinc solubilization before the ZSB were characterized further for other biochemical properties. Out of 28 bacteria isolated from rice soil, ten were found to solubilize zinc. Selected bacterial isolate (SR R-10) was the best ZSB, with the significantly highest solubilization efficiency for zinc oxide (558.33%), zinc carbonate (419.33%) and phosphate solubilization (146.67%). In addition, eight ZSB isolates were able to fix nitrogen, and seven tested positive for siderophores production. In terms of phytohormone production, SR R-10 led the production of indole compounds at 6.140 µg/mL, followed by SR R-2 and SR N-3 at 5.865 and 5.167 µg/mL, respectively. A 16S ribosomal ribonucleic acid (rRNA) molecular characterization revealed that SR R-10 as *Acinetobacter nosocomialis*. This bacterial strain has the potential to be used as a biofertilizer and substitute for chemical fertilizer usage, thus promoting sustainable agricultural practices.

Keywords: Bacteria identification, Biofertilizer, Plant nutrient, Soil microbes, Zinc deficiency

1. Introduction

Rice is one of the main important crops in the world. Rice production is dominated by Asia, which is also a world leader in terms of rice consumption. However, rice production nowadays faces zinc deficiency issues and struggles to find a sustainable and environmentally friendly solution [1]. Waterlogged soil, highly weathered acid, low organic matter content of the soil, the insoluble state of zinc, and excessive liming are the that contribute to the zinc deficiency in soil; these conditions are familiar in rice cultivation [1,2]. Zinc deficiency has been reported to affect the growth of rice seedlings as well as yield. Chemical zinc fertilizer, especially zinc sulphate, has been widely used by rice growers as a fast solution to overcome zinc deficiency. However, the excessive use of chemical zinc fertilizer may result in groundwater contamination and ultimately contribute to climate change and global warming due to greenhouse gas emissions [3].

One of the ways to overcome zinc deficiency in soil is by using a biological method [4,5]. Some bacteria are able to solubilize zinc in the soil. The use of zinc-solubilizing bacteria (ZSB) may provide available and sufficient zinc for rice uptake, thus improving rice growth and yield. Pseudomonas sp. is an example of a bacteria that has been found to assist in the bioavailability of zinc by solubilizing it [6]. One of the advantages of using the ZSB is its subsequent benefits via its biochemical properties and abilities; for example, the ZSB can act as a nitrogen fixer, phosphate and potassium solubilizer, and cellulose degrader [7-9]. By equipping these biochemical properties, ZSB can act as a plant growth-promoting bacteria and biofertilizer, reducing dependency on chemical fertilizer. Pseudomonas pseudoalcaligenes and Bacillus pumilus are examples of bacteria proven to improve some growth parameters for rice [9]. However, additional studies need to be conducted, since there is still a lack of information and reviews on its abilities, efficiency, and effectiveness. More bacteria species may also be discovered with zinc solubilizing potential. Hence, this in-vitro study was conducted to isolate the ZSB from rice soil and screen for its biochemical properties as a plant growth-promoter, before testing for its potential efficiency in sustainable agricultural practices.

2. Materials and methods

2.1 Sample collection

Soil samples were collected to isolate the bacteria from the non-rhizosphere, rhizosplane, and endosphere of rice using composite soil sampling method. The samples were collected at a rice field cultivated with MR 269 rice cultivar in Bukit Rambai, Malacca, Malaysia (coordinates: 2°15′45″ N, 102°9′9″ E). The samples were taken at depth 0-15 cm from the same rice field with an alluvium soil at five different locations. Soil samples collected from different locations in the field were combined into one composite sample. The samples were taken during the vegetative phase (≤ 40 days after sowing) of rice growth, since the bacteria were more active during that phase. The physiochemical properties of the soil are shown in Table 1. The samples were stored in a chiller at 4°C to minimize microbial activity.

Table 1 Physiochemical properties of the soil sample at Bukit Rambai, Malacca.

Type	Alluvium
pH	6.42
N	0.19%
P	5.035 mg/kg
K	76.70 mg/kg
Zn	4.821 mg/kg
C	3.98%

2.2 Isolation of zinc solubilizing bacteria (ZSB)

Non-rhizosphere bacteria isolation was performed by taking 10 g of soil sample into 90 mL distilled water and homogenized. Then, 0.1 mL was isolated and gently swirled on a nutrient agar (NA) plate. Rice roots were used to isolate bacteria from the rhizoplane. Each root was initially cut and gently washed with sterile water to eliminate the excess soil while maintaining those attached to the root. One g of root was placed into nine ml of sterile distilled water and shaken for a few min to totally homogenize the sample. Then, 0.1 mL was pipetted onto the NA plate and spread by using a sterile glass spreader. For endosphere bacteria, the rice plant root was soaked in 70% ethanol for two min, before being treated with sodium hypochlorite for five min, rinsed with sterile water, and dried on a sterilized filter paper. One g of root was taken and scrubbed with a sterile glass rod in a test tube, then added with nine ml distilled water. The sample was shaken for fully homogenized before 0.1 ml was isolated directly on NA plate [10]. The isolates were incubated at 28°C for a couple of days before being sub-cultured into a new NA plate to obtain a single colony of bacteria. Pure cultured bacteria were preserved at 4°C for further studies.

The isolates were inoculated into mineral salts medium (MSM) agar plate containing the following composition per litre of distilled water: glucose 10 g; $(NH_4)_2SO_4$ 1 g; KCl 0.2 g; K_2HPO_4 0.1 g; $MgSO_4$ 0.2 g; agar 15 g and supplemented with 0.1% of insoluble zinc salt [6]. Zinc oxide and zinc carbonate were used as sources of insoluble zinc salt. The plates were placed in an invert position and incubated at 28°C for 72 h. The diameter of the bacterial colonies and halo zones were recorded for the determination of zinc solubilization efficiency using formula SE = (solubilization diameter/growth diameter) × 100 [11]. Bacteria with high solubilization efficiency were indicated as efficient zinc solubilizers.

2.3 Nitrogen fixation activity

A nitrogen-free solid malate medium was used to test the nitrogen fixation activity by ZSB isolates [12]. Overnight grown of bacteria isolates were inoculated into the N-Free malate plate containing the following composition per litre of distilled water: malate 5 g; K_2HPO_4 0.5 g; NaCl 0.1 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; $CaCl_2 \cdot 2H_2O$ 0.02 g; micronutrient solution 2 mL; bromothymol blue solution 2 mL; FeEDTA solution 4 ml; KOH 4.5 g; agar 15 g. The bacterial plates were incubated at 30°C for 24 h. The formation of a blue colour from green indicated the presence of nitrogen fixation activity by the ZSB in the plate.

2.4 Phosphate solubilization ability

Bacterial isolates were inoculated into National Botanical Research Institute's Phosphate growth medium (NBRIP) with the composition per litre of distilled water: glucose 10 g; $(NH_4)_2 SO_4$ 0.1 g; KCl 0.2 g; $Ca_3(PO_4)_2$ 5 g; $MgCl_2{\cdot}6H_2O$ 5 g; $MgSO_4{\cdot}7H_2O$ 0.25 g; agar 15 g [13]. The media were adjusted to pH 7 before being autoclaved (ALP, Japan). The bacteria were then allowed to grow in an incubator at 30°C for 48 h. The diameters of the bacterial colonies and clear zones around the colonies were measured and the solubilization efficiency was calculated using a formula SE = (solubilization diameter/growth diameter) × 100 [11]. The highest solubilization efficiency indicated the most efficient phosphate solubilizer.

2.5 Indole compounds production

Indole compounds production was tested for five selected isolates (SR N-2, SR N-3, SR R-2, SR R-10 and SR R-12) using a colorimetric method [14]. Salkowski reagent was made by mixing one ml of 0.5 M $FeCl_3$ with 50 ml distilled water and 30 ml H_2SO_4. Bacteria isolates were inoculated in 100 mL nutrient broth (NB) and incubated at 28°C for 24 h. Then, one mL of bacterial culture was pipetted into a new 100 mL NB added with five mL L-tryptophan and incubated at 28°C for 24 h. A non-inoculated broth culture was kept as a control. One and half mL bacterial cultures were then transferred into a microfuge tube and centrifuged at 7000 × g for seven min. One ml of supernatant was slowly taken and transferred into a cuvette. Then, four mL of Salkowski reagent was added and allowed to stand for 15-30 min for pink colour development, which indicates the presence of indole compounds. optical density $(OD)_{530}$ was read using a visible (VIS) spectrophotometer (Dynamica Halo VIS-10), and the concentration of indole compounds produced by the bacteria was calculated using the standard curve equation.

2.6 Siderophores production

Siderophores production was measured using chrome azurol s agar (CAS) agar media [15,16]. Zinc-solubilizing bacterial isolates were inoculated into a CAS agar plate and incubated for 72 h at 33°C. The plates were observed for colour changes from blue to orange, which indicated that iron was removed from the dye complex by a strong iron chelator (siderophores).

2.7 Identification of bacteria

Bacteria identification was done by using a 16S rRNA gene sequencing [17]. The genomic Deoxy ribonucleic acid (DNA) of the selected bacterial isolate (SR R-10) was extracted by using HiYield™ Genomic DNA Mini Kit (Bacteria) (Real Biotech, Taiwan). The 16S ribosomal ribonucleic acid (rRNA) gene sequence was amplified through polymerase chain reaction (PCR) using thermal cycler (peqSTAR, Germany). The universal forward primer 8F (5'-GAGTTTGATCCTGCTCAG-3') and reverse primer 1492R (5'-GTTACCTTGTTACGACTT-3') [18]. The PCR master mix was prepared for final volume 25 µL per reaction; deionized water 14 µL; 10× reaction buffer containing 15 mM $MgCl_2$ (Lucigen, USA) 2.5 µL; 2.5 mM dNTP mix PCR Grade (Lucigen, USA) 2.0 µL; 100 pmol/µL forward universal primers 0.25 µL; 100 pmol/µL reverse universal primers 0.25 µL; 5 U/mL Taq Polymerase (Lucigen, USA) 0.5 µL and genomic DNA as template (50 to 200 ng) 5 µL.

The PCR thermal cycling conditions were carried out as follows" initial denaturation at 94°C for two min, followed by 35 cycles at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for one min and final extension at 72°C for 10 min. The amplified product was purified using HiYield™ Gel/PCR DNA Mini Kit (Real Biotech, Taiwan) and sent for sequencing (Apical Scientific Laboratory). The sequences were analyzed and aligned using a software (BioEdit 7.2.5). The aligned partial 16S rRNA sequence SR R-10 (1425 bp) was compared with the genes from Basic Local Alignment Search Tool (BLAST), NCBI Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EziBioCloud (https://www.ezbiocloud.net/). The multiple alignment and phylogenetic tree were developed using Molecular Evolutionary Genetics Analysis program (MEGA, version 10.1.6, USA). The phylogenetic tree was constructed using neighbour-joining algorithm and 1000 bootstraps of replicates [19,20].

2.8 Statistical analysis

The triplicates data were tested for normality before analysis using one-way analysis of variance (ANOVA), and significance differences between means were compared using Post hoc Tukey's HSD at 5% probability, using Statistical Package for the Social Science (SPSS) software (version 28.0.0.0).

3. Results

3.1 Zinc solubilization ability

A total of 28 bacteria were isolated. Sixteen were from the rice rhizoplane, nine from the bulk soil, and three from the endosphere sample. Ten isolates (SR E-2, SR N-2, SR N-3, SR R-2, SR R-9, SR R-10, SR R-12, SR R-12, SR R-15 and SR R-16) were found to be able to solubilize both zinc oxide and zinc carbonate. (Figure 1). shows the halo zone formation on the MSM agar supplemented with zinc oxide inoculated with selected isolates.

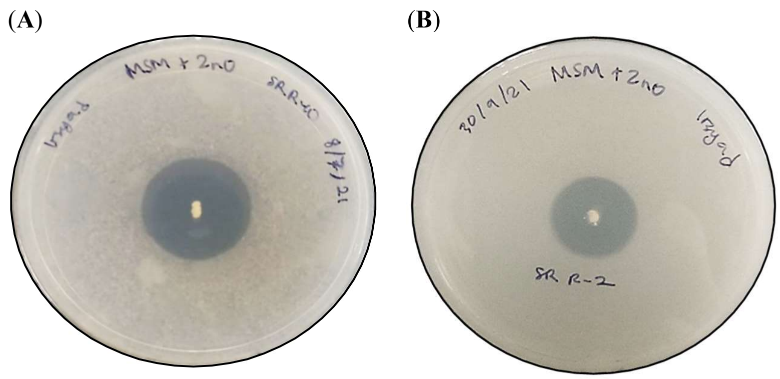

Figure 1 Halo zone formation on mineral salts medium with zinc oxide by bacterial isolates SR R-10 (A) and SR R-2 (B).

As shown in (Figure 2), isolate SR R-10 was the best zinc solubilizer, as it recorded solubilization efficiency of 558.33% for zinc oxide and 419.33% for zinc carbonate, the highest efficiency among the isolates. Meanwhile, isolate SR R-12 and SR N-3 were the second and third best zinc oxide solubilizers, as they achieved 384.33% and 102.33% solubilization efficiency, respectively. Meanwhile, SR N-3 showed consistency in the solubilization efficiency for both insoluble zincs as compared to the other isolates. As an overall for zinc solubilization ability between all ten bacterial isolates, SR R-10 was concluded to be the most efficient zinc solubilizer.

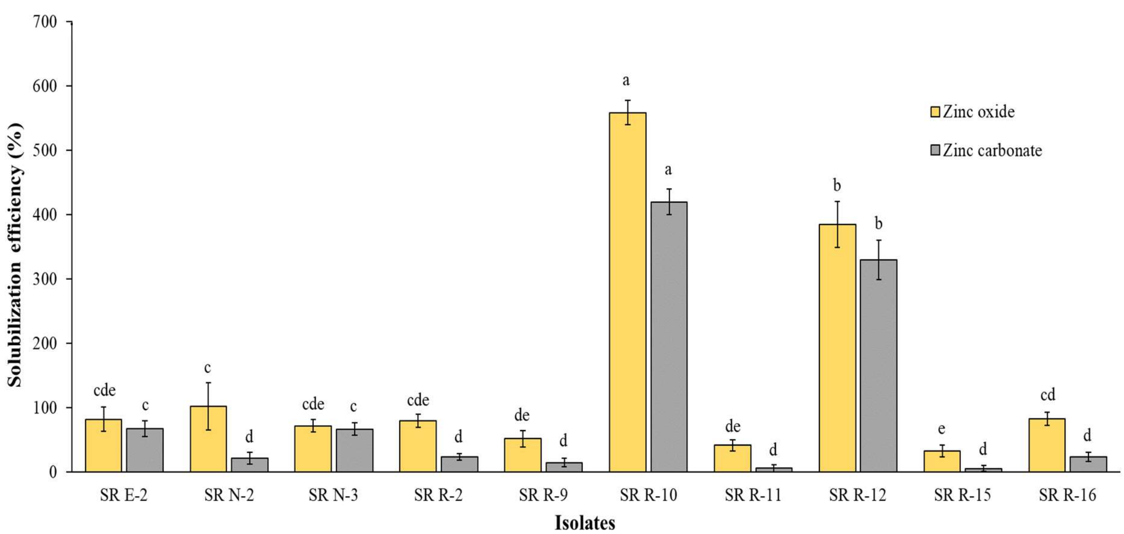

Figure 2 Zinc oxide and zinc carbonate solubilization efficiency by bacteria isolates. Error bars referred to the standard error with multiplier of 2. Bars with the same letters for a zinc source are not significantly different according to Tukey's HSD at $p = 0.05$.

3.2. Characterization of zinc-solubilizing bacteria

The ZSB isolates were further screened for their biochemical properties as a plant growth-promoter. The results are shown in (Table 2). 80% of the bacterial isolates were found to fix nitrogen since they show a positive result in the nitrogen fixation ability test. Only two isolates (SR E-2 and SR R-11) failed to fix nitrogen, as they did not exhibit the formation of blue colour on the N-free malate plate after a day of incubation. Those ZSB which were found to be able to fix nitrogen formed a blue colour on the plate, as shown in (Figure 3A).

Table 2 Biochemical characteristics of zinc-solubilizing bacterial isolates.

Isolates	Nitrogen fixation (+/-)	Phosphate SE (%)	Siderophore production (+/-)
SR E-2	-	23.00^{e}	-
SR N-2	+	52.33^{cd}	-
SR N-3	+	37.00^{de}	+
SR R-2	+	61.00^{c}	+
SR R-9	+	67.00^{bc}	+
SR R-10	+	146.67^{a}	+
SR R-11	-	22.00^{e}	+
SR R-12	+	80.67^{b}	+
SR R-15	+	22.00^{e}	-
SR R-16	+	52.00^{cd}	+

Note: (SE) Solubilization efficiency, (+) Presence and (-) Absence. Values are means ± standard error. Isolates with the same letters are not significantly different according to Tukey's HSD at $p = 0.05$.

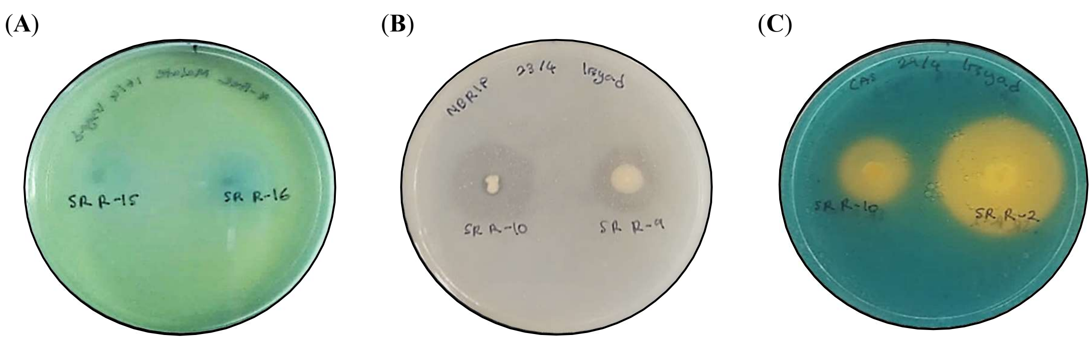

Figure 3 (A) Blue colour formation by isolates SR R-15 and SR R-16 on nitrogen-free malate medium for nitrogen fixation test (B) Halo zone formation by isolates SR R-9 and SR R-10 on NBRIP medium for phosphate solubilization test (C) Orange halo zone formation by isolates SR R-2 and SR R-10 on blue CAS medium for siderophores production test.

The highest phosphate solubilization efficiency was isolate SR R-10, while SR R-12 recorded the highest efficiency for potassium solubilization. All ten ZSB isolates showed positive results for phosphate solubilization ability. The phosphate solubilization efficiency was highest at 146.67% for SR R-10 while SR R-11 and SR R-15 had the lowest efficiency at 22.00%. The best results after SR R-10 were those of SR R-12 and SR R-9, with solubilization efficiency 88.67% and 67.00%, respectively. The formation of the halo zone on NBRIP medium is shown in (Figure 3B). It shows the difference between SR R-9 and SR R-10 in terms of the colony diameter and halo zone diameter. SR R-10 has a smaller colony with a larger halo zone as compared to the SR R-9 and this explained the reason for the high solubilization efficiency obtained by isolate SR R-10. (Figure 3C) shows the formation of orange colour zone on a blue CAS agar medium which describes that the siderophore was produced. As shown in Table 2, seven out of ten ZSB were able to produce siderophores as evidenced by the formation of orange colour on the blue CAS agar medium. The three ZSB isolates (SR E-2, SR N-2 and SR R-15) exhibited no changes of colour.

As shown in (Figure 4), five ZSB isolates were tested on their ability to produce the indole compounds. The selected ZSB were SR N-2, SR N-3, SR R-2, SR R-10, and SR R-12. All five of these ZSB isolates exhibited pale pink colour development after being treated with Salkowski reagent for half an hour, which indicates that the indole compounds have been produced. Isolate SR R-10 has the greatest ability in indoles production as it was able to produce 6.140 µg/mL of indole compounds. SR N-3 obtained 5.167 µg/mL, the third-best after SR R-2 which ranked second as it produces 5.865 µg/mL of indoles. Meanwhile, SR R-12, which performed well in the potassium solubilization test, was only able to produce 4.903 µg/mL of indoles, which was better than SR N-2, which had the lowest indoles concentration produced among the tested isolates at only 2.435 µg/mL of indoles. The results from bacteria identification for the best isolate (SR R-10) show that the isolate was identified as *Acinetobacter nosocomialis*. The phylogenetic tree of the strains is shown as in (Figure 5), with 0.002 nucleotide substitution rate per site for both strains. This bacteria strain was deposited in the NCBI GenBank with accession number ON834324.

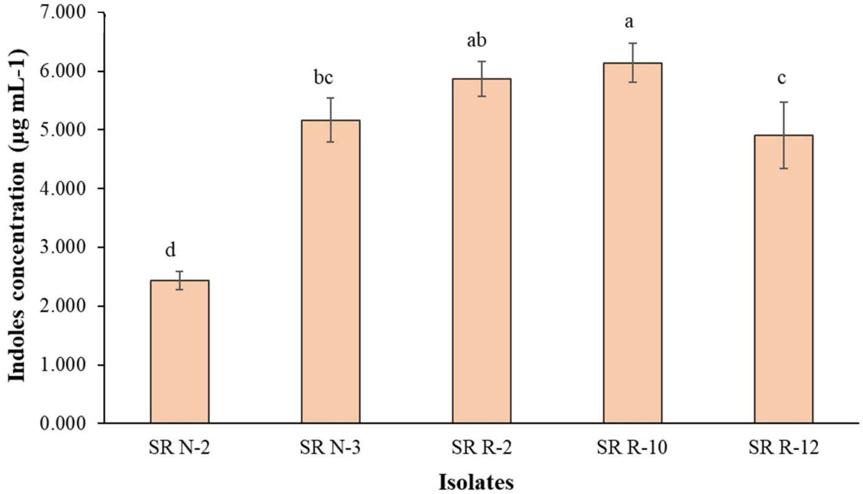

Figure 4 Indole compounds production by selected zinc-solubilizing bacteria isolates. Error bars referred to the standard error with multiplier of 2. Isolates with the same letters are not significantly different according to Tukey's HSD at $p = 0.05$.

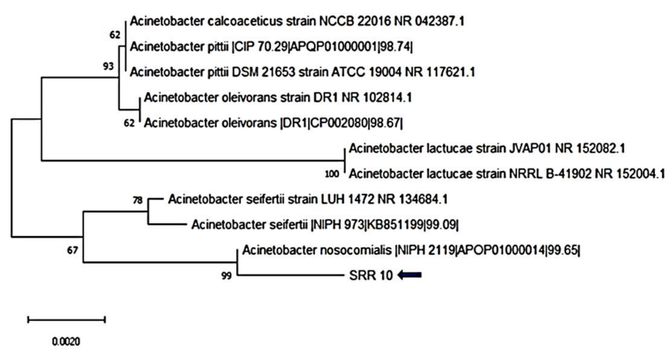

Figure 5 Phylogenetic tree based on 16S rRNA gene sequence comparison for SR R-10 using MEGA 10.1.6 and neighbour-joining analysis with 1000 bootstraps of replicates.

4. Discussion

Soil samples were taken during the vegetative stage of rice, as it is hypothesized that the bacterial community is rich during that stage and in submerged conditions, as compared to the reproductive stage. This has been attributed to the nutrient concentration in the soil sourced from fertilizer application and rhizodeposition, which are favoured for the microbial activity [21]. Some of the bacterial isolates in this study were able to solubilize the zinc through the presence of halo zone formation on the MSM agar when inoculated for a couple of days. Commonly, bacteria were able to solubilize zinc via the production of organic acids [7].

Larger colony diameter was unable to guarantee a larger diameter of halo zone and higher solubilization efficiency, as shown in the results. Similar findings have also reported that the growth rate of the bacteria is not a major contribution to the high solubilization index, as this depends on several factors that affect zinc solubilization by bacteria [6,22,23]. *Bacillus thuringiensis* has higher efficiency in solubilizing zinc carbonate as compared to zinc oxide and zinc phosphate [24]. However, *Bacillus aryabhattai* has been found to be superior in treating zinc phosphate rather than zinc carbonate and was less efficient in solubilizing zinc oxide [25]. This is because every single species of bacteria excretes different types and amount of organic acids, which act differently towards different types of zinc [26].

The results from the biochemical characterization showed most of the ZSB isolates were able to fix nitrogen. A colouring zone was present due to the acetylene reduction activity, which resulted in ethylene production [27]. Similar findings were also reported for bacterial isolated from non-leguminous plants, as a blue colour was formed when tested using various solid and semi-solid assays [28]. Both bacteria and fungi are able to solubilize insoluble phosphates, which will further increase the availability of phosphate in the soil for plant uptake. The genus *Bacillus* was found to solubilize a high amount of phosphate and could potentially be used on plants suffering from phosphate deficiency [10]. In this study, all bacterial isolates which were found to solubilize zinc also solubilized phosphate. The production of organic acids can be considered as the main reason for the solubilization to occur. Bacteria from different species can produce organic acids such as tartaric acid, formic acid, malic acid, malonic acid, lactic acid, gluconic and succinic acid [26]. The organic acids excretion reduces the pH of the medium and is bound to metal ions, thus dissolving the phosphate [29]. However, several studies have reported that this microbial activity of certain species gradually decreases after day 8 and stops at day 10 of the incubation period [29-30]. This could be due to the ability being degraded after generations of culture [29]. Contradictory results have also been reported, as it has also been found that only fungal isolates could acidify the medium, and no decrease in pH was observed in bacterial isolates [31]. In this case, the phosphate solubilization could be due to other mechanisms, such as proton exsorption and phosphatase enzyme production; the factor of incubation period must be considered [11,30].

Meanwhile, it has been found that seven ZSB isolates were able to produce siderophores, since an orange halo was observed on the CAS plate due to the removal of iron from the dye complex by a strong iron chelator. The diameter of the orange halo differed among all the positive tested isolates due to the specific properties of each bacteria. The exchange rate of iron from CAS the siderophore depends on the structure where fast-growing bacteria make larger orange halo and can be controlled through the growth conditions of the bacteria [15]. However, we could not infer the type of siderophore by the ZSB isolates as hydroxamate-type or catechol-type siderophores since we used universal method in the study. However, all strains of endophytic *Methylobacterium* spp. isolated from *Xylella fastidiosa* subsp. *pauca* were negative for catechol-type siderophores, but positive for hydroxamate-type siderophores [32].

The results from the indole compounds production test show that all the five ZSB tested produced indoles. During the tests, L-Tryptophan was used as a precursor to enhance the production of indoles. The production of indoles is attributed to the ability of the bacteria in producing enzyme tryptophanase that will convert the tryptophan into indole compounds through oxidation action and not all bacteria possess this enzyme [33]. From these findings, we can conclude that the tryptophanase produced is directly correlated with the amount of tryptophan which will be converted by the bacteria resulted in greater indoles production. Meanwhile, the presence of cellulase activity by the ZSB isolates may be one of the factors in the selection of the best ZSB isolates, since the bacteria have the potential to penetrate the cellulose of plant roots and thus solubilize the zinc inside.

As the result from this study found that isolate SR R-10, which was evaluated as the best ZSB isolates based on the characterization were identified as *Acinetobacter nosocomialis*. In addition, *Acinetobacter* spp. has been reported as an efficient nutrient solubilizer by a number of studies. These include *Acinetobacter iwoffi*, *Acinetobacter pittii*, *Acinetobacter calcoaceticus* and *Acinetobacter* sp. [8,29]. Gluconic acids have been found to be the main organic acids produced by *Acinetobacter rhizosphaerae* strain BIHB 723 and *Acinetobacter* sp. WR326 and the production were increasing with the incubation time [34,35]. It also has been found that rice inoculated with *Acinetobacter* sp. had increased growth attributes and yield, and it is hypothesized that the mechanism was through the exudation of some organic acids from inoculated PD16 variety, which contributes to higher solubilization of zinc in the soil [7]. These findings prove that the genus *Acinetobacter* has been widely shown to be an effective and efficient solubilizing bacteria, with organic acid production as the main mechanism for solubilization.

5. Conclusion

In conclusion, ten bacteria isolates were found to be ZSB and screened for their biochemical properties. Isolate SR R-10 (*Acinetobacter nosocomialis*) was identified as the best ZSB isolate following the results from the plant growth-promoting characterization. This isolate was the most efficient in solubilization of zinc and phosphate besides positive as a nitrogen fixer, siderophores, indoles producer. Further study needs to be conducted to determine the effectiveness of this isolate as a biofertilizer when inoculated into plant seedlings to observe whether it can provide significant advantages over non-inoculated seedlings.

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