INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
22–0418/2023/29–1–47–54
DOI: 10.17957/IJAB/15.2002
http://www.fspublishers.org

Full Length Article

Isolation and Phosphate Solubilization of Beneficial Rhizobacteria from Cassava Soil

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Received 25 October 2022; Accepted 01 December 2022; Published 30 December 2022

Abstract

Rhizobacteria as the root colonizing microorganisms show a significant impact on plant growth and development. The aim of this study was to isolate rhizobacteria with the ability to solubilize phosphate (P) from cassava (*Manihot esculenta* Crantz) rhizosphere. Soil samples of cassava rhizosphere of Piroon 2 (PR2) cultivar were collected at 6 months after planting (MAP). The population of culturable P-solubilizing bacteria (PSB) indicated that their population (1.92152×10^{11}) was higher than nitrogen fixing (N-fixing) bacteria. The N-fixing bacterial population presented only 12% of PSB. In this study the nine better PSB were isolated from the cassava rhizosphere. These isolates showed the lag and log phases during the 240 and 420 min, respectively. The generation rates of bacterial isolates were in the range of G=15.49 min (NS23) and G=35.70 min (NS24). Isolates showed the significant difference to solubilize P on solid and liquid NBRIP medium. The result showed that isolate NS39 was significantly better than the other isolates. The diversity of isolates to solubilize P was in the range of 0.1 to 0.3 mg/L P. The isolates NS39 and NS14 could be better candidates for field and biotechnological applications.

Keywords: Phosphate solubilizing bacteria; Rhizosphere; Cassava; Phosphorus

Introduction

Rhizobacteria as the root colonizing microorganisms produce the significant impact on plants. This interaction proved to be neutral, pathogenic and beneficial (Beneduzi *et al.* 2012). The importance of many beneficial symbiotic relationships of rhizobacteria have been scientifically documented (Bloemberg and Lugtenberg 2001; Buckley and Schmidt 2003; Birkhofer *et al.* 2008). The term of beneficial rhizobacteria or plant growth promoting rhizobacteria (PGPR) has been interchangeably used (Beneduzi *et al.* 2012). The population of PGPR was estimated to be 2–5% of total bacterial population in the rhizosphere; however, this population can vary in different plant and soil conditions (Girvan *et al.* 2003; Begoude *et al.* 2016).

The economic importance of beneficial rhizobacteria in plant production is mostly related to their application in biofertilizers as well as industrial microbiology and biotechnology. Different types of PGPR were introduced and applied successfully in the agricultural fields (Lin *et al.* 2012). The type of symbiotic relationship of bacteria and plant can be either helping in absorption of nutrients or suppressing pathogenic microorganisms.

One particular nutrient that can get great benefit from PGPR is phosphorus. Phosphorus, beside nitrogen and potassium, is a major plant nutrient requirement for plant growth and development. Soils in agricultural systems, especially in tropical areas contain large amounts of phosphate (P), a considerable part of it related to its accumulation based on the regular application of chemical fertilizers (Malhotra *et al.* 2018). However, a large part of the chemical fertilizers, which is considered as the soluble and plant can uptake that easily, rapidly fixed and transferred to insoluble and ultimately becomes unavailable (Du *et al.* 2020). The phenomenon of precipitation of phosphorus in soil is greatly dependent on pH and soil type. Free oxides and

hydroxides of Al and Fe in acidic soils are highly involved in precipitation of P, however in alkaline soils Ca mostly have this duty to fix P and reduce the soil content of soluble P (Hou *et al.* 2020). Phosphate solubilizing bacteria (PSB) showed that it can be a great asset to solve this problem. A key significant requirement to apply beneficial rhizobacteria or PGPR, particularly PSB, successfully is their ability to affect properly the positive symbiosis interaction with target plant. Therefore, the continuous identification of indigenous rhizobacteria and determining their ability is inevitable.

Cassava (*Manihot esculenta* Crantz) is the most important crop after rice, wheat and maize from the Euphorbiaceae family. It is native perennial shrub from South American region (Francis *et al.* 2013). Cultivation of cassava is for root storage and other parts, mostly in tropical and subtropical countries (Clifton and Keogh 2016). Cassava is propagated with stem cutting and mostly cultured in nutrient deficient acidic or alkaline soils (Bellotti *et al.* 2012). Cassava farmers usually need help to improve it from rhizospheric pathogens and diseases (Thaikert *et al.* 2015; Tappiban *et al.* 2018).

A considerable population of bacterial species is associated with the plant rhizosphere with the ability to solubilize P can apply to produce commercial bio fertilizers in agricultural fields (Oleńska *et al*. 2020). These bacteria are not usually adequate and applications as biofertilizers increased their number in the rhizosphere and help them to compete better with other bacteria. Thus, in the agricultural system, especially for green culture, inocula of plants with species and strain of more effective microorganisms is inevitable (Basu *et al*. 2021). As the root colonization is highly strain- and soil-specific (Sraphet and Javadi 2022), the screening of natural resources to find better and reliable strain linked to identification and detection methods are necessary for inocula production (Hassan *et al*. 2019). Scientific literatures showed the effectiveness of PSB as organic acid producer in combating pathogens (Ricke 2003). The aim of this research was to isolate and identify rhizobacteria from cassava rhizosphere to help in combat the rhizospheric pathogens as well as assimile and solubilize soil nutrients. Specific objective was to find isolates with P solubilizing ability from the rhizosphere of elite cultivar "Piroon2" that shows high yield and low cyanide content cultivated in Thailand.

Materials and Methods

Soil sampling

Soil samples were collected from cassava field at Nakhon Ratchasima Agricultural Research and Development Center, Department of Agriculture, Nakhon Ratchasima (14°52' 46.9704" N: 101°38' 56.7924" E). The samples were taken randomly from cassava field of Piroon 2 (PR2) cultivar at 6 months after planting (MAP) from a depth of 15–20 cm. Three replications of soil samples were collected from the rhizosphere part of cassava root with no non-rhizosphere soil.

Population of culturable bacteria

Soil samples (10 g) were placed in a 200 mL Erlenmeyer flask containing 90 mL DDH_2O. The sample was shaken for 1 h and used for serial dilution and then inoculation of petri dishes containing National Botanical Research Institute's phosphate growth medium (NBRIP) with 1.5% agar media (Javadi Nobandegani *et al.* 2015). NBRIP contained L^{-1}: glucose, 10 g: $Ca_3(PO_4)_2$, 5 g; $MgCl_2W_6H_2O$, 5 g; $MgSO_4W_7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g. The same procedure was applied for the N-free MJV medium. MJV medium contained L^{-1}: Mannitol, 1.0; K_2HPO_4, 0.4; KH_2PO_4, 0.4; $MgSO_4$ $7H_2O$, 0.2; $CaCl_2$, 0.02; Na_2MoO_4, 0.002; $FeCl_3$, 0.01; pH 7.0. Measurement of bacterial population was based on the formula:

Population of bacterial soil = bacterial colony (colony forming unit) × dilution factor

Isolation of beneficial bacteria

Bacterial isolates were obtained from soil samples surrounding the cassava rhizosphere. A 10 g of soil was diluted in 95 mL of sterile water to form a serial dilution up to 10^{-6}. Then, 0.1 mL of the final three dilutions was individually plated on NBRIP media. Bacteria represent clearing halo-zone on the plates were selected and purified on the same media. Nine bacterial isolates were selected for further study based on the high solubilization of P. Colonies of bacterial isolates were observed under the microscope. The colonies on the medium were observed and purified during incubation for 7 days (Javadi Nobandegani *et al.* 2014).

Gram staining

The conventional Gram staining method was applied for staining the colonies of isolates. Briefly, one drop of bacterial culture was put on a slide. In order to fix the bacteria to the slide, the slide was passed through the flame of a Bunsen burner. The primary stain (crystal violet) and secondary stain (safranin) as well as iodine (mordant) added in a timely manner to differentiate the isolates and stain them. Finally, the slides were observed using a compound microscope at 10, 40, 100 to 1000x magnification (Harrigan and McCance 2014).

Bacterial growth curve

An inoculum from bacterial culture was streaked on agar plate by using a sterile loop and incubated at 37°C for 24 h. Single colony of each strain was selected, inoculated into a test tube containing 10 mL of LB broth and incubated at 37°C overnight. Then, 5 mL of bacterial culture were transferred into 100 mL of LB broth in a sterile 250 mL conical flask and incubated at 37°C. The optical density (OD) of culture suspension was measured at 0 h and every 60 min at a

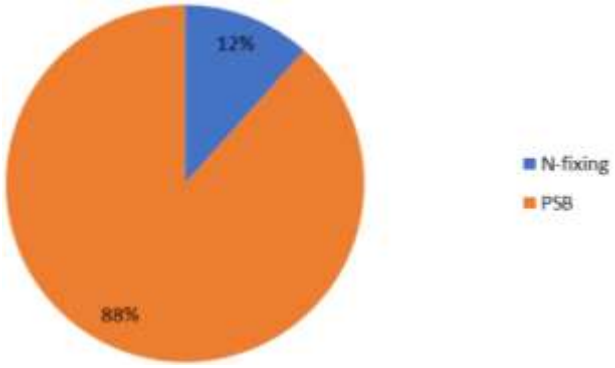

Fig. 1: The pai chart represented the phosphate solubilizing bacterial population in orange and Nitrogen fixing bacterial population in blue

wavelength of 600 nm using a spectrophotometer. The plot of time in min on X axis versus optical density at 600 nm on Y axis was drawn to obtain a growth curve of bacteria. The bacterial generation rate was calculated with equation:

$$Generation = G = \frac{t}{n}$$

$$G = \frac{t}{3.3log\frac{N_t}{N_0}}$$

Determination of P-solubilization on plates and in liquid culture

The isolates were cultured in liquid of NBRIP medium overnight (OD600=1) followed by culture in the wet autoclave disk of overnight re-cultured on plate of NBRIP (1.5% agar/L). The cultured plates of NBRIP were incubated at 37ºC. The halo-zone was measured at 48 h. For determination of phosphorus in liquid the isolates were cultured in flask containing 100 mL NBRIP and incubated in shaker incubator at 37ºC, 1500 rpm, the measurement was done after 7 days followed the Fiske and Subbarow (1925) method with some modification.

Experiment design and statistical analysis

The bacterial growth curve and determination of phosphate experiments were done in a completely randomized design (CRD) with three replicates. Data were analyzed for variance (ANOVA) and Tukey ‘s multiple comparison with SPSS software version 21 (IBM 2021).

Results

Soil sampling and population of culturable bacteria

The soils sampling was done randomly based on the standard procedures and results indicated that PSB population was higher than N-fixing bacteria. The population of PSB in one-gram soil was around 1.92152×10^{11} that was 88% more than the N-fixing bacteria. The population of N-fixing bacteria in 1 g soil was 25×10^8. N-fixing bacteria represented 12% of the PSB population (Fig. 1). It is mentioned here that some bacteria might be in both populations. Moreover, these data presented the only culturable bacteria, which mostly represent the whole bacterial population.

Isolation of beneficial and P-solubilizing bacteria

A total of nine better isolates were purified during this stage. It should mention here that the sensitivity of the nitrogen fixing bacteria was more than P-solubilizing bacteria. As the growth conditions of PSB were not disturbed they were more resistant to harsh conditions. In the purification stage of PSB they showed great solubilization of P on plate (Fig. 2). Isolates NS12, NS14, NS22, NS23, NS24, NS26, NS29, NS32 and NS39 that showed higher activity on NBRIP media (after purification by re-culturing) were chosen for further experiments.

Gram staining

Nine bacterial isolates purified on the solid NBRIP medium, were identified with Gram-staining (Fig. 3). After reviewed under the microscope all of the bacterial isolates showed Gram negative bacilli shape. Gram staining involved in staining the bacterium cell wall.

Bacterial growth

Bacterial growth curves were prepared to find the lag, log (exponential) and stationary phases. The bacterial isolates showed the lag and log phases during the 240 and 420 min, respectively (Fig. 4). The specific time for entering these critical phases was different among the isolates. The rate of growth of bacteria was different, which ranged from G=15.49 (NS23) to G=35.70 min (NS24). After 420 min the bacteria started to enter the stationary phase. Furthermore, the comparison of the bacteria growth showed that there was non-significant difference between the bacteria growth curve ($P \leq 0.05$) (Fig. 4).

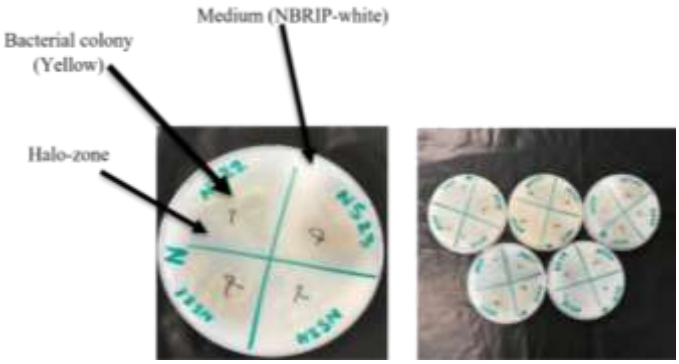

Fig. 2: Bacteria on NBRIP medium during the early stage of purification and re-culture

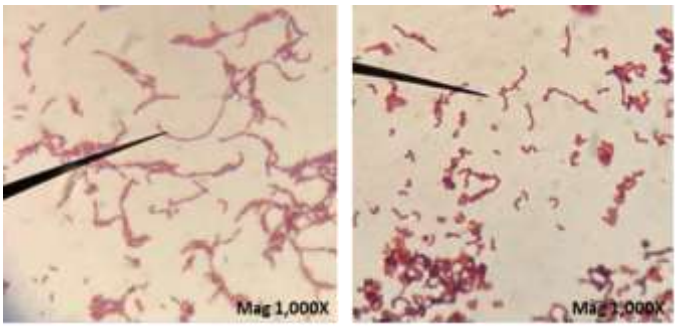

Fig. 3: The gram staining of isolates

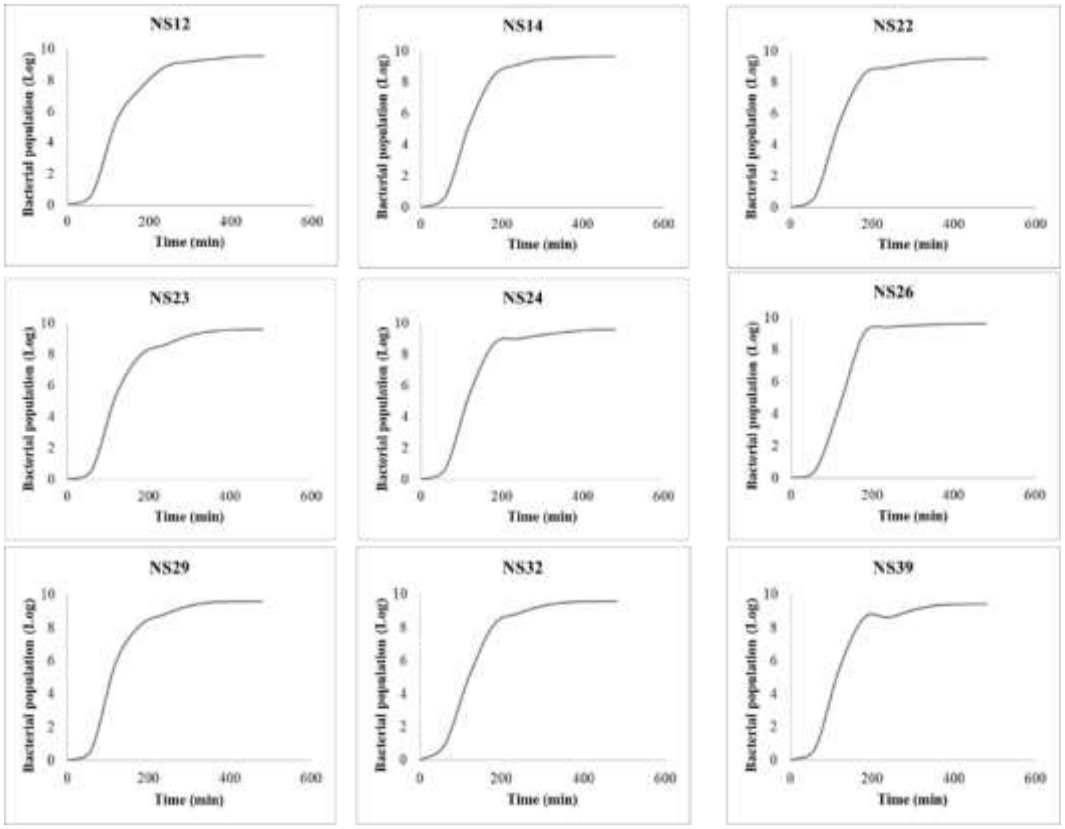

Fig. 4: Bacterial growth curves

Determination of P-solubilization on plate (halo-zone) and in liquid culture

All isolates showed differential ability of P solubility and halo-zone formation on NBRIP media (1.5% agar/L) when incubation at 37ºC for 48 h. NS14 bacterial isolate showed a higher performance and was significantly different compared to others ($P \leq 0.05$). Isolates NS39 and NS26 showed no significant difference (Fig. 5).

Bacterial isolates NS24, NS32 did not show the clear halo-zone although they showed growth on the plates, and were therefore selected for determination of P solubilizing inliquid culture. In liquid culture, the phosphate solubilization by isolates ranged from 0.1 to 0.25 mg/L P (Fig. 5–6). Not all isolates showed this ability in liquid culture. The result showed that isolate NS39 was significantly better than other isolates. However, the isolate NS14 showed no significant difference from NS22. The results of halo-zone and liquid culture were consistent for most of the isolates. Surprisingly, the isolate NS12 showed no P solubilization in liquid culture, however it showed halo-zone. Comparison of these experiments showed different mechanism of P solubilization with isolated bacteria from the cassava rhizosphere. (Fig. 5).

Discussion

Here we showed the diversity of PGPR in Cassava soils, and with the help of identified PSBs, better and more effective strains of PSB, isolated. The production of organic acids is the main mechanism in bacteria to solubilize the soil's mineral P. The colonization of two important genes (PQQ and gabY genes) for organic acid production was reported (Granada *et al*. 2018). Organic acids that are involved and reported in P-solubilizer bacteria are succinic, isovaleric, glycolic, lactic and oxalic as well as isobutyric, malonic and acetic acids (Kour *et al*. 2020). As organic acids have inhibitory effect on PSB growth (Hsiao and Siebert 1999; Pinhal *et al.* 2019), therefore isolated PSB growth condition on three different lag, log and stationary phases were investigated in more details. Overall, the bacterial isolates did not show any significant difference in lag phase, however the specific rate of growth was different and very low compared to the log phase (Fig. 4). This can be related to the bacterial acclimatization to the broth medium, biosynthesis of enzymes and other metabolites and finally adjust to the medium (Trivedi *et al.* 2010; Engelkirk *et al.* 2020).

The bacterial cells are metabolically active, however, the increase in bacterial cells was in very low rates compared to the log phase. In contrast, the bacterial isolates showed very high rate of growth in log phase, as the population double in very fast pried of time (Fig. 4). Generally, in this phase the bacteria are sensitive to external stress and as long as constant nutrient and environment conditions provide, the fast growth is obtained. The stationary phase between the bacterial isolates was observed as soon as the essential nutrients in medium and water oxygen became insufficient (Sastry and Bhat 2016).

Duration of each phase was different among the bacterial isolates however the bacterial growth curve was not significantly different (Fig. 4). The information provided here can help in inoculation of number of bacterial isolates for enhancing the plant growth and producing the organic acids. The information provided in this experiment would be helpful for bacterial growth modeling and DNA extraction (Shigyo *et al*. 2019).

The Gram stain is a basic method to differentiate bacteria to one of two groups (Gram-positive and Gram-negative) based on the structure of bacterial cell walls. Bacterial cell wall is one of the important parts of the bacterium for up taking the nutrients for survival, while the prevailing conditions affect this structure significantly. As calcium phosphate was the important ingredient in NBRIP (made it a selective medium) and cannot be absorbed without solubilization, therefore this condition might force the bacteria to change the growth behavior. At first stage the structure of the membrane would change and follow the sporulation stage to survive. This could be due to changes in peptidoglycan membrane transformations in the early stage of growing on the medium. Nonetheless, the results need to be confirmed with molecular identification with more clear details (Tocheva *et al*. 2013).

The determination of solubilization of P was determined with plate halo-zone and liquid culture, however the results mostly were contradictory (Joe *et al.* 2018; Teng *et al.* 2019). This contradiction showed that to determine the P-solubilization in liquid culture could be more reliable than the halo-zone formation (Billah *et al.* 2019). Furthermore, the bacterial isolates showed no significant difference in growth. This indicated that for industrial application there is a need to calculate the generation rate. Our data showed that generation rate could be important in case of using the isolate for production of organic acids (Mattey 1992; Hsiao and Siebert 1999; De Filippis *et al.* 2018; Pinhal *et al.* 2019).

Beneficial effects of inoculation with P-solubilizing microorganisms on many plants have been documented by different researchers (Billah *et al*. 2019; Rafi *et al*. 2019). The PSB with the ability to fix nitrogen symbiotically are present in the legume nodules. Several publications have showed that P-solubilizing strains of Rhizobium increased growth and P content of many leguminous plants (Kalayu 2019). Mixed cultures or co-inoculation microorganisms showed the most effective way to produce incula for different plants. Therefore, good synergistic interaction between vesicular arbuscular mycorrhizae (VAM) and PSB exists (Soumare *et al*. 2019). This effect was also observed in inoculation with PSB and Azospirillum or Azotobacter (Alaylar *et al.* 2020).

Previously P-solubilizing bacteria have been isolated from rhizosphere by plating serial dilutions of soil extracts in many media, although the first medium was introduced as the Pikovskaya's solid medium (Suleman *et al*. 2022). Most PSB media contain insoluble tri- or bi-calcium P, allowing the isolation of P-solubilizer microorganisms by the formation of "halo" zone around their colonies (Fig. 5). This halo-zone is

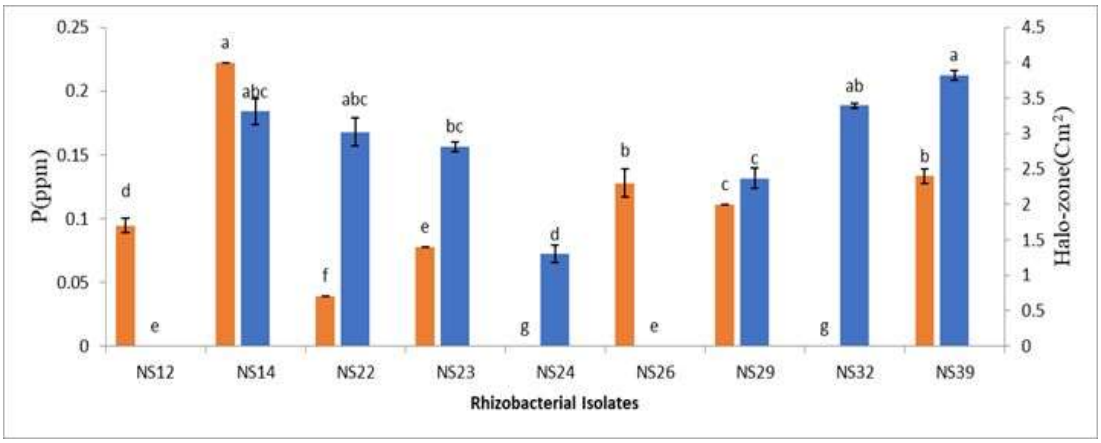

Fig. 5: Phosphate solubilization on NBRIP plates to measure halo-zone (orange) and NBRIP liquid culture (blue) by the isolates. Means with different letters represent significant difference at $P < 0.05$ Tukey's multiple comparison, n = 3. Bars show standard error of the treatment's mean

Fig. 6: Setup for bacterial isolation during phosphors determination by developing blue color before measuring the optical density at 882 nm

due to secretion of organic acids or protons, which drop the medium pH. Although there is instability of P-solubilization by some isolates, most isolates may retain their ability for long time after culturing (Ahmad *et al*. 2022). The identification of better and effective P solubilizers should be done by measuring their P-solubilizing capacities in liquid cultures as determined in our research (Fig. 5 and 6). For better isolation of PSB, NBRIP medium was used, which is known to show higher efficiency compared to other PSB isolation media (Patre and Peter 2021).

Conclusion

The cassava soils entailed a high diversity of PSB. Ability of isolated bacteria to solubilize P (in particular calcium phosphate) in harsh conditions could direct that they could be better candidates for biofertilizers production even for other plants. Molecular identification and characterization of bacterial isolates as well as field survey may help finding clearer distinction for field applications in future.

Acknowledgments

This research project was supported by Mahidol University, Thailand. The authors would like to thank the Faculty of Science and Technology, Suan Sunandha Rajabhat University (SSRU), Thailand. Special thanks to the Chemistry group (Department of Sciences, SSRU) and Science Center (SSRU) for their generous support. Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Thailand are gratefully acknowledged for their kind support.

Author Contributions

KT, NS, RB, SB, CT and SC: Conceptualization, investigation and methodology; TM: Microbial experiments; SS and BJ: Supervision, review and editing

Conflict of Interest

The authors declare that they have no competing interests

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

This work does not involve animals hence ethics approval not required

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