RESEARCH ARTICLE

Isolation and selection of salt-tolerant bacterial strains capable of solubilizing phosphorus and synthesizing phosphatase enzyme from rice-shrimp soil in Mekong River Delta, Vietnam

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Abstract: The aim of this study was to isolate and select salt tolerant bacteria having both functions in phosphorus solubilization and phosphatase synthesis from rice-shrimp farming soil in saline areas in Mekong River Delta of Vietnam. Phosphorus solubilizing bacteria were isolated on National Botanical Research Institute's Phosphate (NBRIP) agar medium containing 1% NaCl and the activity of phosphatase enzyme was determined by disodium p-nitrophenyl phosphate reagent method at a wavelength of 420 nm. The result showed that from 15 saline soil samples, a total of 95 strains of phosphorus solubilizing bacteria were isolated and 19 of them showed their good phosphorus solubilization. The results about phosphatase activities of these 19 strains illustrated that TBT5-3 bacterial strain was the highest phosphatase producing strain with an amount of 0.377 U/mL after 10 days of incubation. This strain showed its best phosphatase producing capacity when cultured in the liquid culture medium containing pH 5, 1% NaCl, glucose and urea under the shaking speed of 120 rpm. Based on 16S rRNA gene sequence analysis this phosphatase synthesizing bacterial strain was genetically identified as species of *Bacillus* sp. TBT5-3 since 100% of this train sequence is affiliated with *Bacillus megaterium*.
Keywords: Saline soil, rice-shrimp soil, phosphatase activity, phosphorus solubilizing bacteria, *Bacillus* sp.

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1 Introduction

Phosphorus (P) is one of the essential elements for plant development and growth. It makes up about 0.2% of a plant dry biomass (Azziz et al., 2012; Tak et al., 2012). P is essential in every aspect of plant growth and development, from the molecular level to many physiological and biochemical plant activities including photosynthesis (Sharma et al., 2013). Soil P exists in various chemical forms including inorganic P (Pi) and organic P (Po). These P forms differ in their behaviors, fate in soils (Turner et al., 2007; Campbell and Racz, 1975), and availability to plants (Brady and Weil, 2008). The main input of inorganic P in agricultural soil is from phosphorus fertilizers. Nearly, 70 to 90% of phosphorus fertilizers applied to soils are fixed by cations and converted to inorganic P (Walpola and Yoon, 2012). P gets immobilized by cations such as Ca^{2+} in calcareous or normal soils to form a complex calcium phosphate ($Ca_3(PO_4)_2$) or with Al^{3+} and Fe^{3+} in acidic soils to form aluminum phosphate ($AlPO_4$) and ferrous phosphate ($Fe_3(PO_4)_2$) (Kumar et al., 2018; Satyaprakash et al., 2017). Salinity-induced P deficiency is individually or both major abiotic stresses in saline-based agroecosystems, that negatively affect almost all facets of plant growth and development (e.g., seed germination, vegetation, flowering, fruiting, and leaf senescence) as well as plant metabolism (e.g., photosynthesis, respiration, protein synthesis, and lipid metabolism) (Yoneyama et al., 2012; Kosová et al., 2013; Carstensen et al., 2018; Mokrani et al., 2020). The insoluble forms of phosphorus consequently become unavailable for plants to uptake. These insoluble forms of phosphorus, under suitable pH conditions, are converted into soluble forms, and with these processes microorganisms in soils play a very important role. Phosphate solubilizing microorganisms (PSMs) are a group of beneficial microorganisms capable of hydrolyzing organic and inorganic phosphorus compounds to form soluble phosphorus. Among these PSMs, bacteria are considered as the most predominant group including *Bacillus*, *Pseudomonas*, and *Rhizobium*, followed by fungi like *Penicillium* and *Aspergillus*, arbuscular mycorrhizae and actinomycetes (Muindi, 2019). The strains *Bacillus* and *Streptomyces* spp. are able to mineralize complex organic phosphates through the production of extracellular enzymes like phosphoesterases, phosphodiesterases, phytases, and phospholipases (Walpola and Yoon, 2012). PSMs can miner-

alize soil organic P by producing some enzymes to solubilize phosphorus such as phosphatase, phytase...They hydrolyze organic forms of phosphate compounds, thereby releasing inorganic phosphorus that will be taken up by plants later. Phosphatase is a group of enzymes that catalyze the hydrolysis of esters and anhydrides of phosphoric acid (Condron et al., 2005), from a wide variety of organic substrates, producing phosphate ions (Tazisong et al., 2015). Phosphatase is present in the rhizosphere and plays a major role in the mineralization of organic phosphorus present in soil (Rodriguez and Fraga, 1999). Alkaline and acid phosphatases use organic phosphate as a substrate to convert it into an inorganic form. The phosphatase enzyme is greatly influenced by culture conditions including salt concentration, temperature, and pH value, and determining the optimal value of these parameters is extremely important (Gomes and Steiner, 2004). Most bacterial phosphatases have an optimum pH in the range from 4.0 to 5.5 (Akuzawa and Fox, 1998). The optimal temperature for phosphatase enzyme activity ranged from 40 to 70°C (Abdallah et al., 1999a, b). However, according to a study by Behera et al. (2017), *Serratia* sp. could produce maximally phosphatase enzyme at the temperature of 45°C, an agitation rate of 100 rpm, pH 5.0, glucose as an original carbon source, and ammonium sulfate as an original nitrogen source. However, most of the previous studies about PSMs did not study enzyme activities involved in phosphorus solubilization. Thus, this research was aimed to isolate salt tolerant and phosphorus solubilizing bacteria capable of producing phosphatase enzymes and to identify the favorable conditions for synthesizing phosphatase.

2 Materials and Methods

2.1 Isolation of phosphorus solubilizing bacteria from saline soils in rice-shrimp farming system

Saline soil samples were collected on rice-shrimp soil at the end of the rice crop with a depth of 0-20 cm in some provinces of Mekong River Delta of Vietnam including Bac Lieu, Ben Tre, Ca Mau, Kien Giang, and Soc Trang with 3 soil samples for each site. An aliquot of 10 grams of each soil sample was put into a 250 mL glass bottle containing 90 mL buffer phosphate and the samples were shaken at 150 rpm/min for 1 hour on a shaker. The composition of buffer phosphate liquid medium (g/L) was 23.99 g NaH_2PO_4 and 15.59 g NaH_2PO_4. After shaking, a series dilution with a factor of 10 was prepared and aliquot 50 μL of each dilution was spread on NBRIP agar medium containing 1% NaCl. The composition of NBRIP liquid medium (g/L) was D-glucose (10 g), $Ca_3(PO_4)_2$ (5.0 g), $MgCl_2{\cdot}6H_2O$ (5.0 g), $MgSO_4{\cdot}7H_2O$ (0.25 g), KCl (0.2 g), $(NH_4)_2SO_4$ (0.1 g), pH=7.0 $\pm$ 0.2 (Mehta and Nautiyal, 2001). Samples were incubated for 5-7 days in the dark under laboratory conditions. After incubation, bacterial colonies showing the halo ring surrounding were selected for further purification on TSA medium consecutively 5 times. Morphological characteristics of selected bacterial colonies including size, shape, color form, elevation and margin were recorded and described accordingly. The composition of the TSA medium includes Tryptone Soya Broth 30 g/L and agar 15 g/L. After purification, biomass of bacterial colonies was stored in 30% glycerol solution for further studies.

2.2 Phosphate solubilizing ability of isolates

Bacterial culture preparation: The bacterial isolates were cultured on Tryptone Soya Broth (TSB) for 3 days under laboratory conditions. The composition of the TSB solution includes Tryptone Soya Broth 30 g/L. Biomass of each bacterial strain was harvested separately by transferring the whole TSB solution. The optical density of the bacterial solution was adjusted with sterilized distilled water to OD = 0.7 by using a spectrometer with a wavelength of 600 nm. This microbial solution suspension was used as a bacterial source for experiments.

Experimental layout: The experiment was conducted in a completely randomized design manner. Each bacterial isolate corresponded to one treatment and the experiment was arranged with 3 replicates for each treatment, corresponding to 3 Erlenmeyer flasks. An aliquot of 1 mL of the microbial solution was added into a 100 mL Erlenmeyer flask containing 49 mL NBRIP liquid medium. The control treatment was performed in the same manner but without bacterial inoculation. Samples were put on the orbital shaker at a speed of 100 rpm in the dark and under laboratory conditions for 15 days. The concentration of phosphorus solubilized in the liquid medium by bacteria was determined after 3, 5, 7, 10, and 15 days of incubation. Optical density was determined at 880 nm with the help of a spectrophotometer (Spectrometer Thermo Scientific, Multiskan Spectrum). The concentration of P_2O_5 in solution was measured with the help of a standard series dilution of P_2O_5 concentration varied between 0 and 1 mg/mL. The isolates showed high capacity in salt-tolerant and phosphate solubilization were selected to determine phosphatase enzyme activity in NBRIP liquid medium containing 1% NaCl.

2.3 Evaluation of the activity of phosphatase enzyme in the NBRIP liquid medium

The experiment was arranged in a 100 mL Erlenmeyer flask in a completely randomized design with 3 replicates corresponding to 3 flasks for each bacterial strain. The Erlenmeyer flask contains 49.5 mL sterilized NBRIP liquid medium, 1% NaCl and 0.5 mL of bacterial solution (OD_{660nm} = 0.7). The control treatment was performed in the same manner but without bacterial inoculation. The sample flasks were shaken on an orbital shaker at 110 rpm, at room temperature in the dark, and the experiment was carried out for 14 days. The phosphatase enzyme activity was determined at day 6, 10, and 14 after inoculation. Phosphate enzyme activity was

determined by the method of Tabatabai and Bremner (1969). Briefly, 1 mL of the bacterial cell-free supernatant was added into a glass tube containing 1 drop of toluene which was used to kill bacteria in the solution, then 4 mL of buffer (Maleic acid and Tris hydroxymethyl aminomethane, pH 6.5) and 1 mL of disodium p-nitrophenyl phosphate 0.5 mM were added later. The mixture was vortexed and incubated at 37°C for 1 h. After 1 h incubation, 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ were added into the solution to stop the reaction, then the sample was filtered with Whatman filter paper No.42. To indicate a positive result for phosphatase, the sample solution should have the yellow color. The p-nitrophenol standard series were prepared the same manner but without addition of toluene and disodium p-nitrophenyl phosphate 0.5 mM. The positive sample which showed the yellow color was determined later for the concentration of p-nitrophenol at 420 nm using a spectrophotometer.

2.4 16S rRNA gene sequence for identification of the best bacterial isolate

The colony morphology of bacterial isolates showing best in phosphatase activity was described through the following criteria: size (mm), color, shape, form, elevation and margin. Moreover, the bacterial specimen for cell morphology was carried out by using a clean sterilized bamboo toothpick to take a small biomass of bacterial colonies, spread the biomass on Lame, dropped some drops of sterile distilled water and mixed well the biomass into water. The sample was passed through the flame to immobilize the bacterial cell, then a drop of Fuchsin dye 0.5% was applied on the position of the fixed bacteria, using Lamella to cover the bacterial pot. The shape and other bacterial cell morphologies were observed and recorded under an optical light microscope.

Gram test: Gram reaction was tested by mixing a loop of bacterial colonies with 1 drop of KOH 3% on a microscope slide by a toothpick. Bacterial strain was considered as Gram negative when a thread was formed between the toothpick and the microscope slide (Suslow et al., 1981).

The 16S rRNA gene sequence: The selected bacteria that showed their highest capacity on phosphatase activities was selected to have 16S rRNA gene sequence. The results of 16S rRNA gene sequence were compared with available databases of gene banks to identify bacterial species of the selected isolate by using the BLAST program (GenBank). Combining the morphological, cell and homologous morphological characteristics of BLASTN to determine the species name of one selected bacterium.

2.5 Evaluation of the effect of cultural conditions on phosphatase enzyme activity of the selected bacteria

2.5.1 Different shaking speeds

To determine the effect of orbital shaking speed on the synthesis capacity of phosphatase enzyme by the selected bacteria, an experiment conducted with five different shaking speeds as follows 0, 60, 80, 100 and 120 rpm as experimental treatments. Each treatment contained their own control treatment without bacterial inoculation accordingly. Bacterial solution was prepared at OD_{660nm} = 0.7. Then, an aliquot of 0.5 mL bacterial suspension was transferred to 100 mL Erlenmeyer flask containing 49.5 mL NBRIP liquid medium. The samples were put on the orbital shakers with corresponding speeds of 0, 60, 80, 100 and 120 rpm in the dark under laboratory conditions for ten days. The phosphatase enzyme activity was determined after inoculation according to the method of Tabatabai and Bremner (1969).

2.5.2 Different NaCl concentrations

The experiment was conducted to evaluate the effect of different concentrations of NaCl on phosphatase enzyme activity of the selected bacteria. The different NaCl concentrations including 0%, 1%, 2%, 3%, 4% and 5% NaCl were used as individual treatment. Each NaCl concentration treatment contained a control treatment without microbial inoculation accordingly. The best shaking speed for phosphatase synthesis from the results of the test in section 2.5.1 was applied in this experiment. The phosphatase enzyme activity was determined after 10 days of incubation by the method of Tabatabai and Bremner (1969).

2.5.3 Different pH values

To evaluate the effect of different pH values of NBRIP liquid medium on phosphatase enzyme activity by the selected bacteria, an experiment was conducted with four different pH values of liquid medium including pH 3, 5, 7 and 9. Each pH treatment had their own control treatment without bacterial inoculation accordingly. The best NaCl concentration from the results of the tests in section 2.5.2 was applied in this experiment and the samples were put on the orbital shaker at the best speed recorded from the section 2.5.1 in the dark under laboratory conditions for ten days. Phosphatase enzyme activity was measured after 10 inoculation days by following the method of Tabatabai and Bremner (1969).

2.5.4 Different carbon sources

Five different carbon sources including 1% of each lactose, sucrose, maltose and glucose were considered as different treatments and the control treatment of each carbon source treatment was conducted in the same manner but without bacterial addition. Each treatment had 3 replicates, corresponding to 3 different incubation flasks. The best pH value and NaCl concentration from the results of the tests in section 2.5.2, and 2.5.3 were applied in this experiment and the samples were put on the orbital shaker at the best speed recorded from the tests in section 2.5.1 for ten days. Phosphatase enzyme activity was measured after 10 incubation days followed by the modified method described by Tabatabai and Bremner (1969).

2.5.5 Different nitrogen sources

This experiment was conducted to find out the best nitrogen sources in NBRIP liquid medium for optimizing phosphatase enzyme activity by the selected bacterial strains. A concentration of 11% of each four nitrogen sources including potassium nitrate, ammonium sulfate, ammonium molybdate and urea as nitrogen sources for bacteria were considered as different treatments. Each nitrogen source treatment had a control treatment without bacterial addition accordingly. The sharking speed, pH, carbon source, and NaCl concentration were applied in this experiment according to the recorded data from section 2.5.1, 2.5.2, 2.5.3, and 2.5.4, respectively. The phosphatase activity by bacteria was determined according to the method by Tabatabai and Bremner (1969).

2.6 Statistical Analysis

The data were analyzed by ANOVA with MINITAB software with 16.2 versions.

3 Results and Discussions

3.1 Isolation of phosphorus solubilizing bacteria from saline rice-shrimp farming system soil in the Mekong River Delta of Vietnam

From 15 samples of saline soil collected on the rice-shrimp farming fields in 5 different coastal provinces of the Mekong River Delta of Vietnam were cultured on NBRIP agar medium containing 1% NaCl. Characteristics of colonial and cell morphologies as well as bacterial Gram stain were described for phosphorus solubilizing bacteria. Based on these characteristics, ninety five phosphorus solubilizing bacteria were isolated from these 15 soil samples. There was a high diversity of colonial morphology of isolated bacteria in terms of color, size, shape, surface and bacterial cell shapes (Figure 1). Among soil samples, samples from Bac Lieu province had 28 phosphorus solubilizing bacteria, accounting for 29.50% of the total isolated bacteria and the lowest number of phosphorus solubilizing bacteria was found in soil samples of Soc Trang province, 7 isolates (accounting for 7.30%) whereas the remaining other soil samples from Ben Tre, Ca Mau and Kien Giang had 19-22 bacteria (accounting for 20-23%). Thus, this result showed that although soil samples were collected on the same rice-shrimp farming system soil which was affected by salinity, the number of phosphate solubilizing bacteria numbers and the diversity of these bacteria in terms of colony morphology were much different. This indicated that the geography and salinity levels in soils may be key factors regulating the difference in the numbers of phosphorus solubilizing bacteria isolated from samples to samples.

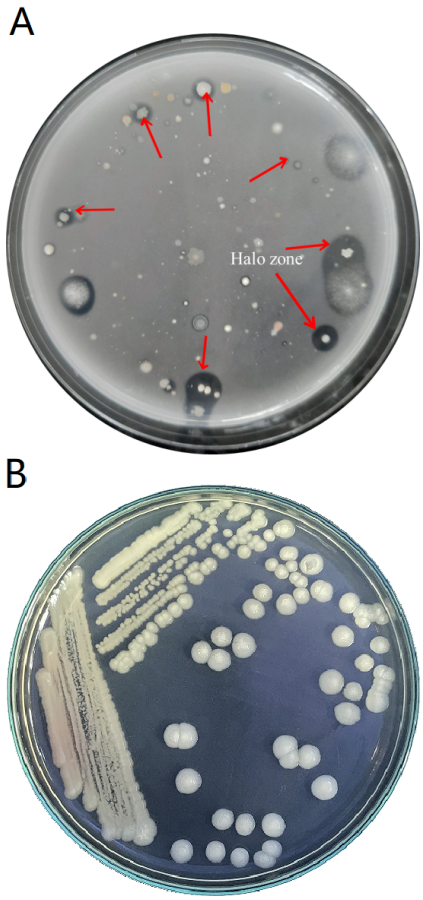

Figure 1. A: Diversity of colonial morphology and the phosphorus solubilizing capacity of bacteria on NBRIP agar containing 1% NaCl; B pure isolate of TBT5-3 on TSA agar medium after 3 days of incubation.

3.2 Phosphorus solubilization capacity of bacterial isolates

The result of study on phosphorus solubilization capacity of 95 different bacteria isolated from different saline soil samples indicated that their capability in phosphorus solubilization in NBRIP liquid medium was widely different. In this part, just the most phosphorus solubilizing bacteria selected to present their capacity in phosphorus solubilization in liquid culture were indicated in Figure 2. There was a big variation of phosphorus solubilization capacity of selected bacterial strains in the liquid medium. The concentration of phosphorus released from bacteria to the liquid solution strongly varied from 163 mg/L to 2,044 mg/L, and was significantly different from each other ($p<0.05$). It means that some isolates had lower capacity in phosphorus solubilization as compared to others and vice versa. Among that, 19 out of 95 strains had good ability in phosphorus solubilizing capacity, accounting for 23.21% of the total tested bacteria isolates. In fact, the best bacterial isolate found to be the highest amount of phosphorus in liquid culture medium (2,044 mg/L P_2O_5) after 15 days of incubation was TBL1-4

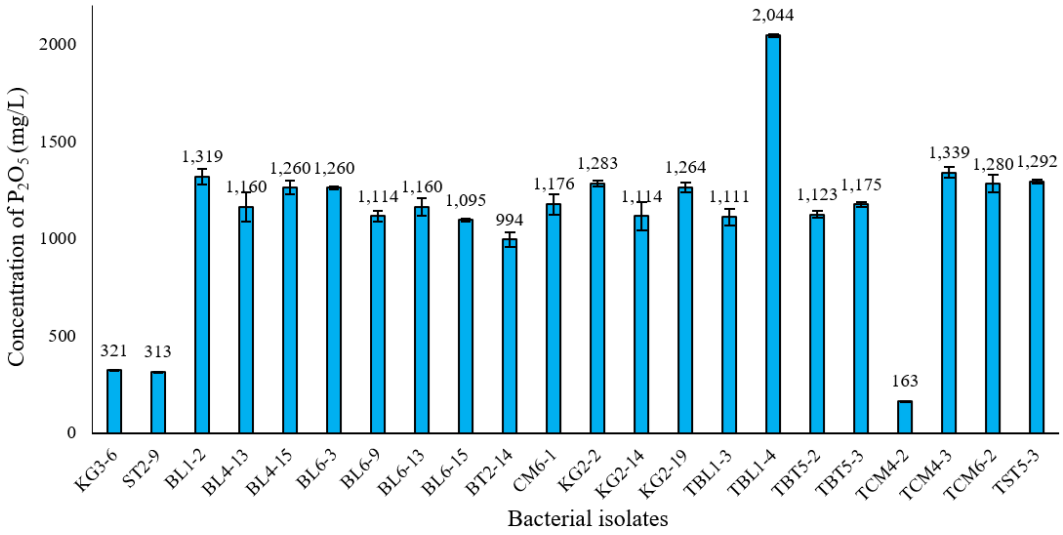

Figure 2. The highest soluble phospohrous concentration in NBRIP liquid culture of selected 22 isolates from 95 bacterial strains isolated within 15 days of incubation.

while eighteen strains from 95 isolated had ability to liberate phosphorus into the liquid culture medium between 163 and 1,339 mg/L P_2O_5 after 15 days of incubation. Therefore these 19 strains of bacteria with the best capacity in dissolving phosphorus were selected to determine the activity of phosphatase enzymes in the next evaluation. In addition, the time to reach the maximum peak of phosphorus concentration in liquid medium of each isolate was also fairly different from each other, but most of the cases the highest peak of P_2O_5 concentration was found at day 15 after inoculation, while 4 others were found highest at day 3 and day 5 after inoculation.

In a comparison with other previous studies, it was found that Wu et al. (2012) isolated a very highly effective phosphorus solubilizing fungus from rhizospheric soil with the maximum amount of 1,252 mg/L of P_2O_5 after 40 hours in a liquid medium containing tricalcium phosphate (TCP) as sole phosphate source. Similarly, Tan et al. (2016) obtained 25 fungal strains from six ferralsol samples of Tithonia and evaluated their solubilizing capacity for insoluble phosphate ($Ca_3(PO_4)_2$) in liquid medium. The result showed that these isolated fungi could dissolve phosphorus from insoluble form arranged 37.25-494.41 mg/L of P_2O_5. Additionally, Walpola and Yoon (2013) isolated phosphate solubilizing bacteria in soil in South Korea, and showed that they dissolved TCP with maximum P solubilization at 720.75 μg/L. Saikrithika et al. (2012) evaluated the amount of phosphorus solubility from TCP of a bacterial strain isolated from vermi-compost applied soil. It was able to solubilize up to 125 mg/L of P_2O_5. For mineral phosphate solubilization capacity, Krishnaraj and Dahale (2014) concluded that 53 isolated strains including bacteria, fungi, actinomyces from many previous studies could solubilize and liberate phosphorus with a range of 100-500 mg P_2O_5/mL. Many studies have shown that P solubilizing microorganisms can secrete a variety of low-molecular organic acids during metabolisms, such as malic acid, propionic acid, lactic acid, acetic acid and citric acid. These organic acid anions could react with calcium ions in the liquid medium to release P from moderately soluble phosphorus (Lin et al., 2001). Besides, some extracellular enzymes especially phosphatase, even ammonium salts and nitrate salts, etc. are released by microbes into liquid medium, leading to dissolve highly insoluble TCP [30] (Krishnaraj and Dahale, 2014).

3.3 Phosphatase enzyme activity of 19 selected strains

The ability to synthesize phosphatase enzymes by 19 selected isolates from different ecosystem habitats is presented in Table 1 and it can be seen that the amount of phosphatase produced by different isolates was significantly different when compared with each other. In fact, 3 strains BL4-15, TCM6-2, TBT5-3 had highest ability in synthesizing phosphatase enzyme as compared to other remaining strains ($p<0.05$) and this enzyme activity of these three bacteria was ranged 0.207-0.377 (U/mL), whereas the other 16 strains had lower enzyme activity of phosphatase (0.003-0.170 U/mL). In which, the strain, TBT5-3 after 6 days of incubation, the synthesized phosphatase was 0.139 (U/mL), but at day 10 the concentration was 0.377 (U/mL) and at day 14, 0.247 (U/mL) of phosphatase was produced and was considered as the highest phosphatase producing isolate as compared with other strains ($p<0.05$). When taking into account the phosphatase enzyme activity and solubilized phosphorus amount in the liquid culture medium of this bacterial strain together, it can be seen that this bacterial strain did not solubilize $Ca_3(PO_4)_2$ well as compared to other strains. Thus this result means that not only phosphatase enzymes but other enzymes and organic acids also were involved in the phosphorus solubilization.

Table 1. Phosphatase enzyme activity of 22 selected bacterial strains in liquid medium (n=3)

No.	Code of bacteria	Phosphatase enzyme activity in liquid NBRIP containing 1% NaCl (U/mL)		
		6 day	10 day	14 day
1	BL1-2	0.130^{a}	0.098^{c}	0.060^{d}
2	BL6-5	0.005c	0.007^{f}	0.003^{g}
3	TBT5-2	0.005^{c}	0.012^{f}	0.005^{fg}
4	BL6-3	0.085^{b}	0.102^{c}	0.033^{e}
5	BL6-9	0.098^{b}	0.029^{ef}	0.022^{ef}
6	BT2-14	0.022^{c}	0.057^{d}	0.021^{ef}
7	BL4-13	0.004^{c}	0.043^{de}	0.019^{efg}
8	TCM4-3	0.009^{c}	0.030^{def}	0.018^{efg}
9	TBL1-4	0.012^{c}	0.031^{def}	0.020^{efg}
10	KG2-2	0.090^{b}	0.107^{c}	0.053^{d}
11	BL6-13	0.020^{c}	0.016^{ef}	0.021^{ef}
12	BL4-15	0.028^{c}	0.029^{ef}	0.207^{b}
13	TCM6-2	0.084^{b}	0.141^{b}	0.214^{b}
14	TBT5-3	0.139^{a}	0.377^{a}	0.247^{a}
15	CM6-1	0.012^{c}	0.019^{ef}	0.014^{fg}
16	KG2-19	0.017^{c}	0.024^{ef}	0.016^{fg}
17	KG2-14	0.010^{c}	0.019^{ef}	0.016^{fg}
18	TST5-3	0.012^{c}	0.013^{f}	0.014^{fg}
19	TBL1-3	0.098^{b}	0.153^{b}	0.170^{c}
	F	*	*	
	CV (%)	100	125	128

Note: Values in the same column with the same letters are not significant difference at 5% level of Tukey's test.

Lin et al. (2001) explained that P solubilizing microorganisms could secrete a high variety of low-molecular organic acids during metabolism, such as malic acid, propionic acid, lactic acid, acetic acid and citric acid. These organic acid anions could react with calcium ions in the liquid medium to release P from modestly soluble phosphates. Moreover, Giles et al. (2018) and Darch et al. (2016) showed that organic anions and phosphatase enzyme could be combined together to solubilize inorganic P from an organic P source. Similarly, according to Clarholm et al. (2015) indicated that the importance of this association was used as the unbutton model. Besides, some extracellular enzymes and even ammonium salts and nitrate salts, etc. were released by microbes into liquid medium, leading to dissolve highly insoluble TCP (Krishnaraj and Dahale, 2014). In short, in this study many phosphate solubilizing bacterial strains including the strains TBT5-3 solubilized well tricalcium phosphate and based on the result phosphatase enzyme was also one of the key factors to control the phosphorus solubilization. Therefore, the TBT5-3 strain was selected to genetically identify and investigate the environmental conditions regulating the phosphatase enzyme activity of this strain.

Several previous studies have reported that insoluble inorganic phosphorus could be dissolved by low molecular weight organic acids (eg, citric and gluconic acids) from phosphorus solubilizing bacteria and fungi (Sashidhar and Podile, 2010; Ogbo, 2010; Patel et al., 2011), and organic phosphorus can be solubilized by extracellular enzymes (eg, phosphatase and phytase) from bacteria (Tan et al., 2016; Neal et al., 2017). Similarly, Palacios et al. (2004) isolated and identified the lactic acid bacteria *Lactobacillus pentosus* CECT 4023 as a strong phosphatase enzyme producer with an activity of 94.4 (U/mL). Besides, Rombola et al. (Rombola et al., 2014), isolated *Burkholderia gladioli* R 3.25 from roots of *Zea mays* in Ponta Grossa-PR-Brazil and this strain had an ability to produce phosphatase ranging 103-113 (U/mL) depending on the substrate and pH medium. Similarly, the *Serratia* sp. PSB-37 was isolated from soil samples obtained from different locations within the mangrove forests of the Mahanadi River delta, Odisha, India with salt tolerance and enzyme production (76,8 U/mL) was recorded after 48 h of incubation (Behera et al., 2017). As compared this study and the previous studies it can see that the phosphatase enzyme activity of bacteria in this study (TBT5-2) was much lower than the previous study.

3.4 16S rRNA gene sequence

Result of 16S rRNA gene sequence of the best selected bacterial isolate, TBT5-3 is presented in Table 2. When comparing the 16S rRNA gene of isolate with the database on the world genbank BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi.), the results showed that this bacterial strain belonged to the *Bacillus* genera. Especially, when combining with colony morphological characteristics like circular colony, convex colony, milky and smooth-face, rod-shape and positive gram cells, it can see that this isolate from saline soil in Ben tre Province had a 100% identification index with *Bacillus megaterium*. Thus, they were identified as *Bacillus* sp. TBT5-3. This result was consistent with the morphological descriptions from other publications (Rahmansyah and Sudiana, 2010; Suleimanova et al., 2015).

Table 2. The genetic identification of strain TBT5-3

Isolated strain	Origins of saline soil	Similarity (%)	Bacteria strain on database		Identified as
			Strain	Registration number	
TBT5-3	Ben Tre	100	*Bacillus megaterium*	*NR_112636*	*Bacillus sp.* TBT5-3

3.5 Affecting the cultural conditions on the activity of phosphatase enzyme

3.5.1 Different sharking speeds on phosphatase enzyme activities

Surveyed results on phosphatase enzyme activity at different shaking speeds 0, 60, 80, 100 and 120 rpm are presented in Figure 3. The result showed that agitation rate had an strongly effect on the synthesis of phosphatase enzyme, however at the speeds of 60, 80, and 100 rpm, the activity of

enzyme was not much different from each other, but at the sharking speed of 120 rpm the activity of phosphatase enzyme was achieved highest (0.444 U/mL), almost 3 times higher than the other shaking speed treatments. Perhaps at this high speed an adequate amount of air was introduced into the medium to supply enough dissolved oxygen requested by bacteria (Behera et al., 2017). In contrast, the phosphatase enzyme activity was found to be lowest at a shaking speed of 0 rpm (0.092 U/mL). At a speed of 60, 80 and 100 rpm, the activity of phosphatase was 0.130, 0.013 and 0.154 U/mL, respectively. The variation in the speeds has been found to influence the extent of mixing of oxygen in the liquid culture in the flasks and also affects the nutrient availability. Variation in agitation or shaking speed has been found to influence the extent of enzyme production (Nascimento and Martins, 2004). Especially, high shaking speed can increase the oxygen content in the liquid culture medium but cannot increase enzyme production because the agitation rate could change the structure of the enzyme (Roychoudhury et al., 1988). In the liquid culture, reducing the aeration rate could reduce enzyme production (Wang et al., 2006). In this study, optimal phosphatase production by the bacterial isolate, TBT5-3, was observed at 120 rpm.

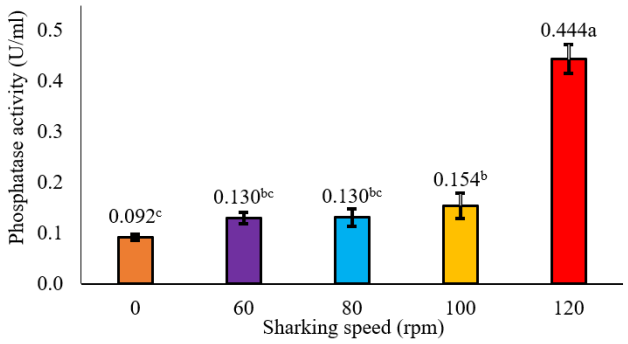

Figure 3. The activity of phosphatase of the strain TBT5-3 at different agitation rates (n=3).

3.5.2 Different sodium chloride concentrations on phosphatase enzyme activities

The results of the experiment to assess the effects of different NaCl concentration on phosphatase enzyme activities of TBT5-3 are presented in Figure 4. It was clear that the highest phosphatase enzyme activities was found in the treatments supplemented with 1% NaCl and 2% NaCl in which the enzyme activities were 0.484 U/mL, 0.464 U/mL, respectively, but not significantly different when compared these two treatments together. In contrast, the lowest phosphatase activity was found in the 3% NaCl treatment (0.111 U/mL). The activity of phosphatase of 0% NaCl and 0.5% NaCl was 0.211 U/mL and 0.228 U/mL, respectively and was not significantly different ($p>0.05$) when comparing these two treatments together. The research showed the concentration of NaCl had an importance for the liquid culture medium to enhance the activity of the phosphatase enzyme.

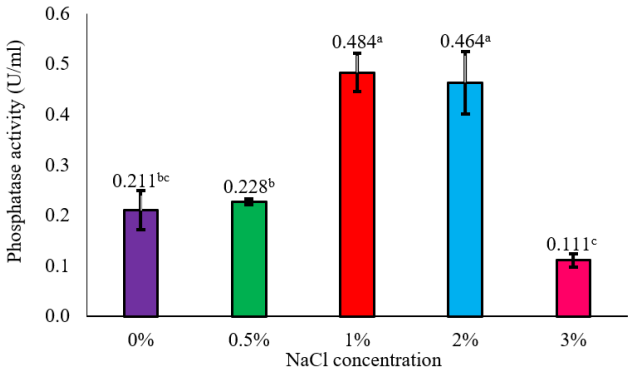

Figure 4. The activity of phosphatase of TBT5-3 strain at different NaCl concentrations in liquid culture medium (n=3).

The results of this study are consistent with the previous studies which concluded that NaCl concentration had a strong effect on enzyme activities. Particularly, Aqel (2012) showed that the maximum and optimum phosphatase activity was at concentration of sodium chloride from 0.5 to 1% for all tested bacteria in their experiments. Similarly, the results by Bylund et al. (1990) showed that the optimal phosphatase activity of *Halomonas elongates* at 1.37 and 3.4 M of NaCl.

3.5.3 Different pH levels on phosphatase enzyme activities

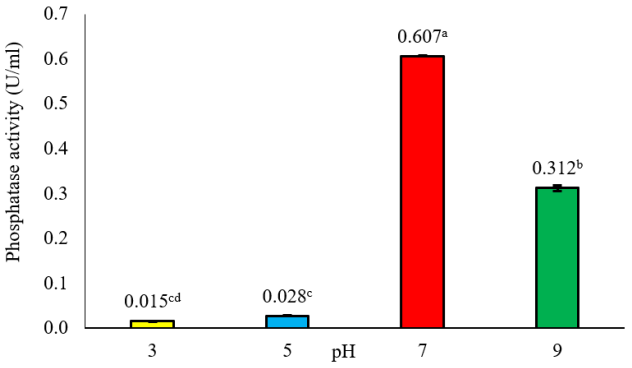

Figure 5. The activity of phosphatase enzyme by strain TBT5-3 at different pH levels of the liquid culture medium (n=3).

Phosphatase enzyme activities in different pH level liquid culture media are presented in Figure 5. It was clear to show that different pH levels gave a significant difference in enzyme activities ($p<0.01$). Noticeably, phosphatase enzyme activity was observed to be highest at pH 7 with an amount of 0.607 U/mL, followed by pH 9 (0.312 U/mL). In contrast, pH 3, and pH 5 treatments gave the lowest phosphatase activity of 0.015 U/mL, and 0.028 U/mL, respectively. It was clear to see that under the acidic environment of liquid culture medium (pH=3, and 5) the activity of phosphatase enzymes

decreased dramatically. This could be explant by the fact that a decrease in pH medium beyond the optimum value could affect the active site of the amino acids as the enzyme which was unable to form an enzyme substrate complex and thus there is decrease in enzyme activity (Sasirekha et al., 2012). Therefore, Padan et al. (2005) suggested that in order to evaluate the maximum crude enzyme production, there was a need to evaluate the optimal growth conditions required. The optimum pH for most aquatic bacteria is between pH 7 and 8.5.

3.5.4 Different carbon sources on phosphatase enzyme activities

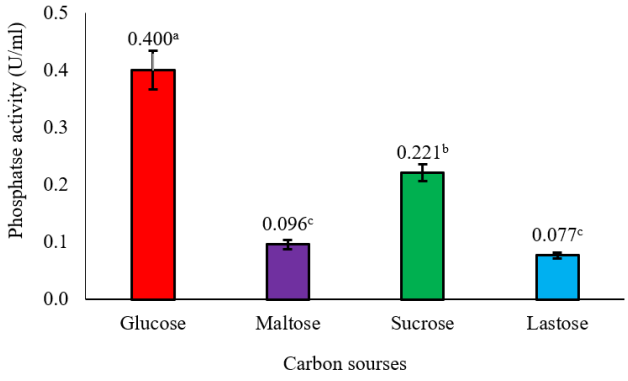

Figure 6. The activity of phosphatase enzyme of strain TBT5-3 with different carbon sources (n=3).

The results presented in Figure 6 revealed that the different carbon sources affected differently enzyme activities produced by *Bacillus* sp. TBT5-3 isolate. During the surveyed period, glucose was found to be the best carbon source in helping to produce phosphatase enzyme activity as compared with other three remaining carbon source treatments showing the enzyme activities of 0.4 U/mL whereas the phosphatase enzyme activity in the treatment containing sucrose was 0.22 U/mL as ranked as the second position. In contrast, two treatments including maltose and lactose had the lowest phosphatase enzyme activity and were not significantly different ($p>0.05$) with each other. These results are also in agreement with Behera et al. (2017) reported that when cultured with *Serratia* sp. PSB-37 in liquid culture containing glucose as an original carbon source, the activity of phosphatase was achieved highest. However, Aqel (2012) reported that most *Bacillus* strains showed their maximum and optimum capacity in synthesizing phosphatase when there was a presence of galactose except for *Bacillus* strain 35 which preferred fructose and sucrose. The presence of either glucose or lactose could lead to inhibit the activity of the enzyme produced from *Bacillus* strain 14. Therefore, the activity of the phosphatase enzyme produced by each carbon substrate differed from each other, depending on the type of carbon source which was preferred by organisms. The presence of individual carbohydrates in the culture media affected the abundance of alkaline phosphatase, indicating the differences in metabolic flexibility among these microorganisms (Elena et al., 1999). For this present study, the bacterial isolate, TBT5-3, only showed its maximum phosphatase production in the liquid culture medium supplemented with glucose as a carbon source.

3.5.5 Different nitrogen sources on phosphatase enzyme activities

Phosphatase enzyme activity of TBT5-3 in different nitrogen sources is presented in Figure 7, showing that there was statistically significant difference among treatment in terms of the phosphatase activity. The highest activity of phosphatase was obtained at 0.613 U/mL in the treatment adding urea as the main nitrogen source for bacteria. In contrast, the lowest concentration of this enzyme was achieved at 0.120 U/mL in the ammonium molybdate supplemented treatment as the major nitrogen source. The average phosphatase enzyme activity in two treatments including potassium nitrate and ammonium sulfate were fairly high and were 0.442 U/mL and 0.317 U/mL, respectively.

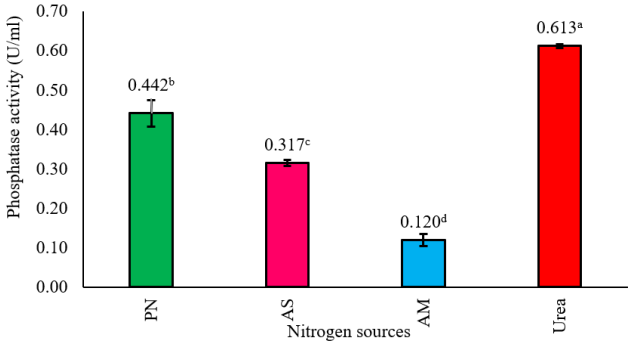

Figure 7. The activity of phosphatase enzyme of strain TBT5-3 in liquid culture media containing different nitrogen sources (n=3).

Note: PN: potassium nitrate; AS: ammonium sulfate; AM: ammonium molybdate.

This result is consistent with the result study of Behera et al. (2017) showed that the strain *Serratia* sp. PSB-37 when cultured in ammonium sulfate containing medium as an original nitrogen source had a maximum phosphatase activity. Moreover, the amount of enzyme activity produced in each different substrate treatments differed depending on the type of nitrogen source preferred by organisms. Investigations on the impact of nitrogen supplements on enzyme production revealed that not all nitrogen sources would act as an enhancer for the production of enzymes (Behera et al., 2017).

4 Conclusion

In summary, bacterial strains isolated from different saline soil samples in many different provinces in the Mekong River Delta of Vietnam had highly different capacities in phosphorus solubilization. From 15 saline soil samples in 5 different

provinces belonging to the Mekong River Delta of Vietnam, 95 bacterial strains of salt-tolerant phosphorus solubilizing bacteria were isolated. Among that, 19 isolates were identified as good phosphorus solubilizers. Especially, TBT5-3 was genetically identified as *Bacillus megaterium* and was found to be the strongest phosphatase enzyme activity. The environmental factors of the culture medium such as pH, carbon source, nitrogen source, NaCl, and sharking speed strongly influenced the enzyme activities of this phosphatase. The optimum medium conditions for this enzyme activity were pH 7, 1-2% NaCl, glucose 1% and urea 0.1% and shaking speed of 120 rpm. Therefore, it is possible to exploit *Bacillus megaterium* TBT5-3 as microbial fertilizer to increase green crops yield, reduce phosphate chemical fertilizers application for a safe and sustainable agricultural production.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data.

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