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Full Length Article

Organophosphorous Solubilizing Mechanism of *Pseudomonas* spp. Isolated from Rhizosphere of Chinese Pine

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Abstract

Phosphorus is an essential element for plants, whereas they cannot use most of phosphorus, which is fixated by calcium, aluminum, iron and organic compounds. Phosphorus solubilizing microorganism (PSM) and its secreting extracellular enzymes have been widely reported in the process of converting soil fixed phosphorus into a bioavailable form. Hence in this study two strains of organophosphate solubilizing bacteria (YP-1, YP-4) were isolated from the rhizosphere soil of Chinese pine (*Pinus tabulaeformis*) in Qingshuihe county (in Hohhot, P. R. China). The organophosphate solubilizing activity was determined to 24.42 ± 0.52 μg mL^{-1} and 23.47 ± 0.38 μg mL^{-1} for YP-1 and YP-4, respectively. YP-1 was identified as *Pseudomonas sp*. YP-4 was identified as *Pseudomonas reinekei* based on phenotypic and molecular characterization, respectively. The most favorite carbon and nitrogen sources were sucrose and $(NH_4)_2SO_4$ for YP-1, whereas glucose and KNO_3 for YP-4 in the cultural medium. The extracellular catalytic proteins secreted by microorganisms have the ability to solubilize phosphorus. The extracellular enzymes of two strains were also extracted and determined by Sephadex G-150 method. The molecular weights were 39.8 kDa (Kilodalton) from YP-1 and 38.0 kDa from YP-4. As for the optimized conditions for enzyme catalyzing activities, except for 40ºC and 60 min reaction time, the variations were discovered at pH 3.0 and pH 9.0 for YP-1 and YP-4, respectively. By using mass spectrometry, it was found that the corresponding enzyme derived from YP-4 could match 25% to alkaline phosphatase. We conclude that the two strains and their extracellular enzymes can solubilize organophosphate. It is envisioned that the two strains PSM and their related enzymes may find wide applications in future agricultural and biotechnological fields. © 2019 Friends Science Publishers

Keywords: Organophosphate; Bacteria; Enzyme; Chinese pine

Introduction

Chinese pine (*Pinus tabuliformis*) is one of the most widespread tree species and the main species for afforestation, with economic, social, environmental, and ornamental values in Northwestern China (Yuan *et al*., 2016). However, the deficiency of available phosphorus in soil environment leads to growth retardation, dark leaves and inhibition of seeding and rooting development system (Li *et al*., 2006; Zeenat *et al.*, 2018). Phosphorus is a main component of soil nutrients and is one of the indispensable elements for plant growth, which profoundly affect the overall growth progress of plants by influencing metabolic rate-limiting enzyme such as cell division and development, energy transport, signal transduction, macromolecular biosynthesis and photosynthesis (Khan *et al.*, 2002; Shenoy and Kalagudi, 2005; Ahemad *et al.*, 2009; Wang *et al.*, 2009; Sadiq *et al.*, 2013; Rubya and Lqbal, 2016). Phosphorus is one of the deficient elements, compared to the other major essential macronutrients (with the exception of N). On average, organophosphate is between 30% and 65% of total phosphorus in mineral soils. In organic soils (>20–30% organic matter), organophosphate can approach up to 90% of the total phosphorus (Jones and Oburger, 2011). In addition, phosphate fertilizer is used to promote plant growth in a long-term and later become fixation insoluble form, which results in low absorption by the plants and large accumulation of phosphorus in the soil (Sundara *et al.*, 2002).

The rhizosphere phosphorus microorganisms are capable of solubilizing organophosphate into a bioavailable form *via* uptake and release of organic and inorganic ions and molecules, including mineral equilibria (the release of complexing or mineral dissolving compounds, such as

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organic acid anions, siderophores, protons, hydroxyl ions, and CO_2) and mineralization (the conversions of phosphorus between organic and inorganic forms mediated by extracellular enzymes) (McGill and Cole, 1998; Sims and Pierzynski, 2005). Almost 1–50% of soil bacteria can be classified as phosphorus converting microorganisms (Gyaneshwar *et al.*, 2002). Organophosphate solubilizing bacteria can efficiently degrade organophosphate, including species of the common soil bacteria (*Pseudomonas*, *Azotobacter*, *Burkholderia*, *Bacillus* and *Rhizobium*). Organophosphate mineralization is that the organophosphate converting bacteria convert organophosphate into a bioavailable form by secreting some enzymes (*e.g*., phosphatase, nuclease and phytase) in soil environment (Jin *et al.*, 2016). Mineralization of organophosphate like nucleic acids and lecithin under favorable conditions are carried out by nuclease and lecithinase. The hydrolysis of both esters and anhydrides of H_3PO_4 can be catalyzed by phosphatases or phosphohydrolases (Tabatabai, 1994). These enzymes are classified as acid and alkaline phosphatases because their maximum activities are related to pH of reaction conditions. The phytic acid to myoinositol and phosphate can be broken by phytase via intermediate inositol polyphosphate (Abul and Charles, 1993; Ramani, 2014). A broad group of enzyme converts organophosphate pesticides into small molecules (non-toxic and soluble in water) by cutting off molecular bonds in organic compounds such as P-F bonds, P-CN (Cheng *et al.*, 1993). These enzymes are widely found in microorganism (*Eschericia coli*, *Bacillus subtilis*, *Pseudomonas*, etc.), animals and plants. In addition, these enzymes probably have potential application for environmental monitoring, pollutant handling, and biosensors (Labry *et al.*, 2005; Fernandez and Kidney, 2007; Tian and Zhou, 2007).

Keeping the above notions in consideration, this study focused on organophosphate solubilizing bacteria identified and characterized from the rhizosphere soil of Chinese pine in Qingshuihe County, Hohhot, Inner Mongolia. Furthermore, their extracellular enzymes to solubilize organophosphate has been purified, identified and characterized, which may have potential industrial, agricultural and biotechnological application.

Materials and Methods

Bacteria Isolation and Biochemical Characterization

Organophosphate solubilizing bacteria were isolated from soil samples which were obtained from mixed soils of the different locations within Chinese pine in Qingshuihe County, Hohhot, Inner Mongolia. Soil samples (10 g) were serially diluted and coated in triplicates on Egg Yolk Solid Medium. The organophosphate solubilizing bacteria can produce phosphoric acid by solubilizing the organophosphate, so that a transparent circle is formed around the colony. Colonies forming clear surrounding halos were selected as organophosphate solubilizing bacteria and named as YP. The morphology of organophosphate solubilizing bacteria was observed by Gram staining. The physiological and biochemical identification was done according to the bacteriological manual, including the contact enzyme test, methyl red test (MR test), acetyl-methyl methanol test (V-P test), indole test, gelatin liquefaction test, starch hydrolysis test, nitrate reduction test, lecithin test, malonate test, citrate utilization test and sugar fermentation tests (Du *et al.*, 2008).

Molecular Identification of the Bacterial Strain

Extraction of genomic DNA from organophosphate solubilizing bacteria by the CTAB (cetyltrimethylammonium Ammonium Bromide) method, was as described by Chen *et al*. (2006). The 16S rRNA gene of organophosphate solubilizing bacteria were amplified by PCR (polymerase chain reaction), using universal 27F forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R reverse primer (5'-CTACGGCTACCTTGTTAC GA-3'). PCR product was purified using TIANgel Midi Purification Kit, TIANGEN, Beijing (China), then ligated with the cloning vector pMD19-T and transferred to *E. coli* (DH5α). Nucleotide sequences were determined by HuaDa Gene Company, Beijing. The final partial sequence was submitted to NCBI gene bank in order to obtain the similar sequence. High sequence similarity was selected as reference strains. The phylogenetic tree of the tested strain and the reference strain were constructed by MEGA7.0 software, using the neighbour-joining DNA distance algorithm with a bootstrap of 1000.

Quantitative Estimation of Bacterial Ability to Degrade Organophosphate

The organophosphate solubilizing bacteria were isolated in triplicate, from egg yolk solid medium after 4 days at 30ºC.The diameter of dissolved phosphorus transparent circle (D) and the diameter of the colony (d) were determined by a vernier caliper. Preliminary determination of the ability of strains to solubilize phosphorus was determined by calculating the size of D/d. The organophosphate solubilizing bacteria were inoculated into the beef extract peptone liquid medium, containing: Beef extract 3.0 g; NaCl 5.0 g; Peptone 10.0 g. The flasks were incubated in an incubator shaker at a shaking speed of 160 rpm for 37℃, the bacteria fermentation broth was measured at 660 nm every half hour until the OD was 0.5. After 1% inoculation was added to the 30 mL Monkina's medium, (glucose 10 g; $CaCO_3$ 5 g; NaCl 0.3 g; KCl 0.3 g;$(NH_4)_2SO_4$ 0.5 g; $MnSO_4 \cdot H_2O$ 0.03 g; $FeSO_4 \cdot 7H_2O$ 0.03 g; pH 7.0 ~ 7.2), the flasks were incubated at 30 ℃ in an orbital shaker at 160 rpm for 6 days. In triplicate, non-inoculated medium served as a control. Subsequently, centrifugation for 20 min

(4°C, 10000 rpm). 5 mL reagent (containing: Ammonium molybdate 1.0 g; H_2SO_4: 15.3 mL; Antimony potassium tartrate: 10 mL; Ascorbic acid; 1.5 g), and 20 mL distilled water was added to 25 mL of the supernatant in 50 mL volumetric flask, and mixed. After setting aside for 30 min at room temperature, the absorbance was measured at 660 nm. The corresponding phosphorus concentration was checked on the standard curve and the phosphorus content was calculated. The difference of soluble phosphorus content between the experimental and the control group was used as the ability of the tested strain to solubilize phosphorus (μg L^{-1}). Three replicates were used. With $(NH_4)_2SO_4$ as the nitrogen source, carbon sources were glucose, sucrose or maltose (the carbon source concentration was 10 g L^{-1}), and with glucose as the carbon source, nitrogen sources were $(NH_4)_2SO_4$, urea or KNO_3 (the nitrogen source concentration was 0.5 g L^{-1}). The phosphorus contents were then detected.

Isolation and Purification of Extracellular Enzyme with Ability to Solubilize Phosphorus

The organophosphate solubilizing bacteria were inoculated in Monkina's medium, 37°C in an orbital shaker at 160 rpm for 48 h. Then centrifuged (4°C, 10000 rpm) for 20 min to obtain cell-free culture supernatant. The supernatant was lyophilized by vacuum freeze-dried and used 5 mL Tris-HCl (50 m*M*, pH 7.0) to dissolve the lyophilized powder. Lyophilized supernatant was purified by filtration chromatography, using the Sephadex G-150 gel (GE Healthcare, Sweden). The elute was Tris-HCl (50 m*M*, pH 7.0) at a flow rate of 23 mL min^{-1}. The peak at 280 nm was detected by a nucleic acid protein analyzer and then collected the elute with protein, thereby obtaining purified extracellular enzyme. The reaction between 1 mL of each peak collection solution and 24 mL Monkina's medium as substrate in erlenmeyer flasks containing were started at 30℃ for 90 min; then added 20% trichloroacetic acid to terminate the reaction, with 5 mL reagent and distilled water to the volume of 50 mL. After setting aside for 30 min at room temperature, absorbance was measured at 660 nm and the phosphorus content was calculated. The purity of the obtained enzyme with ability to solubilize phosphorus was analyzed by 12% SDS-PAGE. The samples were stained with Coomassie Blue R-250. The standard curve constructed by measuring the migration distance between protein standard and target protein with indicator, respectively. The molecular weight of the protein with the ability to solubilize phosphorus was calculated according to the standard curve and was initially identified. A single target protein band was cut for mass spectrometry analysis with a mobile phase: A: 0.1% formic acid solution; B: 0.1% formic acid-acetonitrile solution; flow rate: 300 mL min^{-1}; elution gradient: 3 to 8% B elution for 1 min, 8 to 40% B elution for 5 min, 40 to 85% B for 1 min and finally eluted with 85% B for 1 min; Full MS (resolution: 70000; scanning range: 350-1600); dd-MS2: (resolution: 17500); and injection volume of 10 μL.

Characterization of Partially Purified Extracellular Enzyme

The characterization of partially purified enzymes activity was studied by changing the reaction parameters, such as pH from 3.0 to 11.0, temperature (at 10°C intervals) from 20 to 60°C, and the reaction time of 0 to 120 min. The reaction between 1 mL of each peak collection solution and 24 mL Monkina's medium as substrate in erlenmeyer flasks containing were started at 30°C for 90 min; then added 20% trichloroacetic acid to terminate the reaction, with 5 mL reagent and distilled water to the volume of 50 mL. After setting aside for 30 min at room temperature, absorbance was measured at 660 nm and the phosphorus content was calculated by the formula (Tsang *et al*., 2007).

Statistical Analysis

Data on organophosphorus solubilizing capacity under different conditions were analyzed of multiple comparisons (ANOVA) (SPSS 20.0; Koneri *et al*., 2008). Significant effects of treatments were determined by the magnitude of F value ($P \leq 0.05$).

Results

Isolation and Identification of Organophosphate Solubilizing Bacteria

Organophosphate solubilizing bacteria were isolated from the rhizosphere soil of Chinese Pine. Two strains of organophosphate solubilizing bacteria were isolated and named YP-1 and YP-4. Based on the morphological and biochemical analysis, YP-1 was rod-shaped, gelatin liquefaction, citrate, and fermentation of glucose were positive, whereas gram staining, methyl red, VP tests, indole tests, amylase, nitrate reduction tests, lecithin enzyme tests and fermentation of lactose, sucrose and maltose were negative. YP-4 was found to be resembled with YP-1 about morphological and biochemical analysis, except the fermentation of glucose, which was negative. Further, genus Pseudomonas was confirmed by BLAST analysis data of the 16S rRNA gene sequence. YP-1 showed 99% similarity with the genus *Pseudomonas* spp. (KC355362). YP-4 showed 99% similarity with the *Pseudomonas reinekei* (LT629709.1). By comparing the nucleotide sequences of 16S rRNA sequences of the isolates YP-1 and YP-4 with different *Pseudomonas* spp. from NCBI database, a phylogenetic tree was constructed, we found that the YP-1 is most closely related to *Pseudomonas* spp., and YP-4 is most closely related to *P. reinekei* (Fig. 1).

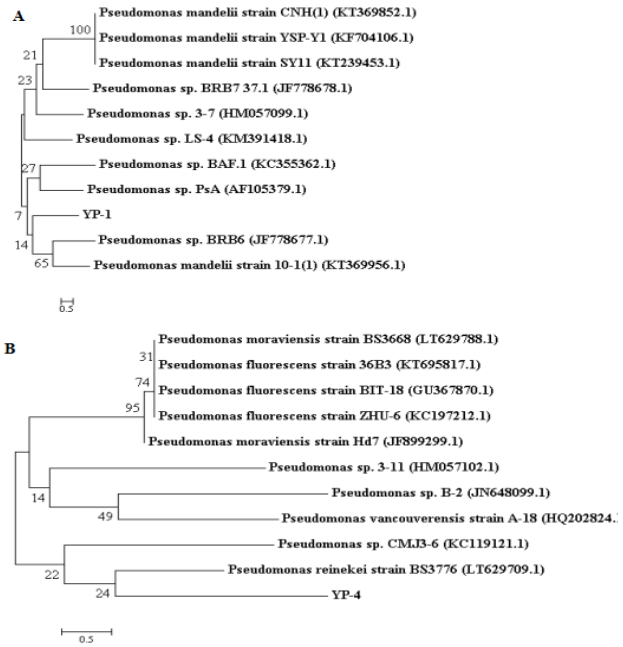

Fig. 1: Phylogenetic tree based on 16S rRNA gene sequences by Neighbor Joining method (using MEGA 7.0), showing the relationship between (**A**) YP-1, (**B**) YP-4 and other members of the *Pseudomonas* spp. The Genbank nucleotide accession numbers are listed next to the strain names. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The scale bars represent 0.5 substitution/site

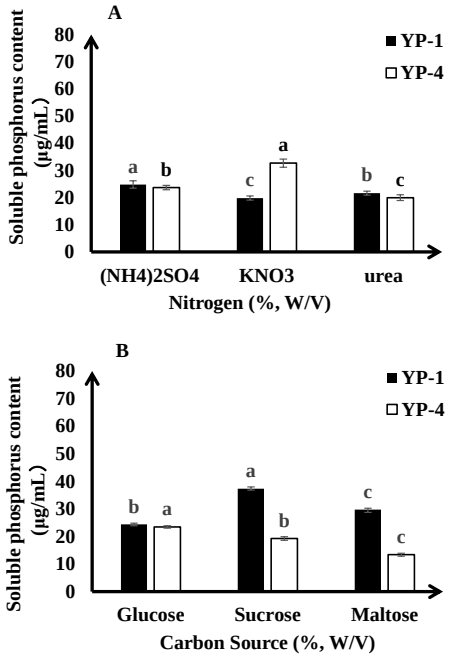

Fig. 2: Influence of different culture medium: (**A**) Nitrogen sources and (**B**) Carbon sources on crude enzymes production by the degrade, YP-1 and YP-4. Different superscripts indicate significant difference among different strain (Lowercase letters are significant, ANOVA, Duncan's test; $p < 0.05$)

Solubilizing of Organophosphate

In evaluation of the organophosphate solubilizing activity of the strain *Pseudomonas* spp., the D/d of the YP-1 was 3.15, and the YP-4 was 2.27. Further, the definite activity of the bacteria was detected by combining with quantitative methods. The soluble phosphorus content in the supernatant was used to indicate ability to solubilize organophosphate of the bacteria. When the carbon source was sucrose, the nitrogen source was $(NH_4)_2SO_4$ in the Monkina's medium, the organophosphate solubilizing activity of the bacteria strain YP-1 was the highest. With the addition of glucose and KNO_3 to the culture medium, YP-4 had highest activity for solubilizing organophosphate (Fig. 2A and B).

Purification of Extracellular Enzyme

The extracellular proteins secreted by YP-1 or YP-4 were isolated and purified by Sephadex G-150 gel (Fig. 3 and 4). It was found that only one protein was secreted into the cell fermentation supernatant by the YP-1, was (21.94 ± 3.82 μg mL^{-1}). The YP-4 was capable of secreting two proteins, while only one of the enzyme had ability to degrade organophosphate, was (16.14 ± 0.98 μg mL^{-1}). The smaller protein of YP-4 had no activity.

Identification of Protein that Solubilize Organophosphate

According to the standard curve of protein electrophoresis (y=-0.9954x+5.1857), the molecular weight of the extracellular enzymes of YP-1 and YP-4 were calculated as 39.8 kDa and 38.0 kDa, respectively (Fig. 5). After mass spectrometry determination, the extracellular enzyme secreted by YP-1 did not find a matching protein, whereas the extracellular enzyme secreted by YP-4 was matched with a alkaline phosphatase, had the highest coverage 25%, protein accession for WP_016986280.1.

Characterization of Extracellular Organophosphate Solubilizing Enzymes

Enzymes activities were greatly influenced by the incubation temperature (Fig. 6A). The optimum temperature for the enzymes activity were 40℃, with enzymes activities of 67.68 ± 0.18 μg mL^{-1} (YP-1) and 23.78 ± 0.59 μg mL^{-1} (YP-4), respectively. The enzymes activities of YP-1 had a large difference at different temperature conditions. The enzymes activities of YP-1 showed "decrease first and then increase" with increasing pH (Fig. 6B). The maximum activity of enzyme was recorded (11.01 ± 0.18 μg mL^{-1}) at pH 3.0. At pH 9.0, the enzyme of YP-4 had the greatest ability to solubilize organophosphate, the content of soluble

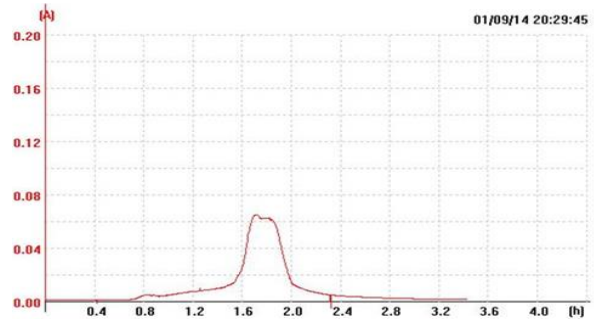

Fig. 3: The extracellular enzyme of YP-1 was purified by Sephadex G-150 gel chromatography. A peak appeared at 1.8 h

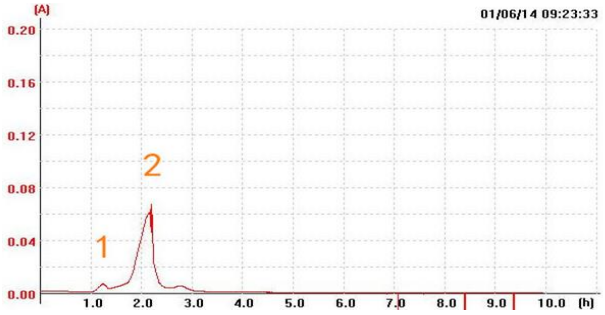

Fig. 4: The extracellular enzyme of YP-4 was purified by Sephadex G-150 gel chromatography. Two peaks (1 and 2) appeared at 1.2 h and 2.2 h

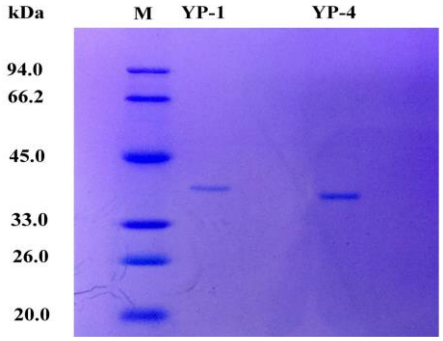

Fig. 5: SDS–PAGE analysis of extracellular proteins was secreted by YP-1 and YP-4. The purified proteins (lane YP-1 and YP-4) were separated by 12% (w/v) SDS–PAGE followed by staining with Coomassie Blue R-250. Lane M: molecular weight markers

phosphorus was 22.05 ± 0.37 μg mL^{-1} in the reaction system. By the assay of enzymes activities with increasing reaction time (Fig. 6C), the reaction between enzyme and organophosphate was accomplished within 30 min.

Discussion

The research of organophosphate solubilizing bacteria is focused on soil microorganisms, and the amount is greater in the rhizosphere (20–40% of the total) as compared to

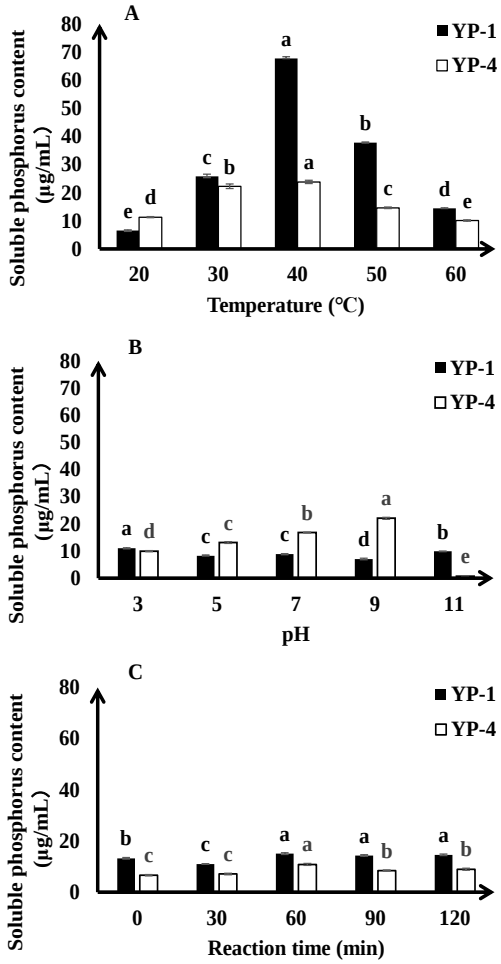

Fig. 6: Effect of different reaction parameters (**A**) Temperature, (**B**) pH, and (**C**) Reaction time on purified enzymes production by the isolate, YP-1 and YP-4. (Lowercase letters are significant. ANOVA, Duncan's test; $p < 0.05$)

non-rhizosphere (10–15% of the total amount) region (Swaby and Sperber, 1958). Two strains of YP-1 and YP-4 with the ability to degrade organophosphate were obtained from rhizosphere of Chinese pine, were *Pseudomonas* spp. and *P. reinekei*, respectively. In comparison to the present study, Jorquera *et al.* (2008) isolated organophosphate solubilizing bacteria from rhizosphere soils of different plants (ryegrass, white clover, wheat, oats and lupines), which exhibited maximum ability of solubilizing phosphorus for 29.92 ± 1.45 μmol L^{-1}. Tao *et al.* (2008) isolated ten organophosphate solubilizing bacteria from subtropical irrigation and temperate non-irrigated soils, which could degrade phosphorus in the range of 13.8 ~ 62.8 μg mL^{-1}. Rhizosphere symbiotic bacteria, *Pseudomonas* spp. and *Bacillus* spp. have strong ability to solubilize organophosphate in the soil (Wei *et al.*, 2014). Lin *et al.* (2000) found that *Bacillus* spp. was the main part of the organophosphate solubilizing bacteria, followed by *Pseudomonas* sp., in the rhizosphere soil of farmland

(wheat-maize), lawn (fescue), woodland (elm) and vegetable land (cauliflower).

According to the size of the halo zone, the YP-4 ability of solubilizing organophosphate was higher than YP-1. However, the organophosphate solubilizing ability of two bacterium were quantitatively determined in liquid culture by molybdenum blue colorimetric method, showing the opposite result. The size of the halo zone around colony is not always accurate to determine the ability about solubilize organophosphate of the bacteria (Behera *et al.*, 2017). This may be due to various diffusion rates of different organic acids secreted by an organism (Nautiyal, 1999). When the lecithin in the Monkina medium became soluble phosphorus and reacted with ammonium molybdate to form ammonium salt, the ascorbic acid was reduced to molybdenum blue (absorbance at 660 nm), organophosphate solubilizing efficiency of microorganisms can be easily detected. Hence, the molybdenum blue colorimetric method is better way to evaluate organophosphate solubilizing efficiency.

The ability to solubilize organophosphate of microorganisms will change as the living environment changes. A wide range of carbon source can be used by microorganisms; differences in carbon sources are significant for specific bacterial strains. As a kind of life element, nitrogen is an important constituent element of proteins and nucleic acids. For this reason, this study further explored the effects of different carbon and nitrogen sources on the organophosphate solubilizing efficiency of YP-1 and YP-4. Similar studies have been conducted by many researchers, they also analyzed the different carbon and nitrogen sources on the growth and physiological function, *Tricholoma mongolicum* Imai (We *et al.,* 2011), *Trichoderma harzianum* (Rajput *et al.,* 2018) and *Beauveria bassiana* (Dale and Shinde, 2018) which indicated the carbon and nitrogen is the major component on the growth and development of organisms.

Determining the catalytic characteristics of enzyme, is not only beneficial to expound the enzyme function and the mechanism of enzyme action, but also has important practical significance in science developing. The activity of purified enzyme is greatly influenced by reaction parameters such as pH, temperature, and reaction time. Many enzyme researchers have also shown that temperature and pH are crucial to enzyme activity (Ortega-Anaya and Santoyo, 2015; Hirata *et al*., 2015; Lorenzen *et al.*, 2017). In order to optimize the soluble phosphorus production, it is necessary to evaluate optimum conditions of these parameters. The pH can affect the ionization of binding and catalyzing sites of enzyme to substrate, alter the charge distribution, thereby affecting activity of the enzyme (Chen *et al.*, 2009; Sasirekha *et al.*, 2012). The extracellular enzyme of YP-1 was stable with the different conditions of pH, thus it is an acid-alkali resistant protein. Since the extracellular enzyme YP-4 had the highest activity at pH 9.0, it is speculated that YP-4 produced an alkaline phosphatase. The number of activated substrate molecules will increase as the temperature increases the reaction speed will accompany by faster. When the temperature reaches the appropriate level, continued increase in temperature will cause the enzyme denaturation, resulting in reaction rate is reduced (Peterson *et al.*, 2007). In addition, it had invalid on enzyme activity with the prolongation of the reaction time, indicating that whole process about the binding and catalyzing of the enzyme and substrate is stable and quick.

In this study, it was found that only one protein was secreted into the cell fermentation supernatant by YP-1, and had the solubilizing organophosphate activity. YP-4 was capable of secreting two proteins, while only one of the enzyme had ability to solubilize organophosphate. These two purified enzyme were detected by SDS-PAGE, protein bands were single, indicating that the protein contains sufficient purity. The corresponding enzymes derived from YP-4 can match 25% to alkaline phosphatase. This result is consistent with the previous speculation that enzyme derived from YP-4 had best activity with pH 9.0. Unfortunately, as the protein sequence is unknown, the enzyme secreted by YP-1 did not find a matching protein. The reliable protein sequence will be detected by others proteomics technologies in the next study. The gene encoding phosphatase will be tried to be cloned from the genome of YP-4 and YP-1 by PCR, then the structure of phosphatase will be analyzed to understand solubilizing mechanism of organophosphate in details in the future study.

Conclusion

In this study, the organophosphorous solubilizing bacteria were isolated in the rhizosphere soil of Chinese pine (*Pinus tabulaeformis*) in Qingshuihe County (in Hohhot, P. R. China). Two strains were identified as *Pseudomonas sp.,* and *P. reinekei.* We found that the bacteria were secreted extracellular enzymes to solubilize organophosphorous. Futher, the extracellular enzymes were isolated and purified, and had great abilities of organophosphorous solubilizing. It may have probable use as bio-inoculants to increase soil fertility by minimizing fertilizer application, which can promote sustainable agriculture and help to meet future needs.

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