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Conversion of spent mushroom substrate to biofertilizer using a stress-tolerant phosphate-solubilizing *Pichia farinose* FL7

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ABSTRACT

To develop high-efficient biofertilizer, an environmental stress-tolerant phosphate-solubilizing microorganism (PSM) was isolated from agricultural wastes compost, and then applied to spent mushroom substrate (SMS). The isolate FL7 was identified as *Pichia farinose* with resistance against multiple environmental stresses, including 5–45 °C temperature, 3–10 pH range, 0–23% (w/v) NaCl and 0–6 M ammonium ion. Under the optimized cultivation condition, 852.8 mg/l total organic acids can be produced and pH can be reduced to 3.8 after 60 h, meanwhile, the soluble phosphate content reached 816.16 mg/l. The *P. farinose* was used to convert SMS to a phosphate biofertilizer through a semi-solid fermentation (SSF) process. After fermentation of 10 days, cell density can be increased to 5.6×10^8 CFU/g in biomass and pH in this medium can be decreased to 4.0. SMS biofertilizer produced by *P. farinose* significantly improved the growth of soybean in pot experiments, demonstrating a tremendous potential in agricultural application.

1. Introduction

Phosphorus is one of the most important macronutrients for plant growth (Sharpley, 1995). It is required in many cellular processes such as energy transfer, signal transduction, macromolecular biosynthesis, photosynthesis and respiration (Shenoy and Kalagudi, 2005). Soils are short of soluble form of phosphorus in most areas; therefore, the application of chemical phosphate fertilizer has become increasingly popular in many countries. Unfortunately, most of chemical fertilizers can be immobilized by Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} quickly after applied and become unavailable to plants (Bayer et al., 2001). In addition, the large-scale utilization of chemical fertilizers accelerates the degradation of soil structure and the reduction of organic substrate, and causes other environmental problems, like soil harden, eutrophication and heavy metal pollution (Gyaneshwar et al., 2002). Moreover, the high cost of chemical fertilizers is another limiting factor. Although soluble phosphate (P) is extremely deficient in soils, many lands have a high storage of total P (Zou et al., 1992). In order to utilize total P, P-solubilizing microorganisms (PSM) with the capability of transforming the insoluble P to soluble forms (HPO_4^{2-} and $H_2PO_4^-$) have been used as fertilizers to increase soluble P content (Vassilev et al., 1997; Goenadi and Siswanto Sugiaro, 2000; Narsian and Patel, 2000; Reddy et al., 2002; Oliveira et al., 2009). Biofertilizers have been found to enhance crop yield and prevent P loss, thus promoting the sustainable development of agriculture industry. Many microorganisms have been utilized as solubilizing insoluble P inoculants in the past. Among them, many P-solubilizing fungi, such as *Penicillium* (Whitelaw et al., 1999; Xiao et al., 2008), and *Aspergillus* species (Vassilev et al., 1997; Reddy et al., 2002; Ogbo, 2010) have been studied in details. Biofertilizers with PSM can transform the insoluble P to soluble forms by acidification, chelation, exchange reactions, and polymeric substances formation (Son et al., 2006). However, P-solubilizing processes are severely affected by environmental factors such as temperature, pH, NaCl concentration (Leyval and Barthelin, 1989). For example, in high salinity alkaline environments, PSM growth is constrained, resulting in low efficiency of P solubilization. Therefore, it is necessary to isolate new high salt- and pH-tolerant PSM for improving P-solubilizing capability. Unlike chemical fertilizers, biofertilizers can be produced by utilizing a wide range of raw materials including wastes from agricultural processing in order to drive the cost down. Spent mushroom substrates (SMS), the byproduct of mushroom industry, consist of cellulose, hemicellulose, lignin, remnant of edible fungi and carbohydrate, so it could be a suitable material for cultivating P-solubilizing microorganisms. As estimated, there are about 2 million tons of SMS produced in China each year, the potential economic impacts of recycling SMS are significant (Qiao et al., 2011).

In this study, isolation and characterization of a stress-tolerant PSM which could solubilize insoluble P at high efficiency have been

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studied. Preliminary application showed that SMS biofertilizer produced by *Pichia farinose* can significantly improve the growth of soybean in pot experiments, demonstrating a tremendous potential in agricultural application.

2. Methods

2.1. Isolation of P-solubilizing microorganisms (PSM)

Agricultural wastes compost was obtained from Tianjin, PR China. The compost from different fermentation stages were suspended in sterile water with 0.9% NaCl and shaken for 30 min on a rotary shaker. The serially diluted water samples were inoculated on the modified Pikovskaya (MPVK) agar plate (glucose 10 g, yeast extract 0.5 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.3 g, KCl 0.3 g, $MgSO_4{\cdot}7H_2O$ 0.3 g, $CaCl_2{\cdot}2H_2O$ 0.1 g, $FeSO_4{\cdot}7H_2O$ 0.03 g, $MnSO_4{\cdot}H_2O$ 0.03 g, distilled water 1000 ml, pH 7.0∼7.5, agar 20 g) containing 5.0 g tricalcium phosphate ($Ca_3(PO_4)_2$) as sole P source (Son et al., 2006). After 5 day incubation at 30 °C, PSM were selected by detecting the dissolving zone diameter/colony diameter (D/d). Colonies with a bigger ratio (D/d) were picked and further purified by plating again on MPVK agar plate supplemented with $Ca_3(PO_4)_2$.

Each of the purified strain was inoculated in 250 ml conical flask containing 100 ml of MPVK medium supplying with 0.5 g $Ca_3(PO_4)_2$. The flasks were incubated for 12, 24, 36, 48, and 60 h at 25, 30, and 35 °C in shaking beds (200 rpm), respectively. The cells were harvested by centrifugation at 4000 rpm for 10 min at room temperature. Soluble P content in supernatant was determined by molybdate blue colorimetric method (Fiske and Subbarow, 1925; Nautiyal, 1999), with sterile uninoculated medium as negative control. The microorganism with the greatest soluble P content was selected for further analysis. All the experiments were conducted in triplicates.

2.2. Identification of the isolate

Isolate FL7 with the highest P-solubilizing ability was identified based on the 18S rDNA sequence. Genomic DNA was extracted from FL7 using tissue cell genome DNA extraction kit (Biomiga) according to the manufacturer's instructions, and was used as polymerase chain reaction (PCR) template for 18S rDNA amplification. The 18S rDNA fragments were amplified with primers P1: 5'-ATCTGGTTG ATCCTGCCAGT-3' and P2: 5'-GATCCTTCCGCAGGTTCACC-3'). Amplified products were sequenced and then Blast searched against GenBank, EMBL, DDBJ, PDB databases (Altschul et al., 1997). The 18S rDNA sequence of FL7 has been deposited in GenBank under the accession numbers HQ242650.

2.3. Culture conditions for phosphate solubilization

Isolate FL7 was shaken (200 rpm) in Erlenmeyer flasks (250 ml) containing 100 ml of MPVK liquid medium with different concentrations of NaCl (0%, 5.0%, 10.0%, 15.0%, 20.0%, 21.0%, 22.0%, 23.0%, 24.0%, and 25.0%), NH_4^+ (0–7 M NH_4^+) at pH 7.0 and 30 °C respectively. In addition, FL7 was cultivated at different temperatures (5–45 °C) and initial pH (3–10). Under these conditions, insoluble P-solubilizing activity of FL7 was measured. 0.5, 1.0, 1.5, 2.5, 3.0, and 3.5 g of $Ca_3(PO_4)_2$ were added to the medium to determine the effect on the solubilization of insoluble P by the isolate.

Insoluble P solubilization experiments were carried out in flasks with 100 ml of MPVK liquid medium and 0.5 g $Ca_3(PO_4)_2$ sample as sole P source. Flasks were shaken at 200 rpm, 30 °C for 60 h in a rotary shaker unless stated otherwise. Sterile medium served as negative control. All the experiments were conducted in triplicates.

2.4. Evaluation for insoluble phosphate solubilization, pH, organic acids, acid phosphatase and phytate activity

The experiments were carried out in flasks with 100 ml of the optimal liquid medium supplemented with 0.5 g $Ca_3(PO_4)_2$ as sole P source. Flasks were shaken at 30 °C for 132 h. Cultures were harvested every 12 h in order to record the soluble P content, pH, organic acids, acid phosphatase and phytate activity. Soluble P in culture supernatant was determined by molybdate blue colorimetric method (Fiske and Subbarow, 1925; Nautiyal, 1999). The pH was measured using a pH-meter (SG8, Meitler Toledo). Cultures were centrifuged at 12,000 rpm for 20 min and passed through 0.22 μm nylon filter, and then organic acids were analyzed. Organic acids were detected and quantified using a high performance liquid chromatogram (HPLC) equipped with a C18 column and a UV detector (dionex ultimate 3000). The mobile phase was 2.5 mM/l KH_2PO_4 and 1% acetonitrile with a constant flow rate of 0.5 ml/min. Elutes were analyzed at λ 210 nm and identified by comparing the retention time with that of the pure organic acids. The organic acids were quantified by peak areas obtained for the standards for lactic acid, oxalic acid, malic acid, succinic acid, formic acid, citric acid. Extracellular acid phosphatase and phytate activity were determined according to the method described by Golovan et al. (2000). All the experiments were conducted in triplicates.

2.5. Biofertilizer preparation by semi-solid fermentation (SSF)

The SSF medium was prepared mainly from sun-dried SMS, which was ground and then passed through 20 mesh screen. The analysis showed that the SMS contains 70.2% moisture, and the dry SMS contain 59.6% organic matter, 1.2% total nitrogen, 1.56% total potassium and 0.75% total P. No available P was detected in SMS. Fermentation was performed by inoculating about 1×10^6 test PSM of exponential phase (the strain FL7) into 30 g of autoclaved media (1.5 g corn flour and 28.5 g ground dried SMS) in 250 ml Erlenmeyer flasks at an initial moisture content of 65–70%. The compost was turned over every 8 h for 10 days. The pH of the SSF was determined on 5 g sample homogenized in 15 ml of distilled H_2O using a pH meter. Fungal growth was estimated by counting colony-forming units on agar plates. All the experiments were conducted in triplicates.

2.6. Efficiency of biofertilizer

Ten different soils for this experiment were gathered from Tianjin, China. They were air-dried, passed through a 20 mesh screen, and analyzed for pH, organic matter content, available P and total P (Bray and Kurtz, 1945). The basic properties of the soil were as follows: Soil 1: pH 7.2, organic matter content 0.49%, total P 628.35 mg kg^{-1}soil, available P 0.00 mg kg^{-1}soil. Soil 2: pH 7.4, organic matter content 0.42%, total P 542.87 mg kg^{-1}soil, available P 0.00 mg kg^{-1}soil. Soil 3: pH 7.5, organic matter content 0.40%, total P 434.69 mg kg^{-1}soil, available P 0.00 mg kg^{-1}soil. Soil 4: pH 7.1, organic matter content 0.48%, total P 580.73 mg kg^{-1}soil, available P 0.00 mg kg^{-1}soil. Soil 5: pH 6.9, organic matter content 0.53%, total P 731.12 mg kg^{-1}soil, available P 4.80 mg kg^{-1}soil. Soil 6: pH 7.3, organic matter content 0.43%, total P 853.25 mg kg^{-1}soil, available P 5.30 mg kg^{-1}soil. Soil 7: pH 7.1, organic matter content 0.50%, total P 701.47 mg kg^{-1}soil, available P 4.80 mg kg^{-1}soil. Soil 8: pH 7.0, organic matter content 0.51%, total P 776.29 mg kg^{-1}soil, available P 5.00 mg kg^{-1}soil. Soil 9: pH 7.2, organic matter content 0.46%, total P 681.31 mg kg^{-1}soil, available P 5.00 mg kg^{-1}soil. Soil 10: pH 7.3, organic matter content 0.44%, total P 476.74 mg kg^{-1}soil, available P 0.00 mg kg^{-1}soil. A pot culture experiment of soybean seeding was carried out in greenhouse with temperatures of 20–35 °C (day) and 16–24 °C (night) and a natural