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Utilization of microorganisms in the solubilization of low-quality phosphorus raw material

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ABSTRACT

Presented paper reports the possibility of utilization of microbial solubilization process of renewable phosphorus resources for production of phosphorus biofertilizers. The aim of this work was to find the effectiveness of the solubilization process in the relationship with the used material that undergoes the solubilization, that was performed by soil bacteria *Bacillus megaterium*. Three from four, used materials are recognized as a renewable, such as poultry bones, fish bones as well as ashes from incineration of waste sludge form III° stage of biological treatment of wastewater. It was found that the composition of produced acids is strongly related with the material used as a source of phosphorus. The soluble P_2O_5 concentration in the medium ranged between 68.9 and 1289 mg/L with variations among different sources of phosphate used in the experiment. The highest maximum P_2O_5 concentration was found for poultry bones and fish bones 1299 mg/L and 955 mg/L, respectively.

1. Introduction

Intensive agriculture practices achieving high yields require large quantities of chemical fertilizers, which are costly and their utilization creates environmental problems. The extensive use of chemical fertilizers in agriculture is currently under debate due to environmental concerns and fears for consumer health (Yu et al., 2012; Rodríguez and Fraga, 1999; Behera et al., 2014; Rudresh et al., 2005).

Research efforts are concentrated on elaboration of techniques that involve the use of less expensive, though less bio-available sources of plant nutrients such as phosphate rock (one traditional method is the acidulation of phosphate rock (PR) with small amounts of H_2SO_4 or H_3PO_4 to produce partially acidulated phosphate rock (PAAR) (Vassilev et al., 2001)) as well as low quality wastes that contain high content of phosphorus and by application of phosphorus solubilizing bacteria (PSB) to obtain an alternative to currently used fertilizers (Chen et al., 2006).

Usually, one gram of fertile soil contains 10^1–10^{10} bacteria, and their live weight may exceed 2000 kg ha^{-1}. The PSB are ubiquitous with variation in forms and population in different soils (Behera et al., 2014).

Most of phosphorus in low quality renewable raw material is present as non-exchangeable form, which is not directly available to plant. Phosphorus solubilizing bacteria could effectively colonize plant roots and promote plant growth through various mechanisms that include increased mobilization of insoluble nutrients and subsequent enhanced plant uptake (Yu et al., 2012; Rudresh et al., 2005). Organic and mineral acid metabolite production and decrease of medium pH appear to be the major mechanisms for phosphorus renewable raw material solubilization. The role of organic acids in solubilization of phosphates was firstly reported by Sperber (1958). It is generally recognized that organic acids solubilize phosphates through protonation and/or chelation reactions. An alternative P-bearing sources, mechanism and factors affecting phosphates solubilization, and particularly the effect of animal phosphorus bearing sources on organic acid production was reported in the literature (Mendes et al., 2013, 2014; Vassilev et al., 2013b). Additionally, it was mentioned that some elements that may be released during phosphorite solubilization could affect in microbial metabolism and consequently the composition of produced organic acids (Mendes et al., 2013, 2014). Besides the acid strength, the type and the position of the ligand determine the effectiveness of the organic acid in the solubilization process (Vassilev et al., 2001). Microorganisms able to solubilize and

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mineralize phosphate pools in soils are considered to be vital in phosphorus cycle in the environment (Rudresh et al., 2005). A biological approach for liberating phosphates from P-bearing materials by organic acid producing microorganisms was proposed as a less expensive and lower-energy technique compared with the conventional chemical techniques (Vassilev et al., 2013a).

The results of co-inoculation of phosphate-solubilizing bacteria (PSB) and nitrogen-fixing bacteria (NFB) on solubilization of phosphate rock (PR) demonstrated that this approach could be a promising and alternative option for utilizing this potent source as P fertilizer in e.g. walnut plants and maintaining greater nutrient availability in soils (Yu et al., 2012).

The aim of this work was to evaluate effects of utilization of *Bacillus megaterium* in solubilization of phosphates from different raw materials such as fish bones, poultry bones, ashes and phosphorite by the identification of organic acids synthetized by microorganisms and their concentration in the broth medium.

2. Materials and methods

2.1. Bacteria and culture medium

Phosphate sources were treated with *B. megaterium* (PCM 1855) as a phosphate – solubilizing microorganism. Bacteria were obtained from Polish Collection of Microorganisms located at the Institute of Immunology and Experimental Therapy in Wroclaw (WDCM106). For cultivation of bacteria, growth medium containing: 10 g glucose; 0.5 g $(NH_4)_2SO_4$; 0.2 g NaCl; 0.1 g $MgSO_4{\cdot}7H_2O$; 0.2 g KCl; 0.002 g $MnSO_4{\cdot}H_2O$; 0.002 g $FeSO_4{\cdot}7H_2O$; 0.5 g yeast extract was used (per liter of distilled water) (Tao et al., 2008) prepared for *B. megaterium* with technical grade reagents (from POCh S.A. Gliwice, Poland).

2.2. Phosphate source

In solubilization experiments, the phosphate sources used were poultry cooked bone, fish bones, ash from waste water sludge (originating from Łyna plant in Olsztyn, Poland), as well as Morocco phosphate rock. All phosphate substrates were ground with a blender and sieved to pass through 1 mm particle size fractions for chemical and solubilization studies.

2.2.1. Extraction of P_2O_5

In order to investigate efficiency and consequently bioavailability of phosphorus (expressed as P_2O_5) from phosphate sources used in experiments, ammonium citrate and water extracts were determined. The experiment was carried out in two stages, by 1 h at 65 °C for ammonium citrate and 30 min at 25 °C for water, according to Regulation (EC) No 2003/2003 of the European Parliament and of the Council relating to fertilizers (method 3.1.4 Extraction of phosphorus which is soluble in neutral ammonium citrate, and 3.1.6 Extraction of water soluble phosphorus). The contribution of soluble and available phosphorus was evaluated by colorimetric vanadophosphomolybdate method described elsewhere (Wyciszkiewicz et al., 2015).

2.3. Experimental and analytical methods

The solubilization experiment was conducted in Erlenmeyer's flasks (capacity 500 mL) with 250 mL medium and 30 g per 1 L of phosphate source at 34 °C under sterile conditions. The medium solution with the phosphorus sources was inoculated with bacteria from agar slant by inoculating loops and incubated as batch cultures. During 8 days of cultivation/solubilization culture medium was shaken at 120 rpm and incubated at 34 °C, (Thermoshake Gerhardt). Samples of microorganism suspension from all culture groups (four groups: poultry cooked bone, fish bones, ash from waste water sludge and Morocco phosphate rock) were collected at the same time. The reaction mixture was filtered through filter paper and permeates were used for the estimation of pH and P_2O_5 concentration, that was measured by colorimetric vanadomolybdophosphoric acid method. pH measurements were conducted with pH-meter Mettler-Toledo (Seven Multi) equipped with an electrode InLab413 with compensation of temperature.

2.4. Analytical methods

2.4.1. Cell growth

The biomass concentration of *B. megaterium* was measured spectrophotometrically (Labuda et al., 2012). Culture was sampled daily to determine its optical density. The optical density was the absorbance of samples at 550 nm (OD550) in a UV/vis spectrophotometer (Varian Cary 50 Cone). Each sample was diluted to make an absorbance less than 1.0, if the optical density was greater than 1.0. The concentration of *B. megaterium* was estimated by equation describing the relationship between the absorbance A_{550} and the concentration of dry weight, Eq. (1):

$$C_s = 0.00532 \cdot A_{550}, \quad R_2 = 0.922\ \text{mg/L} \tag{1}$$

The biomass was dried at 60 °C for three days (Manufacturing of medical and laboratory equipment, WAMED; Warsaw, Poland) and weighed.

The specific growth rate, μ, 1/day of *B. megaterium* was calculated using the Eqs. (2) and (3):

$$\mu = \frac{1}{c_S} \cdot \frac{dC_S}{dt} \tag{2}$$

$$\mu = \frac{\ln C_S^t - \ln C_S^0}{t} \tag{3}$$

where: t – time period (in days), after which the culture concentration was measured (assuming $t^0 = 0$), C_S^t – the culture concentration after time t (mg/L), C_S^0 – the initial concentration of the culture (mg/L). Relative growth rate was determined from the graphically depicted correlation of $\ln C_S = f(t)$. The linear regression for logarithmic phase of the growth was described by an Eq. (4):

$$\ln C_S^t = \mu \cdot t + \ln C_S^0 \tag{4}$$

and parameter μ, 1/day is the slope.

2.4.2. Capillary isotachophoresis

The presence and concentration of organic acids was determined by capillary isotachophoresis. Isotachophoretic separations were performed using a EA 202 M isotachophoretic analyzer (Villa Labeco, Slovakia) operated in the double-capillary mode. The chemicals used were of analytical-reagent grade. As a calibration standards the following acids were used: gluconic acid (Sigma–Aldrich, France), acetic acid, 99.5% (Chempur, Poland), lactic acid, 80% (POCh, Poland), propanoic acid, >99.5% (Sigma–Aldrich, France), 15 mmol β-alanine (Merck, Germany), 10 mmol HCl (Baker Analyzed) and 0.1% methylhydroxyethylcellulose (MHEC, HERCULES, Czech Republic) was used as the leading electrolyte (pH 3.6) and 5 mmol caproic acid (Fluka, Germany) as the terminating electrolyte. The measurements were carried out three times.

2.5. Calculations

The arithmetic mean values, standard deviations (SD) and t tests as well as the model parameters of equations describing the experimental data were determined using nonlinear estimation and multiple regression modules of *Statistica* software *ver. 9.0*. Correlation was considered statistically significant at $\alpha < 0.05$.