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Original paper

Solubilization of apatite by microbial consortia from activated sludge and rhizospheric soils

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Abstract

Two microbial consortia from activated sludge and rhizospheric soils could effectively solubilize apatite and released soluble phosphate in National Botanical Research Institute's phosphate growth medium. During 14-d incubation, the microbial concentration increased gradually, whereas the pH decreased sharply. The solubilization of apatite by microbial consortium from activated sludge was more efficient than that of microbial consortium from rhizospheric soils. Various organic acids (mainly gluconic acid) were detected in the solution. The solubilization process was also illustrated by the observation of scanning electron microscopy combined with an energy dispersive X-ray spectroscopy, which showed an obvious corrosion on the apatite surfaces, and most elements, especially elements phosphorus and calcium, were removed from the apatite. A strong positive correlation between microbial concentration and content of soluble phosphate was observed, and content of soluble phosphate also had a significant negative correlation with solution pH.

Keywords

Apatite, Microbial consortia, Solubilization, Activated sludge, Rhizospheric soils.

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Introduction

Phosphorus is one of the most important nutrients for crop growth in agriculture, posing major constraints to meet the increasing needs of global food production (GILBERT, 2009). However, many soils throughout the world are deficient in phosphorus (RODRÍGUEZ, 1999). Therefore, to enhance crop phosphorus uptake, increasing amounts of phosphorus fertilizer have been applied (MACDONALD, 2011). However, a large portion of phosphorus applied to soils in the form of phosphorus fertilizer is rapidly immobilized soon after application and becomes unavailable to crops (SINGH, 1994).

Microorganisms have a fundamental role in phosphorus biogeochemical cycle and bioavailability (KHAN, 2010). Many microorganisms, including bacteria and fungi, have been reported as phosphate-solubilizing microorganisms (ACEVEDO, 2014; ZENG, 2017; CHERCHALI, 2019). These phosphate-solubilizing microorganisms widely exist in soils, mines, and wastewater, etc., and are involved in a range of processes that affect the transformation of phosphorus (HAFIDI, 2013; KLAIC, 2017; TAKTEK, 2017; ADHIKARI, 2019).

With the current tendency for reduced process cost and environmental pollution, interest in phosphate solubilization through the action of microorganisms and its use in agriculture are receiving greater attention recently (VALETTI, 2018; TANG, 2019; HAMIM, 2019). However, most studies on phosphate solubilization were limited in only using single or few known strains isolated from different sources, especially from soils. It was suggested that in bioleaching operations, multiple microorganisms assist bioleaching activities more effectively when presented as symbiotic consortia (QIU, 2011). Hence more researches are needed to understand the usage of various microbial consortia for the phosphate solubilization.

In this study, apatite was used as the sole phosphorus source, and two microbial consortia from activated sludge and rhizospheric soils, respectively, were used to solubilize apatite. Chemical analysis of microbial concentration, pH and the content of soluble phosphate in the solution was performed to follow the overall process of the solubilization of apatite. The analyses of apatite surfaces by scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM-EDX) to assess the corrosivity of the two microbial consortia to the apatite were also examined.

Materials and Methods

1. Phosphate samples

The phosphate samples used in this study was apatite purchased from Fisher Scientific. X-ray diffraction (XRD) analysis showed that the main composition of the apatite was mainly composed of fluorapatite. The sample was ground to a particle size of 100-200 mesh before used.

2. Microorganisms

The microorganisms used in this study were two microbial consortia from activated sludge and rhizospheric soils, respectively. Activated sludge sample was collected

from a municipal wastewater treatment plant in Edmonton, Canada. The sludge was semi-solid, and the main microorganisms were bacterial *Alphaproteobacteria*, *Betaproteobacteria*, *Sphingobacteria* and *Flavobacteria*, and fungal *Saccharomycetes* at the class level sequenced by Illumina MiSeq platform. Rhizospheric soils sample was collected from a farm located in the south campus of University of Alberta (Edmonton, Canada). The wheat plants were uprooted and the solid rhizospheric soils attached to roots were suspended in sterilized water and mixed using a magnetic stirrer for 20 min to separate microorganisms from them. The main microorganisms were bacterial *Alphaproteobacteria*, *Flavobacteria*, *Sphingobacteria* and *Gammaproteobacteria*, and fungal *Saccharomycetes* at the class level sequenced by Illumina MiSeq platform. The two microbial consortia were enriched by a modified National Botanical Research Institute's phosphate growth (NBRIP) medium (pH 7), which contained (per liter): 10 g glucose, 0.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g KCl, 0.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (NAUTIYAL, 1999). About 5 g/L $\text{Ca}_3(\text{PO}_4)_2$ was added to the medium as a sole phosphate source for selectively screening of phosphate-solubilizing microorganisms. The enrichment was carried out at ambient temperature and initial pH 7. After several successive transfers, the culture was harvested and stored at 4°C for further study.

3. Solubilization experiments

The solubilization experiments were performed in 500 mL Erlenmeyer flasks containing 240 mL NBRIP medium and 6 g apatite samples. Each flask was inoculated with 60 mL microbial consortium. The initial pH of the solution was adjusted to 7. The concentration of the microbial consortium in the solution was adjusted to optical density (OD) at 0.6 under 600 nm by a spectrophotometer before used. Autoclaved, uninoculated medium served as control. The solution was stirred by a magnetic stirring apparatus under 200 r/min at ambient temperature for 14 days. Fifty milliliters of solution were collected every day to measure the microbial concentration and pH in the solution, and then centrifuged the solution at $9000 \times g$ for 30 min. The supernatant was used to measure the content of soluble phosphate. To keep the total volume of the solution at 300 mL, 50 mL NBRIP medium was added to each flask after sampling. At the end of experiments, a small number of residues were taken at random from the flasks. The residues were washed three times with phosphate buffer and dehydrated by using a graded ethanol series, air-dried, and gold-coated for SEM-EDX observation. The analysis was performed using a VEGA3 (TESCAN, Czech) SEM equipped with an energy dispersive spectrometer at an accelerating voltage of 20 kV. All experiments were performed in triplicate.

4. Analytical methods

Soluble phosphate in the solution was determined by using the vanadium-ammonium molybdate colorimetric method (EATON, 2005). Microbial concentration was

measured the OD value at 600 nm using a spectrophotometer (UV-3600; Shimadzu Corporation, Kyoto, Japan). The pH was recorded with a pH meter (AR 15, Accumet, Fisher Scientific) equipped with glass electrode (Cat. # 13-620-108, Accumet, Fisher Scientific).

Results and Discussion

1. Solubilization of apatite by microbial consortia

Phosphate solubilizing microorganisms are abundant in the nature, such as soils, wastewater and phosphate mines (NELOFER, 2016; PANDA, 2016). In this study, two microbial consortia were enriched from activated sludge and rhizospheric soils, respectively, and were used to solubilize apatite. The results of microbial concentration, pH, and content of soluble phosphate in the solutions with the two microbial consortia are illustrated in Figure 1.

The microbial concentration changed regularly during the solubilizing processes (Figure 1a), which could be divided into three stages. During the initial 3 days, a slight decrease of microbial concentration in the solution was observed, which was attributed to the attachment of the microorganisms onto the RP surface. After 3 days, the microbial concentration began to increase with time and reached a maximum value of OD 0.91 (activated sludge) and 0.86 (rhizospheric soils), respectively, on day 10. After 10 days, the microbial concentrations were no longer increased, but kept in constant. However, there was almost no change of OD values in the abiotic control without inoculation.

During the solubilization process, the pH initially increased at the first day, and then decreased sharply to a value of 3.92 (activated sludge) and 3.86 (rhizospheric soils), respectively, on day 6 (Figure 1b). After 4 days, the pH was no longer decreased and kept at values of 4-5 for the duration of the experiment. The results were consistent with many reports of the pH reduction during the process of phosphate solubilization by microorganisms (LI, 2015; XIAO, 2013). However, the pH increased to more than 7 and remained almost constant for the duration of the control experiment.

The two microbial consortia could effectively solubilize apatite in the solution compared to the abiotic control, and the phosphorus solubilization increased gradually during 14 days of solubilization experiments (Figure 1c). The finding was in accordance with our previous studies which showed that the two microbial consortia could effectively solubilize rock phosphates (XIAO, 2018; 2019). The results further indicate that the two microbial consortia have a great potential for use as potential solubilizers of insoluble phosphates.

Microorganisms varied with respect to levels of phosphate solubilization achieved (GAIND, 2016). The results in this study agreed with them. The two microbial consortia varied in their capacities to release soluble phosphate from apatite, and the solubilization of apatite by microbial consortium from activated sludge was better than that by microbial consortium from rhizospheric soils.

Moreover, the biomass and pH reduction in the solution with microbial consortium from activated sludge were also better than that with microbial consortium from rhizospheric soils under the same condition. However, except the microbial sources, the phosphate solubilization ability of microorganisms may be determined by various factors, such as phosphorus sources, technological parameter, culture conditions and so on (MENDES, 2014; YADAV, 2013).

Figure 1. Changes in microbial concentration (a), pH (b), and content of soluble phosphate (c) in solutions inoculated with microbial consortium from activated sludge and rhizospheric soils. Results represent the mean of three replicates $\pm$ standard deviation.

2. Detection of organic acids in the solution

It was reported that the major mechanism for microbial solubilization of insoluble phosphates is reported to be the production of low-molecular weight organic acids (BOJINOVA, 2008; RASHID, 2004; VASSILEV, 2007). The organic acids can combine with metal ions, such as Ca^{2+} , and thus facilitated phosphate solubilization (KPOMBLEKOU, 1994). In the present study, organic acids in the solution were detected by high-performance liquid chromatography (HPLC) analysis. Results showed that various organic acids were detected in the solution inoculated with the two microbial consortia, and different organic acids and their concentrations varied with different microbial consortia (Table 1). The organic acids excreted by the two microbial consortia played a vital role in the acidification of the solution, which could be illustrated by

the decrease of pH, and thus further facilitated the solubilization of apatite.

Gluconic acid was reported as the main organic acid produced during the microbial solubilization of insoluble phosphates (CHUANG, 2007; XIAO, 2015). Similar result was achieved in this study. Among the organic acids detected, gluconic acid was predominantly produced by the two microbial consortia (Figure 2). The concentration of gluconic acid in the solution increased significantly during 14 days of microbial solubilization of apatite, although it had obvious fluctuation during the experiment. Moreover, the microbial consortium from activated sludge excreted the higher concentration of gluconic acid than that of the microbial consortium from rhizospheric soils during the solubilizing process of apatite.

Table 1. Organic acids detected and their concentration (mg/L) in the solution inoculated with the two microbial consortia after 14 days of inoculation.

	Gluconic acid	Citric acid	Oxalic acid	Lactic acid	Formic acid	Pyruvic acid	α -keto-glutaric acid	Succinic acid	Fumaric acid	Ascorbic acid	Tartaric acid
microbial consortium from activated sludge	451.2 $\pm$ 18.9	45.2 $\pm$ 5.5	79.1 $\pm$ 6.4	12.5 $\pm$ 0.8	17.5 $\pm$ 1.2	7.5 $\pm$ 0.7	trace	15.9 $\pm$ 1.1	trace	— ^a	—
microbial consortium from rhizospheric soils	418.7 $\pm$ 29.4	47.9 $\pm$ 3.3	48.6 $\pm$ 2.8	20.3 $\pm$ 1.3	9.7 $\pm$ 0.9	trace	trace	8.7 $\pm$ 0.8	trace	—	—

Results represent the mean of three replicates $\pm$ standard deviation

^a Not detected

Figure 2. Concentration of gluconic acid in solution inoculated with the two microbial consortia during 14 days of solubilization of apatite. Results represent the mean of three replicates $\pm$ standard deviation.

3. Analysis of apatite surface by SEM–EDX

To reveal the different activities of the two microbial consortia in the solubilization of apatite, SEM images coupled with EDX spectra were captured, and are shown as Figures 3 and 4, respectively.

The SEM micrograph of the apatite surface after exposure to the microbial consortia for 14 days revealed that the microbial consortia could make different extents of

corrosion of the apatite as compared to the control (Figure 3). It shows deep holes on the apatite sample after 14 days of solubilization by microbial consortium from activated sludge and rhizospheric soils, respectively, and many eroded pits were identified on the apatite surface (Figure 3b and c). Deep spalling was also found on the apatite surface. However, the apatite surface without solubilization appeared unaltered, and no visible corrosion was observed (Figure 3a). This suggests that the corrosion of the apatite surface

is due to the proton attack and thus made it scraggly and formed many chasms (ROJAS-CHAPANA, 2004; XIAO, 2017).

An EDX analysis of the apatite surface indicated that the elemental phosphorus of the apatite solubilized by both microbial consortium from activated sludge and rhizospheric soils was obviously reduced, and the amount of elemental calcium was also decreased after 14 days of

solubilization (Figure 4). It indicated that the main composition of apatite had undergone an obvious transformation, and it was solubilized successfully by the microbial consortia. However, the apatite in the control system without presence of microbial consortia was unaltered throughout the entire 14 day period. Similarly, different extents of transformation were performed with different microbial consortia.

Figure 3. SEM images of typical apatite surface. **a.** Apatite sample without solubilization (control); **b.** Apatite sample after 14 days of solubilization by microbial consortium from activated sludge; **c.** Apatite sample after 14 days of solubilization by microbial consortium from rhizospheric soils.

Figure 4. Typical EDX spectrums and elements content (embedded table) of the apatite surface. **a.** Apatite sample without solubilization (control); **b.** Apatite sample after 14 days of solubilization by microbial consortium from activated sludge; **c.** Apatite sample after 14 days of solubilization by microbial consortium from rhizospheric soils.

4. Regression analysis between microbial concentration, pH and soluble phosphate content

It is generally believed that during the process of the reaction between the microorganism and mineral, the greater microbial concentration would enhance the microbial acid metabolism, then decreased pH in the solution and converted insoluble phosphate in the apatite to the soluble phosphate. However, there have some conflictive reports (GILBERTO, 2014; NARSIAN, 2000). Therefore, based on the results of this study, regression between microbial concentration, pH, and soluble phosphate content were analyzed in this study, and the results were illustrated in Figure 5.

Figure 5a showed the correlations between microbial concentration and soluble phosphate content. The result revealed that there was a positive correlation between

microbial concentration and soluble phosphate content. This is consistent with the previous research (MALBOOBI, 2009). Figure 5b showed the correlations between pH and soluble phosphate content. It showed a negative correlation which is in accordance with the previous study (KPOMBLEKOU, 2003). The results further confirmed that acidification of the surrounding was the major mechanism for the phosphate solubilization (VASSILEVA, 2000). However, phosphate solubilization is a complex process, and may be determined by multiple mechanisms. Given that the two microbial consortia are such a large group of activated sludge and rhizospheric microorganisms, and that a wide diversity of microorganisms in the microbial consortia was difficult to understand their phosphate solubilizing mechanisms.

Figure 5. The correlations between microbial concentration and soluble phosphate content (a), pH and soluble phosphate content (b).

Conclusions

Solubilization of apatite was found to be possible using two microbial consortia from activated sludge and rhizospheric soils, respectively. The two microbial consortia could effectively solubilize apatite in NBRIP medium and released soluble phosphate in the solution, which can be illustrated by an increase in microbial concentration and pH drop in the solution. HPLC analysis indicated the participation of various organic acids produced by the two microbial consortia, with gluconic acid as the principal component in the process of acidification in the solution. The solubilization process was also illustrated by SEM–EDX observations. The microbial consortia displayed trends of release of soluble phosphate that closely matched the increase in microbial concentration and pH drop in solution. A positive correlation between microbial concentration and soluble phosphate content, and a negative correlation between pH and soluble phosphate content in solution were observed from regression analysis results.

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