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Enhanced biosolubilization of mid-low grade phosphate rock by formation of microbial consortium biofilm from activated sludge

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Abstract: Mid-low grade phosphate rock (PR) is a potential source of free phosphate to facilitate crop growth, but a cost effective and environmentally responsible extraction process is required. In this study, the capacity of a microbial consortium from activated sludge to solubilize PR in a laboratory-scale column reactor was investigated. The microbial consortium proved capable of efficiently releasing soluble phosphate in the reactor effluent over the 90-day trial. The microbial consortium grew well in the column system as evidenced by reduced chemical oxygen demand (COD) in the reaction solution. Biofilm formation was identified as critical for biosolubilization of the mid-low grade PR. Imaging of the biofilm by scanning electron microscopy (SEM) revealed a dense network of microbial cells embedded in extracellular polymeric substances (EPS). The biofilm contained both oxic and anoxic zones. The pH decreased significantly in both the biofilm and the reaction solution during operation, indicating healthy growth of the microbial consortium with corresponding acid generation and subsequent enhancement of phosphate solubilization.

Keywords: biosolubilization, phosphate rock (PR), microbial consortium, activated sludge, biofilm

1. Introduction

Phosphorus (as phosphate) is one of the most important nutrients required for plant growth. However, very low quantities of soil phosphate (0.1%) are present in a form accessible to the plant (Hariprasad and Niranjana, 2009). Therefore, large amounts of costly phosphate fertilizer are applied to soil every year to satisfy the demands of agriculture. Phosphate-bearing minerals, especially phosphate rock (PR), are less costly alternatives to phosphate fertilizer (Rajan et al., 1996). However, direct application to soil is not always effective because of the insoluble nature of the phosphate in PR (Sacko et al., 2012). Thus, a suitable process to solubilize phosphate-bearing minerals is required before they can be used as phosphate fertilizer (Biswas and Narayanasamy, 2006; Moharana and Biswas, 2016). Traditionally, PR is solubilized by inorganic acids, such as sulfuric acid or hydrochloric acid. Since RP is a complex raw material and almost eighty percent of all PR extracted globally is mid-low grade, this process is not recommended due to the high cost and associated environmental pollution (Vaccari and Strigul, 2011).

Microorganisms are known to play a critical role in the natural phosphorus cycle, and a large number of microorganisms have been reported to solubilize phosphate, including bacteria and fungi (Rodríguez and Fraga, 1999; Behera et al., 2014; Xiao et al., 2015). These phosphate-solubilizing microorganisms exist naturally in soils, mines and wastewater, and thus can be easily isolated. With the current demand for low processing costs and environmentally benign production, interest is increasing in biosolubilization of mid-low grade PR using phosphate-solubilizing microorganisms (Vassileva et al., 2000; Hamdali et al., 2008; Xiao et al., 2013a and b; Mendes et al., 2014). However, most previous studies used single microorganisms, while few studies have examined the capacity of mixed microorganisms

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or microbial consortia from natural or intentionally engineered environments.

In this study, a microbial consortium enriched from activated sludge of a municipal wastewater treatment plant in Canada was used to solubilize mid-low grade PR in a laboratory-scale column reactor. Detailed analyses of microbial concentration, pH, dissolved oxygen (DO), chemical oxygen demand (COD) and soluble phosphate content in the effluent were performed to monitor the release of phosphate from the mid-low grade PR by the microbial consortium.

Biofilm is the predominant form of microbial growth in nature and in many engineered environments. Biofilms have been observed in a number of environments, but little is known of their applicability for the biosolubilization of mid-low grade PR. In this study, scanning electron microscope (SEM) was used to study biofilm formation on the surface of the treated ore. For a comprehensive understanding of the microbial activity, microsensors were used to detect pH and DO inside the biofilm. The purpose of this study is to develop a microbial consortium capable of releasing phosphate from mid-low grade PR, and to evaluate the technical feasibility of a column reactor for such applications.

2. Materials and methods

2.1. Mid-low grade PR

The sample of mid-low grade PR used in this study was obtained from Kunyang phosphate mines, which belong to Yunnan phosphate group Co., LTD in Yunan province of China. The main elements of the ore sample as determined by X-ray fluorescence (XRF) are shown in Table 1. Ore samples were first crushed and screened to a particle size of 5–10 mm before use in the column reactor. The total ore sample (approximately 2–3 kg) was loaded in the column and pre-treated with culture medium for 10 days in order to neutralise the acid-consuming components and help establish biofilm communities on the surface.

Table 1. The main elements (wt%) in the ore sample

Element	P_2O_5	MgO	SiO_2	CaO	Fe_2O_3	Al_2O_3	K_2O	MnO	Na_2O	F
Content	22.62	6.23	10.38	40.71	0.85	0.81	0.33	0.28	4.70	2.79

2.2. Microorganisms

Activated sludge was collected from a municipal wastewater treatment plant in Edmonton, Canada. The microbial consortium in activated sludge was enriched by a modified Pikovskaya medium of the following composition (per litre): 10 g glucose, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.0001 g $MnSO_4 \cdot H_2O$ and 0.0001 g $FeSO_4 \cdot 7H_2O$ (Pikovskaya, 1948). About 5 g/L $Ca_3(PO_4)_2$ was added to the culture medium as a sole phosphate source for selection of phosphate-solubilizing microorganisms. The enrichment was carried out for 3 days in a 2.5 L tank aerated with ambient air at 45 L/h under ambient temperature and initial pH 7, and subcultured at two-week interval.

2.3. Biosolubilization experiment in column reactor

The schematic of the column reactor system used in this study is shown in Fig. 1. The system consists of a cylindrical reactor 5 cm (Ø) × 40 cm (H), a 6-L plastic container serving as the reservoir for circulating reaction solution, and a 3-L glass container serving as the reservoir for culture medium. The concentration of the microbial consortium in the reaction solution was adjusted to an optical density at 600 nm (OD_{600}) of 0.6 using a spectrophotometer. A layer of supporting gravel of 10–15 mm diameter particle size and low in acid-consuming minerals was placed 3-cm deep at the bottom of the column before the ore sample was loaded. The reactor was operated as a trickle bed without external aeration. In order to attach biofilm on the ore surface, continuous operation was applied. The reaction solution was aerated at 45 L/h and mixed at 200 rpm by a magnetic stirrer at the bottom of the plastic container. The column was fed dropwise with reaction solution and fresh culture medium at 3 mL/min and 0.3 mL/min, respectively, using a peristaltic pump. The influent was introduced from the top of the column

and instilled onto a piece of sterile gauze placed on the top surface of the ore to assist in uniform distribution. The influent passed through the ore sample by gravity and dripped into the plastic container. The reaction solution in the plastic container was re-circulated to the top of the column in a continuous mode by a peristaltic pump. The reaction solution was bleed out as a waste when exceeding 6 L. The entire experiment was conducted under ambient temperature. The effluent from the column was sampled daily and analysed to determine microbial growth, soluble phosphate content, pH and DO. The COD levels of influent and effluent were measured daily for the first 7 days and again after 60 days of operation. All experiments were performed in triplicate.

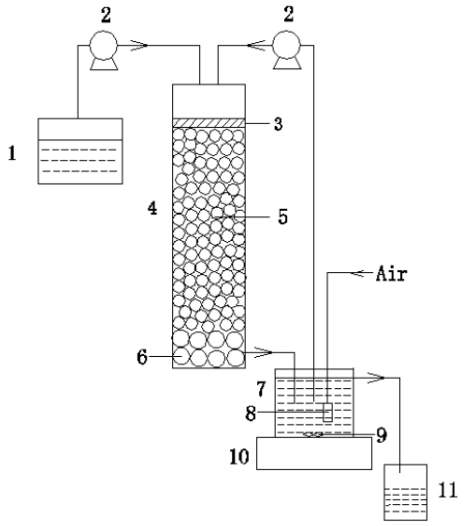

Fig. 1. Schematic of the column reactor system: 1- glass container serving as the culture medium reservoir; 2- peristaltic pump; 3- gauze; 4- column; 5- mid-low grade PR; 6- support gravel; 7- plastic container serving as an internal recirculation reservoir; 8- aerator connected to compressed air tank; 9- mixer; 10- magnetic stirring plate; 11- disposal bottle

2.4. Microsensor measurements

A Clark-type oxygen microsensor with a tip diameter of approximately 15 μm was fabricated and calibrated as described by Lu and Yu (2002). A potentiometric pH microsensor was fabricated and calibrated according to the procedures described by Yu (2000). For microsensor measurements, some pieces of ore with biofilm were taken directly from the column reactor on operating day 60. The samples were acclimated in the culture medium for at least 6 h before the profiles were measured by a microsensor fastened onto a micromanipulator. The microsensor assembly and the samples were put inside a Faraday cage to reduce electromagnetic interference. Each microsensor was calibrated immediately before and after measurements. The biofilm thickness was viewed by a horizontal stereomicroscope and determined by the distance travelled by the micromanipulator. The microsensor tip was advanced into the biofilm and readings were taken at intervals of 50–100 μm. The DO concentration and pH along the biofilm depth were determined from calibration curves. All experiments were performed in triplicate.

2.5. SEM imaging

Ore pieces covered in biofilm were selected at random from the column reactor on operating day 60 and fixed in 2% glutaric aldehyde for 4 h. The fixed samples were washed three times with phosphate buffer, dehydrated by a graded ethanol series, air-dried and gold-coated for SEM imaging using a VEGA3 (TESCAN, Czech) SEM at an acceleration voltage of 20 kV. Control samples were rinsed in water several times to remove the biofilm before SEM imaging.

2.6. Analytical methods

The concentration of soluble phosphate in the effluent was determined using the vanadium-ammonium

molybdate colorimetric method. Cell growth was measured at OD_{600} nm using a spectrophotometer (UV-3600; Shimadzu Corporation, Kyoto, Japan). The effluent pH was recorded with a pH meter (AR 15, Accumet, Fisher Scientific) equipped with a glass electrode (Cat. #13-620-108, Accumet, Fisher Scientific). DO was measured by an LDO 101 model DO probe (HACH Company, USA). The closed reflux colorimetric method was used for COD analysis. All chemical analyses were conducted according to Standard Methods for Examination of Water and Wastewater (Eaton et al., 2005). Values are expressed as mean ± standard deviation for triplicate independent measurements. Means were compared by analysis of variance (ANOVA) and post hoc Duncan's Multiple Range Test (DMRT). A $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characterisation of phosphate solubilization from PR in a column reactor

The dynamics of phosphate solubilization from PR in a column reactor inoculated with a microbial consortium from activated sludge was monitored by daily measurements of microbial concentration, pH, DO and soluble phosphate in the reactor effluent. The results obtained over the 90-day experiment are shown in Fig. 2. As shown in Fig. 2a, the microbial consortium grew well in the reactor. After slow growth for the first 15 days, the microbial concentration increased sharply from day 16 to day 45, akin to a logarithmic growth phase. The microbial consortium appeared to reach a stationary phase after 45 days, with only minor fluctuations in concentration observed thereafter.

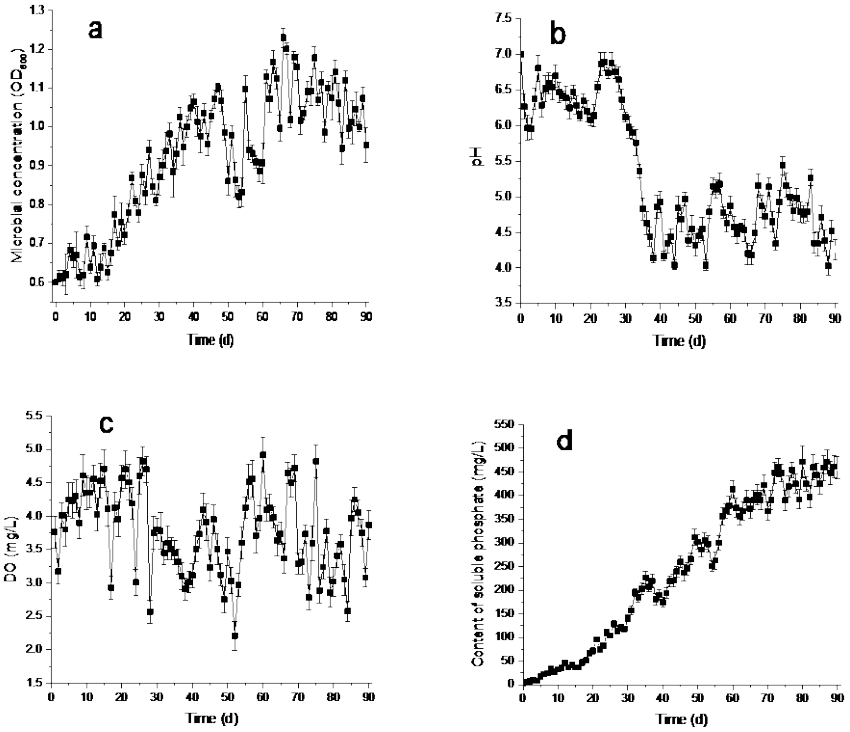

Fig. 2. Changes in microbial concentration (a), pH (b), DO (c) and soluble phosphate (d) in the effluent of the column reactor during PR solubilization by a microbial consortium from activated sludge. Results are the means of three replicates ± standard deviation (error bars)

From the start of the experiment to day 25, the change in effluent pH was negligible. However, the pH decreased sharply from day 26 to day 38, after which it fluctuated between 4–5 (Fig. 2b). The observed pH drop may be due to the release of organic acids as a result of rapid microbial consortium growth. Although organic acids were not measured in this study, it is known that organic acid production constitutes an adaptation strategy by which microorganisms extract limiting nutrients such as phosphorus, calcium and potassium from insoluble mineral matrices through chemical attack on the crystal structure (Banfield et al., 1999; Delvasto et al., 2009). In this case, such a strategy is most likely used by the microbial consortium to solubilize phosphorus-containing zones within the mid-low grade

PR to obtain sufficient phosphorus for further growth.

Aeration of the reaction solution is critical for this column reactor system because there is no external aeration. The DO was supplied by continuous circulation of the reaction solution. However, the reaction solution exhibited irregular fluctuation in DO from 2–5 mg/L during the experiment (Fig. 2c). This might be due to the dynamic and complex solubilizing system in this study, since the microorganisms and PR were all composed of complex compositions.

The effluent also exhibited a relatively steady increase in soluble phosphate concentration throughout the 90-day trial (Fig. 2d), indicating the capacity of the microbial consortium to effectively solubilize phosphate from the mid-low grade PR over sustained periods. However, effluent phosphate concentration did fluctuate moderately during the reaction, reflecting the dynamic nature of adsorption and desorption of soluble phosphate on PR. This could be due to the accumulation of phosphate in biofilm to support microbial growth. Moreover, re-precipitation of metal ions and phosphorus within the biofilm microenvironment may also contribute to transient decreases in soluble phosphate release.

3.2. Removal efficiency of COD

The COD test is commonly used to indirectly measure the concentration of organic compounds in a water sample, and the results provide a useful index of the amount of organic pollutants present (Chen et al., 2012). Reducing COD is one of the most important objectives in any water treatment process and is usually used to evaluate the performance of the treatment (Belmont and Metcalfe, 2003). In this study, COD was quantified in the influent and effluent to evaluate the performance of the microbial consortium. Fig. 3 shows COD concentrations in the influent and effluent of the column reactor during operation from day 60 to day 67. Over this period, the COD removal rate from the influent stabilised at 90%, indicating successful biofilm cultivation and proliferation. Results indicate that biodegradation of organic compounds by the microbial consortium remained efficient during operation of the column reactor.

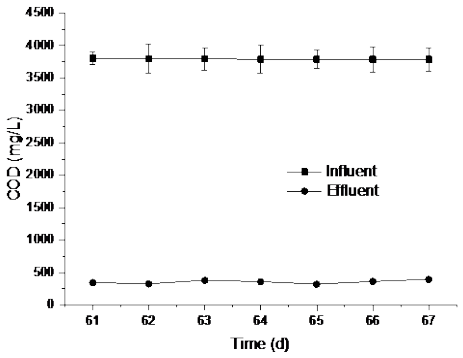

Fig. 3. COD concentration in column reactor influent and effluent from operation day 60 to 67. Results are the means of three replicates ± standard deviation (error bars)

3.3. Microsensor measurements of the biofilm

Microsensors are valuable tools for measuring chemical distributions in biofilms (Yu, 2000). Using microsensor technology, non-destructive measurements can be conducted in situ to reveal spatial variations in microbial activity even in thin biofilms (Lewandowski and Beyenal, 2007). In recent years, microsensors have been developed to measure numerous physicochemical parameters such as oxygen content, pH, redox potential and ammonium and sulfide concentrations in biofilms (de la Rosa and Yu, 2005, 2006; Tan, 2012; Zhou et al., 2011, 2013). In this study, O_2 and pH microsensors were used to measure the local oxygen concentration and pH in the living environment of the microbial consortium. The profiles of O_2 and pH along the biofilm depth in situ on operating day 60 are shown in Fig. 4. The O_2 profile revealed clear oxic and anoxic zone within the biofilm. As shown in Fig. 4, O_2 concentration decreased steeply from the biofilm−liquid interface at 0 μm to a film depth of 600 μm, followed by a more gradual decrease until the film became anoxic at a depth of about 1100 μm.

A slight increase in pH was found between the biofilm−bulk liquid interface and about 600 μm below the interface (Fig. 4), followed by a much more gradual increase at greater depths. However, the total

change was only ~0.3 pH units. The increase in pH within the oxic region of the film indicates greater bioactivity, generating sufficient acids to cause a noticeable change in pH within the film. The pH value in the biofilm (4.4) was close to that of the effluent (4–5) (Fig. 2b), indicating a similar pH in the biofilm and reaction solution.

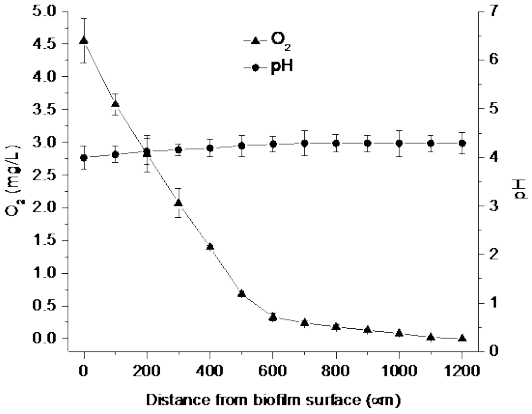

Fig. 4. Profiles of O_2 and pH in biofilm. The interface between the biofilm and bulk liquid is indicated by depth of 0 μm. Results are the means of three replicates ± standard deviation (error bars)

3.4. Biofilm formation by SEM analysis

In some systems, the survival of microorganisms depends on their ability to adhere to a surface. In phosphorus-limited environments, for example, microorganisms colonize ore surfaces containing phosphorus to scavenge it (Banfield et al., 1999), mostly through biofilm formation. To analyse colonization of the microbial consortium, the treated PR surfaces were examined using SEM. SEM micrographs (Fig. 5a) of the PR surface 30 days after inoculation revealed the presence of a microbial

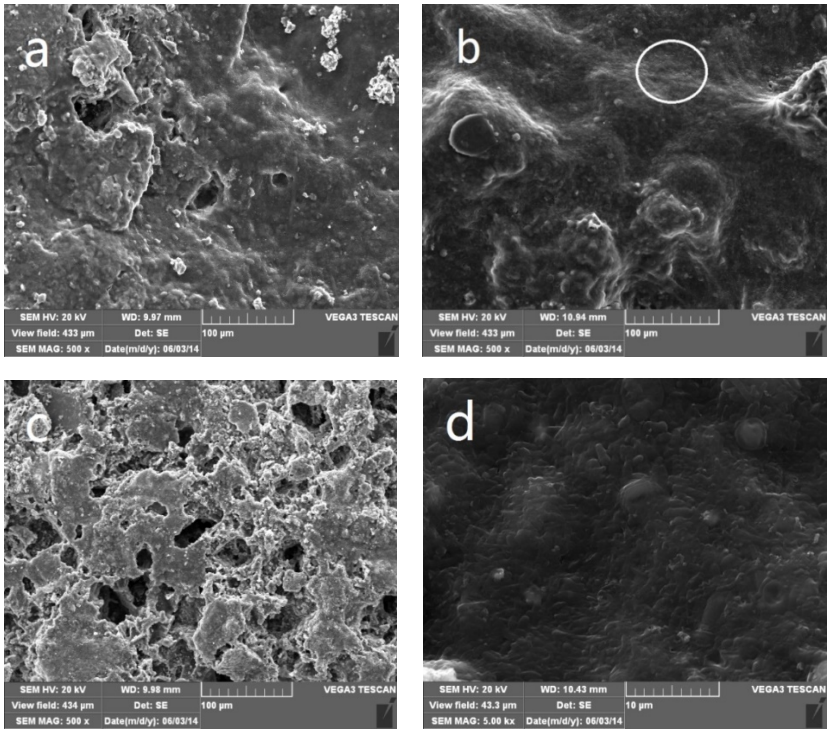

Fig. 5. SEM micrographs of a typical PR surface solubilized by a colonized microbial consortium from activated sludge. (a) PR surface after 30 days of operation. (b) PR surface after 90 days of operation. (c) PR surface in (b) after rinsing to detach the biofilm. (d) High-magnification image showing the details (circle in Fig. 5 (b)) of the dense biofilm formed

consortium attached to the mineral surface and formation of sparsely distributed biofilms. At this time, there were no erosion pits on the PR surface. After 90 days of operation, however, the PR surface was entirely covered by a thick layer of biofilm (Fig. 5b). Fig. 5d shows the detailed structure of the dense biofilm, which appeared as individual microbial cells embedded in a dense network of organic materials. These organic materials may include microbial exopolymers and other metabolites attached to the PR surface (Delvasto et al., 2009). Secretion of extracellular polysaccharide substance (EPS) by microbes colonizing a surface is considered necessary for biofilm formation (Delolme et al., 2011). The EPS facilitates initial surface colonization by acting as a conditioning agent, creating primary or secondary bonds between the surface metal centres and the EPS functional groups (Omoike and Chorover, 2006). The bare ore surface revealed after biofilm removal on day 90 exhibited honeycomb-like holes (Fig. 5c), clear evidence of biofilm attack (solubilization) on the PR surface to generate phosphoric acids.

4. Conclusions

Biosolubilization of mid-low grade PR is possible using a microbial consortium from activated sludge in a laboratory-scale column reactor. The microbial consortium grew well in the column, and was efficient in biodegrading organic compounds. The pH in the reaction solution decreased, which facilitated phosphate solubilization. The formation of biofilm in the column reactor was associated with the rate of biosolubilization. Microsensor measurements showed that biofilm included oxic and anoxic zones. A reduction in pH was also detected in the biofilm, which would further support phosphate solubilization. The soluble phosphate released by the microbial consortium can be easily absorbed by plant. The findings indicate that biosolubilization of mid-low grade PR by the microbial consortium from activated sludge has a potential to serve as a microbial phosphate fertilizer. This biological approach for liberating phosphate from mid-low grade PR by microorganisms is proposed as a less expensive and lower-energy technique compared with the conventional chemical techniques.

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