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Research paper

Semi-quantitative analysis of microbial production of oxalic acid by montmorillonite sorption and ATR-IR

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ABSTRACT

Interactions between organic acids and clay minerals significantly influence elemental cycle on Earth. Oxalic acid has been recognized as one of the most important secretions of soil microorganisms, for both typical bacterium *Enterobacter* sp. and fungus *Aspergillus niger*. This study examined the ATR-IR spectra of solid and aqueous oxalic acid. Then, sorption of dissolved oxalic acid, microbial secretion, and simulative acid solution onto montmorillonite were also studied by ATR-IR. The sorption significantly elevated intensity of the characteristic peak of oxalate at 1318 cm^{-1}. Then, the intensity ratio (R) of 1318/1635 cm^{-1} was proposed as an accurate indicator for semi-quantitatively analyzing oxalic acid concentration. R values of ~0.13 and ~0.20 (for non-microorganism system) represented the oxalic acid concentrations of 600–800 ppm and ~2000 ppm respectively. Additionally, only oxalic acid with > 800 ppm concentration can be identified appropriately if mixed with montmorillonite for 6 or 12 h, whereas 24 h shaking can decrease the detection line to as low as 100 ppm. Finally, we proposed an equation of $R_{correction} = C_0 * R_{microbe\ oxalic}$ (with a coefficient C_0 of 3.171) to estimate oxalic acid secreted by microorganisms. The coefficient was necessary due to the interference from multiple organic acids in microbial secretion. This equation worked successfully for both *Enterobacter* sp. and *Aspergillus niger*. It therefore is a reliable method for semi-quantitatively estimating microbial production of oxalic acid via montmorillonite sorption and ATR-IR technique.

1. Introduction

Organic acids have great potentials to enhance ion mobility in soil (Renella et al., 2004). They are also key components of water-soluble fractions of organic molecules in rhizosphere. They hence serve as a link between pedosphere and life. The interactions between organic acids from biological species (microbes, plants, and animals) and rock significantly contribute to the global cycle of various elements (Adeleke et al., 2016). For example, they can enhance the release of available P from insoluble phosphates, which are usually unavailable to plants (Drever and Stillings, 1997; Li et al., 2016a). In addition, organic acids are also vital ingredients in common food sciences, e.g., wine, yogurt, and meat. They are hence tightly correlated to human health and sustainable development in ecosystem, e.g., to shape cell structures, coordinate human intestinal flora, sterilize harmful organisms, as well as to control humus components (Khanji et al., 2018; Mani-López et al., 2012).

Many organic acids such as citric, formic, lactic and oxalic acid are common exudates from microorganisms, and have therefore been well studied (Adeleke et al., 2016; Jones and Darrah, 1994). Citric acid is able to inhibit cells through metal chelation, which can enhance pathogen inhibition compared with lactic acid (Mani-López et al., 2012). Formic acid is typically used to digest proteins at a very small scale and could assist the hydrolysis of highly cross-linked collagen (Hong et al., 2018). Additionally, microorganisms tend to sacrifice the efficiency of adenosine triphosphate (ATP) production in order to secrete more lactic acid. This contributes to a more favorable environment for their continuous growth (Soest et al., 1991). Oxalic acid has been identified as one of the most important secretions of soil microorganisms, for both bacteria and fungi (Whitelaw, 1999). For example, strains of *Aspergillus niger* (*A. niger*) and *Enterobacter* sp. are the most effective soil microbes participating in the weathering process due to their high ability to produce organic acids and increase the acidity (Illmer and Schinner, 1995; Li et al., 2018; Whitelaw, 1999). Especially, oxalic acid has the highest acidity constant ($K\alpha_1 = 6.5 \times 10^{-2}$) with respect to other common organic acids, e.g., formic and citric acid (with acidity

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constants of ~10^{-4}) (Bolan et al., 1994). Therefore, oxalic acid, from either fungi or bacteria, usually dominates the acidity caused by microbial secretions.

Clay minerals are the most abundant phyllosilicates both on the Earth's surface and in marine sediments (Vorhies and Gaines, 2009). They play a relevant role in the transport and fate of trace elements in the environment (Martin et al., 2018). The surface chemistry and physical properties, i.e., surface area, cation exchange capacity (CEC) and porosity, of a mineral control the extent of organo-mineral interactions. For instance, clay minerals, together with Fe/Al oxides and amorphous to poorly crystalline minerals, provide the major fraction of surface area in soils for the sorption of organic matters (Kaiser et al., 1996). Montmorillonite (Mt) has been addressed due to its large surface area, swelling ability, high CEC and ability to organize multiple guest species compared with other clays (Intachai et al., 2016; Li et al., 2017; Okada et al., 2014). Moreover, Mt is tightly associated with other clay minerals in geology, such as kaolinite, illite and some non-clay minerals (Şans et al., 2017).

The adsorption of organic molecules onto minerals is of fundamental importance for understanding of environmental processes, catalytic processes on oxides and creation of bio-compatible materials (Thomas and Kelley, 2008). Beyond that, adsorption onto minerals (including clays) can also decrease the biodegradability of natural organic matters (Keil and Hedges, 1993; Keil et al., 1994; Mayer, 1994), which has significant implications for global elemental cycles (Torn et al., 1997). Certain organic acids show the strong affinity for specific mineral surfaces, which has been used extensively as model anions for sorption studies (Hees et al., 2003). For example, the adsorption of humic acid onto goethite was examined under various environmental conditions (pH, salt and free humic acid concentrations) (Saito et al., 2004). In acidic conditions, citrate can enhance the dissolution rate of Mt due to its formation of surface complexes. In neutral conditions, Mt is dissolved with the decreasing activity of Al^{3+} by forming aqueous Al-citrate complexes (Ramos et al., 2011). Additionally, the adsorption of lysine on phyllosilicates follows the order of montmorillonite > illite > kaolinite at equilibrium pH (6.1–8.8) (Yeasmin et al., 2014). As the most representative organic acid, oxalic acid has been confirmed to form two prominent surface complexes on boehmite, i.e., inner-sphere mononuclear five-membered chelate structure and surface complexations with –OH (Axe and Persson, 2001). However, there are still limited literature regarding the adsorption of oxalic acid onto Mt, especially in microbial system.

High performance liquid chromatography (HPLC) is the most common technique of measuring organic acids secreted by microorganisms, while it is time consuming and high-cost. Additionally, sensitivity of analytical columns to samples, clogging problems and expensive repacking limit its application (Strobel, 2001). Attenuated total reflectance infrared method (ATR-IR), a variety of infrared spectroscopy (IR), has been applied widely to study mineral surface (Johnston and Premachandra, 2002; Yan et al., 2013). Compared with common Fourier transform infrared spectroscopy (FTIR), wet and dry samples can both be directly analyzed by ATR-IR with no complicated preparation, e.g., preparation of KBr pellet (Kubicki et al., 1997). Moreover, even tens of seconds will allow a satisfied analysis on samples. Previous studies have roughly investigated the mechanisms and influences of organic acids adsorbed onto minerals by ATR-IR (Axe and Persson, 2001; Evanko and Dzombak, 1999; Yeasmin et al., 2014). However, how to quantitatively measure the concentrations of adsorbed organic acids (secreted by microorganisms) onto Mt is still unclear.

In this study, the ATR-IR spectra of Mt with sorption of pure oxalic acid after various shaking time (at 6, 12 and 24 h) were examined. Then, microbial secreted oxalic acid and simulative acid solution adsorbed by Mt were also investigated. We finally proposed an equation for semi-quantitatively calculating oxalic acid concentrations, by using Mt as a probe and ATR-IR as the primary technique.

2. Experimental section

2.1. Preparation of montmorillonite, oxalic acid, and microorganisms

Montmorillonite (SWy-2) sample was collected from Wyoming, USA. The sample was provided by The Clay Minerals Society (collection at Purdue University, Indiana, USA). It was stored in desiccator and no chemicals or treatments were applied to Mt before the following experiments.

Both the strains of *A. niger* (CGMCC No.11544, Nanjing Agricultural University) and *Enterobacter* sp. were isolated from soybean rhizosphere soil in Nanjing, China (Li et al., 2018; Li et al., 2016b). The spores of *A. niger* were inoculated on the potato dextrose agar medium (PDA) and cultured at 28 °C for five days. The produced spores were then carefully flushed with sterilized ultrapure water. The conidia concentration was adjusted to 107 cfu/mL (calculated by haemacytometer) by 0.85% sterile saline. After the expansion of strains, 1 mL solutions were injected to 50 mL Pikovskaya (PVK) agar medium and shaken for an additional five days at 28 °C.

Enterobacter sp. strains were activated in liquid nutrient agar (NA) medium and shaken at 37 °C for three days. Then, 1 mL solutions of the strains were inoculated in 50 mL PVK medium. After that, *Enterobacter* sp. was incubated at 37 °C in shaker for an additional three days.

The ingredients of the PVK medium (the phosphorus-solubilizing medium) for both *A. niger* and *Enterobacter* sp. were as follows: Glucose 10.0 g, $FeSO_4·7H_2O$ 0.03 g, $MgSO_4·7H_2O$ 0.3 g, NaCl 0.3 g, KCl 0.3 g, $(NH_4)_2SO_4$ 0.5 g, $MnSO_4·7H_2O$ 0.03 g, $Ca_3(PO_4)_2$ 5.0 g, H_2O 1000 mL, and pH value of 7.20–7.40.

2.2. Adsorption experiments

Non-Mt experiment was performed to investigate ATR-IR spectra of the dissolved oxalic acid in water. The solutions (at 100, 200, 400, 600, 800, 1000, 2000, 2500, 5000, 10,000 and 20,000 ppm) were prepared by dissolving solid oxalic acid dehydrate ($H_2C_2O_4·2H_2O$) (AR, Nanjing Chemical Reagent CO. LTD) in ultrapure water (18.23 mΩ·cm, by Barnstead Smart2Pure from ThermoFisher, Inc., USA).

The solutions (ranging from 100 to 2000 ppm) were then mixed with Mt to investigate the adsorption of pure oxalic acid. Batch experiments for adsorption were performed with triplicates in 50 mL tubes (without microorganisms). 30 mL of solution with various oxalic acid concentrations (ranging from 100 to 2000 ppm) was mixed with 50 mg Mt. The mixtures were shaken at room temperature for 6, 12, and 24 h respectively. The suspension was then centrifuged at 8000 rpm for three minutes and filtered. Finally, the samples were air-dried for three days and ground to < 74 µm powder for ATR-IR analysis.

The simulative acid solution was prepared, so that the adsorption of the mixture of organic acids can be investigated. 200 mg oxalic acid and 100 mg citric acid (two most common exudates from microorganisms) were dissolved in 100 mL ultrapure water, i.e., 2000 ppm for oxalic acid and 1000 ppm for citric acid. Adsorption of oxalic acid onto Mt in this artificial solution was performed with the same processes as the above ATR-IR analysis.

The secreted organic acids from the two microorganisms (*A. niger* and *Enterobacter* sp.) after incubation were collected from the liquid phosphorus solubilizing medium (PVK medium), which were centrifugated at 8000 rpm and then filtered (to remove microorganisms). The solution was more complicated compared with the above non-microorganism and simulative experiments, as multiple organic acids were secreted by the microorganisms (Li et al., 2016a; Whitelaw, 1999). The same adsorption steps and sample preparation, except for microbial incubation, were carried as above non-microorganism and simulative experiments.

The ratios (R, 1318/1635 cm^{-1}) of intensities of the two representative ATR-IR peaks (1318 cm^{-1} assigned to oxalic acid and 1635 cm^{-1} assigned to interlayered water adsorbed by Mt) were