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The preferential oxidation of orthorhombic sulfur during batch culture

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ABSTRACT

The formation of sulfur is predicted by the current understanding of the mechanisms involved in mineral sulfide oxidation and observed in studies of the leaching products that accumulate on the surface of the mineral. Sulfur oxidising bacteria can exploit this energy source and can remove a potentially 'rate-limiting' diffusion barrier. In this study on the activity of sulfur oxidising bacteria cultured on mixed solid sulfur allotropes, it was observed that a heterogeneous culture preferentially oxidised the orthorhombic allotrope and no significant growth on the polymeric allotrope could be demonstrated.

1. Introduction

The formation of elemental sulfur and its metabolic conversion to sulfate by bacteria is considered to be an important part of many processes and industrial operations. In our laboratory, heterogeneous acidophilic cultures are routinely maintained in non-sterile minimal media using commercially available elemental sulfur as the sole energy source. A feature of these cultures is that the pH of the medium can fall well below 1 and the solid substrate is never fully consumed. Even after the elapse of some months, planktonic cells abound and the surface of the remaining small sulfur prills appears fully colonised. It is reasonable to expect that the dissolution of shrinking particles such as sulfur prills would accelerate as the surface area to mass ratio increased with the passage of time. The observed deceleration could be due to the intense acidification of the medium. However, when the amount of solid sulfur added and the production of sulfuric acid was reduced, a small fraction of the solid sulfur substrate still remained. This persistence of residual sulfur suggests that the commercial product may not be homogeneous and that this mixed culture may exhibit some specificity for different fractions of the commercial sulfur.

Elemental sulfur can exist in a wide variety of allotropic forms (Meyer, 1964). The amount of each allotrope largely depends on the thermal history of the solid although other factors contribute such as impurities and pressure. Under conditions of standard temperature and pressure the different allotropes will eventually form the stable orthorhombic phase, which is the predominant allotrope found in nature (Meyer, 1977; Meyer 176). Meyer (1964) also reported that highly purified elemental sulfur usually consisted of a mixture of allotropes, mainly orthorhombic and plastic forms. The ability of bacteria to oxidise specific sulfur allotropes has only been reported by Laishley et al. (1986) who observed the rate of oxidation of orthorhombic, polymeric sulfur and a sample of mixed allotrope sulfur by *Thiobacillus albertis* (since reclassified as *Acidithiobacillus albertensis*, Kelly and Wood, 2000). After 21 days exposure, approximately 70% of the orthorhombic and polymeric phases were utilised but only 35% of the mixed phase allotrope. Laishley et al. suggested that the number of favourable binding sites that allow oxidation of the substrate by *T. albertis* was influenced by the mixed allotrope content of the phase; the greater the content, the slower substrate utilisation. With our initial observations and those of Laishley et al., it appeared that the allotropic form of elemental sulfur did exert some impact on the rate of sulfur oxidation by a bacterial culture.

These observations are potentially important in understanding the roles played by sulfur and sulfur oxidising bacteria in any process producing or consuming elemental sulfur in an aqueous environment, particularly during the oxidative dissolution of mineral sulfides. The production of elemental sulfur during the bio-mining of mineral sulfides is now well known and mechanisms for the oxidation process and the catalytic roles played by the acidophilic iron and sulfur oxidising cells have been described (Rohwerder et al., 2003; Schippers and Sand, 1999; Schippers et al., 1996). Also described has been the control that the solid-state characteristics of the solid mineral phase exert on the mechanism of dissolution and the products that accumulate (Tributsch, 1981a,b, 2001). In the 'thiosulfate' and the 'polysulfide'

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mechanisms described by Schippers and Sand (1999), elemental sulfur is formed from the disproportionation of thiosulfate ($S_2O_3^{2-}$) and the partial oxidation of sulfide (S^{2-}) respectively (Rohwerder et al., 2003).

Recent studies examining the microstructure of the solid sulfur phase that forms during bioleaching have focussed mostly on the dissolution of chalcopyrite (Klauber et al., 2001; Parker et al., 2003, 2008; Rodriguez et al., 2003). Klauber (2008) recently reviewed the surface chemistry of the oxidation of chalcopyrite and suggested the initial sulfur species formed are S–S dimers or short chain polymers followed by chain extension to form a polymeric allotrope which is finally transformed into the orthorhombic allotrope. Klauber also found that the major components associated with dissolution inhibition of chalcopyrite are elemental sulfur and jarosite. Whether a sulfur-based diffusion barrier forms on chalcopyrite is disputed but there is strong evidence for the formation of such a barrier on the surface of sphalerite (Fowler and Crundwell, 1999). Solid phase sulfur also forms on pyrite surfaces (Rojas-Chapana et al., 1996) and its metabolism by bacteria, forming and transporting colloidal material into the cell capsule, is an important process.

There is a continuing discussion in the literature concerning the nature of the solid phase sulfur produced and consumed by the wide variety of microbes that interact with elemental sulfur (Lee et al., 2007; Franz et al., 2007; Urich et al., 2006; Prange et al., 2002a,b; George et al., 2002). Given the extent of control these bacteria exert over solid sulfur it could be expected that they would be able to mobilise and oxidise all of the common allotropic forms of sulfur. In this study we endeavoured to repeat the work of Laishely et al. (1986) and performed non-sterile batch cultures using commercially available elemental sulfur and two other allotrope preparations as the sole energy substrate in minimal medium.

2. Experimental

2.1. Equipment and chemicals

All chemicals used in this study were analytical grade reagents unless otherwise stated and all solutions were prepared with distilled water. The culture used in these experiments was routinely maintained by serial transfer to and from shake flasks at 35 °C. The experiments reported here used large Erlenmeyer flasks containing 5.0 L of medium maintained at 35 ± 0.5 °C and sparged with compressed air and a set of 8 identical shake flasks (100 rpm) containing 100 mL of medium at 35 ± 0.5 °C. The large culture vessel was agitated via a magnetic stirrer bar at sufficient speed to keep the sulfur particles suspended. Cell counts were performed using a Hauser hemocytometer with a grid area of 2.5×10^{-3} mm^2 and a volume of 2.5×10^{-10} L. Samples were taken periodically from the large culture vessel and filtered through Whatman No. 1 papers. The retained sulfur particles were dried on the filter paper at atmospheric pressure then stored at −1 °C. Sample taken for ICP analysis were filtered through a 0.45 µm disposable filter to remove cells and residual sulfur particulates.

Raman spectra were obtained from a Nicolet 6700 FT-IR spectrometer equipped with a NXR FT-Raman module and an InGaAs detector. The indicated intensity of the laser source was 0.4 W, the resolution was 4 cm^{-1} and 256 or 1024 scans were used throughout. The samples were homogenised in a mortar and pestle and then spectra were recorded at 25 °C using 5 mm diameter NMR tubes as sample holders. XRD spectra were collected using a Bruker D8 X-ray Diffractometer ('advance' model). Mass spectra of the sulfur samples were recorded using a Shimadzu gas chromatograph mass spectrometer (GCMS QP2010S model) after direct injection of the solid sample. The pH of the medium in the set of 8 batch culture flasks was continuously recorded by a set of 8 pH probes connected to a high impedance 8 channel voltmeter. Probes were standardised against pH 1.68 and 3.56 buffers (Burkin, 2001).

Polymeric sulfur was prepared following the method of Cataldo (2002). To a stirred 500 mL beaker that contained 200 mL of distilled H_2O and 0.25 g of Triton x100 (Ajax Chemicals) surfactant, 10.0 mL of 98% S_2Cl_2 (Aldrich) was slowly injected via a syringe. The solution immediately went opaque and after a period of 1 h, solid particles with a diameter of approximately 1 mm were recovered by vacuum filtration (Whatman No. 1). Residual surfactant was removed by resuspending the particles in distilled H_2O, filtering and washing with ca. 2 L of distilled H_2O. This process was repeated at least 5 times before drying at the vacuum funnel. This solid material (µ–S synth) was stored in the dark at −1 °C in an airtight container.

Commercially available elemental sulfur (Ajax Chemicals, Ajax–S) was partitioned using a soxhlet apparatus with carbon disulfide as the solvent. The yellow solution was cooled and produced large crystals of the orthorhombic allotrope α–S_8 which were filtered, dried under vacuum and ground in a mortar and pestle before use. The insoluble residue from the soxhlet thimble (µ–S residue), pale in colour, was dried under vacuum before use.

2.2. Growth medium

Three component solutions were prepared in bulk and used to produce a minimal growth medium as required. Solution A, was prepared by dissolving $FeSO_4.7H_2O$ (2.78 g) in 1 L of distilled water acidified with concentrated H_2SO_4 to ca. pH 1.8, with the final pH adjusted to pH 1.5 after the salt dissolved. This solution was stored at 5 °C. Solution B, a macro-nutrient solution was prepared from the following salts, $(NH_4)_2SO_4$ (5.00 g), K_2HPO_4 (2.50 g), $MgSO_4.7H_2O$ (2.50 g) and $CaCl_2.0.5H_2O$ (0.100 g) dissolved in 1.00 L of distilled water and adjusted to pH 1.50 ± 0.05 with concentrated H_2SO_4. Solution C, a solution of micro-nutrients was prepared from the following salts, $CoSO_4.7H_2O$ (2.49 g), $CuSO_4.7H_2O$ (2.81 g), $MnSO_4.H_2O$ (1.69 g), $(NH_4)_6Mo_7O_{24}.4H_2O$ (1.77 g), $NiSO_4.6H_2O$ (2.62 g) and $ZnSO_4.7H_2O$ (2.87 g) dissolved in 1.00 L of distilled water and adjusted to pH 1.50 ± 0.05 with concentrated H_2SO_4.

Medium for batch cultures was prepared by adding to distilled water acidified with H_2SO_4: solution A (1.0 mL L^{-1}; macro-nutrient solution B (20 mL L^{-1}); and micro-nutrient solution C (1.0 mL L^{-1}). Concentrated H_2SO_4 was added to achieve the desired pH in the range of 2.0–2.3. Elemental sulfur in the form of crystalline α–S_8, µ–S residue, µ–S synth or Ajax–S was added at 0.50 g L^{-1} (15 mM). The inoculum was prepared by filtering 100 mL of routine maintenance culture through a Whatman No. 1 filter (25 µm), then through a Millipore membrane filter (0.45 µm). The cells retained on the membrane were resuspended in a buffer solution of H_2SO_4 at a pH of 1.5 then used to inoculate the batch experiment.

The routine maintenance culture used throughout was initially selected from a slurry of mixed iron, copper and nickel sulfide minerals by serial transfers in the sulfur medium. This stable culture is of heterogeneous morphology and has been maintained for more than 5 years. These bacteria were used to inoculate the large (5.0 L) and small (0.1 L) medium described above.

3. Results and discussion

3.1. Preparation and characterisation of sulfur substrates

Approximately 17% (w/w) of the commercially available Ajax–S was insoluble in carbon disulfide and remained in the soxhlet thimble

Table 1
Prominent Raman shift vibration and stretch frequencies for sulfur allotropes.

α–S_8		µ–S	
cm^{-1} (observed)	cm^{-1} (literature)	cm^{-1} (observed)	cm^{-1} (literature)
472.5	475 (a)	456.3	460 (a) (b)
436.7	440 (a)	421.7	425 (a) 427 (b)
246.6	248 (a)	274.2	275 (a) 285 (b)
218	220 (a)	ca. 260 shoulder	260 (a) 268 (b)
153.6	150-160 (a)		

(a) Eckert and Steudel (2003), (b) Steudel and Eckert (2004).