ATP-sulphurylase: An enzymatic marker for biological sulphate reduction?

Oluwaseun Oyekola and Brett Pletschke

Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown 6140, South Africa

Abstract

Adenosine triphosphate-sulphurylase (ATPS) plays a major role in dissimilatory sulphate reduction. In this study, the level of ATPS activity was monitored in a time course study using a biosulphidogenic batch bioreactor system. A coincident decrease in ATPS activity with a decline in sulphate concentration and an increase in sulphide concentration as biosulphidogenesis proceeded was observed. Flask studies further showed sulphate to be stimulatory to ATPS, while sulphide proved to be inhibitory. The effect of ions (Ca^{2+}, Cl^{-}, Fe^{2+} and Zn^{2+}) on the ATPS activity was also investigated. Most of the ions studied (Ca^{2+}, Cl^{-} and Fe^{2+}) were stimulatory at lower concentrations (40–120 mg/l) but proved toxic at higher concentrations (>120 mg/l). In contrast, Zn^{2+} was inhibitory even at low concentrations (≥40 mg/l). ATPS may potentially be used as an enzymatic marker for biological sulphate reduction in sulphate-rich wastewaters and natural environments (anaerobic systems such as soils and sediments found in freshwater and marine systems), providing all residual sulphide and interfering ions are removed using a simple preparative step.

Keywords: ATP-sulphurylase; SRB; Sulphate reduction; Sulphate-reducers; Sulphate-reducing bacteria

Sulphate-reducing bacteria (SRB), a physiologically diverse group of anaerobic microbes (Castro et al., 2000), are involved in dissimilatory sulphate reduction, resulting in the liberation of sulphide (Loubinoux et al., 2002). These microorganisms have been isolated in various environments including deep offshore petroleum reservoirs (Jeathon et al., 2002), petroleum hydrocarbon (PHC) contaminated aquifers (Kleikemper et al., 2002), terrestrial hot springs (Castro et al., 2000), anaerobic sludge (Manz et al., 1998) and even in the digestive tracts of humans and animals (Loubinoux et al., 2002). This group of bacteria has found application in the management of a range of sulphate-rich industrial effluents (Moosa et al., 2002), and are very important in the sulphur cycle in natural environments (e.g. in acid sulphate soils, in particular during the inundation of these sulphate rich soils) (Dent and Pons, 1995) where they initiate the reduction of sulphate to sulphide (Gavel et al., 1998; McLeod et al., 2002).

ATPS plays an important role in dissimilatory sulphate reduction, being the first enzyme involved in the activation of the inactive sulphate substrate (Pletschke et al., 2002).

Pletschke et al. (2002) have previously reported that ATPS activities correlate closely with levels of sulphate reduction. The potential of using these enzymes as marker enzymes for sulphate reduction taking place in natural environments and anaerobic bioreactors was therefore investigated.

A mixed SRB-containing consortium (mainly of the *Desulphovibrio* genus), previously obtained from Grootvlei mine, Gauteng province, South Africa and stored at Environmental Biotechnology Research Unit (EBRU), Grahamstown, South Africa was grown in a 10 l anaerobic batch bioreactor flask (Fig. 1) containing modified Postgate's medium B (Atlas, 1993). The bioreactor flask was seeded with 10% (v/v) (1 l) inoculum of the actively growing SRB consortium, sealed with a rubber stopper and covered with aluminium foil to maintain strict anaerobic conditions and prevent any photosynthetic reactions. High rate N_2 gas was spurged through port D (Fig. 1) in oxygen impermeable Tygon® tubing (G) (Swiss Lab, South Africa) into the reactor to maintain anaerobic conditions during sampling. The bioreactor was incubated in the dark at 30 °C while shaking at 100 rpm. H_2S produced in the reactor was trapped in the hydrogen sulphide trap (A). Samples (100 ml) were withdrawn daily for 23 days to monitor the levels of ATPS activity, pH, sulphate and sulphide.

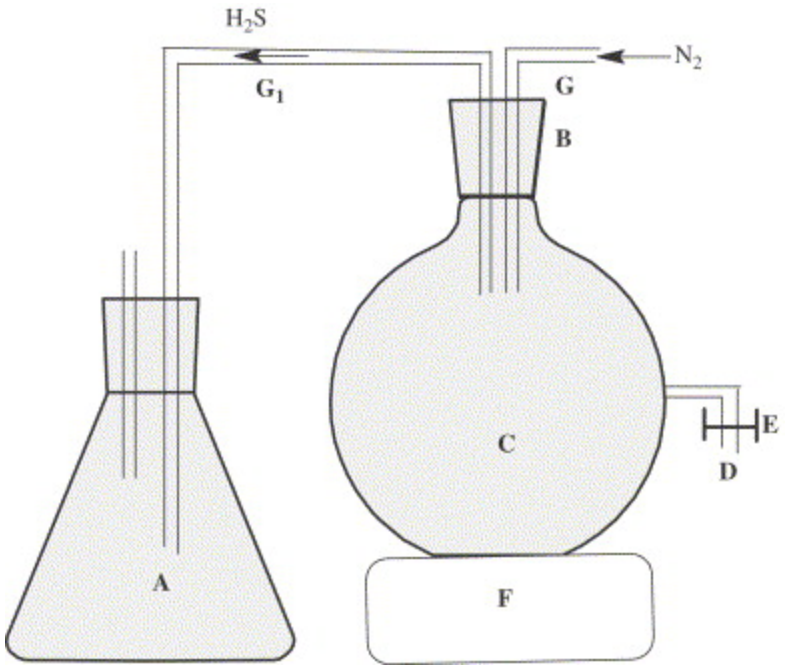

Fig. 1. Schematic diagram of the laboratory-scale biosulphidogenic batch bioreactor: (A) 10% (w/v) zinc acetate solution serving as hydrogen sulphide trap; (B) rubber stopper; (C) 10 l glass tank; (D) sampling port; (E) metal clamp; (F) shaker; (G and G_1) oxygen impermeable Tygon® tubing.

Samples (100 ml) were obtained from the bioreactor followed by pH adjustment to 5.0 using 1 M HCl, so as to liberate the dissolved sulphide as hydrogen sulphide. This sample was then centrifuged (10 000*g*, 10 min, 4 °C). The supernatant was discarded. The pellet was washed three times with distiled water (1:2 w/v), to remove all soluble impurities that could interfere with the assay, followed by resuspension in 0.4 M sodium Tris–HCl buffer (pH 8.0) (1:2 w/v). The resuspended pellet was sonicated for 4×30 s intervals (10 W) on ice using a Virsonic-100 sonicator (VirTis, Co, Inc., USA), followed by centrifugation (3220*g*, 10 min, 4 °C) to remove debris and unlysed cells. ATP sulphurylase activity was measured in the supernatant obtained using the classical colorimetric molybdolysis assay as described by Segel et al. (1987). One unit of ATPS activity was defined as the amount of enzyme that produced a change in absorbance of 0.1 under the assay conditions specified. Sulphide was quantified using a Merck® spectroquant test kit while sulphate was estimated using a SulfaVer® Sulfate reagent (Hach, USA).

The effects of different effectors on ATPS activity were investigated at concentration ranges 0–200 and 0–1000 mg/l, by pre-incubation of the samples with the different effectors (in the dark with minimal headspace to prevent oxidation) for 15 min at 30 °C, before the assay was initiated by the addition of 0.4 ml of the reaction mixture. Suitable controls (containing the effectors at the various concentrations investigated and substituting the enzyme sample with distiled water) were prepared to assess the effects of these effectors on the various assay components.

ATPS activity and sulphate levels correlated closely from days 1 to 15 (Fig. 2a). The major part of sulphate reduction occurred during the first 9 days, with a corresponding decline in ATPS activity and sulphate concentration. This could be attributed to the decreasing availability of the substrate (sulphate). Interestingly, after the initial burst in sulphate reduction from 3257 to 1059 mg/l over the first 9 days, there was a sudden increase in the sulphate concentration in the reactor from day 10 onwards with a corresponding increase in ATPS activity. A possible reason for the increase in sulphate concentration after a marked decrease in the starting concentration is that the reverse reaction of the sulphate activation resulting from a limitation in ATP and an accumulation of APS was occurring. O' Flaherty and Colleran (1999) reported a similar trend in the fluctuation of sulphate concentration in an anaerobic digester treating propionate, butyrate and ethanol containing wastewater. In their investigation, sulphate concentration increased from 500 to 2000 mg/l after the initial reduction of sulphate from approximately 3500 mg/l down to about 500 mg/l; this increase was followed by another phase of sulphate reduction. This was attributed to reverse inhibition of SRB by sulphide. The sulphate concentration reached a plateau on day 17 and no additional sulphate reduction was subsequently observed. At the same time, an immediate inverse relationship was observed between the ATPS activity and sulphide production during the first 12 days of the study (Fig. 2 and Fig. 3). ATPS activity reached baseline after 15 days, probably due to the inhibitory effect of the sulphide produced in the first 15 days. This agrees with the results obtained in Fig. 4, in which sulphide proved to be strongly inhibitory to ATPS activity. Reactor pH was also observed to increase from 6.4 to 8.62 over time (23 days); this was a further indication of SRB activity. Previous results have

also shown that, during sulphate reduction, the pH of the system becomes more alkaline as a result of an increase in concentrations of HCO_3^-, OH^- and HS^- ions (Whiteley et al., 2003). The decrease in sulphate concentration, increase in sulphide production and increase in pH during this course of study all indicated to the proper activity and maintenance of the SRB culture. In a similar investigation by Pletschke et al. (2002) consequent ATPS production during sulphate reduction within the first nine days was also reported with a similar removal of 1700 mg/l sulphate as compared to 2198 mg/l sulphate removed in this study.

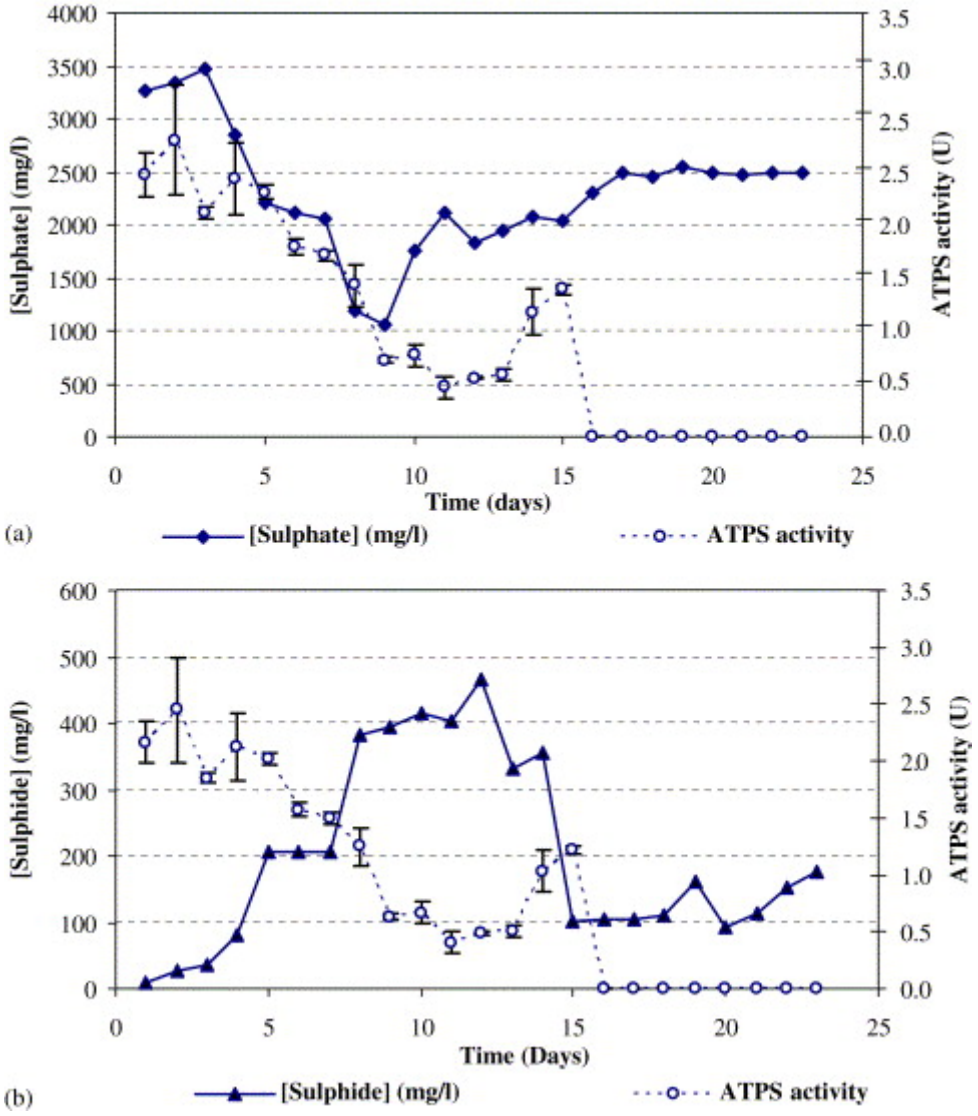

Fig. 2. ATPS activity under biosulphidogenic conditions. (a) ATPS activity versus a reduction in sulphate concentration; (b) ATPS activity versus sulphide production. Each data point represents a mean value±SD, *n*=3.

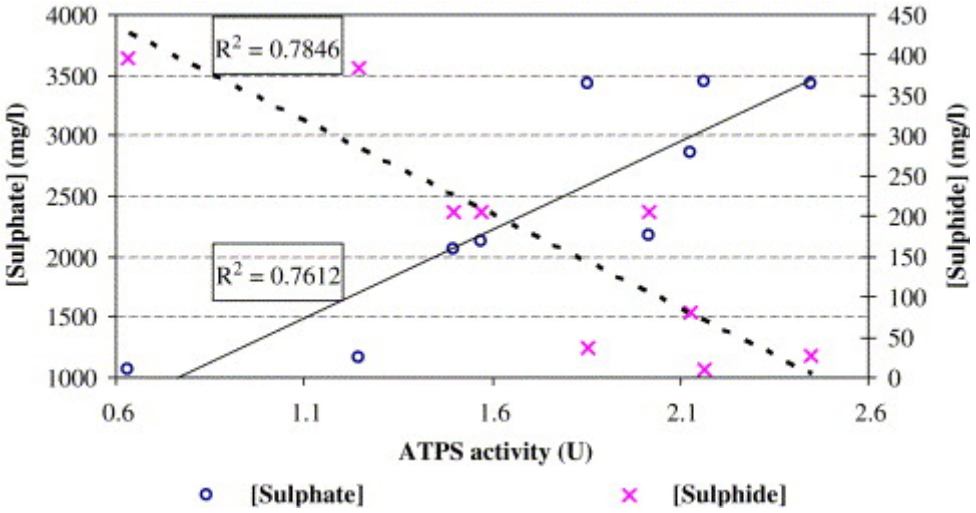

Fig. 3. Correlation of ATPS activity to sulphate and sulphide levels during sulphate reduction. Each data point represents a mean value±SD, *n*=3.

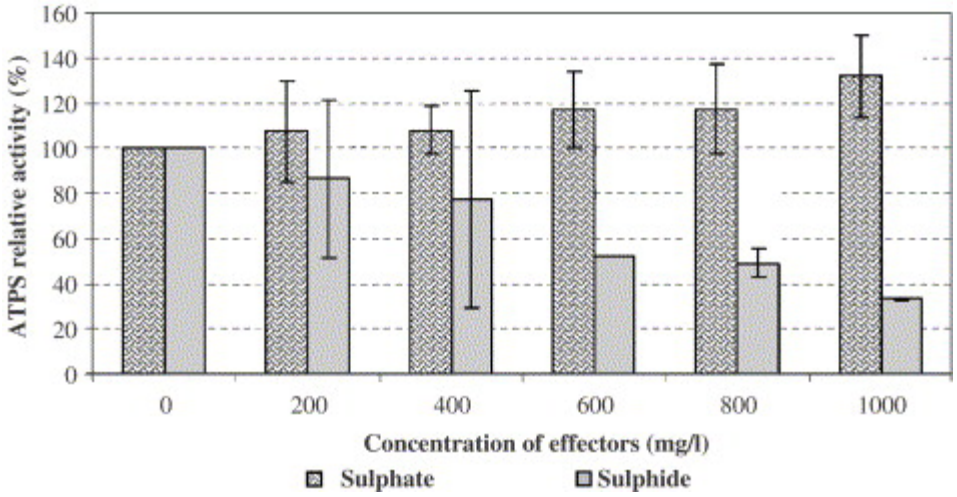

Fig. 4. The effect of sulphur-containing compounds on ATPS activity. Each data point represents a mean value±SD, *n*=3.

At a low concentration range (0–200 mg/l) there was no significant effect of sulphide and sulphate on ATPS activity with increasing concentration. Conversely, at higher concentrations (200–1000 mg/l), sulphate appeared to be stimulatory on ATPS activity, resulting in ~1.3-fold increase of the actual activity, at 1000 mg/l, while sulphide was inhibitory with a maximum inhibition of the actual activity by approximately 66% at

1000 mg/l (Fig. 4). Many enzymes are inhibited by their products (product inhibition) and stimulated (activated) in the presence of substrate, and this may explain the observed effects of the substrate (sulphate) and product (sulphide) on ATPS in this study. Sulphide has also been reported to be toxic to SRB at high levels (>100 mg/l) (Pletschke et al., 2002). In contrast to the observations made in this study, Pletschke et al. (2002) reported that sulphide and sulphate had no effect on ATPS activity at the same concentration range (0–1000 mg/l) using the reverse MgATP-synthesis reaction assay, while Segel et al. (1987) showed sulphate to be strongly inhibitory to ATPS activity.

Ca^{2+}, Cl^{-} and Fe^{2+} ions were stimulatory at the low concentrations (40–120 mg/l), but proved toxic to ATPS activity at higher concentrations (>120 mg/l), except for Zn^{2+}, which was inhibitory even at concentrations as low as 40 mg/l (Fig. 5). Ca^{2+}, Cl^{-} and Fe^{2+} ions are frequently found in acid sulphate soil environments and will therefore all lead to an overestimation of ATPS activity in samples that contain these ions. In tidal swamps and marshes, bacteria reduce sulphate from the tidewater and Fe (III) oxides from the sediment to yield pyrite (FeS_2) as the main end product. Following drainage, pyrite generates sulphuric acid and Fe^{2+} ions (Dent and Pons, 1995). The inhibitory effect of Zn^{2+} on ATPS further supports findings by Pletschke et al. (2002). In another investigation, Gavel et al. (1998) observed ATPS's from two *Desulphovibrio* species to be metalloproteins, containing both Zn^{2+} and Co^{2+}, which were required for catalytic activity. Gavel et al. (1998) showed that the activity of these pure ATP sulphurylases isolated from SRB from the genus *Desulphovibrio* could not be increased by adding Co^{2+}, Zn^{2+}, or both. Cobalt and zinc are therefore necessary as trace elements but can be toxic at higher concentrations (Gavel et al., 1998). It is possible that these Zn^{2+} ions were toxic to the ATPS activity in this study, even at the very low concentrations employed. Due to the inhibitory effects of the metal ions studied at high concentrations, metal-laden wastewaters, though sulphate-rich, can therefore still exhibit low levels of ATPS as a result of metal accumulation.

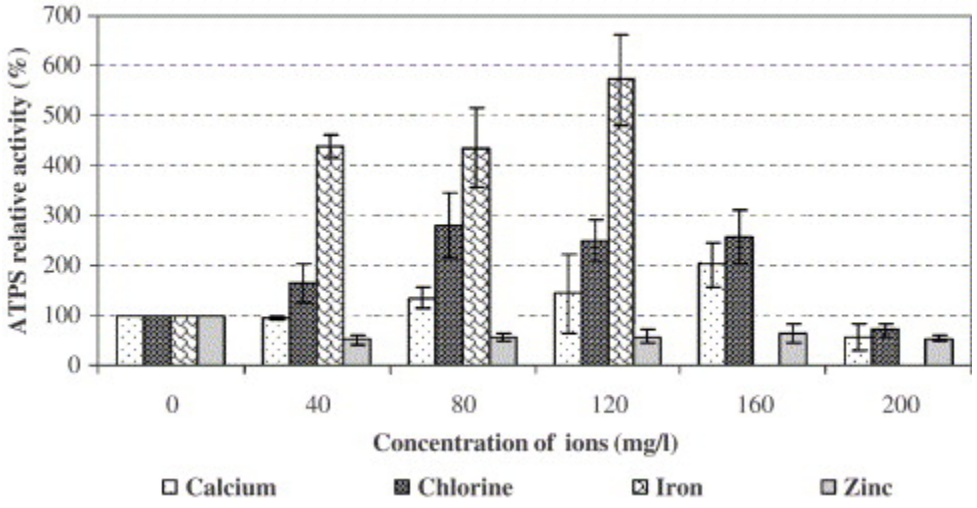

Fig. 5. The effect of various ions on ATPS activity. Each data point represents a mean value±SD, *n*=3.

In conclusion, then, it is evident from the correlation of ATPS activity to sulphate and sulphide levels (during sulphate reduction in the presence of an active SRB population) that ATPS is a key enzyme involved in dissimilatory sulphate reduction. ATPS may potentially be used as an enzymatic marker for dissimilatory sulphate reduction processes occurring in natural anaerobic environments, such as acid sulphate soils (Dent and Pons, 1995), which form large areas of coastal regions in many countries worldwide (in addition to sulphate-rich wastewaters and biosulphidogenic bioreactors). Sulphate reduction is an important process in the global sulphur cycle, and can dominate anaerobic terminal oxidation of organic matter in coastal marine sediments rich in sulphate (Purdy et al., 2002). This process can degrade up to 50% of all organic matter in these sediments (Purdy et al., 2002). ATPS activity has been shown in this study to be affected by the relative concentrations of its substrates and products (e.g. sulphate and sulphide), as well as certain ions (Ca^{2+}, Cl^{-}, Fe^{2+} and Zn^{2+}) known to be present in sulphate-rich environments such as estuarine and coastal sediments (Purdy et al., 2002) and acid sulphate soils (Dent and Pons, 1995). Therefore, all samples of interest should first be prepared in such a way so as to remove all residual sulphide and other potentially interfering ions prior to performing the ATPS assay for monitoring of the ATPS activity and the associated level of dissimilatory sulphate-reduction.

Acknowledgements

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