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The degradation of lignocellulose in a chemically and biologically generated sulphidic environment

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Abstract

Acid mine drainage waters are characterised by a low pH, high concentrations of heavy metals, high levels of sulphate salts and low concentrations of organic material. The biological treatment of these waters has been a subject of increasing focus as an alternative to physico-chemical treatment. The utilisation of lignocellulose as a carbon source has been restricted by the amount of reducing equivalents available within the lignocellulose matrix. This present study demonstrated that lignocellulose could be utilised as a carbon source for sulphate reduction. It was shown that the initial reduction of sulphate observed using lignocellulose as a carbon source was due to the easily extractable components. This degradation resulted in the production of sulphide (~500 mg/l), which further aided in the degradation of lignin (observed as a release of aromatic compounds), allowing greater access to cellulose (and release of reducing sugars).

Keywords: Anaerobic; Aromatics; Lignin; Sulphate reducing; Sulphide

1. Introduction

Acid mine drainage (AMD), also known as acid rock drainage (ARD), originates from mining activities where pyritic minerals have been exposed to water and oxygen. Both chemical and bacterially mediated reactions occur (Lovell, 1983; Johnson, 1995; Cocos et al., 2002), and the various steps involved in the formation of AMD may be summarised as follows (Johnson and Hallberg, 2003):

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (1)$$

AMD is characterised by a low pH, high concentrations of dissolved heavy metals and high levels of sulphate salts. However, not all AMD is acidic and some mine drainage waters may contain high concentrations of metals at near neutral pH values (Harris and Ragusa, 2000; Younger, 2001). Metals that commonly occur in AMD include iron, aluminium, copper, zinc and manganese. The low concentration of organic material (<20 mg/l) presents a problem for the unaugmented bioremediation of these wastewaters (Wittman and Förstner, 1976, 1977; Johnson and Hallberg, 2003).

Although it has been suggested that lignocellulose is not degraded in anaerobic environments (Björdal et al., 2000), Tuttle and co-workers as early as 1969 had shown that after mine water passed through a bed of sawdust, sulphate was reduced in the water (Tuttle et al., 1969). Benner et al. (1985) found that in wetland ecosystems alkaline conditions are necessary for the optimal degradation of cellulose. These findings were confirmed by Pareek et al. (1998, 2001) who investigated the degradation of newspaper under sulphate reducing conditions.

Benner et al. (1985) demonstrated that acidic conditions lower the degradation rate of lignocellulosic compounds compared to more neutral environments. The bio-degradation of organic matter under sulphidogenic conditions was demonstrated to be superior to methanogenic conditions by observing the rates of Avicel, filter paper and unprinted newspaper degradation. Both the rate and percentage

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solubilisation of the material was observed to be higher under sulphidogenic conditions (Kim et al., 1997). In addition, the degradation of lignocellulose was found to be more rapid under sulphidogenic conditions than methanogenic conditions (Pareek et al., 1998).

The Environmental Biotechnology Research Unit (Rhodes University) has demonstrated that the release of aromatic compounds (including the hydrolysis of complex carbohydrates) is enhanced during biological sulphate reduction (Whittington-Jones, 2000; Madikane, 2002). What was not clear from these studies was whether the mild alkaline sulphidic conditions generated by sulphate reducing consortia were instrumental in the lignocellulose degradation process alone, or whether additional biological effects were involved. The abiotic studies reported here were undertaken to investigate the influence of the non-biological components of the bio-sulphidogenic process on the lignocellulose complex, where wood tissue constitutes the electron donor and carbon source. The possible contribution of the sulphate reducing microbial consortia to the anaerobic degradation of wood tissue was also investigated.

2. Methods

2.1. Abiotic experimental set-up

For the purpose of this study, abiotic was defined as having no known living organisms in the flasks. Aerobic organisms were excluded by maintaining an anaerobic atmosphere (sparging with nitrogen). Any fluctuations observed in reducing sugar levels may be attributed to the non-sterility of the whole wood powder. Autoclaving was avoided as it would lead to ultra-structural changes to the wood tissue. Residual microbial activity consuming released carbohydrates may therefore have been present.

Degradation studies were performed in triplicate in 250 ml Erlenmeyer flasks for each of the extracts. Each flask contained 5% (w/v) of pinewood powder; with flask 1 containing tap water with pH adjustment to 8.5 with 1 M NaOH and flask 2 containing tap water, with addition of Na_2S to a final concentration of 500 mg/l sulphide. This concentration of sulphide was chosen to determine the gross chemical effect of sulphide on the wood material. All flasks were sparged with nitrogen and sealed to maintain an anaerobic environment. The flasks were placed on an orbital shaker (Labcon) (90 rpm) at 25 °C for the period of the study.

In order to evaluate the effects of sulphide and the alkaline environment on the degradation process (where pH adjustment was not performed on a daily basis) a second study was performed on pinewood powder using 0.2 M phosphate buffer at pH 8. All the supernatant liquid in the experimental flasks was removed and replaced with freshly buffered solution every 7 days to maintain sulphide levels at 300 mg/l and to prevent an accumulation of the end products of lignocellulose breakdown. Flasks were incubated at 30 °C for comparison with biotic studies, where the optimum temperature for SRP growth was applied. The same percentage of wood powder was used as before, but the experiments were carried out in 500 ml buffer in 1 l Erlenmeyer flasks. The flasks were incubated on an orbital shaker at 100 rpm. Flask 3 contained 0.2 M phosphate buffer (pH 8), without adjustment after the addition of the wood powder. Flask 4 contained 0.2 M phosphate buffer (pH 8), with adjustment of the pH with 1 M NaOH after addition of the wood powder. Flask 5 contained 0.2 M phosphate buffer (pH 8) containing Na_2S in a final concentration of 300 mg/l as sulphide. All flasks were sparged with nitrogen gas to maintain anaerobic conditions.

2.2. Biotic experimental set-up

In the wood powder degradation studies, 5% pinewood powder (w/v) was used in minimal medium (Postgates Medium C, according to Atlas and Parks, 1993) containing no other carbon source and 2000 mg/l sulphate at pH 7.5. All flasks were connected to zinc acetate (5% ZnAc, w/v) to collect H_2S. The reactor was covered with foil to prevent the growth of photosynthetic bacteria and incubated at 30 °C on a Labcon shaker at 90 rpm (flask 6).

2.3. Analysis

Reducing sugars released from the wood tissue were measured using the method adapted from Wood and Bhat (1988). The absorbance at 540 nm was read on a Beckman DU® 530 Life Science UV/vis spectrophotometer. Suitable controls were performed and the absorbance values were converted into glucose equivalents using a glucose standard curve.

The release of aromatic compounds from the wood material was monitored using a Beckman System Gold HPLC, module 126, with detection by a Beckman photodiode array (PDA); module 168 was used together with a Waters Symmetry C_{18} column (4.6×250 mm) with 5-μm particle size. The mobile phase was composed of acetonitrile (HPLC-grade, Merck) and 0.1% acetic acid: water in a ratio of 60:40 (HPLC-grade, Sigma). Naphthalene (Sigma) was used as the internal standard. The flow rate was set at 1 ml/min.

Sulphate concentrations for flask 6 were determined using ion chromatography (IC) with a Hamilton PRP-X100 column (15 mm × 4.1 mm). The mobile phase was 4 mM *p*-hydroxybenzoic acid, 2.5% (v/v) methanol, pH 8.5. The flow rate was maintained at 1 ml/min with a Waters 510 pump and detection was performed with a Waters 430 conductivity detector. Prior to chromatography, samples were cleaned by mixing in a 1:1 ratio of sample: ZnAc (10.44% w/v) to allow for removal of sulphide, followed by filtration through 0.45 μm nylon filters (to remove particulates) then passing it through a 25 mg C_{18} Isolute® solid phase extraction column to bind contaminat-

ing organics (Rein, 2002). Sulphate concentrations were determined using a sulphate standard curve. Sulphide analysis was performed using a Merck Spectroquant® Kit.

3. Results and discussion

Fig. 1a shows the maximum release of aromatic compounds from whole pinewood powder of 806 mg/l without addition of sulphide (flask 1). The release of wood aromatic compounds in the presence of sulphide (flask 2) was two-fold higher (1570 mg/l) (Fig. 1b). In both cases the release of aromatic compounds increased gradually with time until the release of aromatic compounds reached a plateau. In the absence of sulphide, a maximal level of release was reached on day 14 (796 mg/l). In the presence of sulphide, the release reached a maximum on day 18 at 1568 mg/l (Fig. 1b). With no sulphide present the rate of aromatic compound release increased steadily from 0.7 mg/l/h on day 1 to 8.4 mg/l/h on day 14. In the presence of sulphide the rate of release was much greater, with 3.8 mg/l/h being released on day 1, and increasing to 12.8 mg/l/h on day 16.

With no sulphide present (Fig. 2a) reducing sugars were not detectable on day 1, by day 4 40.53 mg/l was measured which decreased to 33.49 mg/l on day 8. In the presence of sulphide (Fig. 2b) a rapid increase in reducing sugar content was observed and then followed by a slow decrease, from 258 mg/l on day 1 to 128 mg/l by day 8. It is uncertain as to why, in the abiotic studies, the reducing sugar decreased at certain times in the time study—the possibility exists that these reducing sugars were converted to volatile fatty acids (VFAs). The levels of VFAs were not monitored in this study. Sulphate reducing prokaryotes (SRP) and methanogen producing bacteria (MPB) are generally excluded by having a concentration of sulphide (500 mg/

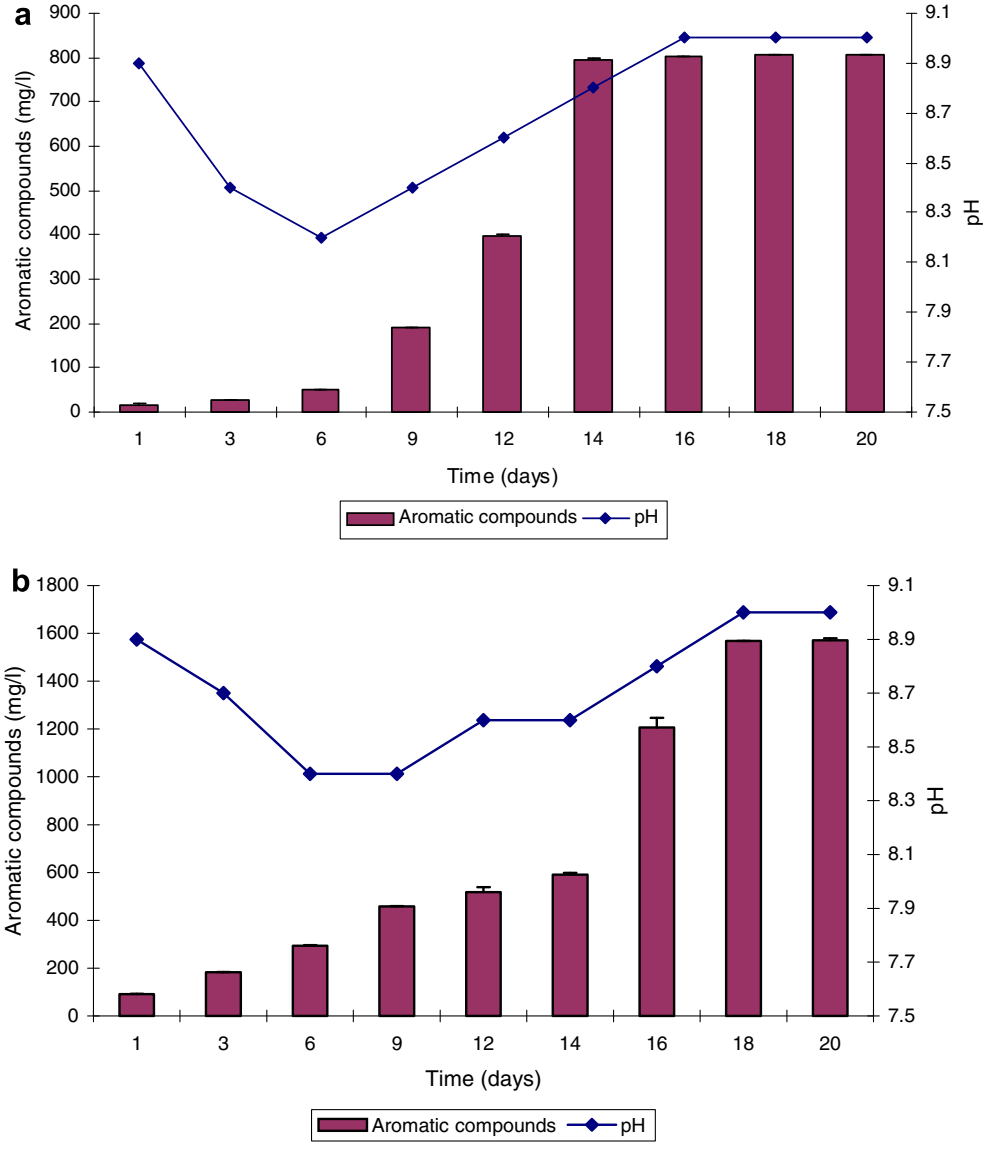

Fig. 1. Flask study of the release of aromatic compounds from: (a) pinewood powder without sulphide and (b) pinewood powder in the presence of 500 mg/l sulphide. Data points represent the means ± SD, $n = 3$.

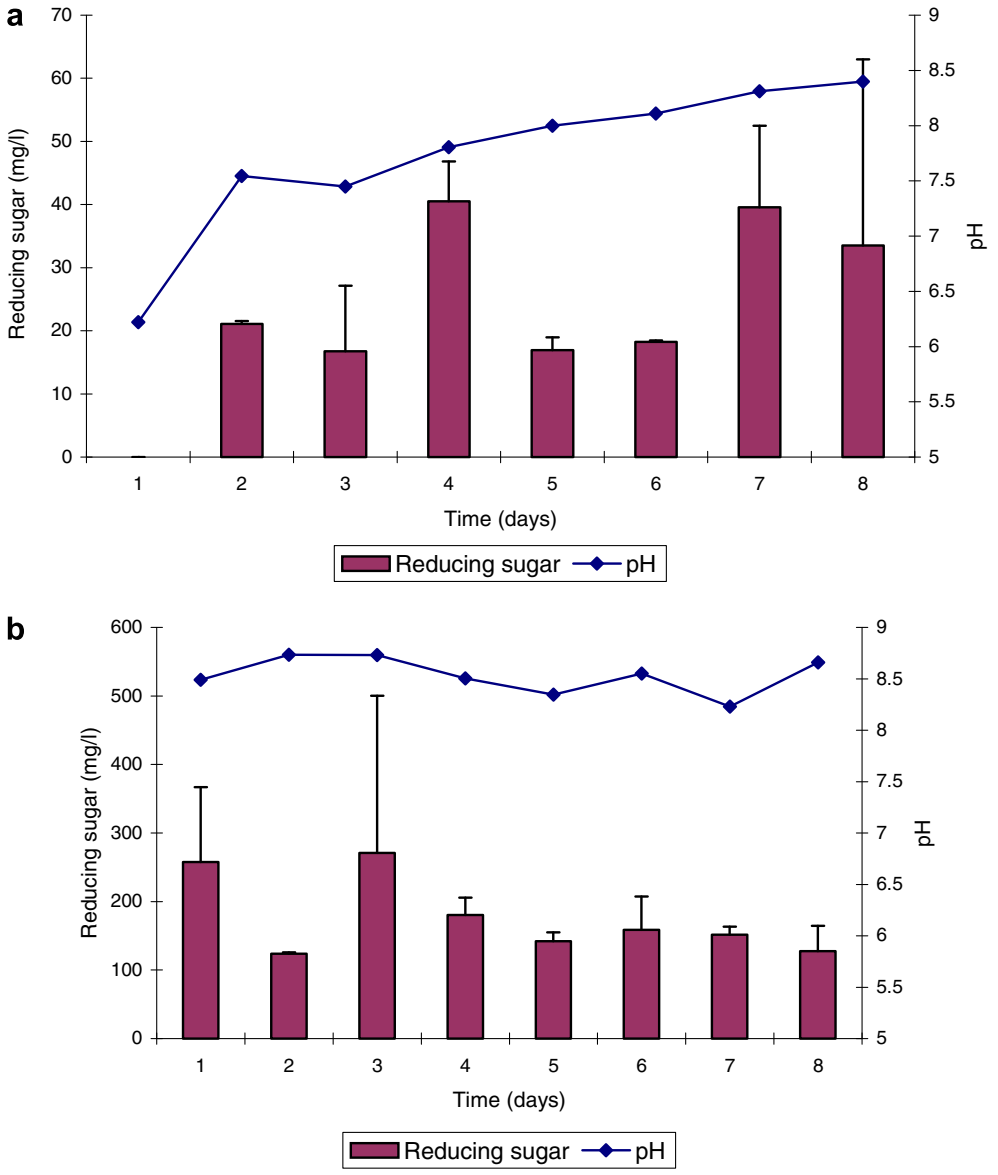

Fig. 2. Flask study of the release of reducing sugars from: (a) pinewood powder without sulphide and (b) pinewood powder in the presence of 500 mg/l sulphide. Data points represent the means ± SD, $n = 3$.

l) present that is toxic to the organisms (Hansford, 2004). However, it is uncertain as to whether sulphide is toxic to these organisms at a pH of 8.5. There were 6.7 times more reducing sugars released in the presence of 500 mg/l sulphide at pH 8.5 than without. The rate of reducing sugar release without sulphide was highest on day 4, with 1.7 mg/l/h released compared to the highest release by day 3 of 6.1 mg/l/h in the presence of sulphide.

From the above experiments it was shown that the pH was unstable in the presence of wood tissue and had to be continuously corrected to maintain stable conditions. In subsequent experiments a buffered solution was used in order to simulate the buffering environment normally maintained within a biological system. As mentioned previously, three flasks were set-up: flask 3 contained the phosphate buffer with no sulphide, flask 4 contained the phosphate buffer with no sulphide but the pH was adjusted daily and flask 5 contained the phosphate buffer and sulphide. All of the flasks demonstrated high levels of reducing sugars after day 1 and up to day 30, and by day 59 no further reducing sugars were detected (Fig. 3a). The possibility exists that these reducing sugars were converted to volatile fatty acids (VFAs). The levels of VFAs were not monitored in this study. Flasks 3 and 4, without any sulphide, showed a steady decline in the release of aromatic compounds after the initial release of 51.03 mg/l and 59.6 mg/l, respectively on day 1 (Fig. 3b). In the presence of sulphide, the release of aromatic compounds increased from 68.9 mg/l on day 1 to 161.5 mg/l on day 23. Thereafter, the release of aromatic compounds decreased to 33.9 mg/l on day 59 (Fig. 3b). Sulphide, therefore significantly increased the levels of the aromatic compounds in the medium ($t = 4.01$,

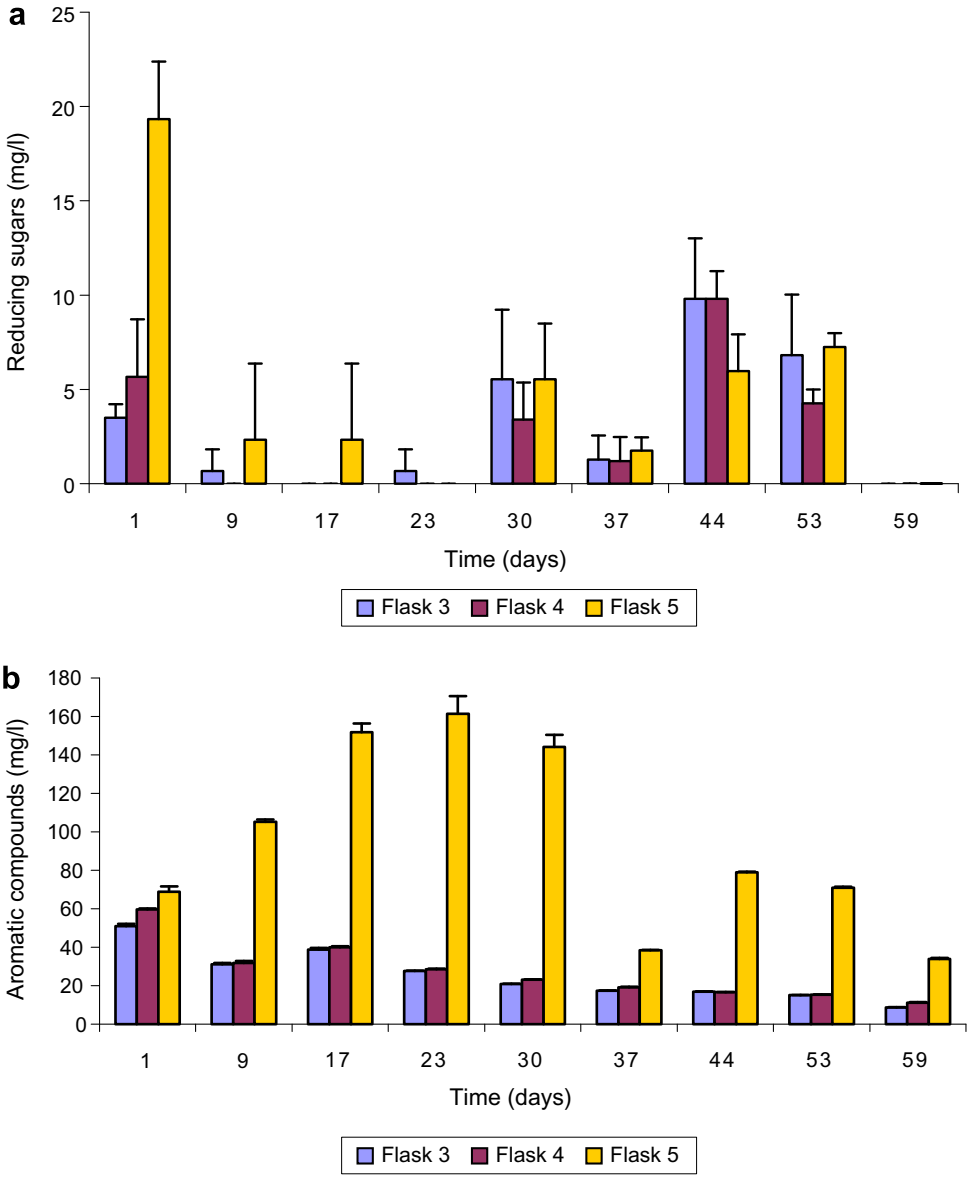

Fig. 3. Release of (a) reducing sugars and (b) aromatic compounds over time. Flask 3, phosphate buffer without sulphide and no pH adjustment; flask 4, phosphate buffer with no sulphide and pH adjustment to pH 8; and flask 5, phosphate buffer with 300 mg/l sulphide. Data points represent the means ± SD, $n = 3$.

$p < 0.05$) (as compared to the levels of reducing sugars in the medium). It was also observed that the levels of wood aromatics (Fig. 3b) were at their greatest (days 9–30), the levels of reducing sugars were at their lowest (Fig. 3a). As the levels of wood aromatics decreased, the levels of reducing sugars increased (between days 30 and 53) (Fig. 3a and b). This could be due to the possible separation of the aromatic fraction from the carbohydrate fraction and the fact that these two processes may occur independently from one another (Sun and Cheng, 2002).

Fig. 3b clearly shows that sulphide had an enhanced effect on the release of wood aromatics over a prolonged period. Initially the release of wood aromatics was similar for all three treatments, but by day 9 there was already a marked enhancement of wood aromatic release observed in flask 5, which continued to the end of the study. Dilute alkali pre-treatments to remove lignin for the utilisation of cellulose have shown that it leads to a swelling of the wood material as well as a separation of the linkages between lignin and hemicellulose (Sun and Cheng, 2002). This swelling of the lignocellulose material also leads to an increase in the release of soluble aromatics, which is evident by the very high levels of wood aromatics present from day 17 to day 30 (Fig. 3b). The decrease in wood aromatic levels after day 30 was possibly due to the exhaustion of soluble aromatics and the slow hydrolysis rate of lignin.

In the biotic study (flask 6), inoculated with the sulphate reducing microbial consortium, sulphate was reduced to zero in 27 days by the sulphate reducing microbial consortium (Fig. 4). After spiking with an additional 1750 mg/l

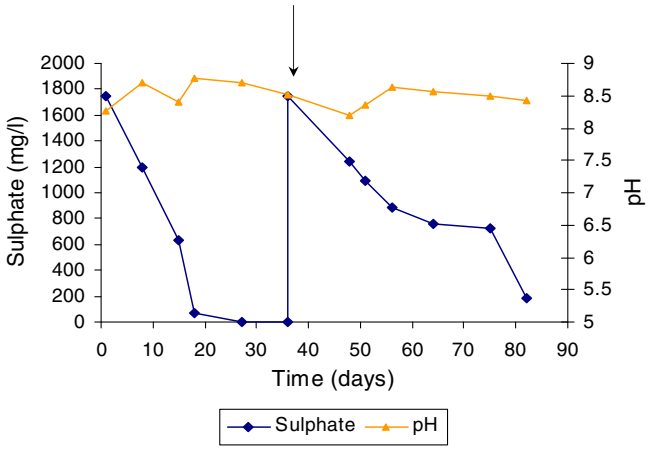

Fig. 4. Sulphate and pH profile in flask 6 (biotic study) over the 82 days of the wood biodegradation study. The arrow indicates spiking with additional sulphate (1750 mg/l) on day 36.

sulphate on day 36, further reduction occurred to 187.1 mg/l over a further 46 days. After spiking with the initial concentration of sulphate on day 36 the rate of sulphate reduction was observed to be 0.2 mg/g wood/day between days 36 and 56, and the pH stabilised around pH 8.5. Concomitantly, sulphide increased from 286.3 mg/l on day 27 to 534.3 mg/l by day 59 (data not shown), thus establishing comparable conditions to the abiotic study (i.e. level of 500 mg/l).

The release of aromatic compounds from pinewood powder (Fig. 5a) exhibited a cyclical pattern of production and consumption with peaks observed on days 16, 35 and 52. Fig. 5b shows production and consumption of reducing sugars (conversion to VFAs) within the batch reactor with a peak around day 18 and again at day 58 and 72, and may be related to the activity of the sulphate reducing microbial consortium. As in the abiotic study, the levels of aromatic compounds released were far greater than the corresponding levels of reducing sugar released into the medium.

In conclusion, then, the abiotic studies demonstrated that aromatic compounds were released to a greater extent than reducing sugars from pinewood powder. In both the

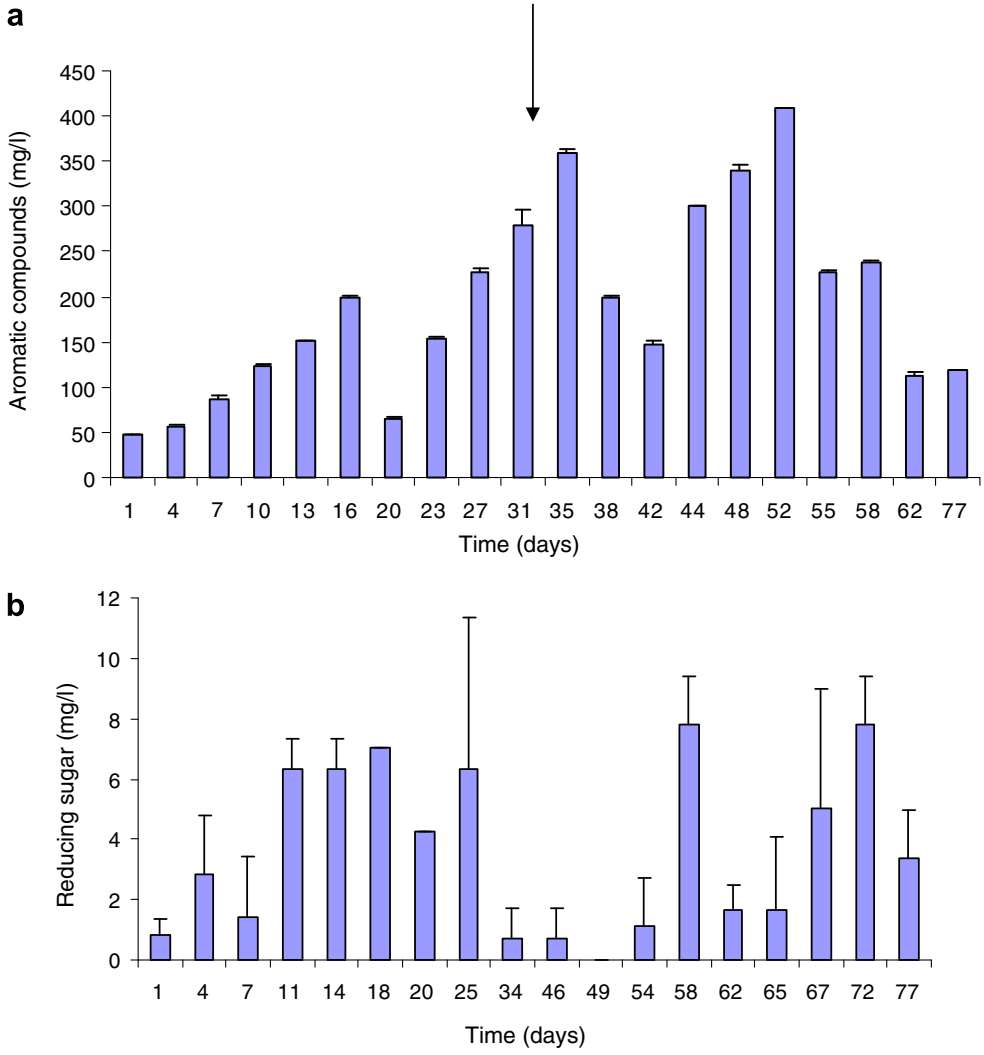

Fig. 5. The release of (a) aromatic compounds and (b) reducing sugar from the biotic flask batch reactor (biotic flask study). The arrow indicates spiking with additional sulphate (1750 mg/l on day 36). Data points represent the means ± SD, $n = 3$.

presence and absence of sulphide the levels of aromatic compounds were far greater than the levels of reducing sugars. For the biotic study (flask 6) a similar result was obtained (Fig. 5a and b). The results obtained for an abiotic system provides an indication of events occurring in wood-packed sulphate reducing biological reactors treating sulphate-rich wastewaters. It was demonstrated that sulphide and alkalinity increased the levels of reducing sugars and aromatic compounds from wood material compared to water treatment on its own. The results obtained for the biotic study suggest that the hydrolysis of lignin is uncoupled from the hydrolysis of the cellulose component. The easily extractable aromatic component contributes to the initial sulphidogenesis that takes place.

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