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Mechanism of bioleaching of coal fly ash by *Thiobacillus thiooxidans*

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Abstract

Bioleaching of aluminum and iron from coal fly ash (CFA) by *Thiobacillus thiooxidans* (*T. thiooxidans*) bacteria is considered. The interactions between bacteria, metabolic products, CFA particles, and leaching products were studied. It is demonstrated that bacterial growth and the amount of metals leached from the CFA are coupled through biological and chemical interactions, which involve the various components in this system.

Bioleaching experiments were performed batch wise by suspending up to 10% (w/v) CFA in *T. thiooxidans* growth medium containing cell inoculum for a typical 3 week period of time. Samples were taken periodically from leached suspensions and relevant parameters including metals' concentrations, cell counts, pH and extracellular polymeric substances (EPS) were determined.

The results show that under the same conditions, similar leaching levels are obtained by sulfuric acid and bioleaching of CFA, and the contribution of other metabolites is insignificant. CFA inhibits the growth rate through two major effects. The first is due to the alkaline components released by the CFA that cause a rise in the pH, and a corresponding delay in growth. The second is attributed to the random attachment of the bacteria to both the sulfur particles (the energy source) and the barren CFA particles, resulting in a so-called "dilution effect" of the sulfur particles, and an inhibition of the initial growth rate. However, after an adaptation period of the bacteria the subsequent growth rate, the maximal cell concentration and minimal pH were similar to those obtained in the control experiment, irrespective of CFA content. Enhanced excretion of EPS was observed in the presence of CFA as well as in calcium and barium enriched growth media. It is presumed that the mechanism of EPS production is related to the presence of the particulate solid phase.

Keywords: Coal fly ash; *Thiobacillus thiooxidans*; Bioleaching; Microbial growth; Bioprocess; Solid-waste treatment

1. Introduction

The growing amounts of coal fly ash (CFA), which are produced by coal-operated power plants worldwide, call for their increased utilization. In this context CFA can be used as a source for metal extraction within appropriate economical constraints [1–4]. Applying biohydrometallurgical technologies, for the recovery of valuable metals from solid residues including fly ash was recently considered [5–7]. The leached metals can then be further processed while the residues may be safely deposited or utilized for construction purposes. The advantage of bioleaching is the relatively low cost and mild conditions of the process, and the subsequent low demand for energy or landfill space compared with conventional technologies [7]. However, slow kinetics and insufficient selectivity with respect to specific metals, particularly aluminum [3,8,9], can offset the advantages of bioleaching.

Among the variety of microorganisms that are known to facilitate metal bioleaching reactions, the autotrophic *Thiobacilli* species are perhaps the most common [7,10]. The bacterium *Thiobacillus thiooxidans* (*T. thiooxidans*) is active at low pH and can endure harsh conditions that exist in concentrated solutions of metals [11]. These particular characteristics make it a suitable microorganism for bioleaching. Specific examples of *T. thiooxidans* growth in environments containing high concentration of metals are: Zn and Cd up to 600 and 400 mM, respectively [12], As^{3+} and As^{4+}: 67 and 534 mM, respectively, iron: Fe^{2+} 537 mM and Fe^{3+} 180 mM [13], and aluminum: 370 mM [14]. In the case of zinc and cadmium, specific proteins were produced, which were capable of binding these metals [12]. The presence of high content of particulate matter can impede bacterial growth. Kandemir [15] showed that for *T. ferrooxidans* in a sulfide mineral medium, particulate matter content above 16% becomes the main growth-limiting factor. Here, the impeding effects were due to a slow down in the rates of oxygen transfer and decreased removal of metabolic products of the bacteria.

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T. Thiooxidans oxidizes sulfur as its principal metabolic process. Like other *Thiobacilli* species, which utilize insoluble metal sulfides and/or sulfur as an energy source, the bacteria developed a mechanism that facilitates the attachment of cells to the surface of sulfur/sulfidic particles [10,16–28]. This mechanism and the conditions that enhance attachment of *T. thiooxidans* to solid surfaces are not well understood. They involve effects of electric charges, surface irregularity, such as cracks or high local concentration of a specific mineral [16,17], as well as cell membrane characteristics [18–20]. In addition, it involves the excretion of extracellular polymeric substances (EPS), which mediate the contact between the cell and the sulfidic energy source [10,21,22]. Besides sulfuric and sulfidic surfaces [23–25], *Thiobacilli* species are also capable of attaching to other solid substances such as coal [26–28] zeolit and glass [18–28].

In this work we study the process of bioleaching of CFA by *T. thiooxidans*. The study is concerned with the effects of CFA content in suspension on growth of *T. thiooxidans* and the subsequent bioleaching of aluminum and iron. In this context, cell attachment to the suspended CFA particles and EPS production including the conditions that enhance this phenomenon, are also investigated.

2. Materials and methods

2.1. Bacterial strain, growth medium, and bioleaching tests

T. thiooxidans ZYR-1 bacteria was obtained from the Ness-Ziona Biological Institute of Israel. Bacteria were cultivated in 2 l Erlenmeyer flasks containing a standard growth medium [29] and 0–10% (w/v) CFA (Israel Electric Corporation from the Rutenberg Power Plant). After adjusting the pH to 5 by sulfuric acid (analytical grade, Frutarom, Israel), the medium was inoculated (approximately 5×10^6 cell/ml — 1 week old) and incubated at 28°C and constant agitation (200 rpm, AK 15, INFORS AG HT shakers). Growth was monitored by cell counts (Petroff hauser count cell and Zeiss, Axiolab light microscope), pH (Consort r301 pH controller) and sulfate accumulation (4500 — SO_4^{2-} C gravimetric standard method [30]). In addition the following parameters were determined periodically: extracellular protein (protein assay kit — Bio-Rad, USA), oil and grease (the 5520 B, oil and grease partitioning gravimetric standard method [30]), and extracellular carbohydrates (the modified sulfuric acid–phenol method [31,32]), that were calibrated using glucose as a standard. Sulfur content in the growth medium was determined gravimetrically after roasting at 600°C.

Aluminum (3000 mg/l), iron (Fe^{2+}, 500 mg/l), calcium (100–1000 mg/l) or barium (20–1000 mg/l) were added to the growth medium as chloride salts, one salt at a time as described below. Other additions consisted of different types of particulate: 5% (w/v) of 5–70 μm glass beads, 5% sea sand, and 5.0 μm alumina powder, purchased from Buehler (USA).

2.2. Leaching tests with sulfuric acid

Leaching tests with sulfuric acid were carried out using a procedure similar to that used for the bioleaching tests with the required addition of concentrated sulfuric acid.

2.3. Pre-leaching conditioning process

A pre-leaching conditioning of the CFA was carried out in the following manner. A sample of CFA was dispersed in HCl solution at constant pH 4 and agitated for 24 h, the CFA was then removed from the suspension, washed with deionized water, and dried at 110°C.

2.4. Chemical analyses

Samples of 1.5 ml suspension were taken periodically from the flasks, centrifuged at 12 000 g for 5 min, and the supernatant was submitted for elemental analysis by flame atomic absorption spectrometry (FAAS) (Perkin Elmer 460) and by Inductively Coupled Plasma (ICP) (Perkin Elmer, Optima 3000 DV). CFA elemental analysis was conducted according to the American Society for Testing and Materials procedure (The American Society for Testing and Materials — ASTM, designated D 3683-78, 1989). The National Bureau of Standards, standard reference material (SRM 1633a) was used primarily as a calibration standard for trace elements.

2.5. Cell attachment to suspended particulate matter

Measurement of cell attachment to suspended particulate matter was performed using a fresh culture. A 2 l, 2 week old cell culture was filtered on a GF/A Whatmann filter in order to remove residual sulfur. The filtrate was centrifuged at 12 000 g for 10 min, and the supernatant was removed thereafter. The bacteria were washed with deionized water, resuspended in a fresh sulfur-free growth medium, and then the liquid cell concentration (X_t) was determined. A known weight of solids was added to a measured volume of cell suspension. After shaking the cell suspension for 10 min (or otherwise as specified), the particulate matter was removed by centrifugation at 100 g, and washed with fresh growth medium. The cell concentration in the liquid phase (X_l) was determined once more. The difference between the initial and final cell concentrations was defined as the concentration of attached cells, X_a.

The calculation of attachment density in terms of cells per unit solid surface area (cell/cm^2), required determination of mass density (measured by a picnometer) and particle size distribution, in terms of number density of particles in each size fraction. The attachment density, Φ, is

defined by

$$\Phi = \frac{X_a}{6m\sum_{i=1}^{k} f_i d_i^2 / \rho \sum_{i=1}^{k} f_i d_i^3}$$

where m and ρ denote solids content (g/ml), and their density, respectively, and f_i and d_i, the number frequency and diameter of particles in the ith size fraction, $i = 1, \ldots, k$, respectively. The concentration of attached cells, X_a, has the same units as X_t, i.e. cells per unit volume of liquid growth medium.

Note that $6\sum_{i=1}^{k} f_i d_i^2 / \sum_{i=1}^{k} f_i d_i^3$ is the surface to volume ratio of the k size fractions, and m/ρ is the volume of solid particles per unit volume of suspension.

2.6. Solid phase characterization

Samples from growth suspensions were submitted to scanning electron microscopy (SEM) and energy dispersive X-ray spectrometry (EDS) analyses. The samples were prepared by membrane filtration (0.2 μm, Poretics polycarbonate), and washed for 1 h with a 2% glutaraldehyde solution followed by a series of washings with mixtures of water/ethanol. The samples were coated with gold or carbon (EDS tests only) and examined by SEM (Jeol 5400), and EDS (Voyager of Noran).

Particle size distributions were measured by a Galai-Cis 1 system. A plastic cuvette containing the sample suspended in 4 ml of deionized water was placed in the instrument and stirred during data acquisition. The obtained data was further processed as described above.

3. Results and discussion

The bioleaching system studied in this work involves *T. thiooxidans* bacteria and CFA in suspensions containing a relatively high content of suspended solids (up to 10% CFA). This system consisted of bacterial cells, their metabolic products, CFA particles and the leaching products. It is demonstrated that bacterial growth and metal leaching from the CFA particles are coupled through biological and chemical interactions, which involve the different components in this system. The leaching rate of CFA was dependent on the amount of sulfuric acid produced by the bacteria [3]; acid concentration was dependent on cell growth rate, and finally, cell activity was dependent on suspension composition (suspended solids, pH and leaching products).

3.1. Effect of CFA on T. thiooxidans

The presence of CFA was found to inhibit the initial growth rate of *T. thiooxidans* (Fig. 1). The major inhibiting factor is the rise in pH due to dissolution of alkaline components of the CFA. Therefore, in order to enable cell growth,

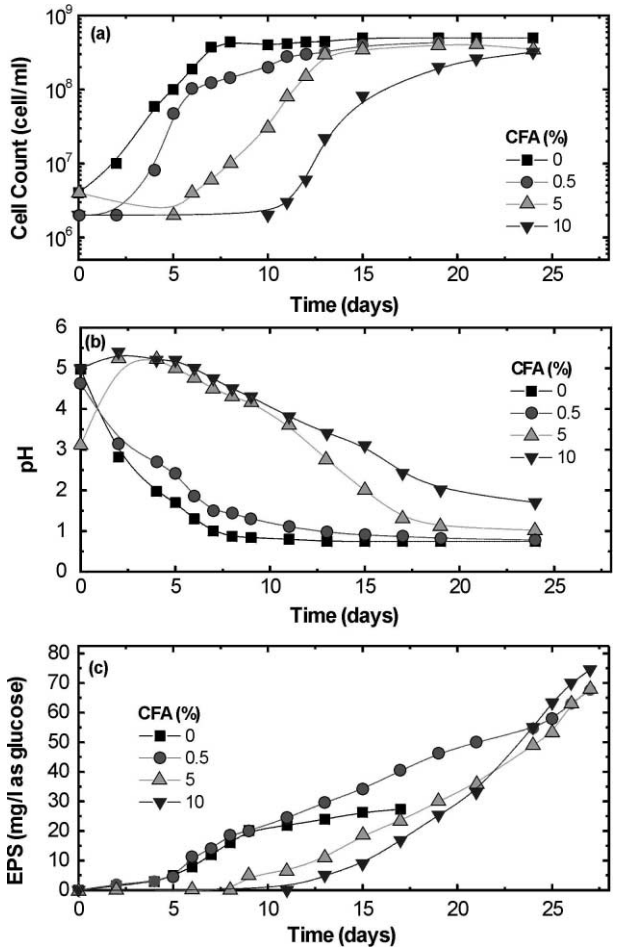

Fig. 1. Batch growth profiles of *T. thiooxidans* cultures in media containing 0, 0.5, 5 and 10% (w/v) CFA expressed in terms of: (a) cell count, (b) pH, and (c) EPS concentration. The concentration of EPS was determined as polysaccharides using glucose as a standard.

the pH was not allowed to exceed 5 where the growth rate was slow. Nevertheless, following a period of adaptation, the cell growth rate in suspensions containing up to 10% CFA resumes the level observed in the control medium (log phase lasts approximately 5–6 days, regardless of CFA content). Maximum cell concentration reached 5×10^8 cell/ml. Generation time ranged from 18.2 h (0.5% CFA) up to 27.9 h (5% CFA), and the average time was 24.3 ± 4.2 h.

In this context, a high concentration of leached metals may affect growth rate. Nevertheless, *T. thiooxidans* is known to grow in harsh environments containing high concentrations of metals. Thus, cultures that passed the lag phase were able to develop in the presence of the leached metals, and subsequently reach the final concentration of the control culture.

Significant increase in the rate of EPS production was observed during the log phase of cell growth in the presence of CFA (Fig. 1c). At extended growth times, this rate was enhanced as CFA content increased. The rate of EPS production followed the pattern of cell growth rate: In both

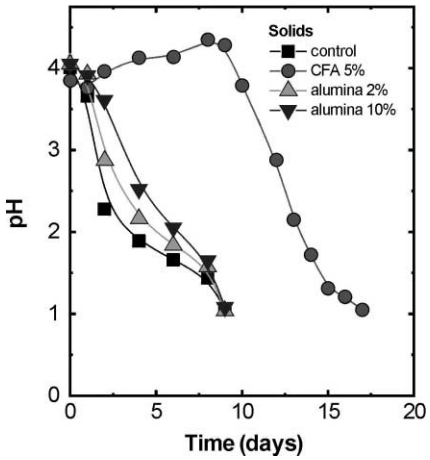

Fig. 2. Effect of different suspended particulate matter on *T. thiooxidans* growth rate (expressed as change in medium pH vs. time).

cases a rapid increase in concentration occurs during the log phase.

The growth of *T. thiooxidans* cultures, in the presence of 2 and 10% aluminum oxide particles, was inhibited compared to the control medium but enhanced compared to the CFA containing medium (Fig. 2). In the presence of conditioned CFA (pre-leached in hydrochloric acid solution at pH 4 for partial removal of alkaline compounds and calcium in particular), the growth rate of cultures was increased relative to those that grew in the presence of unconditioned CFA (data shown in [3]). These results suggest that under the prevailing experimental conditions, suspended solids were not dominant in suppressing growth rate, e.g. as compared to the buffering capacity of the CFA and effect of leached toxic metals. Furthermore, precipitation of calcium sulfate, and random attachment of bacteria to CFA particles are likely to affect growth rates (see discussion below).

3.2. Excretion of metabolites

In addition to sulfuric acid *T. thiooxidans* excretes other metabolites including EPS. In order to identify factors that enhance EPS production, different materials were added to the growth medium and their concentrations were set to reflect the properties of leached CFA suspensions (regarding metal concentration and suspended solids). These included aluminum (3000 mg/l), iron (500 mg/l), glass beads (5%) and sand (5%). None of these materials enhanced EPS production (data shown in [3]). In contrast, a medium containing 1000 mg/l calcium and the 5% CFA medium produced a comparable increase in EPS excretion. The EPS production in the presence of calcium increased significantly with incubation time after the 6th day (Fig. 3). A maximum of EPS excretion was observed at calcium concentration of 1000 mg/l and then it leveled off beyond 5000 mg/l.

Enhanced EPS production was detected also in barium rich growth medium (data shown in [3]). In this case, maximum EPS production of 28 mg/l (after 14 incubation days),

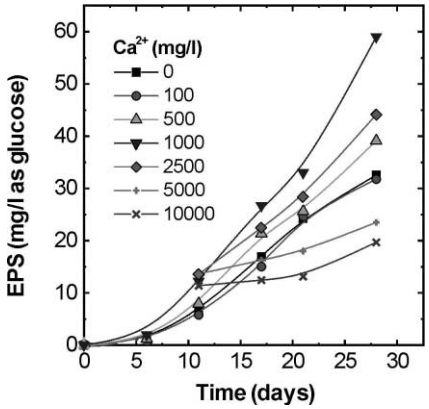

Fig. 3. Effect of calcium concentration on the production of EPS by *T. thiooxidans*. The concentration of EPS was determined as polysaccharides using glucose as a standard.

and 11 mg/l (after 7 incubation days), were observed in the presence of concentrations as low as 20 and 100 mg/l of barium, respectively.

As both calcium and barium sulfate are sparingly soluble (the solubility products of calcium and barium sulfate at 25°C, are 2.45×10^{-5} and 1.08×10^{-10}, respectively, [30]), the EPS production can be linked to the formation of precipitates. The greater ability of barium to stimulate EPS production is attributed to the lower solubility of barium sulfate. These findings can be explained with respect to the role of EPS in mediating the contact between *Thiobacilli* and their sulfuric/sulfidic energy source [10]. It is therefore postulated, that the mechanism stimulating EPS production is primarily related to the presence of a solid phase, and it depends to a lesser extent on specific characteristics of this solid.

In addition to carbohydrates, the concentration of extracellular protein and lipids were also determined. However, no significant changes in the concentrations of these components were detected. In this context, the complexity of solution composition, and insufficient sensitivity of the analytical methods may have prevented detection of relatively small changes in concentration.

3.3. Attachment of T. thiooxidans to CFA

T. thiooxidans is known to adhere to the surface of sulfur and other solid substances including, as was verified experimentally, CFA (see Fig. 4). Attachment of bacteria to the CFA took place a short time after the latter was introduced into the growth medium (Fig. 4a), and persisted after 10 incubation days in this medium (Fig. 4b). The initial attachment density was found to be independent of pH in the range 1.2–6. In a suspension comprising 2×10^8 cell/ml and 10% CFA, approximately 80% of the cells were attached to the CFA with corresponding density of 3×10^6 cell/cm^2. Similar cell densities were observed when the CFA was replaced by 5 μm alumina, and 2.45 μm magnetite particles. Different mechanisms seem to be involved in the attachment at short-

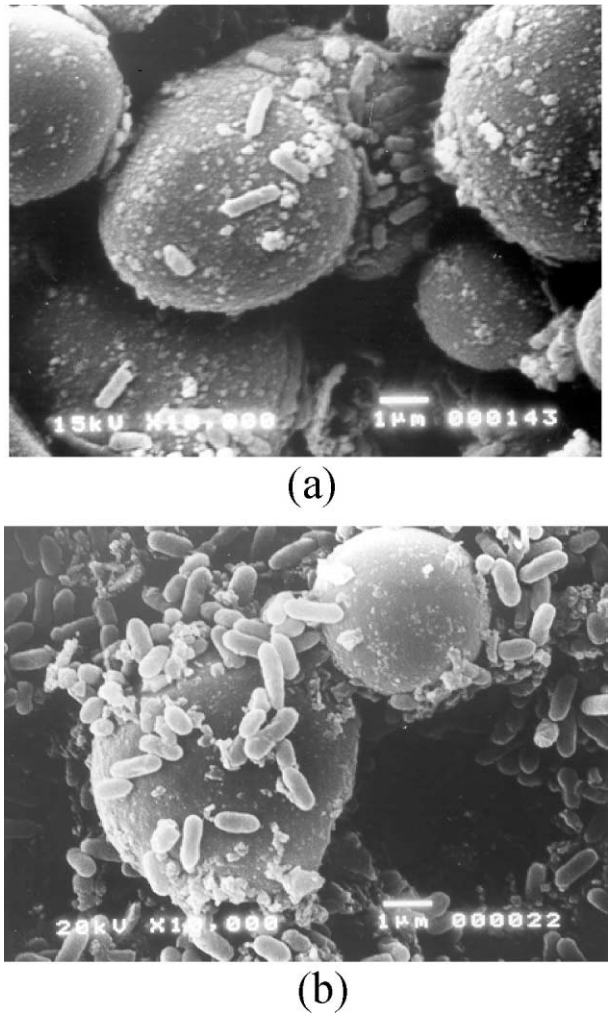

Fig. 4. Attachment of *T. thiooxidans* to CFA. SEM micrographs of cultures obtained after: (a) 30 min and (b) 10 days incubation time. Magnification ×10 000.

and long-term exposure of the cell to the solid phase. Initially, surface/cell interactions are controlling (e.g. surface charge, hydrophobic interactions), whereas at a later stage, EPS is likely to be significant in this process, i.e. following cell adaptation to the growth medium and the subsequent accumulation of EPS in the suspension.

Considering the size of a free cell and CFA particles (mean size 1–2 μm, see Table 1) it was expected that cell attachment to the CFA would increase the latter's size. This was verified by particle size measurements that show this change

Table 1
Mean particle size and standard deviation of suspensions of CFA, *T. thiooxidans*, and their mixture

	Mean diameter ± standard deviation (μm)
CFA	1.87 ± 1.64
T. thiooxidans	1.20 ± 0.77
CFA + *T. thiooxidans*	1.95 ± 1.82

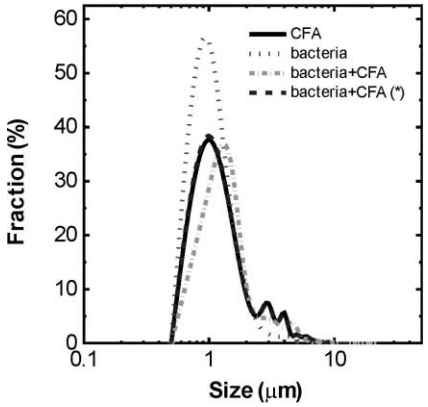

Fig. 5. Particle size distribution of suspensions of CFA, *T. thiooxidans*, and their mixture. Calculated distribution of the mixture is also shown (∗).

in terms of size distribution of the free cells, CFA particles, their mixture, and the calculated values of the combined populations (Fig. 5 and Table 1).

In view of the fact that only cells that attach to sulfur can multiply [23], even a temporary attachment to the CFA is expected to inhibit the growth rate initially, when cell concentration is still low (5×10^6 cell/ml). This delay is attributed to the randomness of motion and attachment of the bacteria in the suspension. Consider, e.g. a 10% CFA suspension having mean particle diameter of 1.87 μm, and density of 2.21 g/cm^3. This gives a surface area (assuming spherical particles), of 1.45×10^{11} μm^2/ml-suspension. If cell dimensions are 1×0.5 μm^2, then 1% surface coverage of the CFA is equivalent to 2.9×10^9 cell/ml-suspension. In a growth medium of 1% sulfur, consisting of 29.9 μm particles and density of 1.95 g/cm^3 [23], the densest monolayer is equivalent to 2.1×10^9 cell/ml suspension. In this context, Konishi et al. [23], specified maximum adsorption of 4.75×10^{10} cell/g sulfur, which here translates into 4.57×10^8 cell/ml suspension.

Thus, the 1% surface coverage in a 10% CFA suspension being considered here is approximately equal to a complete surface coverage of the sulfur. In other words, the expected number of occupied "attachment sites" that can be available from the CFA and sulfur are of the same order. The number density of CFA particles is estimated as 1.3×10^{13} particles/ml-suspension, and that of the sulfur as 3.7×10^8 particles/ml-suspension. This implies that the probability of collision between a cell and a CFA particle is very likely, and even brief attachment of cells to CFA particles produces a significant growth hindrance. This effect is more pronounced at low (initial) cell concentration (5×10^5–1×10^6 cell/ml), where the surface coverage of the sulfur is still low. Thus, the time lost in a barren attachment of cells to the CFA is translated into growth lag. However, as the cell concentration in the growth medium increases, and the sulfur content decreases, the shrinking surface area of the remaining sulfur becomes the controlling growth factor. For example, if half of the sulfur is consumed by the

cells (say from 1 to 0.5%) in a process where the size of each sulfur particle is equally reduced then $(d_2/d_1)^3 = 0.5$, where d_1 and d_2 are diameter of the sulfur particles before and after they have been consumed. This leaves 0.63 of the initial surface area for further attachment of the increased number of cells.

A similar effect was observed in the presence of alumina and conditioned CFA (Fig. 2). In both cases part of the observed delay in growth is attributed to the random attachment of cells to the suspended particulate mater. In this context, the combined effects of alkalinity, toxic leached products and attachment to the CFA particles may be considered as causing the overall delay in the rate of growth of the bacteria.

3.4. Effect of deposits of calcium sulfate on T. thiooxidans

Leached calcium from the CFA may precipitate as calcium sulfate once the activity product of calcium and sulfate in solution exceeds the K_{sp} (2.45×10^{-5} [30]). Enhanced precipitation of calcium sulfate is expected to take place at the leaching sites due to the high concentration of calcium produced there [33]. Similarly, high local concentration of sulfate generated near the bacteria is expected to enhance precipitation of calcium sulfate on the bacteria surface. The deposition of such precipitates on the cell membrane is expected to affect the bacteria growth rate by limiting transfer of nutrients to the cell, and the removal of metabolites away from it. Moreover, calcium sulfate deposition on the cell and/or on the surface of sulfur particles can interfere with cell attachment to the sulfur particles resulting in suppressed growth rates.

It was found that in the absence of CFA, the growth rate was unaffected by the presence of calcium sulfate in the growth medium (up to 10 000 mg/l). However, addition of calcium to a growth medium containing 5% CFA inhibited the growth rate. The ability of the cells to withstand relatively high concentrations of calcium in the absence of CFA can be linked to the EPS, which is excreted by the cells in the presence of both CFA and calcium, and was found to bind precipitates as well as particulate material [3]. The presence of CFA increases the surface area to which the EPS binds and decreases its efficiency as a "precipitate capturing agent". This may increase the amount of deposition of calcium sulfate on the cell and sulfur particles.

3.5. Aluminum and iron bioleaching from CFA

Aluminum and iron bioleaching from CFA by *T. thiooxidans*, occurred concurrently with cell growth and acid production (see Figs. 1 and 6). The rate of leaching followed the rate of cell growth and the decrease in pH. This applies to the lag phase and the rapid increase in cell growth during the log phase until the maximum leaching levels of approximately 3000 and 400 mg/l for aluminum and iron, respectively, were reached. This shows that acid concentration is

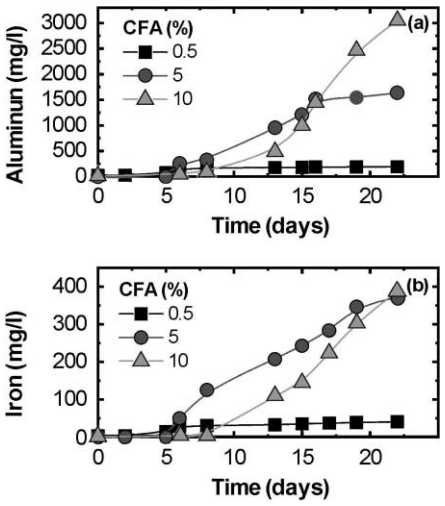

Fig. 6. A plot of aluminum (a) and iron (b) bioleaching from CFA by *T. thiooxidans*.

the major controlling parameter of both the leaching rate and extraction level.

Upon comparing biological and chemical leaching of CFA with sulfuric acid, under the same experimental conditions (i.e. temperature, agitation, CFA content, pH and rate of change of pH), no significant differences between the results of bio- and sulfuric acid-leaching were observed. Thus, it was verified that acid concentration is indeed the governing parameter of the process (data shown in [3]).

The differences between the leachability of aluminum and iron (Fig. 6) are attributed to their different contents in the CFA (155 mg/g-CFA and 37 mg/g-CFA, for aluminum and iron, respectively, [33]), and their different solubility vs. pH (aluminum is more soluble at $1.5 < \text{pH} < 4$ [4]). The percentage of the leached fraction after 3 weeks of bioleaching time was approaching 25% for aluminum and between 15 and 22% for iron, depending on CFA content. However, in contrast to aluminum the trend of the iron curve implies that this fraction could be further increased if leaching time was longer.

Precipitation of calcium sulfate on CFA particles that was observed during sulfuric acid leaching of CFA [3,4,33] was also noticed in the bioleaching process by *T. thiooxidans*. The so-called self-inhibition mechanism involves the formation of precipitates on the surface and within CFA particles, which serve as barriers and hinder mass transfer. Removal of alkaline components from the CFA by hydrochloric acid prior to bioleaching enhanced the subsequent leaching rate (data shown in [3]). However, there was no significant change in the maximum extraction level.

4. Conclusions

The alkaline CFA used in this study produced a delay in the growth rates of *T. thiooxidans* cultures grown in

suspensions containing up to 10% (w/v) CFA. This delay is attributed primarily to the buffer capacity of the CFA. Following a period of adaptation, the bacteria regained its capacity to multiply at the same rate as the control culture, and reached the same maximum cell concentration of 5×10^8 cell/ml. Although, the metals leached from the CFA did not prevent cell growth, they may have contributed to extension of the adaptation period.

The *T. thiooxidans* bacteria that grew in a medium containing CFA, produced up to three times the amount of EPS, as compared to the control medium (25 mg/l polysaccharides in the control medium and 55–70 mg/l in the presence of CFA). Media that was enriched with either calcium or barium, which formed the respective sparingly soluble sulfates, induced similar effects. Thus, it is assumed that this phenomenon was related to a mechanism, which involves solid–bacteria interactions.

T. thiooxidans bacteria was found to adhere to CFA particles in a fresh medium as well as in a 10 day old culture. The random attachment of cells to CFA and sulfur particles is believed to contribute to the initial delay of bacterial growth. This is due to the dilution of the energy source (sulfur particles) by CFA particles. Precipitation of calcium sulfate on the cells and on the surface of sulfur particles may also interfere with cell growth.

Similar rates and extraction levels of major metal components from the CFA particles (i.e. aluminum and iron) were obtained by sulfuric acid and *T. thiooxidans*, under the same leaching conditions ($0.8 \leq \text{pH} \leq 5$ and same rate of pH decrease). It is concluded therefore, that leaching of CFA particles by *T. thiooxidans* takes place indirectly by sulfuric acid, which is the main cell metabolite, and the effects of other metabolites on the bioleaching are relatively insignificant.

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