Metal removal rate of *Thiobacillus thiooxidans* without pre-secreted metabolite

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**Abstract**

Industrial microdevices are mostly manufactured by micromachining technology. Materials can be micromachined by various means, including mechanical, optical and chemical. The concerns of environmental impact and production costs are the inspirations behind one alternative method of micromachining, the use of bioleaching. The evaluation of a potential microorganism for the bioleaching machining technology is conducted in the current study. The dissolution of metals by the chemolithotrophic bacteria *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* is based on the physical contact between the bacteria and their extracellular secreted metabolites and metals. Cell growth quality during the machining process is also an important issue. The specific metal removal rate is a key criterion to characterize any industrial machining process, defined here by the mass removed in milligrams from 1 cm² of machined area in 1 h by a solution with a cellular concentration of $1 \times 10^8$ cells/ml. The authors find that the specific metal removal rates for copper, aluminum and nickel are 0.5 mg/h, 0.06 mg/h and 0.93 mg/h, respectively.

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1. Introduction

Various micromachining methods have been pursued for making miniaturized devices after the advent of manufacturing of microelectronics revolutionized by the industry. Machining is a process of removing the material from the bulk or surface and leaving the remaining material in the designed shape and dimensions. The specific material removal rate is considered a characteristic subject to the machining method, which determines the rate of material removal when certain process parameters are selected. Various techniques have been applied to micromachining, including mechanical, optical and chemical. The innovative use of microbes for microscopic metal removal to achieve microfeatures is considered more environmentally friendly than other means (Zhang and Li, 1998, 1999). A few microbes demonstrate their ability in leaching metals. A fundamental assessment of the metal removal rates for various metals in use of different microbes is needed for further development of the associated micromachining technology.

*Acidithiobacillus thiooxidans* (AT) is an extremely acidophilic bacterium, meaning it is remarkably tolerant in the acidic environment at a pH of 1 or below, and thus differs from *Acidithiobacillus ferrooxidans* (AF) (Chen and Lin, 2004). The metabolite, mainly sulfuric acid, which shows growth in the exponential phase, plays a major role in bioleaching. Bioleaching of heavy metals is considered a complex process. Various parameters such as temperature, pH, nature of the bacteria,

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sulfur content, cell concentration and the surface area of metals, etc., play important roles in determining the machining rate (Battaglia et al., 1994; Tischer et al., 1993; Wong and Henry, 1998). The previous studies showed the optimal growth condition for AT, but other criteria need to be optimized in the process development.

The specific metal removal rate of indigenous AT with or without pre-secreted metabolites was not the same in previous studies (Liu et al., 2003a). The rate of oxidation of a number of minerals is markedly accelerated by Acidithiobacilli. These bacteria play important roles in leaching of metals due to their acidophilic characteristics (Krebs et al., 1997). Not only does the indirect electrochemical metabolite leaching by means of a secreted biofilm in the formation of the cell capsule during the cell cultivation play a part (Gehrke et al., 1998; Rojas-Chapana et al., 1996; Tributsch et al., 1998), but also the direct metal removal, which requires the physical contact between the bacteria and metals, was in action under aerobic conditions (Silverman, 1967). The method used in this machining process needs the abscission of the pre-secreted metabolites in culture and control of the microbes. The specific metal removal rate without the pre-secreted metabolite in culture was normalized by cell concentration and metal area in this paper.

The adopted process is different from other existing bioleaching methods. The machining process will be applied to microdevices machined by direct physical contact of the bacterial on the targeted location, but not by the bacterial metabolite requiring costly lithography and masking in advance.

A sketch of the machining example is shown in Fig. 1. The desired cross micropattern on the sample can be machined by applying the bacteria in direct contact with the workpiece in targeted area. The bacteria targeting can be guided by various disclosed means.

2. Materials and methods

2.1. Growth of indigenous A. thiooxidans

A. thiooxidans (BCRC 15616) of Taiwan local strain obtained from Food Industry Research and Development Institute (FIRDI) were used in this study. The basal 317 medium contained the followings per liter of glass-distilled water: 0.3 g (NH₄)₂SO₄, 3.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, and 0.25 g CaCl₂. The pH was adjusted to 4.5 with sulfuric acid. 1.0% (weight/volume) elemental sulfur was pre-sterilized and added to 317 medium for growth and maintenance of A. thiooxidans. The pH was reduced from 4.5 to 1.4 after 10 days of successful enrichment cultures. The flask culture was used as an inoculum (10% volume/volume) after 10 days at 30°C, with 150 rpm shaking incubation before used in the following experiments. The chemicals were obtained from Sigma Chemical Co., St. Louis, USA.

2.2. Cell harvest

After 10 days scale up incubation, the culture was centrifuged at 2500 × g for 10 min. Cells were washed twice with fresh 317 medium without elemental sulfur and centrifuged again, 10,000 × g for 20 min. The cell pellet was re-suspended in fresh 317 medium at a concentration of 10⁸ cells per ml. The cell suspension could be stored for 1 week without losing much of the bioleaching activity. The cells were counted by the optical density (OD) method. The OD of 0.3 at 545 nm corresponded to approximately (6.0–6.3) × 10⁸ cells/ml of the culture (Gupta and Agate, 1986). This process was carried out each time after cells were harvested and obtained from this good growth of exponential phase.

3. Biomachining experiment

The workpiece materials were sheets of copper (purity 98.5%) and aluminum in thickness of 0.2 mm, but the nickel was in thickness of 2.0 mm. A gem quality sample of pyrite was used in this study. All metals were cut to square of 1 cm on each side. They were rinsed with distilled water and 95% ethanol and dried before use.

In order to control the direction and region of two-dimensional bioleaching, a photore sist patterned test piece was made. It was drawn with Auto-CAD software on computer, then the graphic data were transported to a laser plate-making device to make a photographic plate, called photomask, of the micrograph. The tested piece was covered with the photomask and coated the surface with a layer of photore sist. Thus, the non-coated area was flushed out by developer to expose metallic surface in the region, and the test piece was completed.

The metals were covered with 200 ml of growth medium containing 10 g/l element sulfur and 0.1 g/l nystatin in 500 ml flask. The cell suspension harvested from 10 days pre-cultivation culture by centrifugation at 10,000 × g for 20 min was added to achieve the designed density, and the supernatant with extracellular secreted sulfuric acid metabolite was discarded. A blank experiment without cells was included as the control. The flasks were placed in a shaker at 30°C and 150 rpm for the experiment. After exposure to the machining solution, the metals were removed and gently rinsed with de-ionized water, and placed in oven at 50°C to remove the retaining moisture. During the course of biomachining, the metal removal rate was measured by a balance machine at constant time intervals.
4. Results and discussion

4.1. Cell growth

Fig. 2 shows the growth of AT exhibits an exponential phase during days 5–15. The pH level in successful enrichment cultures with element sulfur can fall from the initial culture medium 4.5–1.4 after 10 days of incubation. The experiment was conducted in triplicate.

Fig. 3 shows the cell concentration has the positive effect on the total metal removal rate in the range from $0.125 \times 10^8$ cells/ml to $1 \times 10^8$ cells/ml and the effects become saturated beyond that. The increasing cell density can affect the total removal rate, particularly at the cell density beyond $2 \times 10^8$ cells/ml. Hence the cell density should be maintained at an appropriate level.

4.2. Specific metal removal rate

The specific material removal rate (SMRR) is defined as follows:

$$\text{SMRR} = \frac{\text{amount of metal removal (mg)}}{\text{machining time (h) \times cell concentration (1 \times 10^8 \text{ cells/ml}) \times machined area (1 \text{cm}^2)}}$$

To determine the effect of the indigenous AT on metal removal, the metal was oxidized at different cell concentration and in the absence of the cell culture; the results are shown in Fig. 4. The total amount of copper oxidized was 24 mg and 31.5 mg after 48 h of metal removal in $1 \times 10^8$ cells/ml and $2 \times 10^8$ cells/ml cell culture and 5.6 mg of abiotical leaching from 1 cm² of machined area, respectively. The amount of aluminum and nickel was removed as the same condition as copper. The total amount of aluminum oxidized was 2.9 mg and 5.6 mg after 48 h of metal removal in $1 \times 10^8$ cells/ml and $2 \times 10^8$ cells/ml cell culture and almost no mass loss in control for aluminum, respectively. The total amount of nickel removed was 45.1 mg and 94.7 mg after 48 h of metal removal in $1 \times 10^8$ cells/ml and $2 \times 10^8$ cells/ml cell culture and 12.3 mg in control for nickel. The efficiency of metal removal with cells was much higher than that without cells.

The initial specific metal removal rates for copper, aluminum and nickel in 48 h are shown in Fig. 5. They are 0.5 mg for Cu, 0.06 mg for Al and 0.93 mg for Ni per hour with $1 \times 10^8$ cell/ml cell concentration from 1 cm² of machined area. The culture through the experiment was obtained after 10 days of pre-cultivation when cells were in exponential growth phase during the metal removal process. As shown in Fig. 4, the efficiency of biotic metal removal is much higher than that of abiotic process. The amount of copper oxidized biotically was four times higher than that of oxidized abiotically at 48 h, respectively. The similar result for nickel is 3.6 times. Fig. 6 shows the process of biomachining experiment with a photosist patterned test piece of copper. The workpiece was put into a flask in which the above-mentioned culture fluid of AT was held. The flask was put into the environmental incubator shaker, under the above-mentioned culture conditions. Thus the two-dimensional machining process began to remove the exposed metal. The machining depth was measured with a dial indicator after 24 h and 48 h of machining and was approximately 15 µm per day. The amount of undercut was very small and influences the degree of biomachining during the overall machining process. The aspect ratio under the bioleaching condition of AT was around 1.85.

To identify the effect of metal surface area of metal removal by indigenous AT, the metal was cut to square sections in 1.0 cm × 1.0 cm, 2.0 cm × 2.0 cm and 3.0 cm × 3.0 cm and machined in $1 \times 10^8$ cells per ml cell culture. The control was in medium without cells. Fig. 7 shows the amount of copper and aluminum removal increases with the exposed machining surface area and the specific metal removal activity.
was consistent during the initial metal removal experiment and saturated after 72 h.

The specific metal removal rates at fourfold and ninefold enlarged surface area were consistent in copper in the abundant machining solution, but have increasing rate in aluminum during 24–72 h.

A previous study has shown that the presence of about 10 ppm and 50 ppm of Cu$^{2+}$ has a slightly positive effect on the growth of AT. In contrast, Cu$^{2+}$ inhibits the growth of AT when the concentration is above 300 ppm (Liu et al., 2003b). The effects are not clear in use of other metals. To increase the metal removal, the microbial stress exposure should be minimized. The removal of toxic Cu$^{2+}$ or other metals during the metal removal process needs to be taken into account to obtain a better machining efficiency.

Fig. 4 – Specific metal removal rate (cell culture after 10 days pre-cultivation and harvested by 10,000 × g centrifugation) of (A) copper; (B) aluminum; (C) nickel.

Fig. 5 – Specific metal removal rate after 48 h.

Fig. 6 – SEM photograph of biomachined grooves (80 μm in depth and 45 μm in width) on a pure copper piece.
As the production of acid is considered as a rate limiting step in overall biomachining process (Tichy et al., 1993), the rate of metal solubilization is affected by the rate of acid production and the affinity of physical contact between the metal and bacteria.

5. Conclusion

The current paper reveals the metal removal rates of Cu, Al and Ni are markedly accelerated by Acidithiobacilli. Metal removal was found more effective by the bacterial method than chemical leaching of these metals. The cell concentration enhanced the machining rate, and while the effect was saturated at $1 \times 10^8$ cells/ml cell density in copper while not observed in aluminum and nickel. Nickel is the most readily solubilised metal with high chemical leaching in control, while aluminum was poorly solubilised. The oxidation of metals and sulfur by AT is found to be competitively inhibited by cells. The nature of cell–cell competitive inhibition is not clear but is probably related to the mechanism of sulfur oxidation and metals dissolution occurred at the cell surface as a limiting step. The specific metal removal is measured by normalization of the surface area and cultivation time at the same cell concentration of $1 \times 10^8$ cells/ml. The specific metal removal of copper is 0.5 mg and 0.93 mg for nickel and 0.06 mg for aluminum, respectively.

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References


