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Stability of copper tolerance in *Thiobacillus ferrooxidans*

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Abstract

A strain of *Thiobacillus ferrooxidans* MAL-4-1 was adapted to grow at higher concentrations of copper by repeated subculturing in the presence of increasing levels of added cupric ions in 9K medium. The strains adapted to copper were found to be more efficient in bioleaching of copper from concentrates. When copper tolerant strains were back cultured repeatedly in 9K medium without cupric ions, the initially developed metal tolerance was observed to be lost. This indicates that the copper tolerance developed is stress-dependent and not a permanent trait of the adapted strain.

Introduction

Thiobacillus ferrooxidans is a chemolithotrophic acidophilic bacterium most widely implicated in the bioleaching of several sulphide minerals (Brierley 1978). During bioleaching of sulphide ores and concentrates, heavy metal ions accumulate in the leach liquor and beyond certain concentrations they become toxic to the organisms and thereby affecting dissolution rates. It has thus become necessary to develop metal-tolerant strains of *Thiobacillus ferrooxidans* for efficient leaching (Natarajan and Iwasaki 1983; Natarajan et al. 1983; Natarajan 1985). For example, by serially subculturing *Thiobacillus ferrooxidans* in an acidic medium containing increased concentrations of cupric ions, it is possible to develop strains that are tolerant to higher levels of copper in solution (Brahmaprakash et al. 1988).

However, it is not clearly understood as to whether such an acquired metal tolerance could be permanently retained by the organism under bacterial leaching conditions. Is it absolutely essential to maintain continuously the developed high copper tolerant strains in a medium containing high copper concentrations so as to retain their metal tolerance? Is the acquired metal tolerance a permanent feature under all conditions of bacterial growth and leaching?

This work was undertaken to assess the copper tolerance of *Thiobacillus ferrooxidans* under different conditions of maintenance and subculturing.

Materials and methods

A pure strain of *Thiobacillus ferrooxidans*, MAL-4-1 isolated, characterized (Vishniac 1974) and cultured from Malanjkhand copper mines, India was used. Stock cultures were routinely grown and maintained in a mineral salts medium supplemented with ferrous iron at pH 2.0 (Silverman and Lundgren 1959). Filter-sterilized ferrous sulphate solution (60 ml) was added aseptically to 140 ml of autoclaved 9K mineral salts medium. A 10% of the inoculum (72 h old culture) was then added to the medium and the inoculated flasks were incubated on a rotary shaker (240 rpm) at 30 °C. Bacterial growth was monitored by estimating the residual ferrous iron in the medium at different intervals of time.

A strain of *Thiobacillus ferrooxidans* was made tolerant to higher levels of copper by repeated subculturing in the 9K medium in the presence of different concentrations of added copper sulphate. Ferrous iron oxidation as a function of time was monitored in the presence of different copper concentrations. Adapta-

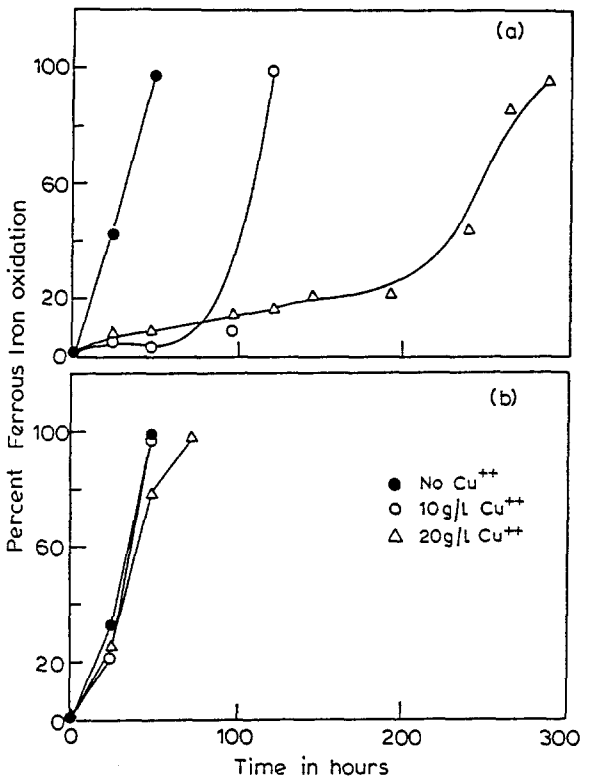

Fig. 1. Ferrous ion oxidation by *Thiobacillus ferrooxidans* strain MAL-4-1 in 9K medium: *(a)* in the presence and absence of Cu^{++} before adaptation; *(b)* after adaptation to 10 g/L and 20 g/L of Cu^{++}.

tion to a particular concentration of copper was considered achieved when the growth curves of the organism in the presence and absence of copper in the medium are similar i.e., when the copper-adapted strain could oxidize the ferrous iron in the medium at the same rate as unadapted strain. After complete adaptation to a particular level of copper in the medium, the organism was again subcultured repeatedly into 9K medium containing still higher levels of copper. By this process the organism acquired the enhanced metal tolerance. The cultures developed from a single strain but acquired tolerance to various levels of copper were routinely maintained in 9K medium containing same concentration of copper.

In another set of experiments, copper-adapted cultures were serially subcultured in 9K medium without any copper. Such back-cultured strain was once again inoculated into 9K medium containing different concentrations of copper. Growth measurements were made to check the ability of this culture to oxidize ferrous iron after each subculturing in the presence and absence of different levels of copper.

Results and discussion

Thiobacillus ferrooxidans is known to tolerate higher levels of heavy metals. In the present study, *T. ferrooxidans* MAL-4-1 was grown at different levels of cupric ion as illustrated in Fig. 1(a). The organism when grown in the absence of copper could oxidize all the available ferrous iron in 9K medium within 48 hours. But in the presence of 10 g/L and 20 g/L of cupric ions, it could oxidize only less than 10% of the available iron during this period of time. The organism could however overcome growth inhibition caused by copper on prolonged incubation in the presence of higher copper concentrations. The lag period increased at higher levels of copper. Approximately 120 and 300 hours of incubation were necessary to completely oxidize all the available ferrous iron in the 9K medium in the presence of 10 g/L and 20 g/L of copper, respectively.

However, by repeated subculturing of the organism in the presence of the above levels of copper, the growth inhibition caused by copper could be overcome (Fig. 1(b)). Such adapted strain could oxidize all the available ferrous iron in the medium in presence of copper at the same rate as unadapted strains (MAL-4-1) in the absence of copper. The strain tolerant to the above indicated levels of copper were subcultured, as described above in the absence of added copper to 9K medium. After each such subculturing in 9K, the organism was again allowed to grow in the presence of different levels of copper in a separate set of flasks. The growth patterns of tolerant and unadapted strains of *Thiobacillus ferrooxidans* were similar after first few series (4 to 5) of subculturing in the absence and presence of copper, indicating that the organism had not lost its ability to tolerate copper, even after growing in the absence of the toxic metal. However, after 5 to 6 repeated subculturing in the absence of cupric ions, the organism started loosing its ability to tolerate higher levels of copper. For example, after nineth subculturing in the absence of copper, it took 120 h and 168 h to oxidize all the available ferrous iron, in the presence of 10 g/L and 20 g/L, of copper respectively (Figs 2a and 2b). Thus, the strain completely lost its ability to tolerate 10 g Cu^{++}/L, while the loss was only partial in case of the strain adapted to 20 g Cu^{++}/L. It could then be inferred that after continued subcultures in the absence of 20 g Cu^{++}/L, this reduced tolerance to copper ions may also be lost in the case of 20 g/L copper-tolerant strain.

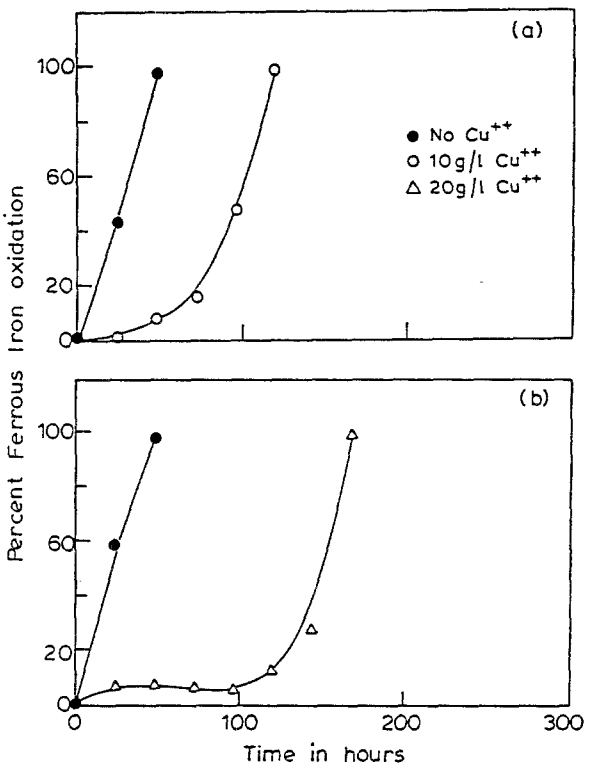

Fig. 2. Ferrous iron oxidation in 9K medium: *(a)* by 10 g/L Cu^{++} adapted strain and *(b)* by 20 g/L Cu^{++} adapted strain after nine subcultures in the absence of Cu^{++}.

Thiobacillus ferrooxidans, when grown in the presence of Cu^{++} ions showed varied levels of lag periods depending upon the levels of copper present in 9K growth medium (Tuovinen et al. 1971; Brahmaprakash et al. 1988). Two hypotheses were proposed to explain such prolonged lag periods (Tuovinen et al. 1971). In the first place, a long lag period may be needed during which a membrane-associated system develops which enables the cells to oxidize ferrous-iron for energy; i.e., all cells can oxidize iron after some structural rearrangement of molecules associated with the semipermeable cytoplasmic membrane. A second possibility is that the lag phase may represent the time when selection of metal-tolerant cells takes place while other cells unable to oxidize iron in presence of copper do not survive and are thus eliminated. It is thus likely that in a population of *Thiobacillus ferrooxidans* strain MAL-4-1, some metal tolerant cells do exist and after adding Cu^{++} (or any other heavy metal ion, for that matter) to the medium, the development of these cells would be favoured. We have tried to address this aspect in the present study. If Cu^{++} tolerant cells are present in a population, they will outgrow other cells during repeated subculturing in the presence of indicated levels of Cu^{++}. Cells which are not capable of growth in the presence of Cu^{++} would then be phased out gradually, allowing only copper-tolerant cells to grow. When Cu^{++} tolerant strains are developed, the entire population should then contain only metal-tolerant cells. If this hypothesis were to be true, then even after repeated subculturing of a copper-tolerant strain in the absence of Cu^{++}, the tolerant cells should not at any point of time loose their ability to oxidize Fe^{++} in the presence of Cu^{++}, as all of them are metal-tolerant. Also, there should not be any lag period as there is no need for selection. Contrary to this, our results indicate that due to repeated back culturing of an initially copper-tolerant strain in the absence of Cu^{++}, the cells lost their ability to oxidize Fe^{++} in the presence of Cu^{++}. The above observation then imply that the ability of the copper-tolerant strain to oxidize ferrous iron in the presence of higher concentrations of copper is a developed trait and is stress-dependent. These results support the first hypothesis, i.e., all cells in a population can tolerate Cu^{++} and oxidize Fe^{++} after some structural rearrangement of the cell material associated with cytoplasmic membrane. The increasing lag periods observed at higher levels of Cu^{++} do suggest that the cellular mechanism when exposed to metal cations is specifically affected by different levels of cations and the protecting mechanism is metal-specific to some extent. Brahmaprakash et al. (1988) also observed similar results wherein the lag periods differed for Cu^{++} and Zn^{++}, indicating that the protecting mechanism is probably metal specific.

More studies are needed to understand the mechanism of copper tolerance in *T. ferrooxidans* and the cellular component involved. Genetic studies including genome analysis and identification and characterization of plasmids encoding metal resistance if any, are the need of the hour. The ability to gain or loose metal tolerance may involve hopping genetic elements called insertion sequences (Yates and Holmes 1987; Yates et al. 1988). Because of genomic rearrangement due to hopping of these transposable genetic elements, a strain may gain or loose its ability to oxidize iron depending on the presence or absence of metal ions as well as the amount and type of metal ions present in the system. Thus changes at genetic and biochemical levels may play a role in determining the extent and stability of protection mechanism required to counter the action of toxic heavy metal ions. This protection mechanism may involve the enzymes implicated in the

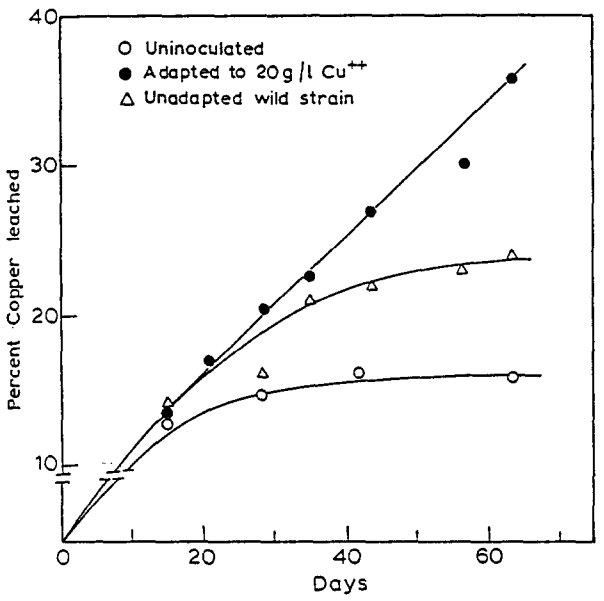

Fig. 3. Bioleaching of chalcopyrite-pyrite mixed concentrate using copper-tolerant and unadapted strain of *Thiobacillus ferrooxidans* strain MAL-4-1.

oxidation machinery or many other related proteins and components.

However, it is clear that the copper-adapted strain of *Thiobacillus ferrooxidans* is useful in bioleaching as illustrated in Fig. 3, wherein the results of bioleaching of a mixed chalcopyrite-pyrite concentrate by adapted and unadapted strain of *T. ferrooxidans* are shown. Better leaching kinetics and higher copper recoveries from the concentrate were obtained using copper-tolerant strain compared to the strain without adaptation. The metal tolerant strain for bioleaching was maintained and subcultured in the presence of 20 g of Cu^{++}/L.

Acknowledgement

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