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Immobilisation of *Thiobacillus ferrooxidans* cells on nickel alloy fibre for ferrous sulfate oxidation

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Abstract The immobilisation of the iron-oxidising bacteria *Thiobacillus ferrooxidans* on nickel alloy fibre as support is described. This matrix showed promise for application in iron oxidation under strongly acidic conditions. The influence on the colonisation process of *T. ferrooxidans* exerted by the initial pH of the medium and by temperature has also been studied. Results showed that immobilisation of *T. ferrooxidans* cells was affected by changes of temperature between 30 °C and 40 °C and in pH from 1.4 to 2.0.

Introduction

Thiobacillus ferrooxidans is an acidophilic iron-oxidising bacterium that can be found naturally in acid mine drainage. These bacteria catalyse the oxidation of iron pyrites to ferric sulphate and sulphuric acid, increasing the reaction rate for chemical oxidation. At the iron oxidation step in such a system, a high density of cells of iron-oxidising bacteria is essential for rapid iron oxidation.

In recent years, most studies of *T. ferrooxidans* have been aimed at improving the rate of oxidation of Fe (II) (MacDonald and Clark 1970). Many types of reactors operating under both batch and continuous regimes have been tested with a view to obtaining better results (Nemati et al.1998). However, continuous operating systems have to work with low rates of dilution to prevent washout of the cellular population. For this reason, the use of immobilised biomass improves results with respect to the rate of oxidation and, at the same time, high cellular concentrations are achieved inside the reactor.

Immobilisation technology of various kinds of cells has rapidly developed and has led to various applications (Abbot 1976; Chibata and Tosa 1977; Brodelius 1978; Colowick and Kaplan 1987; Champagne 1996; Arroyo 1998). Immobilisation of whole cells is characterised by stable maintenance of an extremely high density of living cells in and/or on the immobilisation matrices. Therefore, immobilised cells are available effectively over a longer period in systems having a small number of free-living cells.

Various matrices have been used for immobilisation of *T. ferrooxidans* by adhesion, for example to surfaces of glass beads (Grishin and Tuovinen 1988), ion-exchange resin (Karamanev and Nikolov 1988), activated carbon (Grishin and Tuovinen 1988; Carranza and García 1990; Halfmeier et al. 1993) or by entrapment within calcium alginate, agar, κ-carrageenan and gerlite (Lancy and Tuovinen 1984; Wakao et al. 1994). Other supports, such as polyurethane foam (Armentia and Webb 1992), combine the advantages of adhesion with those of entrapment.

The use of nickel alloy fibre for the immobilisation of viable cells of *T. ferrooxidans* is an innovation that has not been reported to date; further, it represents a useful alternative to the use of non-metallic support materials, mainly because it combines the advantages offered by the entrapment of the derivatives of the cellular adhesion. This support provides an extensive surface available for adhesion, and the microorganisms have a certain affinity for the nickel present in its composition (Kai et al. 1995).

The main objective of this study was to determine the feasibility of using this support for immobilisation of *T. ferrooxidans* and, at the same time, to establish a procedure for immobilization, on a laboratory scale, that is simple, fast and easily reproducible, and that can be suitably adapted to the industrial scale. Our previous work (Gomez et al. 1996; Gómez and Cantero 1998),

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using freely suspended cells of *T. ferrooxidans*, showed that ferrous iron oxidation is influenced by temperature and pH. However, there is limited information available on the influence of pH and temperature on immobilised *T. ferrooxidans*. Recently, Nemati and Webb (1997) have investigated the effect of temperature on the kinetics of ferrous iron oxidation by immobilised cells of *T. ferrooxidans* at 20 °C and 30 °C, respectively. Experimental results showed that the kinetics by biofilms at 30 °C are significantly faster than those at 20 °C. The work reported here summarises results on how immobilisation of this bacteria is affected by temperature (from 30 °C to 40 °C) and pH.

Materials and methods

Microorganism and growth conditions

The strain of *Thiobacillus ferrooxidans* used in this study was isolated from the Rio Tinto mines of Huelva (Spain) and kindly made available by the Biohydrometallurgy Group of the University of Seville (Spain). This strain has the same properties and characteristics as the strain used by Nemati and Webb (1997), obtained from National Collection of Industrial and Marine Bacteria (NCIMB 9490).

The bacteria were grown in a medium proposed by Silverman and Lundgren (1959): $(NH_4)_2SO_4$ 3.0 gl^{-1}; $MgSO_4$ 0.5 gl^{-1}; K_2HPO_4 0.5 gl^{-1}; KCl 0.1 gl^{-1}; $Ca(NO_3)_2$ 0.01 gl^{-1} and a variable concentration of $FeSO_4$, depending on the experiment to be performed.

Solid medium for subcultures was proposed by Johnson and McGinness (1991) and was called FeTSBo. This medium was prepared as follows: the three separately sterilised solutions (tryptone soya broth/basal salts, ferrous sulphate and agarose) were combined, thoroughly mixed and the complete medium divided 2:1 by volume and held at 50 °C. To the large volume was added 2.5%(v/v) of an active culture of heterotrophic acidophile (*Acidiphilium ferrireductans* SJH), the solution was again thoroughly mixed and 20 ml aliquots dispensed into sterile Petri plates. When the agarose had gelled, it was covered with a thin layer (10 ml) of sterile FeTSB (which had been heated to its melting point of 50 °C).

Characteristics of the support material

Nickel alloy fibre was used as the immobilisation matrix. This material is available commercially from Scotch Brite 3 M (Spain) and consists of an alloy of nickel and stainless steel, in the form of coiled metallic ribbons. The characteristics of this material are given in Table 1.

Immobilisation procedure

T. ferrooxidans was immobilised on nickel alloy fibre according to the following procedure: a fixed amount of support was placed in a 1-l flask with 600 ml of liquid medium (Fe^{2+} concentration over 2.0 gl^{-1}) and 10%(v/v) inoculum. To adjust the pH to 1.8, H_2SO_4 5 mol l^{-1} was added. Cultures were incubated at 30 °C on a rotary shaker at 200 rpm. When the ferrous iron concentration reached a minimum, the flask was drained and the medium was replaced without any intermediate inoculation. Several consecutive batches were run on a "draw and fill" basis until steady-state biomass levels had been achieved.

Table 1 Matrix characteristics

Real density (g/cm^3)	7.83
Apparent density (g/cm^3)	0.58
Porosity (%)	92.6
Specific surface (m^2/m^3)	8831.18
Ribbon width (mm)	1
Prime spiral diameter (mm)	2.5
Ribbon thickness (mm)	0.1

Analytical methods

Ferrous sulphate oxidation was monitored by determining the residual ferrous iron concentration at various intervals. The 1,10-phenanthroline method of Vogel (1986) was used. In order to determine the ferrous iron concentration, a 10-μl sample was placed in a tube and diluted with 1.0 ml of distilled water. The pH was adjusted to between 3.0 and 6.0 with 2 mol l^{-1} sodium acetate, 0.8 ml of 1,10-phenanthroline solution was added and, finally, an additional 10 ml distilled water. The absorbance at 515 nm was measured after 5–10 min. In order to determine total iron concentration, 1.0 ml hydroxylamine chloride was added to the sample instead of 1.0 ml distilled water and the same procedure followed. A calibration curve of known $FeSO_4$ concentrations was used to calculate the iron concentrations. The concentration of iron (III) in solution was calculated by subtracting the average iron (II) concentration from the total iron concentration measured at each point in time.

Total cell number

In order to measure the total biomass adhered to the matrix support, a known amount of nickel alloy fibre was placed in a flask with 5 ml of oxalic acid 10%(w/v), at each "draw and fill" cycle. After 10 min, the support was rinsed with 5 ml of distilled water for 10 min. Then, the rinsings were added to the previous cell suspension obtained.

The biomass concentration was determined by direct counting using a Neubauer chamber counter (0.02 mm depth and 1/400 mm^2) under an optical microscope. In some cases, it was necessary to dilute the samples with basal salt solutions because of the high biomass concentrations. Each measurement was made in duplicate to minimise the experimental errors inherent in working with microbial populations.

Scanning electron microscopy

Samples of the matrix material were removed during and at the termination of experiments for scanning electron microscopy (SEM). The samples were gently washed with 0.1 N sulphuric acid and fixed with 2.5% glutaraldehyde solution (Sigma, St. Louis, Mo.) for 1 h at 4 °C. After, the samples were washed three times with 0.1 M cacodylic acid solution (pH 7.0) for 15 min. The samples were fixed with 1% osmium tetroxide (pH 7.0) for 1 h, followed by dehydration with acetone and critical-point drying. All samples were mounted on specimen tubs with a silver paint, gold-coated, and examined by SEM under a Jeol JSM-820 model. All preparation steps, which involved washing, fixation, and dehydration, were carried out with minimum physical disturbance of the sample material.

Packed-bed reactor column

Ferrous sulphate oxidation by immobilised *T. ferrooxidans* cells was studied using a glass column (1 × 0.05 m) with an inlet for medium and air at the bottom and an outlet for effluent at the top. Nickel alloy fibre with immobilised *T. ferrooxidans* was placed in the column and a layer of sintered glass was placed at the bottom of the matrix layer to keep this inside the column. A working volume of 1350 ml and aeration rate of 0.675 ml min^{-1} were used. The temperature was maintained at 30 °C and flow rates of both inlet

and outlet were regulated with automatically controlled peristaltic pumps. Sampling of the effluent was performed from the end of the effluent tubing at the reservoir and the samples were analysed for ferrous iron and total iron.

The column was operated in batch mode until near steady-state conditions were achieved, then the reactor was switched to continuous operation. Steady-state operation was considered to be established when the ferrous iron concentration varied by less than 5% during a period of time equal to the theoretical retention time.

Results

In order to study the colonisation of the support matrix by *Thiobacillus ferrooxidans* cells, concentration of Fe (II) and Fe (III) were determined daily, together with the concentration of biomass adhering to the support at the end of each cycle. Figures 1 and 2 show the development of the concentration of these variables.

In Fig. 1 the different cycles of colonisation, with the consecutive charging and discharging of the medium, can be observed. After an initial cycle of longer duration for the cells to become acclimatised to the new culture medium, it only took 24 h for all the available substrate to be consumed.

Analysing the data from Fig. 2, it can be seen that the biomass adhering to the support increases rapidly in the first culture cycle, after which it reaches a maximum value of biomass concentration that does not then vary with time. As a result, only 70 h after inoculation, it can be seen that the nickel alloy fibre is capable of retaining a population of 8.4×10^8 cells per gram of support. Subsequently, the process of colonisation continues until reaching a value of around 9×10^8 cells per gram of support, at which level it stabilises.

In Figs. 3, 4 and 5, it is possible to see that *T. ferrooxidans* forms a microbial aggregate (biofilm), consisting of bacteria and an inorganic substance called jarosite (precipitates of ferric iron complexes), on the surface of the support matrix. This biofilm has a thickness of 2–3 μm and the precipitates may limit

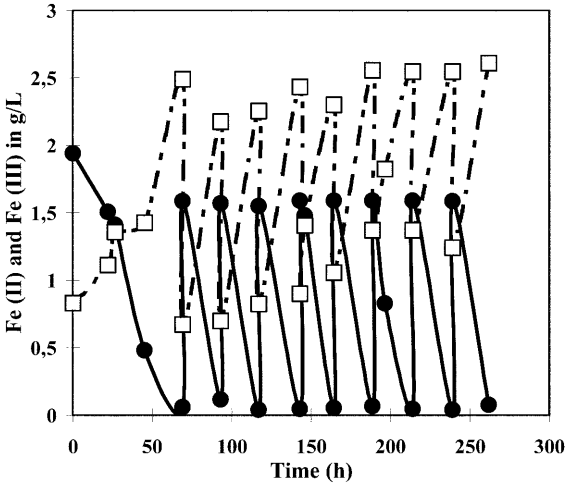

Fig. 1 Evolution of ferrous (●) and ferric (□) iron concentration in solution as a function of time during repeated-batch oxidation

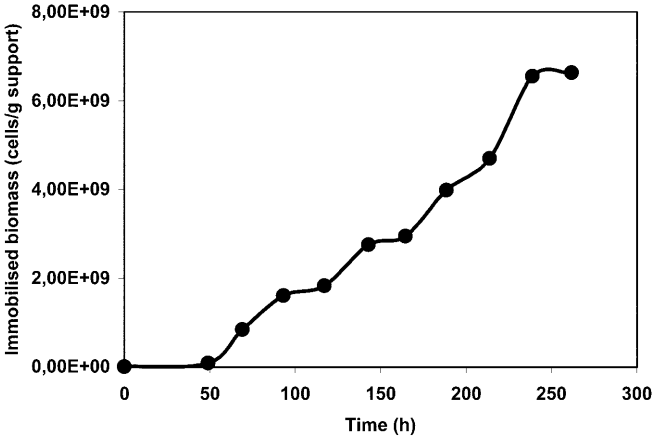

Fig. 2 Evolution, in logarithmic scale, of adhered biomass concentration during process colonisation

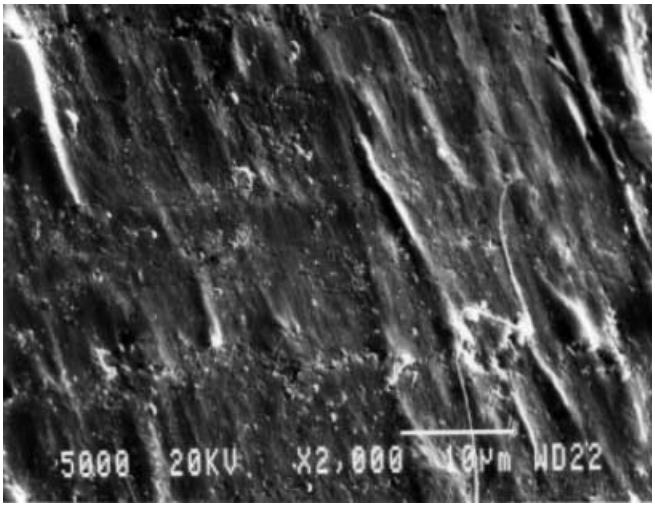

Fig. 3 Scanning electron micrograph of nickel alloy fibre surface before immobilisation

Fig. 4 Scanning electron micrograph of biomass support particles removed during immobilisation process

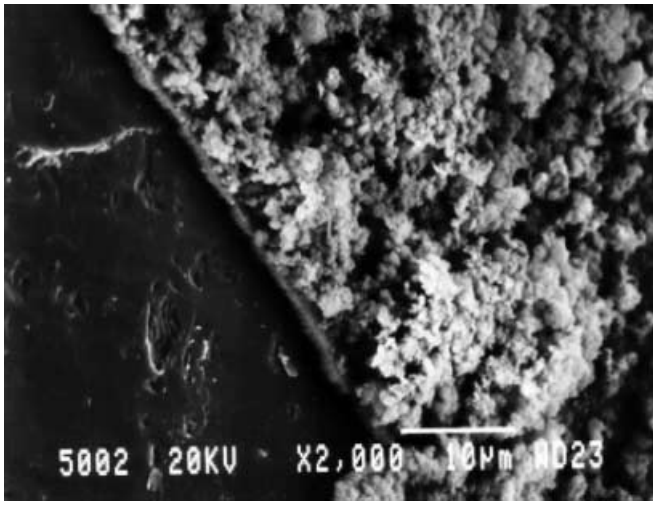

Fig. 5 Scanning electron micrograph of biofilm formed over nickel alloy fibre surface

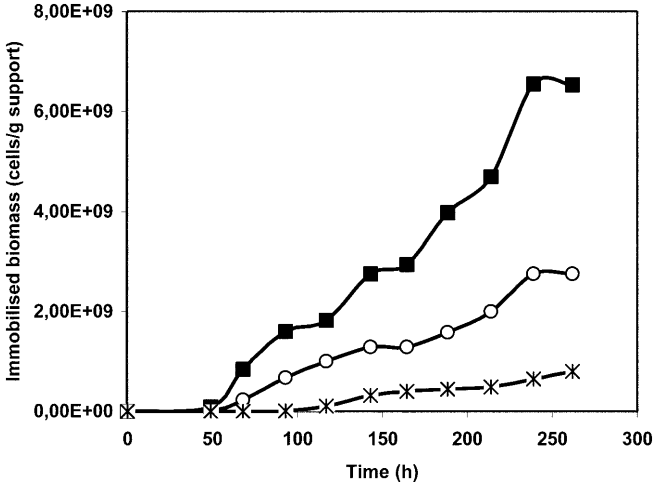

Fig. 7 Progressive changes in adhered biomass in repeated batches cultures as function of pH value: 2.0 (■), 1.7 (○), 1.4 (×)

immobilised biomass concentration because they occupy matrix surface that would otherwise be available to the bacteria. Thus, it is important to work at pH values that minimise formation of these compounds. This pH value was established as 1.8 in previous work (Gómez and Cantero 1998).

In general, it is well known that surface roughness is essential for a successful immobilization. In this case, precipitates that accumulate on the surface promote the adhesion of *T. ferrooxidans* cells. It is therefore necessary to establish a compromise between pH value and precipitate quantity in order to obtain adequate adhesion of the microorganism.

Results obtained for evolution of immobilised biomass along the process at different temperatures and pH values are shown in Figs. 6 and 7. It can be seen that the effect of temperature on growth is important because employing higher temperatures decreases the growth of the immobilised bacteria. As the temperature is increased the effect is more significant and the final immobilised biomass is lower.

In order to compare the results obtained in batch experiments with those from the application of this support material in continuous reactors, a quantity of nickel alloy fibre, colonised according to the procedure previously described, was placed in a reactor column. This reactor was initially filled with fresh medium and set into batch operation until residual levels of substrate were reached. At this point, the feed was connected and continuous operation was started. Figure 8 shows the evolution of the concentration of iron (II) and iron (III) in an experiment for $D = 0.25\ h^{-1}$, in which 93% of the

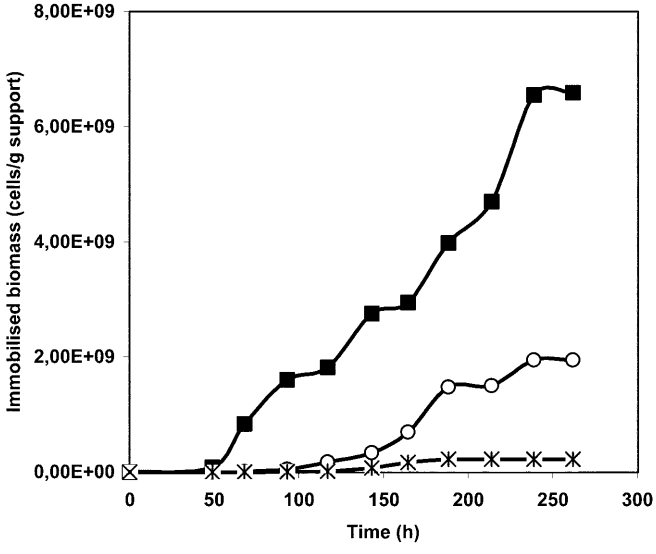

Fig. 6 Progressive changes in adhered biomass in repeated batches cultures as function of temperature: 30 °C (■), 35 °C (○), 40 °C (×)

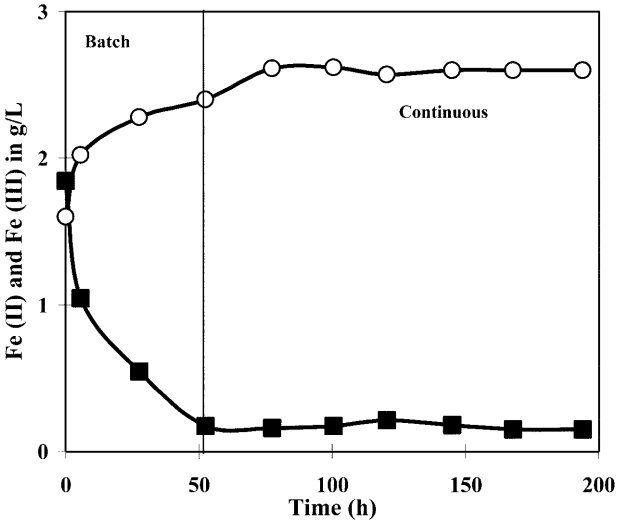

Fig. 8 Continuous oxidation of ferrous sulphate by immobilised *Thiobacillus ferrooxidans* cells in a column reactor: Fe^{2+} (■) and Fe^{3+} (○)

initial iron was oxidised in steady-state conditions. These results show that nickel alloy fibre is a suitable support material for the oxidation of ferrous sulphate by *T. ferrooxidans* in continuous operation.

Discussion

The results in Fig. 2 can be explained by the physical properties of the support matrix. The nickel alloy fibre has, relative to its volume, a very large surface area, which favours the development of the natural tendency of the microorganism to adhere to solid surfaces; this produces a high rate of colonisation in the first cycles of operation.

The results obtained for the rate of colonisation of this support are comparable with the data published by Armentia and Webb (1992) for the colonisation of *Thiobacillus ferrooxidans* cells on polyurethane foam. Under the same conditions, the process of immobilisation on nickel alloy fibre is similar to that on particles of polyurethane foam; however, the latter support requires a longer incubation time to reach the same levels of adhered biomass density.

In other comparisons, the rate of colonisation of nickel alloy fibre is three times faster than the formation of biofilms on low-grade sulphide mineral, which is considered one of the most suitable immobilisation supports for this microorganism (García et al. 1989), or four times faster than the colonisation rate on PVC cylinders (Nikolov et al. 1988). In the case of the sulphide mineral, the colonisation process needs 25 days to reach a high cellular density; in the PVC cylinders, 30–35 days are necessary to achieve a concentration of bacteria adhering to the support of 1.28×10^3 to 6.50×10^4 cells. This concentration is very low in comparison with that reached on nickel alloy fibre and, furthermore, the authors of the study pointed out that formation of the biofilm is not homogeneous over the full surface of the support; rather, there are certain zones where the adhesion of the microorganisms occurs preferentially.

Results obtained for immobilised biomass as a function of temperature are in agreement with those obtained by Gómez and Cantero (1998) for submerged cultures, and by Nemati and Webb (1997) for immobilised *Thiobacillus ferrooxidans* in biomass support particles. However, the effect of temperature on growth is weak, unlike when ferrous iron is oxidised in suspended culture, where temperatures over the optimum result in the maximum specific growth rate falling to zero. This shows that these bacteria change their activity when they grow in a fixed state, but the mechanism and nature of this change are still unclear. It is probably due to certain physiological alterations of the microorganism during fixation.

Experimental data demonstrating the effect of pH on the colonisation process are shown in Fig. 7. It can be seen that there are significant differences between the results of experiments at pH 2.0 and at 1.7–1.4 for rate of immobilisation and maximum adhered biomass concentration. As can be expected, a comparison of final immoblised biomass showed that at pH 2.0 it was more than twice that at pH 1.7 and eight-fold that at pH 1.4. In this case, the rate of oxidation is smaller because of poorer growth and the reduction of viability at this pH (Gómez and Cantero 1998).

Furthermore, if we consider that the biofilm consists of *T. ferrooxidans* cells attached to the surface of the pores of an inert porous substance (jarosite) and that there is a sharp reduction of precipitates as the pH is reduced, then a decrease of adhered biomass per gram of support is inevitable.

Thus, as Nemati and Webb (1997) reported, temperature and pH can be used as effective parameters to control the level of immobilised biomass and to control operational problems, such as plugging and channelling in bioreactors, that may arise from uncontrolled growth of bacteria.

Lastly, it is significant that the adhesion of the cells of *T. ferrooxidans* to the nickel alloy fibre is stable, and that the bacteria conserve their oxidative capability during storage under normal environmental conditions. It is thus possible to re-start a reactor using particles of nickel alloy fibre colonised by *T. ferrooxidans* after storage for 10 weeks, without the addition of substrate. These results are important for industrial-scale operation; with respect to the practical operation of a reactor, it may be stopped and re-started at any time without too much trouble. This possibility has been previously referred to by Olem and Unz (1980), working with rotating biological contactors, and Armentia and Web (1992), working with polyurethane foam support particles.

Therefore, it can be concluded that nickel alloy fibre as a support material for the immobilisation of *T. ferrooxidans* is a suitable alternative to other, non-metallic support materials, mainly as a result of its ease of handling, low cost, possibility of re-utilisation, high specific surface and stability of the biofilm formed. In addition, the protocol for the immobilisation of this microorganism on a laboratory scale is simple, fast, without the need for special physico-chemical agents, and easily reproducible; thus, its implementation on an industrial scale should be quite practicable.

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