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Microalgal-facilitated bacterial oxidation of manganese

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In the presence of unicellular microalgae, bacterial manganese oxidation was increased by up to ten times the rate produced by bacterial oxidation alone. Azide-poisoned controls demonstrated that the manganese-oxidizing bacteria were active in the algal-bacterial oxidation of manganese. Scanning electron microscopy showed that oxide formation occurred in a number of structurally different deposits on the surface of the alga. Studies involving algal cell fractionation showed that bacterial manganese oxidation was facilitated by the algal cell wall, possibly via Mn^{2+} adsorption. Variations in growth conditions had an effect on algal-bacterial oxide formation and composition. High nutrient (yeast extract, peptone and/or sucrose) levels favored microbial growth but lowered oxide formation, whereas optimal levels of manganese oxide formation required minimal media. High concentrations of either organic nutrients or mineral salts promoted manganese carbonate precipitation.

Keywords: manganese oxidation; microalgae; algal cell wall; media effects

Introduction

Manganese can form a variety of soluble and insoluble compounds [10]. The oxidation mechanisms involving soluble manganese (Mn^{2+}) are often complex in natural environments [23,27]. Chemical oxidation of Mn^{2+} proceeds slowly and usually involves low reactant concentrations [20,21]. Microorganisms play an important part in manganese cycling, through binding, oxidation and reduction processes [22,25]. Microbial manganese oxidation mechanisms may be either direct or indirect, which Nealson *et al* [26] described as: i) oxidation mediated by a cell component; and ii) changes in the extracellular Eh and pH caused by microbial growth and metabolism, respectively.

Many studies on microbial manganese oxidation have dealt with the biogenesis of natural deposits [9,14,17], characterization of the microorganisms involved [6,11,24] and the reaction mechanisms involved [3,10,25]. Few reports have explored the possibility of exploiting these microorganisms for the macro-production of commercially useful manganese oxides. Most quantitative studies [1,7,8] have resulted in low oxidation rates because of the use of low Mn^{2+} concentrations, as high Mn^{2+} concentrations are inhibitive.

Greene [16] showed that relatively high amounts of manganese oxidation could be achieved using a high Mn^{2+} concentration (60 mM). The addition of an acid-tolerant unicellular microalga (*Chlamydomonas* sp) and urea to bacterial cultures (*Pseudomonas* and *Arthrobacter* sp) increased the oxidation of Mn^{2+} by ten-fold at neutral pH [18]. Fourier transform infra-red (FTIR) spectroscopy and X-ray diffraction techniques identified the products of algal-bacterial manganese oxidation as a semipure disordered manganese oxide (γ-MnO_2) [18,31]. Greene and Madgwick [18] showed that the microalga could be used in either a metabolically active (viable) or inactive (autoclaved) state with only minimal differences in oxide formation, suggesting a physicochemical role for the alga.

The present work describes the effect of varying nutrition and physical condition on algal-bacterial manganese oxidation. The role of microorganisms and their cellular components in Mn^{2+} removal and oxide formation were also investigated.

Materials and methods

Algal growth

The acid-tolerant alga, *Chlamydomonas* sp MKA [16] was grown in an artificial minewater [30] containing: $(NH_4)_2SO_4$ (BDH) 1.2 g, KH_2PO_4 (BDH) 0.2 g, $MgSO_4 \cdot 7H_2O$ (BDH) 0.2 g, $FeSO_4 \cdot 7H_2O$ (BDH) 0.3 g, $MnSO_4 \cdot H_2O$ (BDH) 0.3 g and biotin (Sigma) 0.02 μg in 1 L of 0.01 M H_2SO_4 (pH 2). All other algal species (Table 1) were grown in 0.01% Aquasol (Hortico Ltd) in

Table 1 The effect of different microalgae on the removal of Mn^{2+} and oxide formation in algal-bacterial oxidation

Algal-bacterial oxidation[a]	Mn^{2+} removed from solution (g L^{-1})	Oxide formed[b] (g L^{-1})
Chorella sp WB	0.70 ± 0.07	0.33 ± 0.03
Ulothrix sp	0.68 ± 0.09	0.21 ± 0.04
Scenedesmus sp	0.67 ± 0.07	0.25 ± 0.05
Haemaetococcus sp	0.73 ± 0.08	0.21 ± 0.04
Chlamydomonas sp	0.69 ± 0.10	0.17 ± 0.05
Chorella sp	0.55 ± 0.07	0.18 ± 0.05
Chlamydomonas sp MKA	0.88 ± 0.09	0.58 ± 0.06

[a]Bioreactors were inoculated with *Arthrobacter* sp MK-2 and incubated for 30 days at 30°C using 30 mM Mn^{2+}.
[b]Concentration of manganese oxides [4].

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H_2O (pH 6). The algae were grown in aerated (approximately 1 L min^{-1}) glass tubes at 25° C. A light : dark cycle of 18 h: 6 h was provided with Grolux (Sylvania) fluorescent lamps at a average photon flux of 10 μmol m^{-2} s^{-1}. All algal cultures were grown to a biomass of 0.6 g L^{-1} (dry wt). For *Chlamydomonas* sp MKA this was equivalent to an $OD_{310\,nm}$ of 5.0.

Bacterial growth

Bacterial strains, Mix-5 (mixed culture) and *Arthrobacter* sp MK-2 isolated from the alga *Chlamydomonas* sp MKA [16] were used as inocula in studies of algal-bacterial oxidation. These isolates were inoculated into sterile bacterial growth medium [16,30] containing, $MnSO_4 \cdot H_2O$ (BDH) 5 g, peptone (Oxoid) 2 g, yeast extract (Oxoid) 1 g and mineral salts KH_2PO_4 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $MgSO_4 \cdot 7H_2O$ (BDH) 0.02 g, $(NH_4)_2SO_4$ (BDH) 0.1 g, NaCl (BDH) 0.2 g in 1 L of H_2O (pH 6.5) and incubated for 48 h at 30°C on an orbital shaker at 100 rpm.

Algal-bacterial manganese oxidation

Aerated column bioreactors were used for algal-bacterial oxidation studies [16,30]. A 100-ml portion of the sedimented algae (400 ml of *Chlamydomonas* sp MKA was adjusted to pH 6.5 and allowed to sediment) was added to a column bioreactor, containing 1 ml of Antifoam C emulsion (Sigma) (3% w/v solution) and autoclaved. A 190-ml portion of filter-sterilized oxidation medium containing: $MnSO_4 \cdot H_2O$ (BDH) 5 g, urea (BDH) 10 g, and mineral salts in 1 L of H_2O (pH 6.5) was aseptically added to the bioreactors. Bioreactors were then inoculated with 10 ml of bacteria culture. Sterile controls were obtained with added 15 mM NaN_3 (Sigma).

Bioreactors were incubated at 30°C and aerated with filter-sterilized (Millex-FG_{50}, Millipore) air at 1000 ml min^{-1}. The volume was maintained at 300 ml by the addition of sterile distilled water. Bioreactors were incubated for 4 weeks and analysed weekly for pH, Mn^{2+} and oxide concentrations.

Optimizing algal-bacterial oxidation

A variety of nutritional and physical conditions was tested to improve the algal-bacterial oxidation of manganese. The incubation temperatures used were from 15° to 50°C, initial Mn^{2+} concentrations were from 0.4 to 10 g L^{-1} and added nutrients were yeast extract and peptone. The carbon sources used were sucrose and a combination of sucrose with yeast extract and peptone. Mineral salts were KH_2PO_4, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ and NaCl. Iron (Fe^{2+}) additions were 0.5, 2.5 and 5.0 g L^{-1}.

Light/electron microscopy

Algal-bacterial oxidation was viewed at different stages of culture by phase contrast microscopy (Olympus BH light microscope). At higher magnification, scanning electron microscopy (SEM) was used to study changes in oxide morphology during oxide formation. Samples were prepared by freeze-drying, according to Willison and Rowe [32] using a Balzer BAF 400. Freeze-dried samples were either chromium-coated using a Dynavac Zenosput 2000 or alternatively carbon-coated with an Edwards Speedivac when performing elemental analysis. Samples were viewed either with a Cambridge Stereoscan 360 or a Hitachi S900 field emission scanning microscope. The qualitative elemental composition of the samples was determined using a Kevex Delta 5 EDAX spectrometer.

Fractionation of algal cells

Preliminary work [19] had shown that algal fractions had active roles in facilitating the algal-bacterial oxidation of manganese. Algal cell walls were prepared according to Fleet and Phaff [12,13] and substituted for whole algal cells in standard algal-bacterial oxidation. Algal cell walls were isolated by disruption of whole algal cells (400 ml) using a Gaulin homogenizer (Model 15M 8TA) in 0.1 M Tris buffer. The homogenate was separated by centrifuging it ($102 \times g$ per 20 min) (Beckman J-21B centrifuge), with the cell walls being recovered by washing them six times in buffer and three times in H_2O. All fractionation work was carried out at 4°C.

Manganese oxide analysis

Fourier transform infrared (FTIR) spectroscopy (Nicolet MX-1E spectrometer) was used to characterize the oxides tentatively by comparison with a range of standard oxides [18]. Crude precipitates were washed with distilled water, dried (105°C per 24 h) and then finely ground in an agate mortar before analysis.

Chemical analysis

Soluble manganese (Mn^{2+}) was analysed by atomic absorption spectroscopy (AAS) (Varian AA-1475). pH was determined using a combination electrode (Activon). Manganese oxide concentration was determined semi-quantitatively by the benzidine acetate assay [17]. Total manganese content in oxide precipitate was determined via acid-microwave digestion and measuring the Mn^{2+} by AAS. Mn(IV) content was determined indirectly by measuring the oxidizing capacity of the oxide [4].

Results

Algal-bacterial oxidation of manganese

Figure 1 shows the combined effect of algal and bacterial cells (algal-bacterial oxidation) on manganese oxidation. Both the amount of Mn^{2+} removed from solution and oxide formed were increased compared to bacterial oxidation alone and to NaN_3 poisoned controls. Mn^{2+} uptakes were approximately different, whereas oxide formation showed a lag in production up to 14 days. The bulk pH during algal-bacterial oxidation increased initially to 7.2–7.5 after 7 days, then decreased with increased oxidation and stabilised between 6.4–6.8 after 30 days.

Table 2 shows the effect of varying culture conditions on algal-bacterial oxidation and oxide composition. Mixed culture inocula (Mix-5 and sterile algae) and non-sterile conditions (non-sterile algae and no inoculation) gave similar amounts of oxide formation and Mn^{2+} removal to that found using pure culture (MK-2 and sterile algae) algal-bacterial oxidation. However, chemical analysis of these crude precipitates showed that the oxide composition changed according to the culture conditions. The Mn(IV)

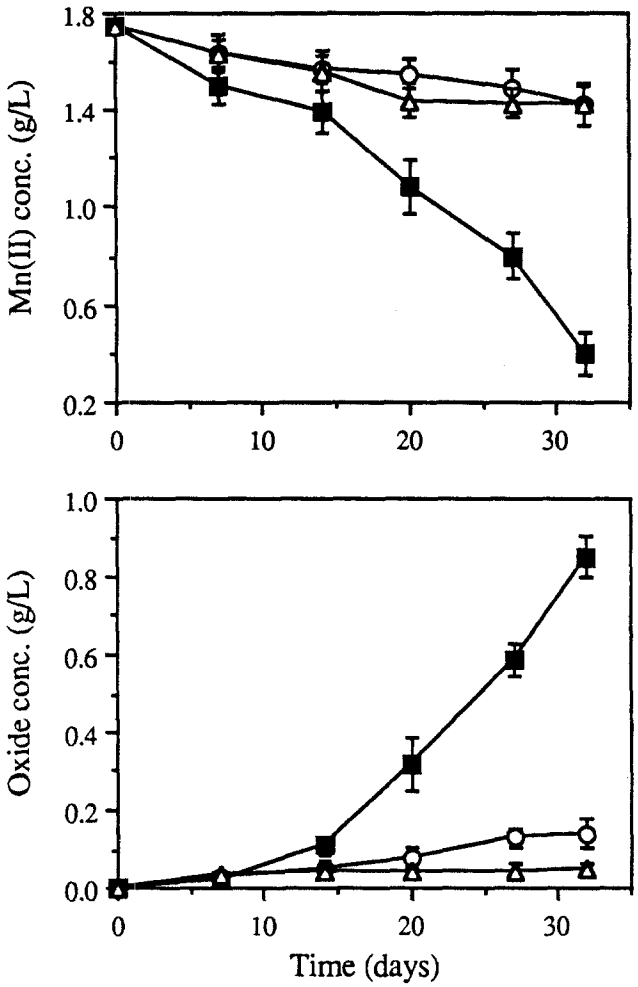

Figure 1 Time course oxidation of manganese (30 mM) at 30°C. Algal-bacterial oxidation with *Arthrobacter* sp MK-2 (■), bacterial oxidation with *Arthrobacter* sp MK-2 (○), and NaN_3-poisoned (15 mM) algal-bacterial oxidation (Δ).

Table 2 The effect of different culture conditions on algal-bacterial oxidation of manganese (30 mM)

Algal-bacterial oxidation[a]	Mn^{2+} removed from solution (g L^{-1})	Oxide formed[b] (g L^{-1})	Crude precipitate	
			Total Mn (%)	Mn(IV) (%)
MK-2 + sterile algae[c]	1.36 ± 0.08	0.85 ± 0.05	41	14
Mix-5 + sterile algae[c]	1.39 ± 0.09	1.06 ± 0.07	35	6
Non-sterile algae[d]	1.46 ± 0.11	0.92 ± 0.06	31	6

[a]Bioreactors were incubated for 30 days at 30°C.
[b]Concentration of manganese oxides [4].
[c]*Chlamydomonas* sp MKA was autoclaved.
[d]No bacterial inoculation.

content was reduced by 50% when mixed culture and non-sterile conditions were employed.

A variety of different microalgae were tested to determine if algal-bacterial oxidation was dependent on the algal species. Table 1 shows the effect of different algal species on Mn^{2+} removal and oxide formation. The removal of Mn^{2+} from solution was generally slightly less than with *Chlamydomonas* sp MKA, whereas oxide formation was decreased by about 50% or greater. The use of NaN_3-poisoned controls also inhibited oxidation.

Effect of temperature on manganese oxidation

Variations in oxidation temperature, exhibited a temperature optimum at 30°C for both Mn^{2+} removal and oxide formation (Figure 2a). The use of higher or lower oxidation temperatures decreased both the removal of Mn^{2+} and oxide formation. However, the ratio of oxide produced to Mn^{2+} removed (oxide yield) varied with temperature. Lower oxidation temperatures resulted in lower oxide yields, whereas at higher temperatures oxide yields were unchanged.

Effect of initial Mn^{2+} concentation on oxidation

Figure 2b shows that over a range of initial Mn^{2+} concentrations, Mn^{2+} removal and oxide formation were unrelated. The removal of Mn^{2+} from solution increased linearly with increasing Mn^{2+}, whereas the amount of oxide formed increased to a maximum at 3.3 g L^{-1} (60 mM) Mn^{2+} and then remained constant. However, the ratio of oxide produced to Mn^{2+} removed reached a maximum at 1.6 g L^{-1} (30 mM) Mn^{2+}.

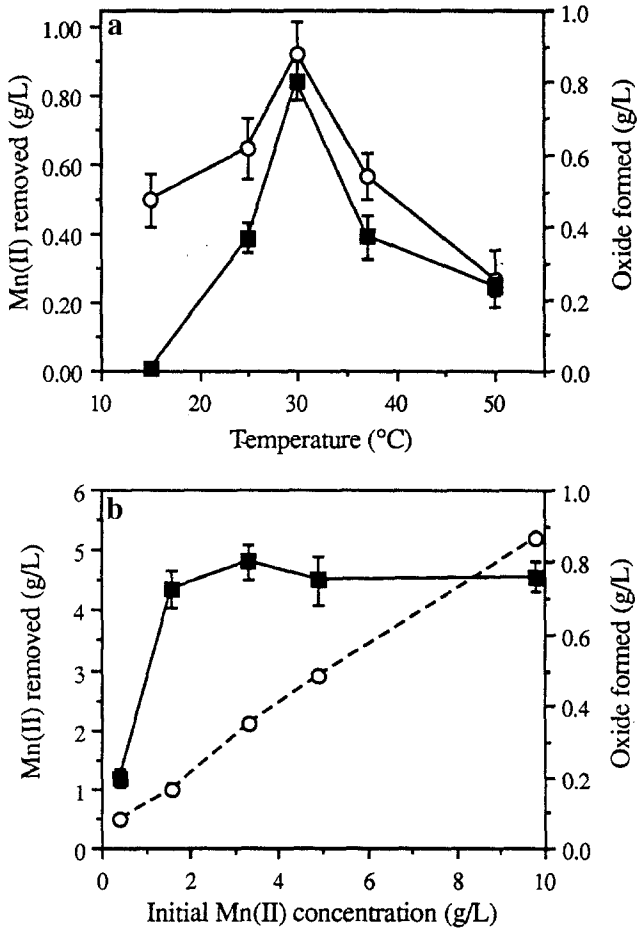

Figure 2 The effect of temperature (a) and initial Mn^{2+} concentration (b) on Mn^{2+} removal (○) and oxide formation (■) in algal-bacterial oxidation for 30 days.

Effect of medium composition on oxide production

Changes in medium composition had a marked effect on algal-bacterial oxidation. The addition of nutrients, yeast extract, peptone and/or sucrose suppressed oxide formation and with increased concentrations, also promoted the precipitation of manganese carbonates ($MnCO_3$). The FTIR spectrum (Figure 3a) of an algal-bacterial oxidation with added nutrients shows a major peak at 1500 wavenumbers, indicating a manganese carbonate compound, whereas with no nutrient addition (Figure 3b) a strong MnO_2 peak is present at 570 wavenumbers.

While oxide formation was reduced due to organic nutrient addition (yeast extract, peptone and/or sucrose), the amount of Mn^{2+} removed from solution was usually unaffected. The addition of excess organics often caused the initial rapid removal of Mn^{2+}, resulting in precipitation of $MnCO_3$.

Addition of varying mineral salt concentrations also suppressed oxide formation, whereas when no salts were added a slight drop in oxidation occurred (Table 3). Oxide inhibition was noted when individual salts were added at high concentrations [30]. However, some salt (eg NaCl) addition gave comparable amounts of oxidation and Mn^{2+} removal to algal-bacterial oxidation with mineral salt addition. The addition of Fe^{2+} (0.5, 2.5, 5.0 g L^{-1}) had a similar effect on oxide formation as mineral salt addition. Low Fe^{2+} concentrations increased oxide formation, whereas at higher concentrations oxidation was suppressed [30]. Chemical analysis showed that Fe^{2+} addition reduced the total Mn and Mn(IV) contents in the crude precipitates.

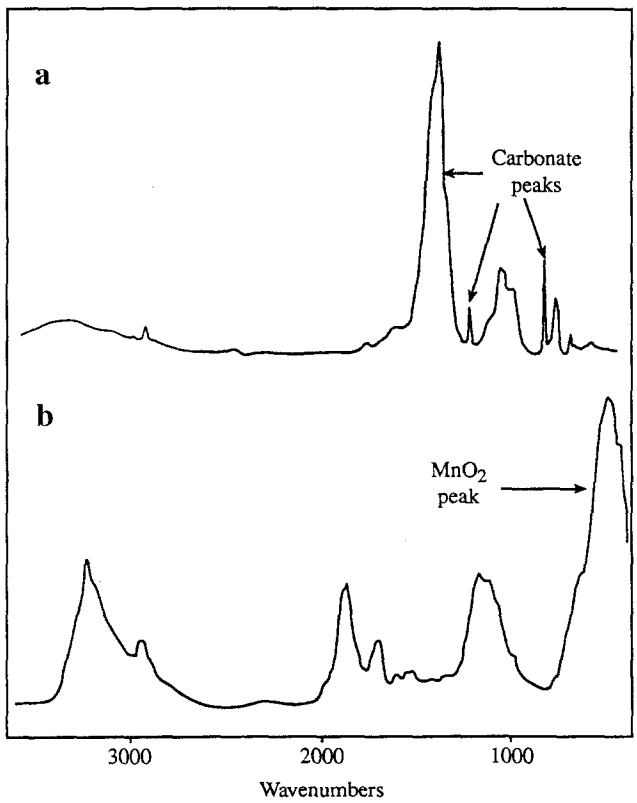

Figure 3 FTIR spectra of precipitate formed by algal-bacterial oxidation of manganese (30 mM) at 30°C for 30 days with added nutrients (a), yeast extract 2.5 g L^{-1} and peptone 4 g L^{-1} and with no added nutrients (b).

Table 3 The effect of mineral salts on Mn^{2+} removal and oxide formation in algal-bacterial oxidation

Algal-bacterial oxidation[a]	Mn^{2+} removed from solution (g L^{-1})	Oxide formed[b] (g L^{-1})
Mineral salts[c]	0.83 ± 0.07	0.75 ± 0.07
Mineral salts[d]	1.05 ± 0.09	0.90 ± 0.06
No added mineral salt	0.85 ± 0.08	0.75 ± 0.05
NaCl 2 g L^{-1}	0.90 ± 0.10	0.85 ± 0.04

[a]Bioreactors were inoculated with *Arthrobacter* sp MK-2 and incubated for 30 days at 30°C using 30 mM Mn^{2+}.
[b]Concentration of manganese oxides [4].
[c]Mineral salts: KH_2PO_4 0.1 g, $CaCl_2{\cdot}2H_2O$ 0.2 g, $MgSO_4{\cdot}7H_2O$ 0.04 g, $(NH_4)_2SO_4$ 0.2 g, NaCl 0.4 g L^{-1}.
[d]Mineral salts: KH_2PO_4 0.05 g, $CaCl_2{\cdot}2H_2O$ 0.1 g, $MgSO_4{\cdot}7H_2O$ 0.02 g, $(NH_4)_2SO_4$ 0.1 g, NaCl 0.2 g L^{-1}.

The morphology of algal-bacterial oxides

Under a light microscope, algal-bacterial deposits appeared to be a mixture of oxides, algal and bacterial cells. The oxides had a black-brown appearance, closely associated with the alga. Using higher magnification SEM, oxide formation was observed on the surface of the alga. Many different sized and shaped precipitates were present. Elemental analysis of the surface precipiates confirmed the presence of manganese and oxygen as the major constituents on the algal surface, with other minor signals.

Time sequence studies (Figure 4) showed that oxide formation exhibited a number of changes in morphology with time. Initially (Figure 4a) a large amount of extracellular material was present due to polysaccharide and protein release by lysis of the alga at pH 6.5. At 7 days (Figure 4b) bacterial cells and oxide could be seen attached to algal surfaces and by 14 days (Figure 4c) the algal surfaces were partly or completely encrusted with oxides. Changes were also observed in the size and shape of oxide crystals. Smaller crystals (Figure 4d and e) were observed initially, while larger more sheety precipitates (Figure 4f) formed with time.

Substituting algal cell walls in algal-bacterial oxidation

The substitution of isolated algal cell walls for whole algal cells confirmed that manganese-oxidizing activity was associated with the algal cell wall. The amounts of Mn^{2+} removed from solution, oxide formed and crude precipitate composition are shown in Table 4. When NaN_3 was used with isolated algal cell walls, both Mn^{2+} uptake and oxidation were reduced.

Discussion

The demonstration of high amounts of manganese oxide formation shows that large-scale biological MnO_2 production is possible. Previous studies dwelt mainly on purified single microbe systems using low Mn^{2+} concentrations. Using algal-bacterial oxidation, high amounts of bioformed oxide were possible using high Mn^{2+} concentrations at neutral pH. The advantage in using a combination of algal and bacterial cells for manganese oxidation

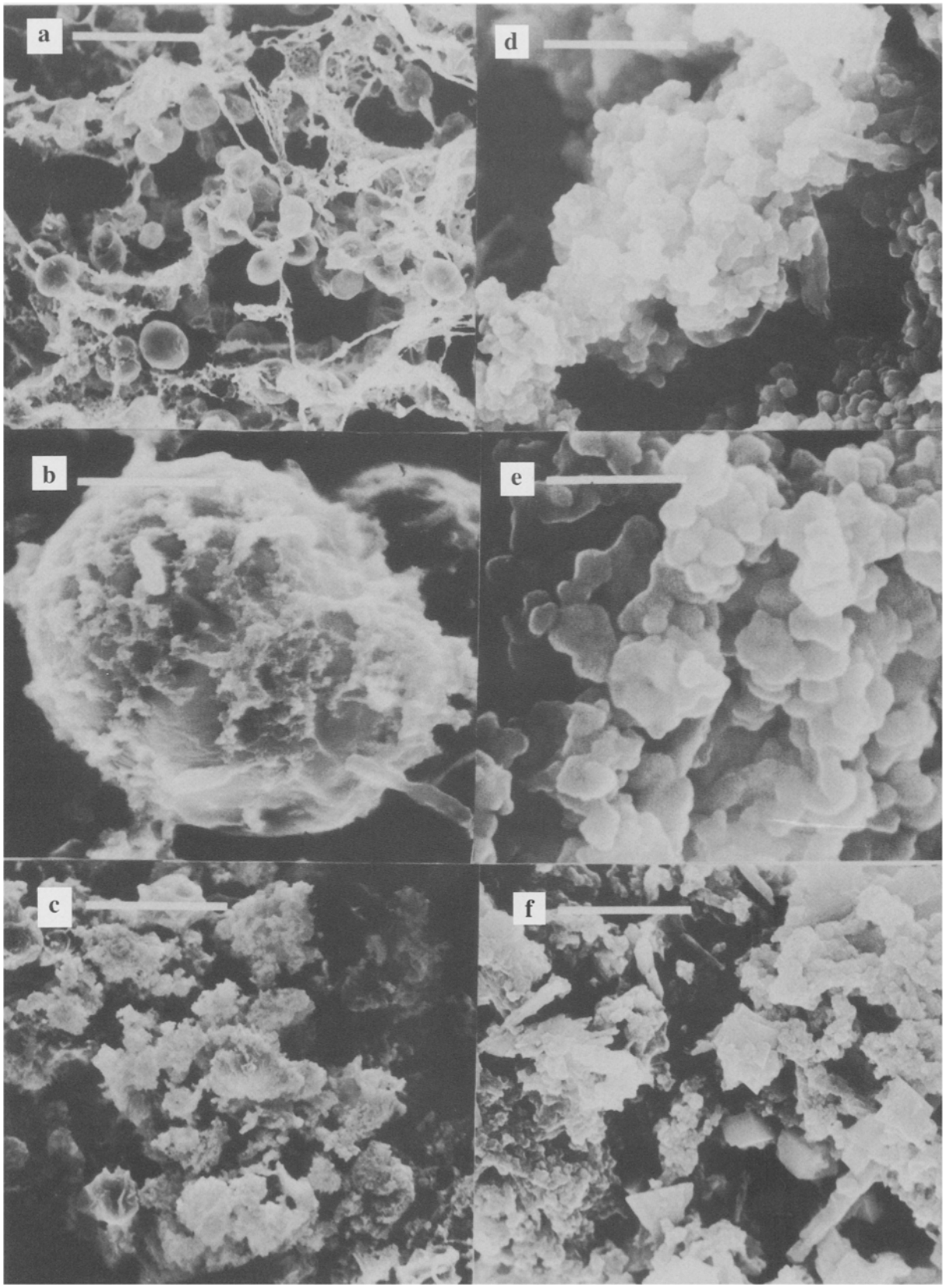

Figure 4 Scanning electron micrographs of algal-bacterial cultures involved in oxidation of manganese (30 mM) with time at varying magnifications. (a) 0 days, bar 15 μm; (b) 7 days, bar 1.66 μm; (c) 14 days, bar 8.6 μm; (d) 22 days, bar 0.5 μm; (e) 22 days, bar 0.2 μm; (f) 42 days, bar 1.5 μm.

Table 4 The effect of substituting isolated algal cell walls for whole algal cells in algal-bacterial oxidation

Algal-bacterial oxidation[a]	Mn^{2+}removed from solution (g L^{-1})	Oxide formed[b] (g L^{-1})	Crude precipitate	
			Total Mn (%)	Mn(IV) (%)
Whole algal cells	1.05 ± 0.10	0.75 ± 0.06	40	14
Isolated cell walls	1.25 ± 0.09	0.79 ± 0.05	37	13
Isolated cell walls + NaN_3	0.22 ± 0.09	0.03 ± 0.01	ND[c]	ND[c]

[a]Bioreactors were inoculated with *Arthrobacter* sp MK-2 and incubated for 30 days at 30°C using 30 mM Mn^{2+}.
[b]Concentration of manganese oxides [4].
[c]ND: not determined, insufficient sample.

appears to be due to creating and maintaining a more suitable oxidative environment.

A variety of different physical and nutritional conditions were tested to further improve and gain a better understanding of the roles of microorganisms in Mn^{2+} removal and oxide formation. The addition of NaN_3 to algal-bacterial oxidation showed that viable bacteria were the primary catalysts in oxide formation, whereas the algal cells played a major role in the adsorption of Mn^{2+} from solution. The neutral pH throughout the oxidation, comparative low temperature of incubation and the effect of Mn^{2+} concentrations on oxide productivity, all support a bacterial rather than a simple chemical oxidation of the Mn^{2+}. The substitution of other unicellular algae for *Chlamydomonas* sp MKA also confirmed the general potentiating effect of the algal surface properties in the oxidation of manganese.

Algal-bacterial oxidation of manganese was directly influenced by the medium components. High concentrations of nutrients to algal-bacterial oxidation promoted bacterial growth and created unsuitable oxidizing conditions. This resulted in inhibition of oxide formation and in some cases the chemical precipitation of $MnCO_3$ due to the rapid bacterial decomposition of the carbohydrates to CO_2 and H_2O, suggesting a non-oxidizing metabolism by the bacterial cells. Maximum algal-bacterial manganese oxidation occurred using minimal nutrient addition. Whether the oxidising bacteria may be able to derive some energy from the respiration of the conversion of Mn(II) to Mn(IV) is open to further investigation.

Scanning electron microscopy showed that manganese oxidation occurred on the surface of the algae. The oxide precipitates were formed in a number of morphological stages and the final deposits were mixtures of different precipitates. Chemical analyses of the crude manganese oxides supported these findings that the algal-bacterial deposits consist of a mixture of Mn(IV) species, Mn(III) intermediate oxidation states, absorbed Mn^{2+} ions and organic material of microbial origin.

Preliminary fractionation of the *Chlamydomonas* sp MKA cells showed that the oxidizing activity was associated with the algal cell solids rather than soluble material [19]. The substitution of isolated algal cell walls in algal-bacterial oxidation confirmed that the cell wall most likely mediates the oxidation of the manganese probably by binding and stabilizing the Mn^{2+}, but does not necessarily stimulate the final oxide composition. Previous reports [2,5,15,28,29] showed the adsorption of Mn^{2+} to negatively-charged polymers prior to its oxidation to MnO_2 by an unknown biocatalyst.

The presence of both microalgae or their cell walls and viable bacteria were essential for high levels of manganese oxide formation. The combined effect of these components appears to be in maintaining a favorable micro-oxidative environment. The algal cell walls most probably provide sites for binding and stabilising Mn^{2+} in some form of surface complex. This complex then presumably allows the bacteria to oxidize and precipitate the manganese on the algal cell surfaces.

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