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Bioleaching of manganese from low-grade ores using manganese-reducing microbial cultures

A. M. MOHOD, P. B. SALUNKHE AND K. M. PAKNIKAR*
Division of Microbial Sciences, Agharkar Research Institute, G. G Agarkar Road, Pune 411004, India.
email paknikar@vsnl.com, Phone: +91-0212-354357, 353680; Fax +91-20-5651542

Abstract

Citrobacter sp. isolated from sediment sample of a polluted creek was found to reduce and hence solubilise manganese from manganese dioxide The optimum conditions for manganese reduction were. pH (9.5), temperature (35°C), inoculum size (10^9 cfu/ml), and glucose (10 g/l) When the culture was used for leaching of manganese from a low-grade pyrolusite ore in a stirred tank reactor, >90% manganese could be extracted in 13 days at 1% (w/v) pulp density The process has the potential of becoming an efficient alternative to the conventional processes for leaching of low-grade pyrolusite ores

Keywords: Pyrolusite, bioleaching, manganese reduction

1. Introduction

Manganese is one of the elements of the first transition series with abundance of 0.1% in the earth's crust.[1] The element can exist in the oxidation states of 0, +2, +3, +4, +6 and +7. However, in nature the +2 and +4 oxidation states are commonly found. Of these, the +2 state can occur as a free ion in solution, whereas +3 and +4 states occur as insoluble oxides. This is a metal of particular industrial importance in the production of steel and alloys. Manganese dioxide-containing minerals such as pyrolusite are a major source of manganese. Traditional processes for extracting manganese from such minerals make use of reducing agents such as sulphur dioxide which can be a dangerous pollutant at high concentrations. Therefore, there is a need for developing alternative environment-friendly methods. In this respect, recovery of manganese using manganese-reducing bacteria is an interesting alternative process for the future.

There are two known mechanisms by which microorganisms can effect reduction of manganese, viz. direct and indirect. The direct mechanism of manganese reduction involves the oxidation of organic matter coupled to microbial respiration, with manganese oxide serving as an electron acceptor in the absence of oxygen.[2-7] However, in indirect reduction, a large number of organic and inorganic compounds are involved. In this reduction mechanism, physical contact of microorganisms with the mineral particle is not necessary. Stone and Morgan[8,9] examined reduction and dissolution of Mn(III) and Mn(IV) oxides by 27 organic compounds found in nature. Indirect Mn(IV) reduction may also occur through the production of inor-

*For correspondence.

ganic compounds such as ferrous iron,[10] sulphide[11] or through hydrogen peroxide formed during aerobic respiration.[12]

Microbial reduction also has the potential to be important in the formation of reduced minerals, release of dissolved manganese into sediment pore and ground waters, and the release of trace metals bound to manganese oxides.[13] Biogeochemical action of subsurface microorganisms on manganese compounds may be economically important in mineral conservation and in new hydrometallurgical extraction methods, especially for low-grade ores.

The present investigation attempts to study the feasibility of using manganese-reducing microorganisms isolated from subsurface environments for the extraction/solubilisation of manganese from pyrolusite ores.

2. Materials and methods

2.1. *Sediment samples*

Sediment samples were collected aseptically from a saline lake (Lonar lake, Maharashtra), freshwater dam (Bhavanapadu dam, Andhra Pradesh) and polluted creek (Thane creek, Maharashtra). Samples were transported on ice to laboratory where they were stored at 4°C until further processing. All the sediment samples were analysed for pH and the presence of iron, manganese, cobalt, nickel, copper, zinc, selenium, tellurium and cadmium.

2.2. *Enrichment and isolation of manganese-reducing cultures*

For selection and screening of efficient manganese-reducing microorganisms, experiments were set up by inoculating 1 g aliquots of sediment samples in 250-ml Erlenmeyer flasks containing 100-ml Bromfield medium (composition in *g*/l: ammonium sulphate, 1.0; potassium dihydrogen phosphate, 0.5; magnesium sulphate, 0.05; glucose, 10.0; and yeast extract, 0.15) supplemented with 0.1% (w/v) manganese dioxide. The flasks contained medium adjusted to the respective ambient pH and were incubated on rotary shaker (120 rpm) at 30°C for 15 days. Microbial reduction of manganese dioxide was detected by visually observing disappearance of particulate manganese dioxide and increase in turbidity due to bacterial growth. A loopful of culture from these flasks was transferred to freshly prepared Bromfield medium containing 0.1% (w/v) manganese dioxide. After 2–3 successive transfers, a loopful of culture was streaked on nutrient agar (Hi Media) plates and the plates were incubated at 30°C for 48 h. Isolated colonies developing on nutrient agar plates were subcultured on nutrient agar slants for preservation in a refrigerator.

2.3. *Screening, selection and identification of efficient manganese-reducing culture*

Each of the isolated culture was grown in 250-ml flasks containing 100-ml Bromfield medium (pH 7.5) without manganese dioxide at 30°C on a rotary incubator shaker (120 rpm) for 24 h. The cells were harvested by centrifugation (10,000 × g for 10 min; Sorvall RC 5B plus, USA), washed twice and resuspended in physiological saline. This saline suspension was used as inoculum for further experiments.

Experiments were performed in 250-ml Erlenmeyer flasks containing 100-ml sterile Bromfield medium (pH 7.5) and supplemented with 0.1% (w/v) manganese dioxide. The final cell

density in all the flasks was adjusted to 10^6 cfu/ml. The flasks were incubated at 30°C in a rotary incubator shaker (Gallenkamp, England) for 96 h at 120 rpm. After incubation, the contents of the flasks were centrifuged at 10,000 × g for 10 min and the supernatant was used for manganese estimation.

The most efficient manganese-reducing culture was selected for further studies and identified by carrying out morphological, cultural and biochemical tests as specified in the Bergey's Manual of Systematic Bacteriology.[14]

2.4. *Factors affecting manganese reduction*

The factors affecting manganese reduction were investigated in a series of batch culture experiments supplemented with 0.1% (w/v) manganese dioxide. The effect of various factors such as pH (5.5–10.5), temperature (25–40°C), inoculum size (10^5–10^9 cfu/ml) and carbon source (glucose, 5–50 g/l) was checked by running different sets of experiments wherein one parameter was varied keeping the others constant. The flasks were incubated at 30°C in an orbital shaker incubator at 120 rpm. Appropriate controls were run and cell protein and solubilisation of manganese were monitored periodically. From the data obtained optimum parameters, viz. pH, temperature, inoculum size and glucose concentration were determined.

2.5. *Bioleaching of pyrolusite-flask experiments*

The pyrolusite samples collected from manganese mines of Orissa were used. The chemical analysis of the ore samples is given in Table I. The ore samples were crushed and sieved to get average grain size of <1.25 mm mesh. The leaching experiments were performed in 250-ml Erlenmeyer flasks containing 100-ml Bromfield medium under previously optimised conditions of pH, temperature, inoculum size and concentration of carbon source. The pulp density of the ores was 1, 5, 10, 15, 20, 25 and 30% (w/v). Samples were periodically removed from flasks, centrifuged and the supernatants were analysed for soluble manganese content. The data were used to select an appropriate ore sample and optimum pulp density for further bioleaching experiment in a continuous mode.

2.6. *Bioleaching of pyrolusite in stirred tank reactor*

The stirred tank reactor comprised a borosilicate glass bottle containing 1-l Bromfield medium. The reactor was set up at optimum parameters of pH, glucose concentration and pulp density

Table I
Chemical composition of manganese ores

Ore	pH	MnO_2	Total Mn	Phosphate	Iron
		g%	g%	g%	g%
D-76	5.09	42.30	43.51	0.203	3.000
D-102	6.79	41.90	33.42	0.170	13 600
D-106	6.38	33.50	14.81	0.158	26.250
D-118	6.26	39.90	26.70	0 175	12.100
D-123	8.21	25.00	25.12	0.165	8.300
D-125	6.55	40.60	33.79	0.210	32 000
D-127	8.02	41.70	32.91	0.195	15.950

of the selected pyrolusite ore. The selected manganese-reducing culture was inoculated at a final cell density of 10^9 cfu/ml, the contents of the reactor were stirred with a magnetic stirrer and air was sparged into the reactor. After 3 days of stabilisation, the stirred tank reactor was switched to a continuous mode by passing Bromfield medium at the bottom of the reactor with the help of a programmable peristaltic pump (Ismatec, Model MCP 552, Switzerland). The flow rate of the solution was maintained at 1 ml/min. The effluent containing solubilised manganese was removed from the top at the same flow rate using the peristaltic pump. The reactor was monitored for a period of 13 days. The effluent samples from the reactor were collected daily and analysed for pH, cell density and manganese content.

2.7. *Analyses*

Appropriate amount of dried sediment samples was digested with a solution of concentrated hydrochloric and nitric acid in a ratio of 3:1 (v/v) at 300°C for 5 h and analysed for the presence of manganese, iron, copper, cobalt, selenium, tellurium, cadmium, nickel and zinc by using atomic absorption spectrophotometry.

The extent of manganese reduction was determined in all the experiments by measuring the solubilised manganese produced over time as described by Burdige and Nealson.[2] Accordingly, 1 ml of the experimental suspension was mixed with 1 ml of 10-mM copper sulphate (pH 4.6). The mixture was then allowed to equilibrate for 2 h, filtered through a 0.2-μM membrane filter and soluble manganese in the filtrate measured by atomic absorption spectrophotometry.

Total manganese from ores was determined by digesting appropriate amount of ore in concentrated hydrochloric acid and manganese content was estimated by using flame atomic absorption spectrophotometry. Manganese dioxide was estimated by sodium oxalate method.[15]

Protein content of the cells was estimated spectrophotometrically using Bradford's reagent after digestion with 2 N NaOH followed by heating at 70°C for 60 min. Bovine serum albumin was used as a standard.[16] Serial-dilution Agar-plating procedure was performed to determine the total viable count of the culture.

3. Results

3.1. *Sediment samples*

Table II shows the chemical composition of sediment samples. The sediment sample (T1) collected from Thane creek showed the highest concentration of iron, manganese, zinc, copper, nickel and cobalt at the level of 10,266, 219, 164, 102, 11.7 and 89.2 mg/kg. Tellurium and cadmium were absent in all the sediment samples. The pH of the samples varied from 6.7 to 10.0.

3.2. *Enrichment and isolation of manganese-reducing cultures*

The enrichments set up at pH 7.5 and incubated at room temperature (28±2°C) showed manganese reduction and increase in turbidity within 8 days. After repeated subculturing and plating of the growth on nutrient agar plates, six morphologically distinct colony types were isolated.

Table II
Chemical analysis of sediment sample

Sediment sample #	Source	pH	Presence of metals (mg/kg)						
			Mn	Fe	Zn	Cu	Se	Ni	Co
C7	Dam	8 3	31.4	2667 00	18.30	6 8	49 80	27 7	30.3
C8	Dam	7.2	18.9	12 30	7.66	4.3	20 60	33.1	29.2
C9	Dam	8.2	29 4	301 40	20 50	5 3	48 30	18 5	9 3
T1	Creek	7 8	219.0	10266 00	164 00	102 0	10 30	11 7	89 2
T2	Creek	8 8	135 0	8798 00	128 00	35 5	110.00	14 0	89.0
L	Lake	9.5	121 0	1196 00	135.00	96 6	43 60	31.1	47.8

Dam: Bhavanapadu Dam, Andhra pradesh, Creek Thane creek, Maharashtra; Lake Lonar lake, Maharashtra.

3.3. *Screening, selection and identification of efficient manganese-reducing culture*

The six isolated cultures showed manganese reduction at pH 7.5. However, the reduction exhibited by the cultures varied widely (Table III). It was observed that isolate #2 could reduce manganese at pH 7.5 with the highest efficiency, i.e. 185 mg/l in 96 h. Therefore, this isolate, identified as *Citrobacter* sp., was selected for further studies.

3.4. *Factors affecting manganese reduction*

Figure 1 shows the relationship between growth and manganese reduction at different pH. It could be seen that the growth and manganese reduction by *Citrobacter* sp. occurred over a wide range of pH (5.5–10.5). The optimum pH for the culture was found to be 9.5 at which 235 mg manganese/l could be solubilised. The culture could grow at temperatures ranging from 25 to 40°C; however, the optimum temperature at which maximum growth and manganese reduction (i.e. 278 mg/l) occurred was 35°C (Fig. 2).

Rate of reduction of manganese by *Citrobacter* sp. in Bromfield medium was directly proportional to cell number (Fig. 3). At 10^9 cfu/ml and under optimum conditions of pH and temperature, 302-mg manganese/l was solubilised. Figure 4 shows that 274 mg of manganese/l was solubilised at a concentration of 10 g/l glucose and there was no significant enhancement in manganese solubilisation with further increase in glucose concentration up to 50 g/l.

Table III
Screening of microbial cultures for manganese reduction at pH 7.5

Isolate	Sample #	Reduction of manganese (mg/l) after 96 h
#1	T1	89
#2	T2	185
#3	C7	107
#4	C8	179
#5	C9	117
#6	L	95

Initial concentration of manganese was 631 mg/l

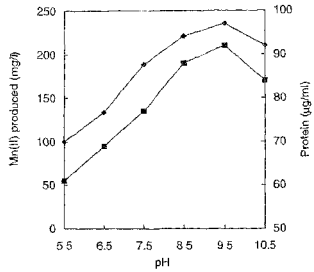

FIG. 1. Effect of pH on manganese reduction by *Citrobacter* sp. at 30°C, with 10^5 cfu/ml. The soluble Mn(II) (◆) and protein (■) were measured after 40 h.

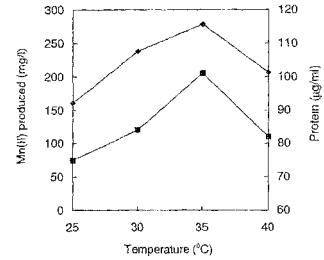

FIG. 2. Effect of temperature on manganese dioxide reduction by *Citrobacter* sp. at pH 9.5, with 10^5 cfu/ml. The soluble Mn(II) (◆) and protein (■) were measured after 40 h.

Therefore, the minimum concentration of glucose at which maximum manganese solubilisation could occur was 10 g/l. Manganese solubilisation was not observed in any of the control flasks.

3.5. *Bioleaching of pyrolusite-flask experiments*

Table IV depicts reduction and dissolution of manganese from pyrolusite ore samples using *Citrobacter* sp. in 60 h. It was found that with the increase in pulp density, the efficiency of manganese reduction decreased. The manganese reduction efficiencies for pyrolusite ores D-76, D-102, D-106, D-118, D-123, D-125 and D-127 at 1% (w/v) pulp density was found to be 1.22, 1.16, 1.31, 1.20, 8.50, 1.85 and 2.68%, respectively. Since the reduction efficiency was the highest (8.5%) for ore D-123, it was selected for further studies.

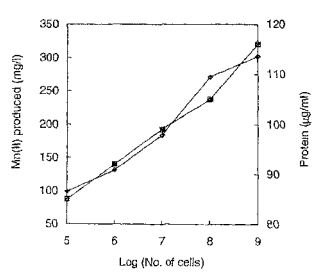

FIG. 3. Effect of initial inoculum size on manganese dioxide reduction by *Citrobacter* sp. at pH 9.5 and temperature 35°C. The soluble Mn(II) (◆) and protein (■) were measured after 40 h.

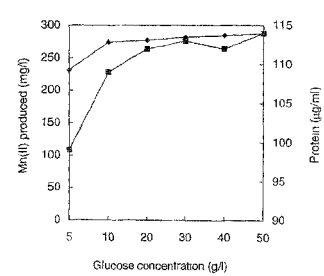

FIG. 4. Effect of glucose on manganese dioxide reduction by *Citrobacter* sp. at pH 9.5, temperature 35°C, with 10^5 cfu/ml. The soluble Mn(II) (◆) and protein (■) were measured after 40 h.

Table IV
Manganese reduction efficiency (g%) at varying pulp density

Ore	Pulp density, % (w/v)						
	1	5	10	15	20	25	30
D-76	1 229	0.638	0.390	0.288	0.239	0.205	0.185
D-102	1 169	0.539	0.272	0.214	0 177	0.159	0.149
D-106	1.313	0.620	0 343	0 260	0.210	0.180	0.170
D-118	1 203	0.511	0.323	0.245	0 214	0.191	0.174
D-123	8 508	1 952	1.115	0.915	0.876	0.738	0.717
D-125	1 850	0 438	0.257	0 249	0.246	0.230	0.231
D-127	2 685	1.160	0.831	0.768	0.613	0.589	0.575

3.6. *Bioleaching of pyrolusite in stirred tank reactor*

The performance of stirred tank reactor over a period of 13 days is shown in Fig. 5. During the initial operation of the reactor in batch mode, it was observed that there was a sharp decrease in the pH of the medium from 9.5 to 4.5 in 48 h with a concomitant increase in soluble manganese concentration. After 3 days operation the manganese content peaked to 336 mg/l. At this stage, the cell density of the culture in the reactor was 42×10^8 cfu/ml. The reactor was then switched to continuous mode that resulted in dilution of the reactor manganese content to 301 mg/l. The manganese solubilisation remained at the level of 301 mg/l between 4 and 6 days after which it steadily decreased to 33.6 mg/l on the 13th day of reactor operation. The cell density of the culture was found to stabilise around 11×10^7 cfu/ml during the entire course of the experiment. The cumulative (total) manganese extraction in 13 days was 2256 mg, i.e. 90%. Although the pH of the medium decreased from 9.5 to 4.5, manganese solubilisation by *Citrobacter* sp. continued.

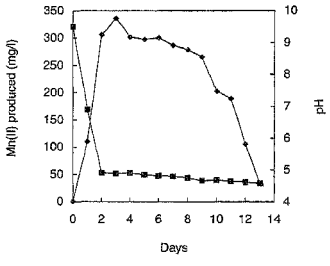

FIG 5. Performance of stirred tank reactor for bioleaching of manganese from pyrolusite ore D-123 at 1%(w/v) pulp density at pH 9.5, with 10^8 cfu/ml using *Citrobacter* sp. The soluble Mn(II) (◆) and decrease in pH (■) were measured up to a period of 13 days

4. Discussion

Manganese-reducing microorganisms have been isolated from diverse habitats such as marine ferromanganese nodules,[17,18] ocean sediments,[2] freshwater sediments,[19-21] and silver-bearing ores.[22] Since strains belonging to subsurface microbial communities exhibit properties potentially useful to industries or in bioremediation or biotechnology.[23,24] It was decided to study the metal-reducing ability of subsurface microflora. The manganese-reducing *Citrobacter* used in the present study is a subsurface strain isolated from a sediment sample obtained from a polluted creek. Since the sediment sample was found to contain metals such as copper, zinc, cobalt, selenium, iron and manganese, isolation of cultures capable of interacting with these metals was expected. The optimum pH for the culture growth was found to be 9.5. This, again was an expected result due to highly alkaline conditions prevalent in the sediments (pH 8.8).

Though there are a few reports on thermophilic manganese-reducing bacteria, [24,25] nearly all of the known manganese-reducing bacteria are mesophilic.[26] The *Citrobacter* used in the present study too is a mesophilic culture with optimum manganese-reducing activity at 35°C. This result is important from the point of process development as the ambient temperatures in India range between 25 and 40°C.

A majority of the previous reports describe manganese reduction under anaerobic conditions.[2,19,27] Manganese reduction by *Citrobacter* sp. in the present study was found to take place only under aerobic conditions. This result supports the findings of Trimble and Ehrlich[6] that oxygen did not interfere with manganese reduction by a marine *Bacillus* species and an unidentified marine coccus. It may however be mentioned that there are no reports on aerobic manganese-reducing *Citrobacter* sp.

In the batch experiments carried out for bioleaching of manganese, it was found that with increasing pulp density (up to 30%), there is no significant increase in lag period (data not shown), possibly due to high metal tolerance of the strain. However, maximum manganese dioxide reduction occurred with 1% (w/v) pulp density as compared to 5, 10, 15, 20, 25 and 30%. This may be due to the availability of a greater surface area for the bacterial action and greater mass transfer of oxygen and nutrients. It is necessary to increase the manganese reduction efficiency at greater pulp densities to make the process economically attractive. One of the ways of achieving this goal could be to use efficient reactor designs that ensure proper mixing of the pulp. It is also essential to check the manganese and iron tolerance of the cultures because normally manganese ores are associated with considerable amounts of iron as well. Moreover, at higher pulp densities the solubilised manganese and the inherent iron concentration might be inhibitory to the culture. The minimum inhibitory concentration of manganese and iron for *Citrobacter* sp. was found to be 3381 and 500 mg/l, respectively (data not shown). From the observation that the minimum inhibitory concentration for manganese was higher (3381 mg/l) than the solubilised manganese in the reactor and a stabilised cell density of 12×10^7 cfu/ml, it could be inferred that the solubilised manganese in the reactor was not inhibitory to the culture.

Citrobacter sp. exhibited reduction and solubilisation of manganese with a significant reduction in the pH (9.50 to 4.50). Ghiorse and Ehrlich[18] observed that intimate contacts between the manganese-reducing isolate *Bacillus* 29 and iron-manganese oxide particles was required.

When *Citrobacter* sp. was inoculated in a flask containing Bromfield medium and a dialysis bag (molecular weight cutoff 12,000) containing manganese dioxide was suspended, manganese was found to be solubilised by the culture (data not shown). These results and the finding that there is drastic decrease in pH of the medium indicate that the *Citrobacter* strain does not require a direct contact between the cells and the manganese ore. The solubilisation of manganese is probably mediated by biogenic low-molecular-weight compounds such as organic acids or hydrogen peroxide. In the case of *Acinetobacter johnsonii* (strain A2), manganese reduction did not require direct contact between cells and manganese oxides and a diffusible substance excreted by the bacteria seems to be involved in the reduction of manganese dioxide.[28] Bacterial metabolites produced during growth such as oxalate or pyruvate could reduce manganese oxides.[8,9]

The reduction rate depends on the mineralogy of manganese oxides.[29] Pseudoamorphous oxides were preferentially reduced by a strain of *Pseudomonas* sp., whereas the oxides included in a crystalline structure were not or only slightly attacked.[29-31] The order of reactivity of the minerals they studied is as follows: Birnessite > δ manganese dioxide >> pyrolusite. In our studies, however, all the ores used were pyrolusite samples, varying in pH, total manganese, phosphate, iron and the content of manganese dioxide. It is interesting that the manganese reduction efficiencies were higher (8.5 and 2.6) for ore samples with alkaline pH (8.21 and 8.02) than ore samples with an acidic pH (below 7). This may be because of the alkaline optimum pH (i.e. 9.5) for the *Citrobacter* sp. used.

Porro *et al.*[32] have used chemoautotrophic bacteria *Thiobacillus thiooxidans* for the recovery of manganese from manganese ore. In these studies, they used suitable reducing substances such as sulphur, ferrous sulphide and ferrous sulphate along with *Thiobacillus thiooxidans* in the culture media. They found that in the presence of reducing substance and *Thiobacillus thiooxidans*, the efficiency of manganese reduction was greater than without reducing agents. These reducing agents can be obtained through the catalytic bacterial oxidations of sulphur or ferrous sulphide to intermediate sulphur compounds. Toro *et al.*[33] used pyrolusite for leaching of manganese in batch and continuous studies. The final release of solubilised manganese was about 7.0 g/l, which accounts for an extraction yield of about 80% (17% manganese content in the pulp). Gupta and Ehrlich[22] obtained a net recovery of 17% solubilised manganese using a *Penicillium* sp. in a stirred reactor within 15 days at 10% (w/v) pulp density. Although most of the workers have used chemoautotrophic organisms for the bioleaching of metals from ores, the use of a heterotrophic metal-reducing bacterium isolated from subsurface environment could be a novel alternative for bioleaching.

Continuous culture studies in stirred tank reactor for manganese bioleaching up to 13 days showed that the culture reduced manganese dioxide with 90% efficiency. Coupling manganese dioxide reduction to consumption of glucose as carbon source is interesting from the point of process development. Although our data refer to the use of glucose, other cheaper carbon sources such as molasses could be valuable alternatives.

The microbial process was found to be highly effective. These results underscore that the bioleaching process developed during the present work is not only highly efficient but also completely eco-friendly. The process reported in the present investigation after further scale-up could have following distinct advantages, viz. (i) no expensive chemical additives required, (ii)

does not produce acid mine waste water and chemical sludge and (iii) would be easy to operate and maintain. The process thus could have the potential of becoming an economical and reliable alternative to the conventional processes employed for the leaching of low-grade pyrolusite ores on a commercial scale.

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