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ORIGINAL PAPER

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Solubilization of cobalt from ocean nodules at neutral pH—a novel bioprocess

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Abstract A marine organism (*Bacillus* M1) isolated from Indian Ocean manganese nodules was characterized. The organism grew well in artificial seawater medium, at near neutral pH, 30°C and 0.25 M NaCl, and showed MnO_2-reducing activity. Growing cultures of *Bacillus* M1 as well as cell-free spent liquor from fully-grown cultures were employed to extract metals from the nodules. The spent liquor of cultures of the organism could dissolve around 45% cobalt (Co) at a pH of 8.2 in 2 h. Co recovery by this treatment was comparable to that in acidic leaching with 2.5 M hydrochloric acid solutions, and was independent of pulp density (w/v ratio). The amount of Co dissolved was beyond the thermodynamic solubility limit in aqueous solution at a pH of 8.2. It is inferred that the metabolites present in the spent liquor played a pivotal role in complexing the Fe (III) phase, solubilizing Co in the process. Partial characterization of spent liquor by spot tests, UV visible spectroscopy and FTIR spectroscopy, showed the presence of siderophore-like phenolic compound(s) with an attached carboxyl group that might form soluble organic complexes with Fe (III).

Introduction

In view of the continuing scarcity of land-based mineral resources, along with increasing domestic consumption of valuable metals across the globe, development of environmentally friendly technologies for exploring alternate sources of metals has become the need of the day. Applying biotechnology to recover strategic metals like Cu, Ni and Co from ocean nodules is one such technology.

Manganese and ferromanganese deposits occur extensively as nodules, crusts and pavements in present-day oceans. Nodules were discovered on the ocean floor by the Challenger Expedition [23]. These ocean nodules are comprised of oxides of manganese and iron together with various valuable metals such as copper (Cu), nickel (Ni) and cobalt (Co). Though the economic potential of recovering valuable metals from nodules has long been recognized, the search is still on for an economically favorable as well as environmentally benign extraction process.

An important step in metal extraction from nodules is solubilization of the desired metals. Since these valuable transition metals are often locked up inside insoluble manganese and/or ferric oxide phases, reductive dissolution of oxides becomes an essential prerequisite for their solubilization. Several researchers have studied the effects of adding reducing agents in mineral acids and ammonia medium on leaching of ocean nodules. The enforced reducing environment results in improved leaching by breaking up the oxide matrices [7, 18, 19, 24, 31, 34]. Such processes have shown varying degrees of success; however, most of them require high temperature pretreatment and/or costly, corrosive reagents to obtain a sizeable amount of metal recovery with favorable kinetics. As the nodules are low-grade ores of Cu, Co and Ni, use of costly chemical reagents as reducing agents may not be economically feasible for large-scale commercialization of the process.

Since the Mn(IV) oxide phase in the nodules harbors Cu, Co and Ni, bacterial reduction of Mn(IV) oxide would result in solubilization of these metals. All the Mn(IV)-reducing bacteria isolated so far from seawater, marine sediments and ferromanganese concretions proved to be heterotrophs. Most of the isolates are aerobes, which can reduce Mn(IV) aerobically or anaerobically [2, 11].

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Ehrlich [8] successfully isolated and characterized a Mn(IV)-reducing organism, *Bacillus* 29, from Atlantic Ocean nodules. Growing cultures of *Bacillus* 29 were able to reduce MnO_2 both aerobically and anaerobically using glucose as an electron donor. However, a component of the electron transport system involved in MnO_2 reduction in this culture was inducible only under aerobic conditions. Both Mn^{2+} and MnO_2 can serve as inducers [9, 32, 33]. MnO_2 reducibility is inducible in all marine cultures tested so far by Ehrlich [10]. Despite differences in the electron pathway from donor to acceptor, the overall reaction involving MnO_2 reduction appears to be similar in all marine organisms tested so far by Ehrlich and his collaborators [10, 12]. As far as the microbial ecology of the Indian Ocean nodules is concerned, there have been no in-depth studies to date; utilizing microorganisms isolated from the nodules themselves to leach out valuable metals from the nodules remains unexplored.

Researchers have started looking into bioprocessing as an alternate route for recovering valuable metals from nodules. Konishi et al. [20] were able to leach valuable metals from nodules using acidiphilic sulfur-oxidizing bacteria, and the thermophilic *Acidianus brierleyi*. Kumari and Natarajan [21] extracted valuable metals from nodules by electro-bioleaching using acid-producing chemolithotrophs like *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. All these processes employ highly acidic environments supplemented with thermal energy or electrical energy for recovery of a sizeable amount of metals, and suffer from the perennial problem of biological extraction processes i.e., slow kinetics.

The major objectives of our present work were to isolate microorganisms from Indian Ocean nodules, characterize and grow the isolates, and ultimately use the growing cells or the growth products for solubilization of Cu, Co and Ni from nodules. The spent liquor of *Bacillus* M1 cultures was partially characterized with the help of UV spectroscopy, FTIR spectroscopy and spot tests. The recovery of metals via the biological route was compared with that of chemical leaching by dilute acids. A probable mechanism of preferential solubilization of Co at near neutral pH is proposed.

Materials and methods

Ocean nodule samples were collected from the bed of the Indian Ocean by the National Institute of Oceanography, Goa, India. These were ground in a mortar and pestle and sieved to obtain appropriately sized fractions for leaching experiments. All leaching experiments were performed with the −50, +75 μm size fraction of nodules, with around 21.8% Mn, 6.4% Fe, 1.1% Cu, 1.1% Ni and 0.2% Co (all values in wt%); 1 g nodules per 100 ml leach pulp (1% pulp density) contained 44.64 mM Mn, 11.42 mM Fe, 1.79 mM Cu, 1.96 mM Ni and 0.3 mM Co.

Isolation of the marine species

The marine bacterium occurred on the nodules as a spore-former. Nodules were first boiled for 30 min in distilled water to get rid of surface contaminants and to germinate the spores [8]. After filtering, the nodule was transferred to an autoclaved porcelain mortar (outer diameter, 150 mm) in an inoculating hood, which had been pre-sterilized for at least 30 min by UV rays, and pulverized using a sterile pestle. About 1 g nodule powder was added aseptically to 20 ml sterilized artificial seawater nutrient broth (ASWNB) containing, per liter distilled water: 28.13 g NaCl; 0.77 g KCl; 1.60 g $CaCl_2{\cdot}6H_2O$; 4.8 g $MgCl_2{\cdot}6H_2O$; 0.11 g $NaHCO_3$; 3.5 g $MgCl_2{\cdot}7H_2O$; 5 g peptone and 3 g beef extract.

ASWNB inoculated with nodule powder was incubated at 30°C for 24 h. From this enrichment medium, a loopful of inoculum was streaked on artificial seawater nutrient agar plates and incubated at 37°C for 24 h. Round, creamy, smooth-surfaced colonies were observed. A colony was picked from the plate and sub-cultured several times on the same medium to obtain a pure strain of the isolate. Since the organism was rod-shaped and was the only one isolated from the marine source we labeled it as *Bacillus* M1. An active culture of the organism is maintained in our laboratory. Periodic streaking was carried out to check the purity of the isolated strain.

Growth of *Bacillus* M1

Bacillus M1 was grown in ASWNB in 250-ml baffled Erlenmeyer flasks at 30°C on a rotary shaker (200 rpm); 10% of an active inoculum (from the late exponential phase) containing at least 10^9 cells/ml was added to sterile ASWNB. Growth was monitored by measuring the cell count using a Petroff-Hauser chamber and by phase-contrast microscopy. The sodium chloride concentration was kept at 0.25 M. Growing cells as well as cell-free spent liquor containing metabolites were used as bioleaching agents.

Leaching experiments

Chemical leaching experiments

Chemical leaching experiments were carried out in 250-ml Erlenmeyer flasks on a rotary shaker (200 rpm) at 30°C to generate baseline data to compare with the bioleaching results; 2.5 M HCl was used as a leaching agent. Glucose was added to the acid medium to introduce a reducing environment in some cases. In all cases, 1 g crushed nodule was used and the nodule:liquid ratio was kept at 1:100 (w/v). The duration of leaching was fixed at 4 h. After leaching, leach liquor was filtered using Whatman 42 filter papers and the collected residue was digested in HCl (1:1) at 60–70°C. Appropriate dilutions of the resulting solution were analyzed for Cu, Co, Ni, Mn and Fe with an inductively coupled plasma (ICP) spectrometer. All chemicals used in these experiments were of reagent grade.

Bioleaching experiments

Leaching with uninoculated medium

Pulverized ocean nodule (1 g) was added to 100 ml ASWNB medium in 250-ml conical flasks. Leaching was carried out on a shaker at 220 rpm at room temperature for 10 h. The leach liquor was analyzed by ICP.

Leaching with growing culture

Pre-sterilized and pulverized ocean nodule (1 g) was added to 90 ml sterilized ASWNB medium in 250-ml conical flasks. Actively growing culture (10% v/v), with 10^9 cells/ml, was added as inoculum. Leaching was carried out on a shaker at 220 rpm at room temperature for 10 h. Growth flasks were taken at appropriate time intervals for analysis of leached metal content in the solution.

Leaching with cell-free spent liquor To obtain cell-free spent liquor, a fully-grown batch culture (after 10 h growth) was centrifuged at 17,675 *g* for 15 min at 10°C followed by pressure filtration through a sterile 0.2 μm Millipore filter. The absence of any cells in the resultant spent liquor was confirmed by phase contrast microscopy.

Pulverized ocean nodule (1 g) was added to 100 ml spent liquor to keep the solid:liquid ratio at 1:100 (w/v; i.e. 1% pulp density) and leaching was continued for 10 h. Recovery of metals in the leach liquor was monitored as a function of time. The effect of solid:liquid ratio on leaching using spent liquor was studied by varying the pulp density from 1% to 10%.

In all the above cases, leach liquor was filtered using Whatman no. 42 filter papers and the residue was digested 1:1 in HCl at 60–70°C. Appropriate dilutions of the resulting solution was analyzed by ICP for Cu, Co, Ni, Mn and Fe.

Characterization of the spent liquor of *Bacillus* M1 cultures

Infra-red and ultra-violet spectroscopy

Spent liquor (10 ml) was heated to dryness and 2–3 ml chloroform was mixed with the dry residue. The insoluble part was dried and then mixed with 2–3 ml methanol, forming a yellow-orange colored solution that was evaporated to dryness and used for infra-red (IR) spectroscopy.

The cell-free spent liquors were collected at 1 h intervals during growth of *Bacillus* M1 and differential ultra-violet (UV) spectra were recorded to observe the changes in spent liquor with increase in period of growth. In all cases, ASWNB was used as a reference.

Results and discussion

Characterization of *Bacillus* M1

Preliminary morphological and physiological examination of *Bacillus* M1 revealed the strain to be Gram-positive, oxidase- and catalase-positive, aerobic, motile and able to reduce Mn(IV).

Growth characteristics of *Bacillus* M1 with respect to pH and E_h of the medium

A typical growth curve, and the variation of redox potential (E_h) measured against a saturated calomel electrode (SCE) of the growing *Bacillus* M1 culture with time, are shown in Fig. 1. Cells started growing immediately in the logarithmic phase, which lasted for about 7 h beyond which growth ceased. Growth was accompanied by a slight increase in pH of the medium from 6.8 to 7.1. The E_h of the medium decreased during growth, indicating the possible presence of some reducing compounds. The growth rate of the cells was calculated to be 5.4 h^{-1}, with a mean doubling time of 0.13 h, indicating the bacterium to be fast growing.

Leaching experiments

Chemical leaching experiments with HCl

Chemical leaching experiments were carried out with 2.5 M HCl to generate baseline data (Fig. 2).

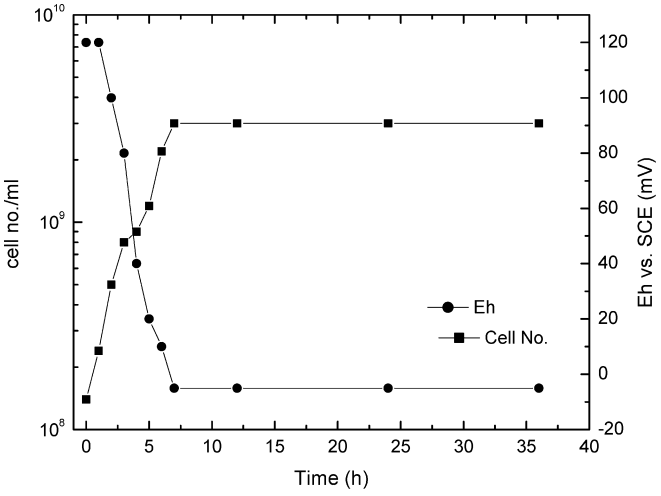

Fig. 1 Growth characteristics of *Bacillus* M1 in artificial seawater nutrient broth (ASWNB)

Although 70–80% Cu and Ni could be recovered from the nodules in about 6 h, only 35% Mn and 45% Co came into solution during this process.

A fraction of transition metals like Co, Cu and Ni may remain entrapped in the Mn(IV) phase of the nodules [4, 6, 16]. When Mn(IV) is dissolved by reducing agents in acidic medium, such associated metal ions are solubilized; addition of 20% glucose to 2.5 M HCl drastically changed the leaching scenario (Fig. 3). Mn and Co dissolution increased to 75–80% from 30–40% while Cu and Ni recoveries were also increased by 5–10%. MnO_2 reduction by glucose [34] in an acidic medium, as shown in the following reaction, facilitated dissolution of the other valuable metals.

$$C_6H_{12}O_6 + 12\ MnO_2 + 24\ H^+ \rightarrow 6\ CO_2 + 12\ Mn^{2+} + 18\ H_2O \quad (1)$$

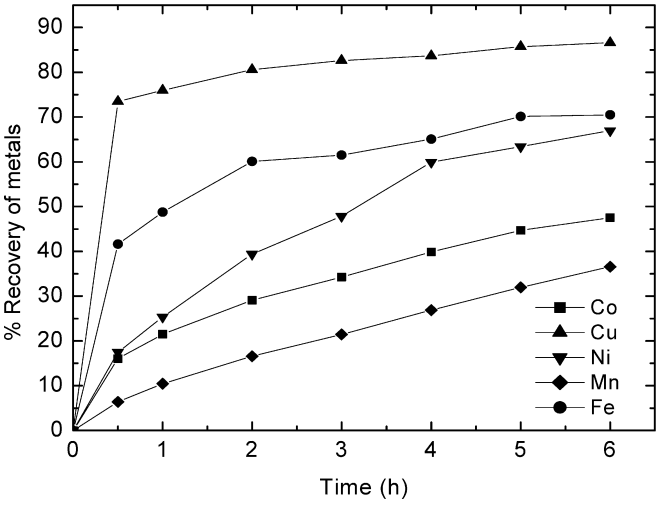

Fig. 2 Recovery of metals during leaching by 2.5 M HCl

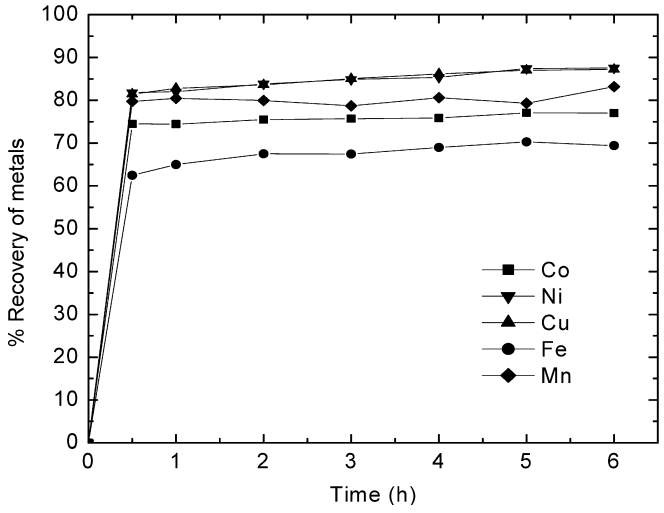

Fig. 3 Recovery of metals during leaching by 2.5 M HCl in the presence of glucose

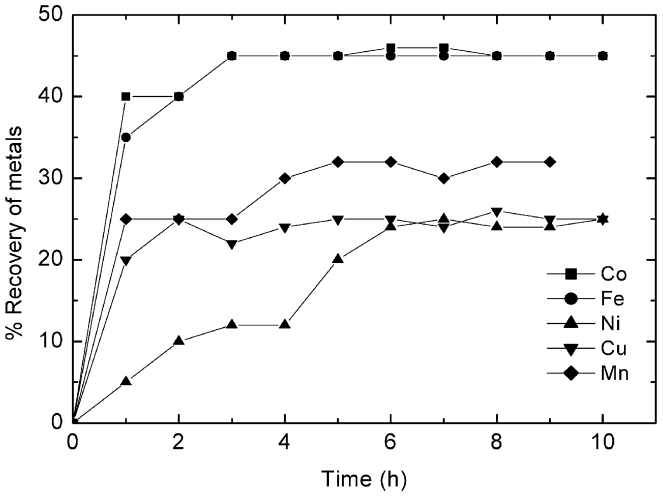

Fig. 5 Recovery of metals during leaching by the cell-free spent liquor of a culture of *Bacillus* M1

Bioleaching experiments

Bioleaching experiments with the nodules were carried out using a growing culture of *Bacillus* M1 as well as the spent liquor obtained after centrifuging out the cells from a fully grown culture. Control experiments were performed with uninoculated medium. All tests were carried out at 1% pulp density signifying 1 g nodules/100 ml leach pulp. Leaching with uninoculated medium showed 10% Cu recovery, while recoveries of Co, Ni, Mn and Fe were negligible. Cu recovery can be related to the complexing effects of peptone in ASWNB and the easy leachablity of the metal [13]. Leaching by the growing culture showed that 35% Co and Fe recovery was achieved after 10 h, while Mn, Cu and Ni recoveries were 25%, 16% and 12%, respectively (Fig. 4). Leaching by the spent liquor is shown in Fig. 5. In this case an increase of almost 10% in recoveries of Fe, Cu, Ni and Co was noted in comparison to recoveries in a growing culture. Assuming metabolites to be primarily responsible for leaching, this enhancement can be related to the greater concentration of metabolites in spent liquor collected after 10 h of growth than is present in a growing culture. With both growing culture and spent liquor, Co and Fe recoveries were greater than those of Cu, Ni and Mn. This can be attributed to the positive correlation of Co with Fe in nodules [4, 6, 16].

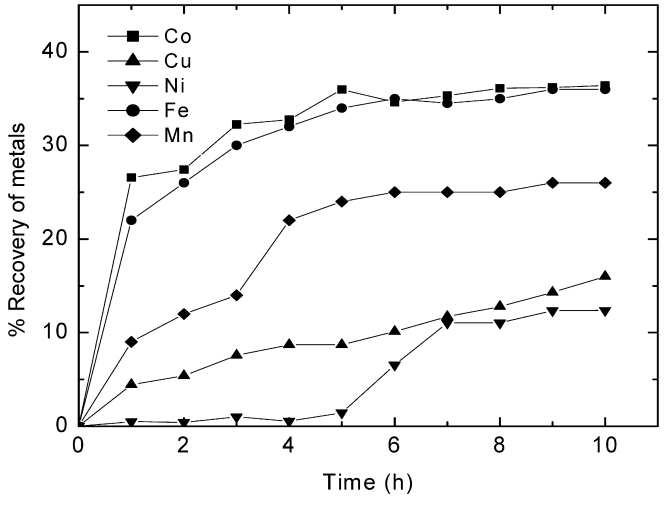

Fig. 4 Recovery of metals during leaching by growing cultures of *Bacillus* M1

Co-dissolution in spent liquor of *Bacillus* M1 at pH values 8.1–8.2 (Fig. 5) was comparable with that in 2.5 M HCl (Fig. 2). At present, acidic dissolution under highly corrosive conditions offers the only route for extraction of metals from nodules. Thus, the bioprocess developed here has enormous potential as an environmentally safe alternative. When pulp density was increased from 1% to 10% (w/v) there was almost a 10-fold enhancement in dissolution of Fe and Co. This advantage can be utilized in scaling up the process. Further work to enhance Co recovery using reducing agents in the system is ongoing.

Mn and Fe oxides and oxyhydroxides form two major phases in ocean nodules [4]. Co dissolution in biological leaching may be tied up with solubilization of the Fe phase, suggesting mineralogical association of some of the Co with Fe oxyhydroxides in nodules. The pH of the leaching medium was 8.1–8.5. The theoretical solubility of all the metals concerned is negligible in that pH range. The solubility of transition metals in the complexed state can be markedly different from that in an un-coordinated state [30]. Instances of complexing ligands secreted by bacteria that help in acquisition of transition metals, especially Fe (III), are known. Sequestration of iron is often achieved by siderophores, a class of microbially produced organic ligands having conditional stability constants in the order of 10^{19}–10^{22} M^{-1}. Most siderophores are of two types, catecholate and hydroxamate [3, 6, 14, 15, 17, 22, 25, 26, 27, 29].

Characterization of the spent liquor of *Bacillus* M1

Confirmation tests for phenolic group

Compounds containing phenolic OH groups yield colored compounds when heated with sodium nitrite. Catechol gives a yellow color with nitrous acid, which changes to an intense orange-red in the presence of excess sodium hydroxide [1]. The test solution was made neutral, 1 ml 10% sodium molybdate solution was then added, followed (in order) by 0.5 ml HCl (0.5 *N*) and 1.0 ml sodium nitrite solution (0.5%). A yellow color immediately developed indicating that catecholate substances were present. Upon addition of 1.0 ml NaOH (0.5 *N*), the yellow color became cherry red.

Monohydroxy derivatives of benzene, e.g., phenol, *p*-cresol, *p*-hydroxybenzoic acid and tyrosine, did not give any insoluble lead compound with lead acetate in neutral solution. Dihydroxy derivatives e.g., catechol, homocatechol, in neutral solution with neutral lead acetate generally give an insoluble lead compound immediately.

UV spectroscopy

Table 1 shows the gradual transition of UV bands over the growth span of *Bacillus* M1. The total spectral range has been divided into three distinct regions: 220–250 nm, 250–275 nm and 300–315 nm. With increasing growth period a band near 308–309 nm and one near 220 nm gradually increased in intensity. Therefore the concentration of species corresponding to these UV bands went up in the spent liquor as the growth of the organism progressed. In first few hours of growth there was a band near 270 nm, which gradually decreased after 6 h. The absorption peaks near 220 and 308 nm strongly suggest a dihydroxy benzoate (DHBA) structure. Chakraborty et al. isolated and partially characterized a catechol-type siderophore produced by *Pseudomonas stutzeri* grown under iron-depleted conditions [5]. The purified siderophore had a UV spectrum (230 nm and 330 nm bands) identical with that of 2,3 DHBA. O'Brien and Gibson [25] isolated five compounds from culture media of *Escherichia coli* and *Aerobacter aerogenes* under conditions of iron deficiency. The compounds, both named 2,3-dihydroxy-*N*-benzoylseine, gave two distinct UV peaks in water, one near 315 nm and the other near 310 nm.

FTIR studies

The solution IR spectra of the spent liquor (Fig. 6) showed the following bands, in decreasing order: 3,099 cm^{-1}, 2,923 cm^{-1}, 2,853.17 cm^{-1}, 1,639.2 cm^{-1}, 1,456.96 cm^{-1}, 1,376.93 cm^{-1}, 1,336.43 cm^{-1} and 1,108.87 cm^{-1}. Peaks at 2,923.9 cm^{-1}, 1,456.96 cm^{-1} and 1,376.93 cm^{-1} are apparently due to the Nujol used in preparing the sample [28] and can thus be ignored. The 3,999 cm^{-1} band was due to an intermolecular hydrogen bonded O–H stretch [28], which is a common feature in phenol compounds; the 1,639.2 cm^{-1} band might be due to C=O stretching. The 1,336.43 cm^{-1} band was an indication of O–H bending while that at 1,108.87 cm^{-1} appeared because of C–H out-of-plane bending. All these bands were indicative of a phenolic compound with an attached carboxyl group. IR spectra of ASWNB (control) revealed none of these specific bands.

All the above tests point to the presence of a phenolic compound with an attached carboxyl group in the spent liquor. The, as yet unidentified, compound resembles the catecholate group of siderophores. We propose a mechanism of Co dissolution by spent liquor of cultures of the *Bacillus* M1:

1 In Indian Ocean nodules a part of the Co may be associated with Fe (III) phases.
2 The spent liquor of the cultures contains siderophore-like phenolic compounds, which are capable of forming soluble Fe (III) complexes near neutral pH. Co associated with the Fe phase comes out into solution in the process.

Conclusions

The following major conclusions can be drawn:

Table 1 UV visible spectra of the spent liquor of *Bacillus* M1 as a function of time

Time period of growth	Peaks recorded (nm), (absorbance)					
(h)	220–230 range		250–275 range		300–315 range	
1	–		250.8	(0.095)	313.2	(0.182)
2	227.4	(0.088)	275.8	(0.105)	310	(0.193)
3	221.6	(0.126)	268.4	(0.082)	312.2	(0.218)
4	–		262.6	(0.083)	308.8	(0.242)
5	–		269.0	(0.069)	313.4	(0.227)
6	229.4	(0.125)	272.6	(−0.130)	309.8	(0.298)
7	233.8	(0.097)	273.6	(−0.154)	309.4	(0.286)
8	233.8	(0.098)	272.2	(−0.126)	309.8	(0.322)
9	233.8	(0.083)	274.2	(−0.240)	308.2	(0.323)

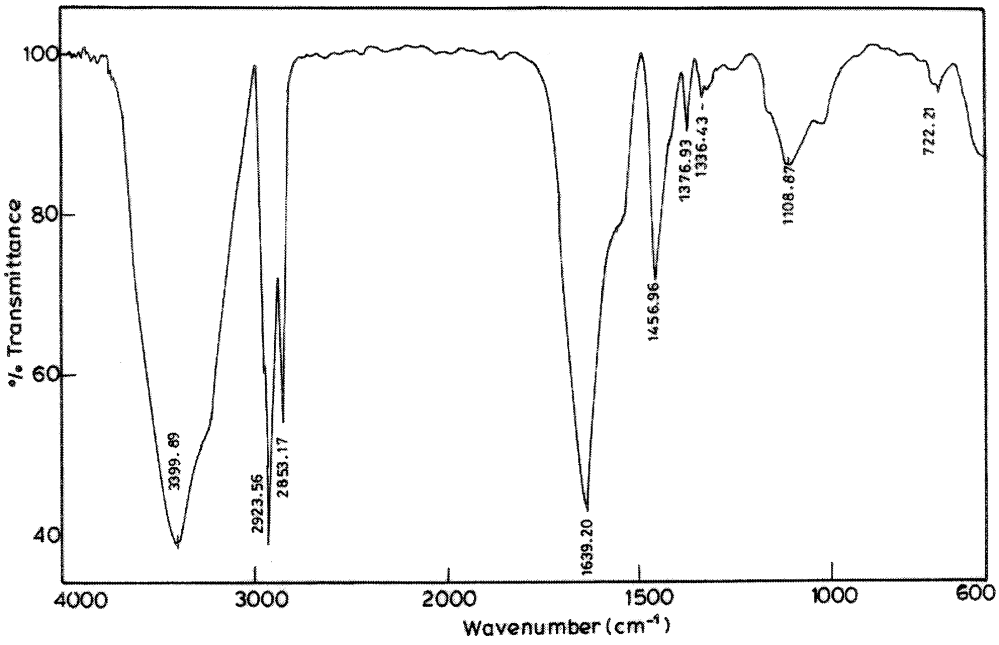

Fig. 6 FTIR spectra of spent liquor of a culture of *Bacillus* M1

1 *Bacillus* M1, a marine organism isolated from Indian Ocean nodules, grew well in artificial sea-water and near neutral pH. The organism is aerobic, Gram-positive and reduces Mn(IV) to Mn(II).
2 About 45% dissolution of Co, comparable to chemical leaching by 2.5 M HCl, was achieved by leaching with the spent liquor of *Bacillus* M1 at near-neutral pH. Around 25% Cu and Ni were also dissolved in the process.
3 When pulp density was increased from 1% to 10%, an enhancement in dissolution of Fe and Co of almost 10-fold was observed.
4 Siderophore-like biomolecules present in spent liquor, having a phenolic structure with an attached carboxyl group, may be able to solubilize the Fe (III) at neutral pH by complexation. Co associated with the Fe phase is dissolved in the process.

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