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Recovery of nickel from chromite overburden, Sukinda using *Aspergillus niger* supplemented with manganese

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Abstract−Oxalic acid is a prominent metabolite secreted by several fungi under specific conditions, which acts as a metal chelating agent. Amongst different fungal species, *Aspergillus niger* is favored as the best option for microbial production of oxalic acid. The present study deals with the oxalic acid over production by *A. niger* in response to manganese supplement to its growth medium, which in turn improves the recovery of nickel from pre-treated chromite overburden(COB) during fungal bioleaching. The metabolic pathway in oxalate bio-synthesis by *A. niger* involves one prominent cytoplasmic enzyme oxaloacetate acetylhydrolase (OAH), which catalyzes the breakdown of oxaloacetate metabolic intermediate to oxalate and acetate. Oxalic acid production was increased due to supplement of manganese to the culture medium of the *A. niger*. Manganese acts as cofactor for OAH enzyme; further, it enhances the catalytic activity of OAH to produce more oxalate. With oxalic acid production by *A. niger,* nickel recovery from pre-treated COB was improved. During the study, a maximum of nickel recovery was achieved up to 38.6% from pre-treated COB by adding 80 ppm of manganese to the culture media, whereas 24.0% of nickel was recovered without supplement of manganese (experiments were performed at 30 °C and the COB pulp density 2% w/v).

Key words: *Aspergillus niger*, Chromite Overburden, Manganese, Nickel, Oxalic Acid

INTRODUCTION

Nickel extractions from lateritic mineral have been extensively studied through minerals-microbes interactions [1-3]. Unlike other microorganisms, fungus solubilizes metals from ores by formation of organic acids: citric acid, oxalic acid, gluconic acid etc. [4]. Filamentous fungi, such as *Aspergillus niger*, *Penicillium*, numerous brown-rot and white-rot basidiomycetes, are able to efficiently produce and secrete large quantities of oxalate [5]. As an alternative to classical methods, laterites were leached by organic acids. Organic acids exert the leaching process by acidolysis, complexation and chelate formation [6-8]. Therefore, selection and development of specific fungal strains involved in metal recovery processes is the prime concern for bio-hydrometallurgical operations.

Among different organic acids secreted by microbes, oxalic acid is a potential leaching agent for laterites [2]. Oxalic acid can be synthesized either by chemical or biotechnological route. The most usual chemical methods followed for production of oxalic acid are from formic acid salts (heating sodium formate and treating the resulting oxides with sulfuric acid) and from oxidation of carbohydrates by nitric acid [9]. In spite of this, the most convenient way is metabolites secreted by several fungi under specific conditions. From the different fungal species of *Aspergillus*, *Penicillium*, *Sclerotium* and *Gleophyllum*, *A. niger* is the best option for oxalic acid production.

A. niger produces different organic acids (oxalic and citric acids etc.) according to the operating conditions [10,11]. Out of several proposed pathways, Kubicek suggested that pyruvate generated during the process of glycolysis is transformed to oxaloacetate, which later on hydrolyzes to oxalate. This process is enhanced by supplying relevant quantities of phosphorous and nitrogen sources to the growth medium of *A. niger* operating at pH close to 7.0 [10]. Application of ultrasonic waves for the pre-treatment of *A. niger* for bioleaching of lateritic nickel has resulted in improved nickel extraction [12]. Similarly, the use of ultrasonic waves has improved biodissolution of metals such as copper, zinc, cobalt, nickel, iron and aluminium from black shale by *A. niger*, because of improved organic acid production by the fungi under controlled conditions [13]. Organic acids secreted by *A. niger* have been proved to be useful in nickel bioleaching [12,14,15].

Oxalate can be formed from oxaloacetate in a C-C bond lysis reaction catalyzed by oxaloacetate hydrolase (oxaloacetate acetylhydrolase, OAH, EC 3.7.1.1) and from the oxidation of glyoxylate and glycolaldehyde [10,16,17]. The widely proposed cellular pathway for oxalate production is through the breakdown of acetoacetate by OAH enzyme [18-20]. The key enzyme oxaloacetate acetylhydrolase (OAH), which is located in the cytoplasm of *A. niger*, catalyzes the conversion of oxaloacetate to oxalate and acetate [10]. The OAH is a member of phosphoenolpyruvate mutase [PEPM/isocitrate lyase (ICL)] family. The members of this super family act upon α-oxycarboxylate substrate to cleave C-C or P-C bonds. All the PEPM/ICL super-family members require divalent metal (manganese or magnesium) cofactors for their catalytic activities, where these cofactors play the role of mediators in the interaction between the enzyme and substrate [21].

It has been estimated that the huge Sukinda COB contains nickel

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(0.8-1.0%) [22], which is a strategically important metal that needs to be exploited. The recovery of nickel present in goethite matrix of the COB needs to be processed through a minimal energy utility and eco friendly mineral processing methodology. However, hydrometallurgical processes are antagonistic to bio-hydrometallurgy in terms of both in energy utilisation and environmental concerns; hence, the microbe assisted bioleaching process has emerged as an alternative [23]. Furthermore, the fungal mediated bioleaching has been investigated to address the challenges in mineral processing of nickel laterites. With a view to extract substantial amount of metal values from COB, thermal pre-treatment of COB was carried out prior to leaching. Thermal pre-treatment changes the mineral structure and brings the mineral phase transformation by dehydroxylation of the goethite matrix in raw chromite overburden [24]. Pre-treatment COB has significant influence on nickel recovery by bioleaching.

The present study aimed to improve the nickel recovery from pre-treated COB through application of manganese cofactor to bioleaching culture medium of *A. niger*. During this study manganese supplemented to the culture medium of the *A. niger* increased the production of oxalic acid through enhanced catalytic activity of OAH. As a consequence of overproduction of oxalic acid, nickel extraction from pre-treated COB was improved by bioleaching.

EXPERIMENTAL

1. Microorganism and Growth Conditions

The fungal strain *Aspergillus niger*, a laboratory stock culture at IMMT, Bhubaneswar, Broomfield medium containing (g/L): $MgSO_4$ $7H_2O$ - 0.75, KH_2PO_4 - 0.25, $(NH_4)_2SO_4$ - 0.25, yeast extract-1.0 and sucrose-20.0, pH 6.8 at 30 °C. The fungal strain was identified by 18s rRNA gene sequencing. For identification, the fungal genomic DNA was isolated by using Fungal Genomic DNA isolation kit (KT125, Genei, India). Using consensus primers, the ~900 bp, ITS1-5.8S-ITS2 region of 18s rRNA gene fragment was amplified using PCR (BioRad). The PCR product was cloned and sequenced using the forward and reverse primers. Sequence data was submitted to GenBank (NCBI) database, aligned and analyzed for finding the closest homology for the fungi.

2. Chromite Overburdens (COB)

The COB samples were collected from Sukinda Mines, Odisha, India. The samples were crushed and sieved to obtain a particle size of −75 μm are taken throughout the experiment. The COB samples were pre-treated at 600 °C for 5 hours in a muffle furnace prior to leaching experiments.

3. Mineralogy Study

For the analysis of metal concentrations in the COB samples, COB samples were air dried and acid digested. The digested COB samples after proper dilutions were subjected to analysis for different metals by atomic absorption spectrophotometer (AAS) (*Perkin Elmer - AA200*). Mineralogical analysis of the COB samples was done by means of X-ray diffraction study [Phillips Diffractometer (PW3710)] with a radiation operating at 40 kV and 30 mA to identify major and minor minerals.

Further, ore morphology and mineralogy of COB was studied by scanning electron microscopy (SEM) and transmission electron microscope (TEM). SEM study was carried out on raw and pre-treated COB samples with a size range of 5×5×5 mm with ultra thin film of gold coating using an ion sputter JFC-1100 and studied under a Japanese-made electron microscope (JEOL-JXA-8100). The working height was maintained at 15 mm with a voltage ranging from 10 kV to 25 kV. Samples for the TEM investigations were imaged in (FEI, TECNAI G^2) 20 (Netherlands), equipped with energy dispersive X-ray spectroscopy (EDX) operating at 100 kV. Samples were dispersed in ethanol and subjected to ultrasonication for 5 minutes. 30 μl of the samples were coated to the carbon-coated gold grid and dried before being inserted into the column of the TEM chamber.

4. Bioleaching Study

Bioleaching experiments were carried out in triplicates with 250 ml Erlenmeyer flasks containing 90 ml of Broomfield medium supplemented with manganese concentrations ranging from 0 to 80 parts per million (ppm). Each flask was inoculated with 10 ml (10^6 spores/ml) of spore suspension with 2% (w/v) pre-treated COB and incubated for 24 days at 30 °C on a rotary shaker at 150 rpm. Initial pH of the medium was 6.8. Samples were drawn at regular intervals and were analyzed by AAS to determine the percentage of nickel leached. Control experiments were also performed without addition of fungal suspension and manganese. Aseptic conditions were maintained throughout the experiment.

5. Leaching by Fungal Culture Filtrate

Sets of leaching experiments were conducted with fungal culture filtrates. The fungal culture filtrates were prepared by culturing fungus *A. niger* in Broomfield medium supplemented with different concentration of manganese (0-80 ppm). The carbon source, sucrose present in the culture medium, is quantitatively converted to organic acids through cellular metabolism. The fungal biomass filtered out from the fungal culture and the remaining culture filtrates were sterilized. Furthermore, the nickel leaching experiments were conducted with these culture filtrates of *A. niger*.

6. Chemical Leaching Study

Oxalic acid (0.1 M) and citric acid (0.1 M) solutions were used to evaluate the chemical leaching of COB. The analytical grade reagents were taken for the preparation of leaching solutions. Further experiments of chemical leaching were performed using a mixture of oxalic acid and citric acid in different proportions (Oxalic acid : Citric acid in the range of 25% : 75%, 50% : 50% & 75% : 25% etc.) to evaluate the cumulative effect of organic acids in leaching of nickel from COB. The leaching experiments were performed for 10 days (150 rpm, 30 °C) with COB at 2% (w/v) pulp density.

7. Bio-analytical Study

High-performance liquid chromatography (HPLC) was used for the quantification of the organic acids produced during bioleaching process [25]. HPLC model Agilent-1100, (Agilent technologies, Waldbronn Analytical Division, Germany) containing Zorbax Eclipse XDB-C18 column (150×4.6 mm, i.d) was used for the organic acid analysis. Two mobile phases (A & B) were involved in the HPLC analysis. Solvent-A was methanol (HPLC grade) and solvent-B was Millipore water containing 0.01 mol/*l* of KH_2PO_4 solution; pH was adjusted to 2.32 using phosphoric acid. The mobile phase used was methanol and water (30 : 70 v/v) with a flow rate of 1.2 ml/min with a quaternary pump (Model No-G1311A) operated at 60 °C with thermostat (Model No-G1316A). HPLC grade standards of oxalic acid and citric acid were used. 20 μl of samples were injected manually in the injection port along with the standard solutions. The organic

acids were quantified by UV detection at 210 nm with diode array detector (DAD, Model-G 1315A).

8. OAH Enzyme Assay

Assay for OAH activity was performed as reported by Lenz et al. [26]. The reaction solution (2 ml) was contained 0.125-1.0 mM oxaloacetate, 0.5 mM $MnCl_2$, crude cytoplasmic extract of *A. niger* containing OAH, 0.1 M imidazole (pH 7.6 and temp 25 °C). The reaction was monitored at 255 nm for the disappearance of enol tautomer of oxaloacetate. Prior to the estimation of reaction rate catalyzed by the enzyme in cell extract of *A. niger*, the rate of oxaloacetate consumption *via* spontaneous decarboxylation was measured to determine the background rate. The actual reaction rate was measured by subtracting the background rate from the reaction rate measured in presence of enzyme. The pH of the leach liquor was measured by a pH meter (Systronics, India). The sucrose concentration in culture medium of fungus during bioleaching was estimated by phenol-sulfuric acid method [27].

RESULTS AND DISCUSSION

1. Characterization of fungi

The nucleotide sequence of ITS1-5.8S-ITS2 region of 18s rRNA gene (Fig. 1) of the fungal strain submitted to the NCBI data base. The fungal strain was identified at species level, which belongs to *Aspergillus niger*. The identified strain was closely related to *A. niger* species documented at NCBI with accession no AM270052.1 and AM270051.1.

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CAGGGAGCAGACCACCGGGATTGCCTCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAATT
TGAAAGCTGGCTCCTTCGGAGTCCGCATTGTAATTTGCAGAGGATGATTTGGGTGCGGCCCC
CGTCTAAGTGCCCTGGAACGGGCCGTCAGAGAGGGTGAGAATCCCGTCTTGGGCGGGGTGT
CCGTGCCCGTGTAAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTG
GTAAATTTCATCTAAAGCTAAATACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGA
AAGATGAAAAGCACTTTGAAAAGAGTTAAACAGCACGTGTTTTTGTTGAAAGGGAAGCGCTT
GCGACCAGACTCGCCCGCGGGGTTCAGCCGGCATTCGTGCCGGTGTACTTCCCCGTGGGCG
GGCCAGCGTCGGTTTGGGCGGCCGGTCAAAGGCCCCTGGAATGTAGTGCCCTCCGGGGCAC
CTTATAGCCAGGGGTGCAATGCGGCCAGCCTGGACCGAGGAACGCGCTTCGGCACGGACGC
TGGCATAATGGTCGTAAACGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATATACGCG
AGTGTTCGGGTGTCAAACCCGTGCGCGCAGTGAAAGCGAACGGAGGTGGGAGCCCCCTTGC
GGGGCGCACCATCGACCGATCCTGATGTCTTCGGATGGATTTGAGTAAGAGCGTAGCTGTG
GGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGCGAAGCCAGAGGAAACTCTGGTGGA
GGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCAAATTTGGGTATAGGGGCGAAAGACTA
ATCGAACCATCTAGTAGCTGGTCCTGCCGAGTTC
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Fig. 1. Amplified nucleotide sequence of ITS1-5.8S-ITS2 region of 18s rRNA gene of *A. niger* strain.

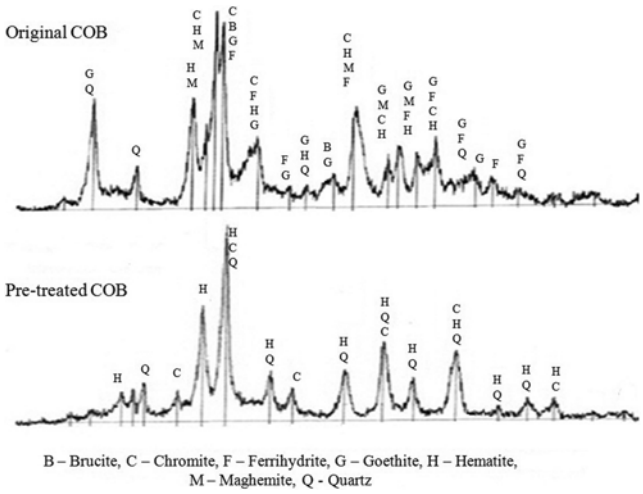
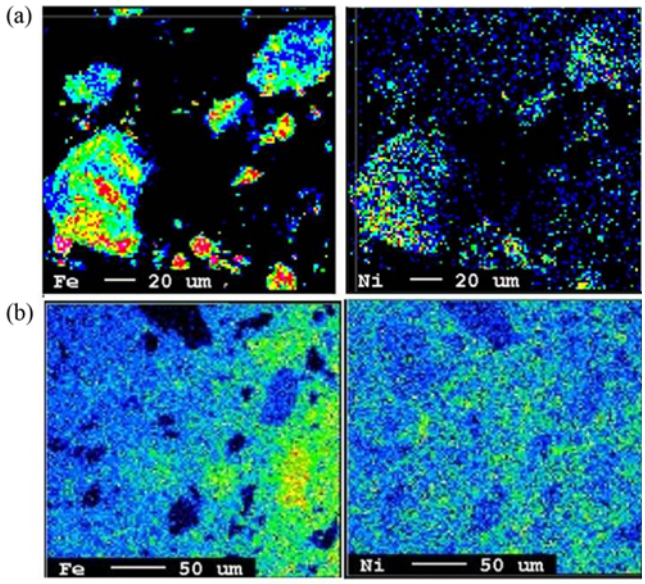


Fig. 2. X-ray diffraction pattern of raw and pre-treated COB.

2. Characterization of COB

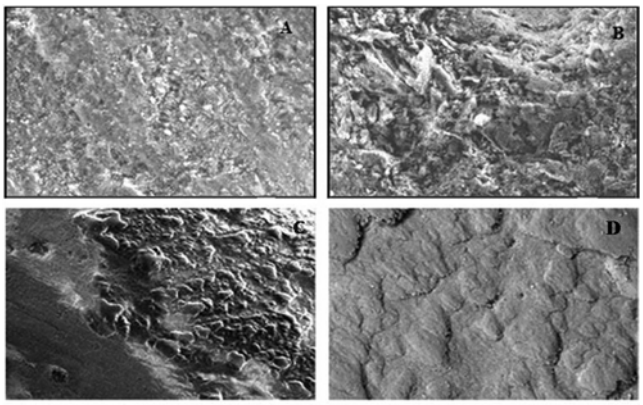
Chemical analysis of COB revealed that the raw COB and pre-treated COB contain 0.99% nickel, 0.03% cobalt, 48.88% iron, 2.59% chromium and 0.21% manganese, and 1.02% nickel, 0.04% cobalt, 50.85% iron, 3.65% chromium and 0.35% manganese, respectively. The X-ray diffraction spectrum (Fig. 2) of COB revealed that, due to thermal pre-treatment, the peaks associated with goethite which was originally present in the raw COB had almost disappeared and there was also a marked increase in intensity of peak bands of hematite in pre-treated COB.

The EPMA study revealed that nickel in raw COB was absorbed within the iron (goethite) matrix (Fig. 3(a)). On pre-treatment of COB nickel was expelled from goethite matrix and homogeneously distributed throughout the COB matrix (Fig. 3(b)). Subsequent study under SEM (Fig. 4) revealed that micro pores and cracks developed on the COB particles due to pre-treatment, which leads to better percolation of lixiviates into the mineral matrix. Similarly, the TEM study (Fig. 5) of COB samples confirmed that pre-treatment of COB



EPMA study of COB (a) Raw COB, (b) Pre-treated COB

Fig. 3. EPMA analysis of raw and pre-treated COB.



SEM study of COB (A & B – Raw COB, C & D – Pre-treated COB)

Fig. 4. SEM study of raw and pre-treated COB.

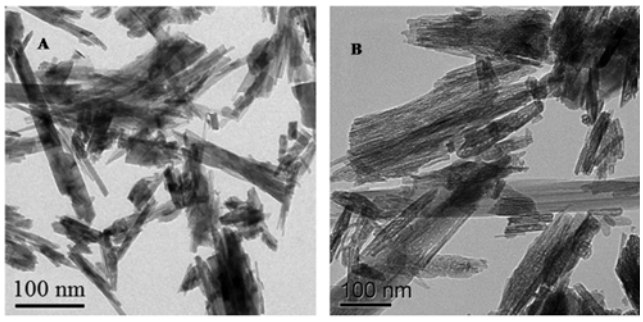


Fig. 5. TEM analysis of raw and pre-treated COB.

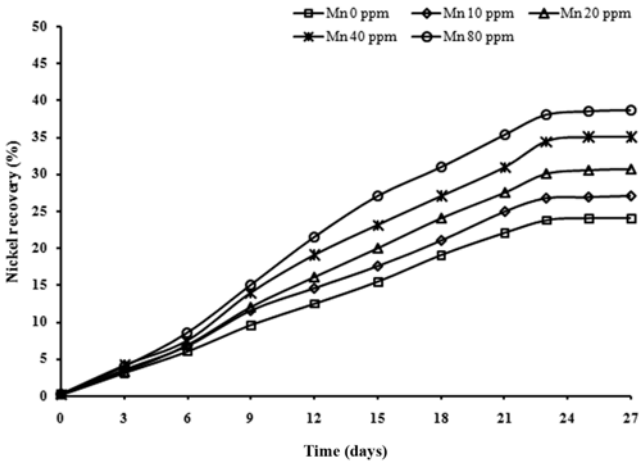


Fig. 6. Nickel recovery from pre-treated COB by *A. niger* at different concentrations (ppm) of manganese supplemented to culture medium (ppm: parts per million).

developed highly porous mineral matrix with discrete rod shaped crystalline structure. Furthermore, the surface area of the ore particles was increased due to pretreatment of COB, as the initial surface area was 23.2 m^2/g, and after pretreatment the surface area of COB particle changed to 45.1 m^2/g.

3. Leaching Study

Fig. 6 is the plot for nickel recovered (%) from pretreated COB with different concentration of manganese as a function of time during *A. niger* mediated bioleaching. All the experiments were conducted at 2% (w/v) pulp density supplemented with 20 g/liter of sucrose. A maximum of 38.6% nickel was recovered when 80 ppm of manganese supplemented the culture medium, whereas only 24% of nickel was recovered without addition of manganese. The addition of manganese to culture medium beyond 80 ppm did not affect significantly the nickel recovery. Previously, it was reported that about 34% of nickel was recovered from thermally pre-treated COB by *A. niger* bioleaching conducted at sucrose concentration of 100 g/liter in culture medium [28]. Our findings reveal that recovery of 38.6% nickel was possible with only 20 g/liter of sucrose in the culture medium incorporating with manganese (80 ppm). As it reduces the requirement of sucrose, i.e., the carbohydrate source for leaching purpose, hence efficiency of the fungal leaching process was improved. The result shows that the nickel recovery gradually increases with rise in manganese concentrations up to 80 ppm by keeping other parameters constant. During the study up to 5th day of leaching the rate of nickel recovery followed a similar trend in all the cases, but beyond that the rate of nickel recovery deviated more and more with increase in concentration of manganese in the medium. It could be anticipated that it (5th day) is the timeline when the organic acids generated by the fungus were affected by supplement of manganese to the medium.

Table 1. Production of Oxalic acid by *A. niger* in response to different concentration of manganese in culture medium

Manganese concentration (ppm)	Oxalic acid production (mg/liter)	Nickel recovery (%)
0	291	24.0
10	298	27.0
20	410	30.6
40	760	35.0
80	986	38.6

Cellular metabolism of *A. niger* converted the sucrose in the medium into a variety of products including organic acids, which leads to lowering the pH of medium. Oxalic and citric acids were detected by HPLC in the leaching medium. These metabolic by-products produced are involved in the dissolution of metals through formation of salts and chelates [3]. In the initial stage of leaching experiments (up to 5th day) both citric and oxalic acids were detected in the culture, but 5th day onwards the presence of oxalic acid was more pronounced. Table 1 represents the HPLC analysis for organic acids produced by *A. niger* in the medium supplemented with different concentrations of manganese. The oxalic acid concentration was significantly increased with the rise of manganese concentrations in the medium up to 80 ppm. Fig. 7 represents the HPLC chromatograms for the organic acid profile of *A. niger* during the bioleaching process. About 291 mg/liter oxalic acid was detected in absence of manganese, whereas 986 mg/liter oxalic acid was detected at manganese concentration of 80 ppm. The oxalic acid concentrations were 291, 298, 410, 760 and 986 mg/liter in 0, 10, 20, 40 and 80 ppm concentration of manganese, respectively. The oxalic acid profile was significantly affected by supplement of manganese to the media. In this context, more elaborate studies were carried out for the production of oxalic acid by *A. niger* in the presence of manganese, since oxalic acid has been reported to be a more potential organic acid for nickel leaching from lateritic COB of Sukinda [1].

On monitoring the growth of *A. niger* during the bioleaching study, it was found that pH level of leaching solution reached to 2.2 when 80 ppm manganese was supplemented, whereas the pH gradient reached up to 2.9 in absence of manganese in medium (Fig. 8(a)). The least extreme of pH was decreased with increase in manganese concentration, i.e., lowest drop in pH during bioleaching was reached in the range of 2.9 to 2.2 with respect to manganese concentration 0 to 80 ppm. This indicates that the total acidity generated was enhanced with increase in manganese concentrations in culture medium of the *A. niger* through elevated production of oxalic acid. The growth study for of *A. niger* shows that about 30 grams of wet biomass was generated in 100 ml culture, both in presence and absence of manganese after complete consumption of sucrose in medium. The presence of manganese in medium do not affect the growth of the fungus;

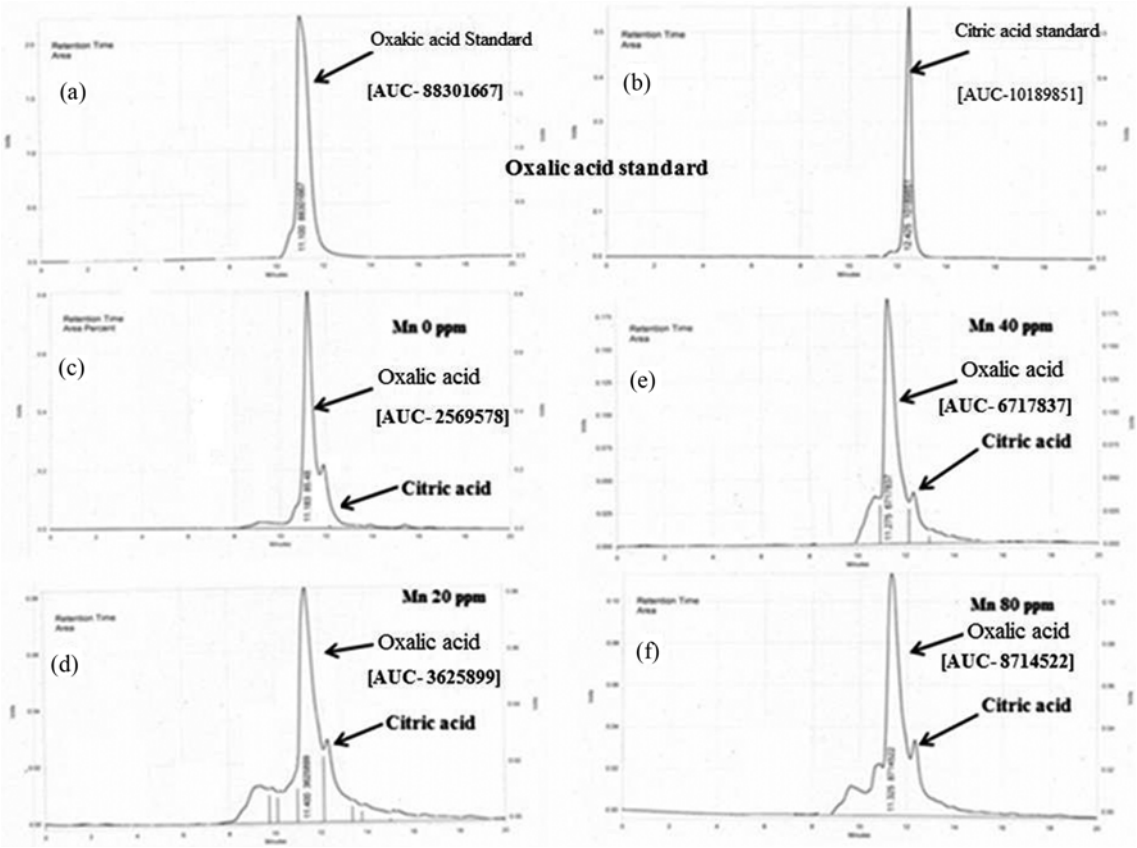


Fig. 7. HPLC chromatograms representing organic acid profile of *A. niger* during bioleaching ((a) standards of oxalic and (b) standard of citric acid; (c), (d), (e) & (f) representing organic acid profile in different concentration of manganese).

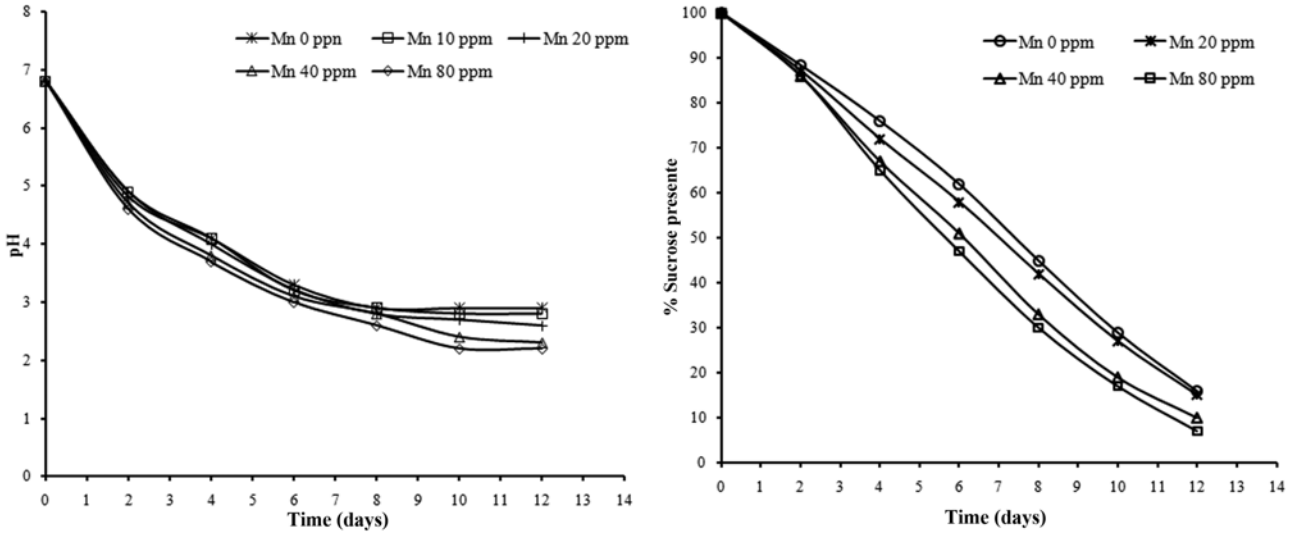


Fig. 8. (a) pH during the growth of *A. niger* in presence of manganese in culture medium, (b) Sucrose concentration in culture medium of *A. niger*.

rather, the rate of sucrose consumption was little faster in case of *A. niger* grown in manganese supplemented medium. In Fig. 8(b) it was reflected that up to the 4th day of culture of *A. niger* the rate of sucrose consumption was the same, but beyond that the rate of sucrose utilization increased with increase in concentration of manganese in medium. The amount of manganese uptake from the culture medium by the *A niger* strain was studied. The culture was filtered using Whatman filter paper and the biomass was dried and then digested in 1 N HCl for AAS analysis for manganese. The maximum amount of manganese absorbed per gram wet weight of the *A. niger* biomass was 0.591 to 0.612 µg during 3rd to 7th day of inoculation. On the 7th day onwards, the uptake of the manganese ceased

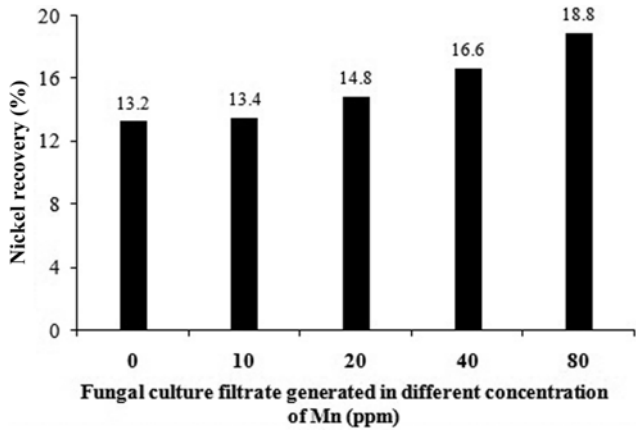


Fig. 9. Nickel recovery from pre-treated COB by using fungal culture filtrates generated in different concentrations of manganese.

by *A. niger*. The manganese uptake capacity of *A. niger* was increased up to the manganese concentration of 80 ppm in culture medium, but beyond that concentration no more manganese was absorbed by the fungal strain.

Fig. 9 shows the nickel recovery from pre-treated COB by using sterile fungal culture filtrates as leaching agents. Nickel recovery (18.8%) was greater in case of culture filtrates of fungus grown in medium containing 80 ppm manganese. Furthermore, the HPLC study confirmed that oxalic acid synthesis by the fungi was more in case of culture medium supplemented with 80 ppm manganese. Similarly, the chemical leaching of pre-treated COB by using synthetic organic acids confirmed the dominating role of oxalic acid in recovery of nickel. The chemical leaching conducted for pre-treated COB by using oxalic acid and citric acid revealed that about 39% nickel was recovered by oxalic acid (0.1 M), whereas 21.5% nickel was recovered by citric acid (0.1 M) leaching (Fig. 10). On subsequent chemical leaching study conducted with a mixture of oxalic acid and citric acid in different proportions, nickel recovery increased with increase in oxalic acid fraction in the mixture of organic acids (Fig. 10). It confirmed that the oxalic acid produced by *A. niger* was playing a prominent role in nickel recovery.

The above study was statistically analyzed through one way ANOVA followed by Duncan's post hoc test. Tables 2 and 3 show

Table 2. One way ANOVA (p=0.05) analysis for the oxalic acid secretion by *A. niger*

Groups manganese (ppm)	Sum	Average	Variance	SD	
0	873	291	1	1.113553	
10	894	298	3	0.87178	
20	1230	410	12	0.87178	
40	2280	760	28	0.52915	
80	2968	989.3333	86.33333	0.6	Significant

The oxalic acid (mg/liter) secretion at 80 ppm manganese supplemented to culture medium of *A. niger* was significant than at manganese concentration of 0, 10, 20, 40 ppm (p=0.05)

Table 3. One way ANOVA (p=0.01) analysis for the nickel recovery by *A. niger* mediated bioleaching

Groups manganese (ppm)	Sum	Average	Variance	SD	
0	72	24	1.24	1.114	
10	81	27	0.76	0.872	
20	91.8	30.6	0.76	0.8718	
40	105	35	0.28	0.5292	
80	115.8	38.6	0.36	0.6	Significant

Nickel bioleaching at manganese concentration 80 ppm in *A. niger* culture medium was significant than at manganese concentration 0, 10, 20, 40 ppm (p=0.01)

ANOVA for oxalic acid secretion by *A. niger* in response to manganese in culture media and nickel recovery from pretreated COB by *A. niger*, respectively.

4. Statistical Analysis

An ANOVA test was performed for the above experiments at p value 0.05 and 0.01. The highest concentration of oxalic acid and nickel bioleaching was at manganese concentration of 80 ppm in the medium of *A. niger*. From Fig. 11 it was inferred that the oxalic acid secretion at 80 ppm was significantly higher than the same at

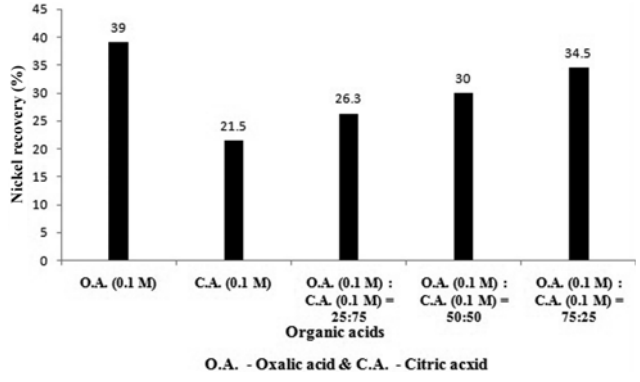


Fig. 10. Oxalic acid and citric acid mediated chemical leaching of pre-treated COB.

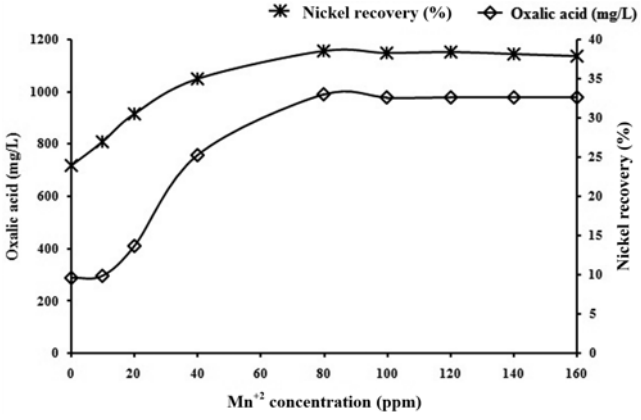


Fig. 11. Nickel recovery and oxalic acid production with respect to manganese concentration in *A. niger* culture medium.

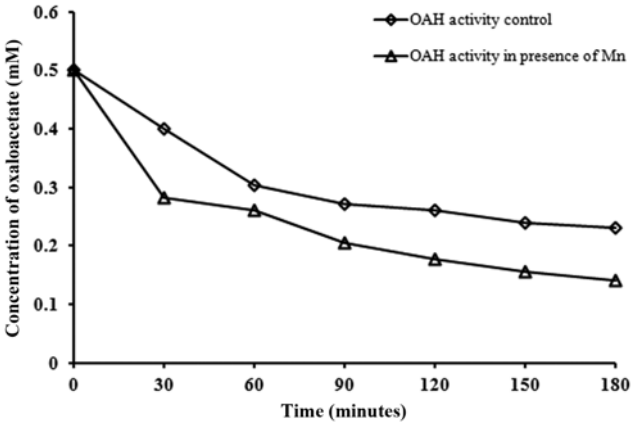


Fig. 12. OAH enzyme activity study for the rate of oxalate synthesis from oxaloacetate by *A. niger* in response to manganese.

manganese concentration of 0, 10, 20, 40 (p=0.05). Whereas, the nickel bioleaching at manganese concentration of 80 ppm was significantly higher than the same at manganese concentration 0, 10, 20, 40 ppm (p=0.01).

5. Oxaloacetate Hydrolase Enzyme Activity of *A. niger* in Presence of Manganese

Chen et al. (2010) reported that among the divalent cations manganese is the most influential metal cofactor for OAH catalytic activity [21]. So the presence of manganese favored the elevation in enzymatic activity for more oxalate production. Manganese cofactor dependent OAH enzyme is located in the cytoplasm of *A. niger*, where it catalyzes the conversion of oxaloacetate to oxalate and acetate [10,20,29]. So the presence of manganese favored the increase in the enzymatic activity to produce more oxalate.

Furthermore, it was observed that the crude cytoplasmic extract of the *A. niger* supplemented with manganese catalyses more and faster breakdown of oxaloacetate into oxalate and acetate in compare to crude cytoplasmic extract without manganese. Fig. 12 represents that addition of manganese favored the breakdown of oxaloacetate and produced more oxalate. The experiment output reveals that the fungal strain *A. niger* mediated bioleaching process may progress by the following manner [25]:

$$\text{Oxaloacetate}+H_2O \xrightarrow{\text{Oxaloacetate acetylhydrolase}} \text{Oxalate}+\text{Acetate} \quad (1)$$

$$C_2H_2O_4 \leftrightarrow (C_2HO_4)^{1-}+H^+ \cdots\cdots (pKa_1=1.27) \quad (2)$$

$$(C_2HO_4)^{1-}+Ni^{2+} \leftrightarrow Ni(C_2HO_4)_2$$
(*Nickel Oxalate Complex*)

$$(C_2HO_4)^{1-} \leftrightarrow (C_2O_4)+H^+ \cdots\cdots (pKa_2=4.20) \quad (3)$$

$$(C_2O_4)^{2-}+Ni^{2+} \leftrightarrow Ni\,(C_2O_4)$$
(*Nickel Oxalate Complex*)

CONCLUSIONS

The aforesaid studies conclude that major nickel content of raw COB was present inside the goethite matrix. From X-ray diffraction study of COB it was found that thermal pre-treatment altered the goethite phase into hematite through thermo-chemical phenomenon. Also the EPMA study revealed that on pre-treatment of COB the nickel was expelled from goethite matrix and distributed homogeneously. SEM and TEM observation reported that pre-treatment of COB developed micropores and cracks and converted the COB into a mesoporous structure, which in turn is more susceptible to leaching agents.

Our studies show the recovery of nickel from the pre-treated COB by the organic acids (oxalic and citric acid) produced by *A. niger.* Among the organic acids, oxalic acid was more effective for recovery of nickel. Furthermore, the nickel recovery was improved with the rise in concentration of oxalic acid in the medium. The oxalic acid production by *A. niger* was enhanced when manganese was added to the culture medium of bioleaching. Manganese was used as a co-factor for the cytoplasmic enzyme oxaloacetate acetylhydrolase of *A. niger*, which converts oxaloactate to oxalate and acetate. Hence, addition of manganese enhanced the bio-synthesis of the oxalate by oxaloacetate acetylhydrolase enzyme of *A. niger*. The HPLC analysis shows that addition of 80 ppm of manganese to the medium maximum amount (986 mg/liter) of oxalic acid was produced by *A. niger.* Concurrently, maximum nickel recovery of 38.6% was obtained when the *A. niger* culture medium was supplemented with 80 ppm of manganese. Further efforts will be required to improve the rate of nickel recovery. The statistical analysis also reveals that the oxalic acid secretion (p=0.05) and nickel bioleaching (p= 0.01) was significantly maximum at 80 ppm manganese concentration.

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REFERENCES

1. L. B. Sukla, V. V. Panchanadikar and R. N. Kar, *World J. Microb. Biot.*, **9**, 255 (1993).
2. B. Ahmad, H. N. Bhatti and S. Ilyas, *Afr. J. Biotechnol.*, **10**(54), 11196 (2011).
3. M. Valix, F. Usai and R. Malik, *Min. Eng.*, **14**(2), 197 (2001).
4. S. Nouren, H. N. Bhatti and S. Ilyas, *Afr. J. Biotechnol.*, **10**(52), 10664 (2011).
5. Dutton and C. S. Evans, *Can. J. Microbiol.*, **42**, 881 (1996).
6. K. Bosecker, *FEMS Microbiol. Rev.*, **20**, 591 (1997).
7. W. Burgstaller and F. Schinner, *J. Biotechnol.*, **17**, 91 (1993).
8. K. Tsekova, D. Todorova and S. Ganeva, *Int. Biodeter. Biodegr.*, **64**, 447 (2010).
9. J. C. Pernet, *Encyclopaedia of chemical technology*, Interscience Publishers Inc., New York (1991).
10. C. P. Kubicek, G. S. Kunar, W. Woèhrer and M. Roèhr, *Appl. Environ. Microbiol.*, **54**, 633 (1988).
11. C. P. Kubicek and M. Rohr, *CRC Crit. Rev. Biotechnol.*, **3**, 331 (1986).
12. L. B. Sukla, K. M. Swamy, K. L. Narayana, R. N. Kar and V. V. Panchanadikar, *Hydrometallurgy*, **37**(3), 387 (1995).
13. F. Anjum, H. N. Bhatti and M. A. Ghaur, *Hydrometallurgy*, **100**, 122 (2010).
14. F. Anjum, H. N. Bhatti, M. Asgher and M. Shahid, *Appl. Clay Sci.*, **47**, 356 (2010).
15. K. D. Mehta, C. Das and B. D. Pandey, *Hydrometallurgy*, **105**, 89

(2010).
16. A. J. Balmforth and A. Thomson, *Biochem. J*., **218**, 113 (1984).
17. K. E. Hammel, M. D. Mozuch, K. A. Jr. Jensen and P. J. Kersten, *Biochemistry*, **33**, 13349 (1994).
18. H. Pedersen, C. Gem and J. Nielsen, *J. Mol. Gen. Genet*., **263**, 281 (2000).
19. H. Pedersen, B. Christensen, C. Hjort and J. Nielsen, *Metab. Eng*., **2**, 34 (2000).
20. G J. G Ruijter, P. J. I. van de Vondervoort and J. Visser, *J. Microbiol*., **145**, 2569 (1999).
21. C. Chen, Q. Sun, B. Narayanan, D. L. Nuss and O. Herzberg, *J. Biol. Chem*., **285**(34), 26685 (2010).
22. L. B. Sukla and R. P. Das, *Trans. Indian Inst. Met*., **40**, 351 (1987).
23. S. Ilyas, R. Chi, H. N. Bhatti, I. A. Bhatti and M. A. Ghauri, *Bioprocess Biosyst. Eng*., **35**, 433 (2012).
24. S. Mohapatra, C. Sengupta, B. D. Nayak**,** L. B. Sukla and B. K. Mishra, *Korean J. Chem. Eng*., **25**(5), 1070 (2008).
25. S. K. Behera, P. P. Panda, S. Singh, N. Pradhan, L. B. Sukla and B. K. Mishra, *Int. Biodeter. Biodegr.*, **65**, 1035 (2011).
26. H. Lenz, P. Wunderwald and H. Eggerer, *Eur. J. Biochem*., **65**, 225 (1976).
27. M. Dubois, K. Gillies, Y. Hamilton, P. Roborts and F. Smith, *Anal. Chem*., **28**, 350 (1956).
28. S. Mohapatra, S. Bohidar, N. Pradhan, R. N. Kar and L. B. Sukla, *Hydrometallurgy*, **85**, 1 (2007).
29. Y. Han, H. Joosten, W. Niu, Z. Zhao, P. S. Mariano, M. McCalman, J. V. Kan, P. J Schaap and D. D. Mariano, *J. Biol. Chem*., **282**(13), 9581 (2007).