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# Screening and characterization of acid producing fungi from different mine areas of Chhattisgarh region

Rinu Khan* and A.K. Gupta
*Microbiology Research Laboratory, School of Studies in Life Science, Pt. Ravishankar Shukla University, Raipur- 492010, Chhattisgarh, India*
**Corresponding author Email*: *khanrinu@gmail.com*
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## ABSTRACT

**Microorganisms are ubiquitous in distribution. They are present in diverse environments including extremophilic regions and adapt physiologically to these environment. In the present study, isolation and screening of acidophilic fungi was carried out from eight mines of Chhattisgarh region *viz*., Laxman coalfield mine, Kusmunda mine, Dalli mine, Rajhara mine, BIOP mine, Hirri mine, Rawan mine, Nandini mine and one ore-dumping site of Bhilai Steel Plant. A total of 64 fungal isolates belonging to nine genera *i.e*., *Aspergillus*, *Alternaria*, *Penicillium*, *Sterila*, *Mucor*, *Trichoderma*, *Fusarium*, *Phoma* and *Paecilomyces* were isolated, identified and evaluated for their acid production ability. Of the 29 positive acidophilic fungi assayed quantitatively, *Aspergillus niger* gr. NFCCI-3303 and *Penicillium* sp 2 NFCCI-3305 were found to be significantly higher producer of organic acids and can be good source for biomining.**

**Keywords:** Acidophilic fungi, Chhattisgarh, mines, organic acids.

## INTRODUCTION

The use of acidophilic microorganisms in mining is a relatively newer and practical approach for extracting trace metals through bioleaching which offers solutions for exploring metals from lean grade ores. Biohydrometallurgy is a process described as being "the dissolution of metals from their mineral sources by certain naturally occurring microorganisms" or "the use of microorganisms to transform elements so that the elements can be extracted from a material when water is filtered through it". Fungi convert metal compounds into their water soluble forms and this ability of fungi to solubilize metals from solid materials gives new prospects for fungal application. Earlier, Krebs *et al*. (1997) reviewed some important fungal species for biohydrometallurgy purpose *viz*., *Aspergillus amstelodami*, *A. clavatus*, *A. ficuum*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *Mucor* sp., *Paecilomyces variotii*, *Penicillium brevicompactum*, *P. cyclopium*, *P. funiculosum*, *P. notatum*, *P. ochrochloron*, *P. oxalicum*, *P. simplicissimum*, *P. spinulosum*, *P.variotii* and *Rhizopus* sp.

Heterotrophic microorganisms are widely distributed all over the mine areas. Researchers have emphasized on isolation of fungi for metal dissolution. Solubilization of metals from ore by acidophilic fungi has been documented by researchers (Bohidar *et al*., 2009 and Behera *et al*., 2011). Bioleaching with fungi is totally based on the organic acid production which serves as leaching agents for the solubilization of metals (Bosshard *et al*., 1996). Fungi are able to achieve this by their metabolic processes where glycolytic pathway converts glucose into variety of products including organic acids (Jain and Sharma, 2004). The acids usually have dual effect of increasing metal dissolution by lowering the pH and increasing the load of soluble metals by complexion/ chelating into soluble organo-metallic complexes. Since mine areas are rich in mineral resources as well as provide acidic environment to the microflora, effort was made to explore the mine areas of Chhattisgarh region which have abundance of mineral resources for the prevalence of acidophilic fungi and their bioleaching capabilities.

## MATERIALS AND METHOD

**Collection of Samples:** Chhattisgarh is a newly carved state from Madhya Pradesh in the year 2000 and is located in central India. It is one of the foremost mineral rich states of India. Eight mines of Chhattisgarh region *viz*., Laxman coalfield mine (22.35° N & 82.50° E) site 1, Kusmumda mine (22.35° N & 82.75° E) site 2, Dalli mine site 3, Rajhara mine site 4 (20.56° N & 81.05° E), BIOP mine (18.60° N & 81.25° E) site 5, Hirri mine (21.98° N & 82.31° E) site 6 , Rawan mine (21.24° N & 81.64° E) site 7, Nandini mine (21.37° N & 81.42° E) site 8 and one ore-dumping site of Bhilai Steel Plant (21.18° N & 81.39° E) site 9 were explored for the study of fungal bioflora. Soil samples were collected in the month of October to February (2012-2014) using aseptic zipped polythene bags, transported to the laboratory and stored at 4°C in the refrigerator till use

**Isolation of Fungi:** The fungi were isolated by direct plate method and serial dilution agar plating method following Waksman and Fred (1922) and Warcup (1950), respectively on Potato Dextrose Agar plates in triplicates. Based on predominance and distinct morphological properties, fungal isolates were selected and purified by repeated sub culturing and streak plating technique.

**Identification of fungi by morphological characteristics:** The fungal isolates obtained from different sites (sites 1- 9) were streaked aseptically on potato dextrose agar plates and incubated at 28°C till the appearance of the colony. All the organisms were identified following Ellis (1976) and Barnett and Hunter (1998). They were further validated through National Fungal Culture Collection of India (NFCCI), Pune and were submitted for accession.

**Screening for Organic Acid Production:** The efficiency of isolated strains for acid production was tested through qualitative and quantitative assay.

**Qualitative acid production assay:** The fungal isolates were qualitatively assayed for acid production using acid indicator medium (AIM) containing 0.04% of bromocresol

purple (Das and Roy, 1998). A loop full of fungal spores was inoculated on Czapek-Dox broth medium containing (g/ l): Sodium nitrate (2.0), Di-potassium hydrogen phosphate (1.0), Magnesium sulphate (0.5), Potassium chloride (0.5), Ferrous sulphate (0.01), Sucrose (30), Bromocresol purple (0.04), and incubated for five days for the production of acid which was confirmed by formation of yellow coloration in the medium.

**Quantitative acid production assay:** The positive fungal isolates were assessed for quantitative acid production by measuring total acidity following Sikandar *et al.* (2001) and strength of acidity was calculated in terms of molarity (M).

**Growth of fungi on potato dextrose agar medium at pH 2:** Five best positive acidophilic fungal isolates *viz*., *Aspergillus niger* gr. NFCCI-3303 (LAK-2), *Penicillium* sp.1 NFCCI-3306 (KUS-2), *Aspergillus niger* gr. NFCCI-3307 (DM-1), *Trichoderma atroviride* Karsten NFCCI-3304 (DM-2) and *Penicillium* sp. 2 NFCCI-3305 (HM-6) were isolated from Laxman coalfield mine (22.35° N & 82.50° E), Kusmunda mine (22.35° N & 82.75° E), Dalli mine (20.56° N & 81.05° E) and Hirri mine (21.98° N & 82.31° E) were characterized for their true acidophilic behavior by growing them on potato dextrose agar medium at pH 2.0 and growth was monitored after 5 days of incubation and was compared with the control plate maintained at pH 6.8.

**Characterization of organic acids produced by fungal isolates by HPLC:** The concentration of organic acids produced by *Aspergillus niger* gr. NFCCI-3303 and *Penicillium* sp strain was determined by HPLC (Agilent). Separation of citric, malic, tataric and oxalic acids was carried out in a C 18 (20 cm x 4.6 mm, 5 micron) column; mobile phase containing a mixture of 0.1M sodium sulphate adjusted to pH 3.5 with dilute sulfuric acid; flow rate 1 ml/ min at ambient temperature. The organic acids were quantified by UV detection at 210 nm.

**Data analysis:** Statistical analyses of data was performed using SPSS 16.0. One way ANOVA and Duncan post hoc test were used for comparing mean.

## RESULTS

A total of 64 fungal isolates belonging to nine genera *i.e., Aspergillus*, *Alternaria*, *Penicillium*, *Sterila*, *Mucor*, *Trichoderma*, *Fusarium*, *Phoma* and *Paecilomyces* were isolated and identified based on colonial and microscopic appearance (**Fig.1**). Twenty-three fungal isolates belonging to seven genera were further investigated and submitted for confirmation of the identity and assigning accession number to National Fungal Collection Centre of India, Pune.

In present study, the dominant fungi included, *Aspergillus niger* gr., *Penicillium* sp. 2 and *Penicillium* sp. aff. *P. camemberti* from all sites except sites 6 and 7. *P. lilacinus* with moderate incidence was recorded from sites 4 and 5 in the present study. *Alterneria alternarina* was recovered from sites 2 and 6. The occurrence of *Mucor* sp., *A. candidus, A. flavus* gr,. *A. nidulans, A. ochraceus*, *A. terreus* gr., *Fusarium moniliforme, Penicillium* sp., *Sterila* sp. gr., *Trichoderma atroviride* and *Phoma* sp. aff. *P. chrysanthemicola* were sporadic in various mine sites.

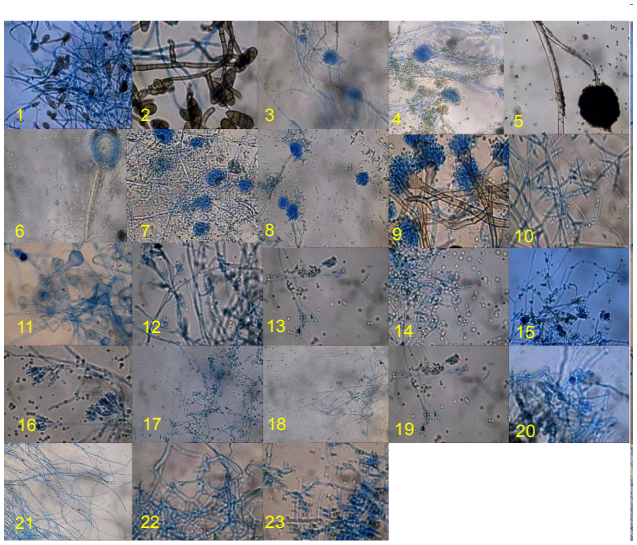


**Fig. 1**: Fungal isolates from different mine areas of Chhattisgarh region
**1**-*Alternaria alternarina* E.G. Simmons NFCCI-3746 (X400), **2**-*Alternaria* sp. aff. *A. pluriseptata* sensu Dingley NFCCI-3747 (X400), **3**- *Aspergillus candidus* Link NFCCI-3759 (X400), **4**- *Aspergillus flavus* gr. NFCCI-3749 (X1000), **5**-*Aspergillus niger* gr. NFCCI-3303 (X400), **6**-*Aspergillus ochraceus* Wilh NFCCI-3757 (X400), **7**-*Aspergillus terreus* gr. NFCCI-3754 (X400), **8**-*Aspergillus terreus* Thom NFCCI-3753 (X400), **9**-*Emericella nidulans* (Eidam) Vuill NFCCI-3758 (X1000), **10**- *Fusarium moniliforme* J. Sheld NFCCI-3752 (X1000), **11**-*Mucor* sp, **12**- *Paecilomyces lilacinus* (Thom) Samson NFCCI-3760 (X1000), **13**- *Penicilium* sp. aff. p.*camemberti* Thom NFCCI-3761 (X1000), **14**-*Penicillium* sp. 1 NFCCI-3306 (X1000), **15**-*Penicillium* sp. 2NFCCI-3305 (X400), **16**- *Penicillium* sp. 3 NFCCI-3751 (X1000), **17**- *Penicillium* sp. aff. *P. corylophilum* Dierckx NFCCI-3750 (X400), **18**- *Penicillium* sp. aff. *P. decumbens* Thom NFCCI-3748 (X400), **19**- *Penicillium* sp.4 NFCCI-3762 (X1000), 20- *Phoma* sp. aff. *P. chrysanthemicola* Hollos NFCCI-3745 (X1000), **21**- *Sterila* sp1, **22**- *Sterila* sp2, **23**- *Trichoderma atroviride* Karsten NFCCI-3304 (X1000).

All the fungal isolates were subjected to primary screening for their acid production ability. Twenty nine fungal isolates showed positive acidophilic activity on the basis of color change of the medium from purple to yellow which indicated the production of acid in the medium (**Fig.2**). The quantitative acid production assay of fungal isolates in the study (**Table-1**) revealed maximum acid production by *Aspergillus* species. *A. niger* gr. (NFCCI-3303) was the most important acidophilic fungus among all the isolates from Lakshman coalfield mine in Korba. *A. niger* gr. NFCCI-3307 from Dalli mine was next in order followed by *Penicilllium* sp. from Hirri mines.

Further, the five fungal isolates were tested for their true acidophilic characteristic feature by growing them on potato dextrose agar medium maintained at pH 2.0 (**Table-2**). The maximum growth was measured in *A. niger* gr. NFCCI-3303 (LAK-2) with a colony diameter of 7.50±0.09 cm followed by DM-1(3.93±0.19 cm) and HM-6 (3.63±0.15 cm). The minimum growth was seen in *Penicillium* sp. 1 NFCCI-3306 (KUS-2) of 3.43±0.25 cm with late sporulation. No growth was seen in *Trichoderma atroviride* NFCCI-3304 (DM-2) after fifth day of incubation period.

**Table 1**: Acid production (M±SE) of positive 29 fungal isolates from selected mine areas of Chhattisgarh region

| S. No. | Code | Fungal isolates | Acid production (in Moles) |
|---|---|---|---|
| 1 | LAK-1 | *A. terrus* gr. | 0.005±0.001$^{h}$ |
| 2 | LAK-2 | *A. niger* NFCCI-3303 | 0.125±0.02$^{a}$ |
| 3 | LAK-4 | *Penicillium* sp. aff. *P. decumbens* Thom | 0.005±0.001$^{h}$ |
| 4 | LAK-5 | *A. terreus* Thom. | 0.011±0.001$^{fgh}$ |
| 5 | LAK-6 | *Penicillium* sp. 1 | 0.005±0.001$^{h}$ |
| 6 | LAK-7 | *Penicillium* sp. 2 | 0.008±0.001$^{gh}$ |
| 7 | KUS-2 | *Penicillium* sp. 1 | 0.018±0.002$^{ef}$ |
| 8 | KUS-1 | *A. terreus* gr. | 0.006±0.001$^{gh}$ |
| 9 | DM-1 | *A. niger* NFCCI-3307 | 0.070±0.01$^{b}$ |
| 10 | DM-2 | *T. atroviride* Karsten NFCCI-3304 | 0.015±0.001$^{efg}$ |
| 11 | DM-5 | *A. alternarina* E.G. Simmons | 0.002±0001$^{h}$ |
| 12 | DM-6 | *Penicillium* sp. 3 | 0.005±0.001$^{h}$ |
| 13 | RM-1 | *A. niger* NFCCI-3307 | 0.028±0.002$^{d}$ |
| 14 | RM-4 | *Penicillium* sp. 4 | 0.005±0.001$^{h}$ |
| 15 | RM-8 | *Penicillium* sp. aff. *P. camemberti* Thom | 0.009±0.001$^{fgh}$ |
| 16 | BIOP-1 | *Fusarium* sp. | 0.006±0.001$^{gh}$ |
| 17 | BIOP-3 | *Penicillium* sp. aff. *P. camemberti* Thom | 0.008±0.001$^{gh}$ |
| 18 | BIOP-11 | *A. niger* | 0.031±0.001$^{cd}$ |
| 19 | BIOP-12 | *A. terreus* Thom | 0.008±0.001$^{gh}$ |
| 20 | HM-1 | *A. niger* | 0.020±0.005$^{e}$ |
| 21 | HM-2 | *A. flavus* gr. | 0.009±0.001$^{fgh}$ |
| 22 | HM-6 | *Penicillium* sp. NFCCI-3305 | 0.037±0.003$^{c}$ |
| 23 | RAW-1 | *A. alternarina* E.G. Simmons | 0.002±0.00$^{h}$ |
| 24 | RAW-4 | *A. flavus* gr. | 0.005±0.001$^{h}$ |
| 25 | RAW-5 | *A. ochraceus* Wilh | 0.002±0.001$^{h}$ |
| 26 | NM-1 | *Alternaria* sp. aff. *A. pluriseptata* sensu Dingley | 0.003±0.001$^{h}$ |
| 27 | BSP-1 | *Sterila* sp. 2 | 0.011±0.001$^{fgh}$ |
| 28 | BSP-2 | *A. terreus* Thom | 0.008±0.001$^{gh}$ |
| 29 | BSP-3 | *Penicillium* sp. 4 | 0.011±0.001$^{fgh}$ |

ANOVA Summary: F = 88.368, df = 28, 58, p= 0.000. Values with different alphabet labels are statistically significant at p< 0.05. Concentration of hydrogen ions in a solution is expressed as moles per liter and used as a measure of the acidity of the solution.

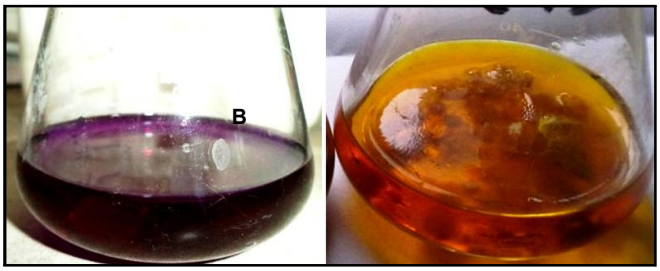


**Fig. 2**: Screening of acidophilic activity in *A. niger* gr.: (A) Control flask without inoculation, (B) Positive fungal isolate

**Table 2:** Growth (colony diameter in cm) of selected acidophilic fungal isolates on PDA at pH 2.0 *vis-à-vis* pH 6.8 (control) in five day incubation period.

| S. No. | Site/ Code | Fungal isolates | Control (pH - 6.8)) | Test (pH - 2.0) |
|---|---|---|---|---|
| 1. | LAK-2 | *Aspergillus niger* gr. NFCCI-3303 | 7.50±0.13** | 7.50±0.09** |
| 2. | KUS-2 | *Penicillium* sp. 1 NFCCI-3306 | 7.50±0.03** | 3.43±0.25* |
| 3. | DM-1 | *Aspergillus niger* gr. NFCCI-3307 | 6.50±0.11** | 3.93±0.19** |
| 4. | DM-2 | *Trichoderma atroviride* NFCCI-3304 | 7.20±0.05** | No Growth |
| 5. | HM-6 | *Penicillium* sp. 2 NFCCI-3305 | 7.50±0.03** | 3.63±0.15** |

** Sporulation; *Late sporulation

Several acidophilic fungi were widely distributed in all the study sites with different pattern of acid production ability. The HPLC analysis revealed that *A. niger* NFCCI-3303 and *Penicillium* NFCCI-3305 produced oxalic and tartaric acids in the culture filtrate. The amount of oxalic and tartaric acid produced by the isolates was found to be 23.96 and 23.37 g/ L, respectively (**Table-3**).

**Table 3:** Quantity of organic acids in culture filtrates of *A. niger* gr. NFCCI-3303 and *Penicillium* sp. NFCCI-3305 following 10 days growth

| Fungal isolates | Organic acids (g / L) | | |
|---|---|---|---|
| | Citric acid | Malic acid | Oxalic + Tartaric acid |
| *Aspergillus niger* gr. NFCCI-3303 | ND | ND | 23.96 |
| *Penicillium* sp. 2 NFCCI-3305 | ND | ND | 23.37 |

## DISCUSSION

The occurrence of genera like *Aspergillus*, *Alternaria*, *Penicillium*, *Sterila*, *Mucor*, *Trichoderma*, *Fusarium*, *Phoma* and *Paecilomyces* in the mine region may be due to their adaptation and associated metal absorption capacities which allow them to prosper in these extreme conditions of high temperature and pH. Klich (2002) reported that *Aspergillus* and *Penicillium* can be isolated from a wide variety of environments including mine areas. Dave and Natarajan (1981) reported the occurrence of *Paecilomyces varioti* with *A. niger* from Indian mine water from Bihar, Rajasthan and Gujarat. Adeleke *et al*. (2010) reported *Alternaria* sp. along with *Penicillium,* and *Epicoccum* from the conglomerate and shale iron ore samples from Northern Cape Province of South Africa. However, Burgstaller and Schinner (1993) reviewed several metal leaching fungi from mine areas and have reported above genera. Krebs *et al.* (1997) reported the presence of both lithotrophic and organotrophic microorganism from the mine areas mobilizing bioleaching. Lopez and Vazquez (2003) suggested that the presence of *Trichoderma* sp. in mine sites can be associated with their bioleaching ability. Jain and Sharma (2004) reported microorganisms producing different organic acids and thus bringing out the metal leaching during fermentation. Several species of *Aspergillus* and *Penicillium viz*., *A. niger*, *A. terreus*, *A. flavus*, *P. brevicomactum*, *P. oxalicum*, *P. purpurescens*, *P. lividum*, *Eupencillium ludwigii* and *P. spinulosum* have been reported by various workers in different mine areas of the world (Gharieb *et al*., 2013 and Amin *et al*., 2014). Xiao *et al*. (2009) reported *Mucor ramosissimus* along with *Penicillium expansum* and *Candida krissii* from phosphate mines in China. Manish *et al.* (2015) isolated *Phoma* sp. from lead contaminated industrial waste water from coal based iron industries, iron casting industry and a petrochemical industry of Raipur, Chhattisgarh. Recently, Verma and Verma (2016) reported *Aspergillus, Fusarium, Penicillium* and *Trichoderma* species from Dalli-Rajhara iron ore mine rhizosphere soil. The study revealed that *A. niger* gr. (NFCCI-3303) was the most important acidophilic fungus among all the isolates from Lakshman coalfield mine in Korba. Several acidophilic fungi were widely distributed in all the study sites with different pattern of acid production ability. The coal mining activity is characterized by the generation of large amount of by-product; one of them is pyrite which tends to acidify the environment. Castro-Silva *et al*. (2003) reported the occurrence of acidophilic fungi in coal mines. Behera *et al* (2011) reported that *A. niger* exhibit good acid producing ability which effectively solubilize nickel and cobalt isolated from Sukinda mines, Orissa. Several species of *Penicillium* sp. have also been implicated in acid production and thus bioleaching of metals like, copper, iron and aluminium (Chaudhary *et al*., 2014; Khan and Gupta, 2015). The HPLC analysis revealed that *A. niger* NFCCI-3303 and *Penicillium* sp. NFCCI-3305 produced oxalic and tartaric acids in the

culture filtrates. Absence of citric acid was found in both the fungus. Gharieb *et al* (2013) in their study reported the presence of oxalic acid and no citric acid in the culture filtrate of *A. niger* both in the presence and absence of hematite and discussed that lack of citric acid might be due to unfavorable experimental conditions, since pH < 2 was reported to be a prerequisite for citric acid accumulation where pH shift in the culture medium towards or above neutrality resulted in considerable accumulation of oxalic acid by *A. niger*. Mandal and Banerjee (2006) reported that the yield of oxalic acid production increased by *A. niger* if pH of the broth was between 6 and 7. The optimum pH for citric acid production by *A. niger* was 2.0 (Auta *et al*., 2014). Fungi are known to produce a variety of organic acids during their metabolic process. *A. niger* and *P. notatum* isolated by Ghorbani *et al*. (2007) produced 14.27 and 10.00 g/ L citric acid and 0.20 and 7.00 g/ L oxalic acid respectively. HPLC chromatograms of organic acids of some important producers are given in **Table 4**. However, the present study was limited to the screening of acidophilic fungi and further work is needed to evaluate the role of fungi in the leaching process.

Table 4: HPLC chromatograms of some important producers of organic acids

| S. No. | Name of the fungus | Organic acids | References |
|---|---|---|---|
| 1 | *Penicillium simplicissimum* | Citric and oxalic acid | Ambreen *et al*., 2002 |
| 2 | *Aspergillus niger* | Citric, gluconic, oxalic, tartaric, malic and phytic acids | Mulligen and Kamali 2003 |
| 3 | *Aspergillus niger* | Citric, oxalic, gluconic, malic and tartaric acid | Mulligen *et al*., 2004 |
| 4 | *Aspergillus niger* and *Penicillium notatum* | Citric and oxalic acid | Ghorbani *et al*., 2008 |
| 5 | *Penicillium notatum* | Citric, oxalic, tartaric and malic acids | Anjum *et al*., 2009 |
| 6 | *Aspergillus niger* | Citric, gluconic and oxalic acids | Bayat *et al*., 2011 |
| 7 | *Cladosporium oxysporum and Penicilluim stoloniferum* | Citric, malic, tartaric, oxalic and phytic acid. | Ibrahim *et al*., 2012 |
| 8 | *Aspergillus niger* | Citric, gluconic and oxalic acid | Behera and Sukla, 2012 |
| 9 | *Aspergillus niger, Eupenicillium ludwigii* | Citric and oxalic acids | Gharieb *et al*., 2013 |

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