# STUDY OF BACTERIAL DIVERSITY OF COAL MINE AREA OF GONDEGOAN OPEN CAST MINE, KANHAN, NAGPUR, MAHARASHTRA

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**Abstract**

Present study based on the isolation of bacterial isolates from soil, Coal and water sample of coal mine area of Gondegaon Open Cast Coal Mine, WCL, Nagpur, Maharashtra. Soil, water and coal samples were collected from three different areas of mine and total 23 different colonies were isolated and purified and further studied for their morphological and biochemical characteristics. More than 75% (18 out of 23) isolates are belonging to Iron Bacteria. The overall study suggested that the Iron bacteria are quite a dominant species in Gondegaon Coal Mine area.

**Keywords:** Iron Bacteria, Open Cast Mine, Iron Oxidizing Bacteria, Microbial Diversity, Bioleaching activities.

## INTRODUCTION

Coal is a very important fossil fuel and complex polymer which is very essential for industrial and domestic purposes. Because of its carbon-oxygen and moisture content it is mostly favourable for microbial growth. Most of the coal reservoirs are rich with different microbial strains like iron bacteria, iron oxidizing bacteria. However, coal rich environments show diverse microbial population with rich functional potentials to convert coal substrates into value-added products and remediation of post-mining sites and industrial deposits. (Akimbekov NS, 2022) (Sudheer P.D.V.N., 2016)

Our nature shows diversity particularly with respect to the microbial flora. The prokaryotic diversity of the nature is an important tool for mankind. As they can be used extensively for a wide variety of purposes. Some prokaryotes show the unique ability to utilize a large number of organic and inorganic molecules for their energy source. (Shelswell, 2004) The 'iron bacteria' were among the first prokaryotes to be observed and recorded by pioneer

microbiologists, such as Ehrenberg and Winogradsky, in the 19th century. (Dworkin, 2012) They were originally considered to be bacteria that catalysed the oxidation of iron II (Fe2+, ferrous iron) to iron III (Fe3+, ferric iron), often causing the zlatter to precipitate and accumulate inside the cells. (Sabrina Hedrich, 2011 ) Various bacterial species have been studied for their coal utilization ability for their growth. Many studies suggests that bacterial isolates belong to coal environments have greater coal-solubilizing ability than exogenous microbial communities (Olawale J.T., 2020)

Soil is the richest source of microbes and shows extreme diversity particularly in the group of prokaryotes and lower eukaryotes. Mine area water, and the minerals are not very favourable for all prokaryotes to grow and adapt in the unusual physiochemical conditions prevailing in them. The pH conditions in mine soils near 5 hence most of the transition metals tend to remain in their higher oxidation state. Such changes usually do not favour mineral transition and transport in to bacterial cells in aerobic conditions. This leads to lower diversity of aerobic organisms in mine area. However, as we go deeper the soil becomes more anaerobic with lower pH that facilitates mineral transport hence; microbial diversity of anaerobes is mostly observable. (Prozorov, 2015)

Microbes belonging to the classes of super oxidizing capacity of minerals mostly predominate in mine areas. This is the reason why sulphur oxidizers, users of inorganic energy sources such as Iron oxidizing bacteria, certain photosynthetic bacteria and obligate lithotrophs are clearly observable. (Ortiz-Castillo, Mirazimi, Mohammadi, & Liu, 2021). Percolation of water through mine areas therefore results in more bioleaching activities. Boon et. al. in 1995 has shown that in pyrite ore areas bacterial iron oxidation occurs with higher ease. (Boon, 1993) (Boon M., 1995) *Thiobacillus ferroxdians* has been shown to oxidize a zinc sulphide almost in the single fashion. (Boon M. &., 1998) scientist have been reported sulphurite concentrates to be very rich in zinc sulphide oxidation by *Thiobacillus*. (Kuenen J. G., 1982) (Fowler TA, 1999)

The role of Iron bacteria in acid mine drainage of Bituminous coal mines is well documented. (Brett J Baker, 2003) (Hallberg, 2010,) Iron bacteria belong to two most common species ***Sphaerotilus*** and ***Leptothrix***. Both are them are sheathed bacteria and are chemoheterotrophs. (The Genera Leptothrix and Sphaerotilus., 2006) (Bertram Schmidt1, 2016) Other iron oxidizers include ***Gallionella*** spp. and ***Thiobacillus ferroxidans***. (Jones DSKohl C, 2015) (Juanjuan Wang, 2009) Stringent growth conditions and their fastidious

nature make them difficult to isolate. Biomineralization procedures are becoming very common which are primarily utilizing iron-oxidizing bacteria. (Farahani S, 2020) (G. F. White, 2016 ) (Singh, 2018)

Diversity of microbes in soil area needs more exotic research as more and more fastidious organisms may be present in that areas. The difficulty of isolation results from the heterogeneous physiochemical conditions required by most of the fastidious organisms. It is mostly observed that a very high material containing up to $10^9$ per ml can be obtained from mine areas. Majority of these cells are unicellular microbes associated with oxides. They were only visible when stained with DNA binding fluorescent dye, acridine orange and viewed by Epi fluorescent microscopy. This explains why cursory examination techniques fail to isolate most of the organisms. (H., 2006) This also explains the fastidious nature of these organisms as they can be isolated under specific conditions. E. G. Hanert, has shown that stalk forming aerobic neutralophilic organisms can be either purified on enrichment or pure culture in laboratory where it can grow only at very low oxygen tension in opposing gradients of oxygen and ferrous ions. (Hanert, 1973) (Chan CS, 2016) Lithotrophically growing $CO_2$ fixers grow to very limited extend as mesotrophic growth.

In the present study a large number of isolates ranging from 35-40 were isolated, when different samplings from coal and water components were carried out. Since most of the isolate were similar in their colony characterization on Nutrient Agar, there was a need of primary screening on visible bases. 23 isolates were screened off and subjected to conventional biochemical identification techniques. However, 18 out of 23 showed positive growth on Isolation Medium for Iron bacteria.

## METHODS AND MATERIALS

The study was divided into 3 major experiments namely,

- Isolation of microbes from soil, coal and water sample.
- To study the Morphological and Biochemical characteristics.
- To screen the isolates for Iron Bacteria.

## 1. Sample Collection


**Image 1: Satellite Image of Gondegaon open cast mine area**

Soil samples were collected from different surface areas of Gondegaon Open Cast Mine, WCL, Nagpur Area, Called FS II (Northen site sliding area), FS III (Eastern Sight) and FS I (western site). 3 samples of coal, water and soil were collected from the above-mentioned sites.

## 2. Purification of strains

The collected samples, were then diluted in 1:10 w/v in sterile distilled water for making suspension. Further 1ml of the suspension was inoculated and separated with sterile spreader on the Nutrient Agar plate. Then it was incubated for 24-48 hours at 37°C in an incubator. Colonies formed after 48 hours incubation period, were further inoculated on nutrient agar slants to get isolated colonies.

## 3. Morphological, Biochemical & Cultural Characteristics

Two major microscopic examination namely Gram staining and motility were carried out. Biochemical characterisation of all the isolates is required to be done for complete identification of these strains, IMViC test, and sugar fermentation, Triple sugar iron agar test, cytrochrome oxidize test, and catalase test and lysine decarboxylase test were carried out for all the strains.

Our aim was to study the microbial diversity of mine area soil and water. Therefore, it was necessary to study each isolate. We were also checked the presence of Iron bacteria. Two Medias namely “Isolation Medium for Iron bacteria”, “Iron Oxidizing Medium” (Twin Pack) were used. As they are specially designed for the isolation of Iron Bacteria only. (As per recommended in HIMEDIA manual).

**Albert’s Stain Reaction for Iron Bacteria**

The twelve strains showing distinct positive growth on isolation medium for Iron Bacteria were subjective to Albert’s stains reactions and the granules were observed

**Results**

**Isolation of the primary isolates from the soil water and coal**

Suitably dilute soil suspension was prepared as per the method describes earlier. 23 distinct isolates were identified on a nutrient Agar Plate based on their colony characteristics. These isolates were marked SA, SB, SC, WA, WB, WC, CA, CB and CC Stains with Subscripts $10^{-1}$, $10^{-2}$ based on the selected site. The colony characteristics were shown in table no 1.1

**Morphological, Biochemical & Cultural Characteristics**

All the 23 isolates were also morphologically characterized on the basis of Gram’s staining and Motility the results were shown in the table number 1.2. Selected isolates were also tested for biochemical characteristics, the results were shown in the table no 1.3. 1.4. 1.5 Cultural characterization were performed on two different media namely “Isolation Medium for Iron bacteria”, “Iron Oxidizing Medium” the results were given in the table number 1.6.

**Albert’s Stain for screening of Iron Bacteria**

The strains showing distinct positive growth in Isolation Medium for iron Bacteria were subjected to Albert’s Stain reaction and the granules were observed microscopically. The results were shown in table 1.7.

**Table 1.1: Characteristics of the Primary Isolates**

| Sr. No | Sample | Colour | Colony Characteristics |
|---|---|---|---|
| 1 | Water A<br>$10^{-3}$<br>$10^{-2}$ | Pale yellow<br>Pale yellow (lemon) | Circular cracky colonies with smooth stretched Surface<br>Having stretched on surface, Concave |
| 2 | Water B<br>$10^{-1}$<br>$10^{-2}$ | Dark yellow<br>Yellow | Plane, pigmented colonies were Observed Smooth, lawn formation |
| 3 | Water C<br>$10^{-1}$<br>$10^{-2}$ | Shiny white<br>Cream colored | Sticky colonies with regular Margin.<br>Lawn formation |
| 4 | Soil A<br>$10^{-1}$<br>$10^{-2}$ | Milky white Colonies<br>Cream Coloured | Sticky colonies with regular margin.<br>Lawn formation |
| **5** | Soil B<br>$10^{-1}$<br>$10^{-2}$ | Cream Colored<br>Cream Colored | Lawn formation<br>Lawn formation |
| 6 | Soil C<br>$10^{-1}$<br>$10^{-2}$ | Pale Yellow<br>Colonies were Observed | Lawn formation |
| 7 | Coal A<br>$10^{-1}$<br>$10^{-2}$ | Pale yellow, White coloured<br>Colonies were Observed<br>Pista coloured, White and pale Yellow. | Circular with regular Margin.<br>Irregular, some are circular. |
| 8 | Coal B<br>$10^{-1}$<br>$10^{-2}$ | Pale yellow, some are dark Yellow.<br>Cream coloured | Stretched, circular.<br><br>Granulated, stretched |

**Table 1.2:Morphological Characteristics of the Isolation**

| Sr. No | Isolates Name | Gram staining | Motility |
|---|---|---|---|
| 1 | CB $10^{-2}$ I | + Bacilli (Short Rods) | Motile |
| 2 | CA $10^{-2}$ IV | + Coccobacilli | Sluggishly Motile |
| 3 | CA III | + Cocci | Sluggishly Motile |
| 4 | CA $10^{-2}$ I | + Cocci | Highly Motile |
| 5 | CA $10^{-2}$ I | + Cocci | Non-Motile |
| 6 | CB IV | + Cocci (in chains) | Sluggishly Motile |
| 7 | CB $10^{-2}$ I | + Coccobacilli | Highly Motile |
| 8 | CA $10^{-2}$IV | + Bacilli (Rods) | Highly Motile |
| 9 | CA III | + Coccobacilli | Sluggishly Motile |
| 10 | CA $10^{-2}$ III | +Coccobacilli | Motile |
| 11 | CC $10^{-2}$ I | -Coccobacilli | Motile |
| 12 | CC $10^{-2}$ II SD | + Bacilli | Motile |
| 13 | CA II | + Coccobacilli | Highly Motile |
| 14 | WB $10^{-2}$ III | +Cocci | Sluggishly Motile |
| 15 | WA II | + Coccobacilli | Motile |

| | | | |
|---|---|---|---|
| 16 | WB I A Bottom | + Coccobacilli | Highly Motile |
| 17 | WB $10^{-2}$ II | + Cocci (Short chains) | Sluggishly |
| 18 | SB | + Coccobacilli | Motile |
| 19 | SA II A Top | +Coccobacilli | Motile |
| 20 | SA I $10^{-1}$A Top | + Cocci (Short bunches) | Motile |
| 21 | SA I A Top | -Coccobacilli | Motile |
| 22 | SB $10^{-2}$A Bottom | +Coccobacilli | Highly Motile |
| 23 | SA III A TOP | + Cocci | Motile |

**+: Gram positive -: Gram positive**

**Table 1.3:" IMViC" TEST**

| Sr. No | Isolated Name | MR | VP | Citrate | Indole |
|---|---|---|---|---|---|
| 1 | CB $10^{-2}$ I | + | - | + | - |
| 2 | CA $10^{-2}$ IV | + | - | + | - |
| 3 | CA III | + | + | - | - |
| 4 | CA $10^{-2}$ III | - | - | + | - |
| 5 | CA $10^{-2}$ I | - | - | + | - |
| 6 | CB IV | - | - | + | - |
| 7 | CB II | - | - | + | - |
| 8 | CB $10^{-2}$II | + | - | - | - |
| 9 | CB V | + | - | - | - |
| 10 | CB III A | + | - | + | - |
| 11 | CC $10^{-2}$ I | - | - | + | - |
| 12 | CC $10^{-2}$ II SD | - | - | - | - |
| 13 | CA II | - | - | - | - |
| 14 | WB $10^{-2}$ III | - | - | + | - |
| 15 | WA II | - | - | + | - |
| 16 | WB I A BOTTOM | - | - | + | - |
| 17 | WB $10^{-2}$ II | - | - | - | - |
| 18 | SB | + | + | - | - |
| 19 | SA II A Top | - | - | + | - |
| 20 | SA I $10^{-1}$A Top | + | - | - | - |
| 21 | SA I A Top | + | + | + | - |
| 22 | SB $10^{-}$ BOTTOM A | + | + | - | - |
| 23 | SA III A TOP | + | + | + | - |

**+ : Test is Positive - : Test is Negative**

**Table 1.4: SUGAR FERMENTATION**

| Sr. No | Isolates Name | Dextrose | | Lactose | | Manitol | |
|---|---|---|---|---|---|---|---|
| | | Gas | Acid | Gas | Acid | Gas | Acid |
| 1 | CB $10^{-2}$ I | - | + | - | + | - | + |
| 2 | CA $10^{-2}$ IV | - | - | + | + | - | - |
| 3 | CA III | - | - | - | + | + | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4 | CA $10^{-2}$ III | - | - | - | - | + | + |
| 5 | CA $10^{-2}$ I | - | + | - | - | - | - |
| 6 | CB IV | + | + | + | - | + | + |
| 7 | CB II | - | + | - | - | - | - |
| 8 | CB $10^{-2}$IV | - | + | - | + | + | + |
| 9 | CB V | - | - | - | - | - | + |
| 10 | CB III A | - | - | - | + | - | + |
| 11 | CC $10^{-2}$ I | - | + | - | - | + | + |
| 12 | CC $10^{-2}$ II SD | - | - | - | - | + | + |
| 13 | CA II | - | + | - | + | + | - |
| 14 | WB $10^{-2}$ III | + | + | + | - | - | + |
| 15 | WA II | - | - | - | - | - | + |
| 16 | WB I A BOTTOM | + | + | + | - | + | + |
| 17 | WB $10^{-2}$ II | - | - | - | - | - | + |
| 18 | SB | + | - | - | + | - | + |
| 19 | SA II A Top | + | - | - | - | + | + |
| 20 | SA I $10^{-1}$A Top | - | + | - | + | - | + |
| 21 | SA I A Top | - | + | - | + | + | + |
| 22 | SB $10^{-2}$ BOTTOM A | + | - | - | - | - | - |
| 23 | SA III A TOP | - | + | - | + | + | + |

**+ : Gas Production** **- : No Gas Production**

## 1.5: LYSINE DECARBOXYLASE TEST, CATALASE TEST AND CYTOCHROME OXIDASE TEST

| Sr. No | Isolates Name | Lysine Decarboxylase Test | Catalase Test | Cytochrome Oxidase Test |
|---|---|---|---|---|
| 1 | CB $10^{-2}$ I | - | + | + |
| 2 | CA $10^{-2}$ IV | - | + | + |
| 3 | CA III | - | + | + |
| 4 | CA $10^{-2}$ III | + | + | + |
| 5 | CA $10^{-2}$ I | + | + | + |
| 6 | CB IV | + | + | + |
| 7 | CB II | + | + | + |
| 8 | CB $10^{-2}$IV | - | + | + |
| 9 | CB V | - | + | + |
| 10 | CB III A | - | + | + |
| 11 | CC $10^{-2}$ I | - | + | + |
| 12 | CC $10^{-2}$ II SD | - | + | + |
| 13 | CA II | - | + | + |
| 14 | WB $10^{-2}$ III | + | + | + |
| 15 | WA II | - | + | + |
| 16 | WB I A BOTTOM | - | + | + |
| 17 | WB $10^{-2}$ II | + | + | + |
| 18 | SB | - | + | + |
| 19 | SA II A Top | - | + | + |
| 20 | SA I $10^{-1}$A Top | - | + | + |
| 21 | SA I A Top | - | + | + |
| 22 | SB $10^{-2}$ BOTTOM A | - | + | + |
| 23 | SA III A TOP | - | + | + |

## Table 1.6: CULTURE CHARATERISTICS OF THE MICROORGANISMS ON DIFFERENT MEDIA:

| Sr.No. | Name of the Isolates | Nutrient Agar | Isolation Medium For Iron Bacteria | Iron Oxidizing Medium |
|---|---|---|---|---|
| 1 | CB $10^{-2}$ I | Granulated yellow coloured Colonies. | Milky white Growth along inoculation. | No Growth |
| 2 | CA $10^{-2}$ IV | Yellow coloured colonies on ocean blue colour. | Sticky milky white growth. | No Growth |
| 3 | CA III | White, granulated uniform colonies. | Milky white growth along inoculation | No Growth |
| 4 | CB $10^{-2}$ III | Greenish pigmented, sticky colonies. | No Growth | No Growth |
| 5 | CA $10^{-2}$ I | Medium changes to green on which yellow colonies | No Growth | No Growth |

| | | | | |
|---|---|---|---|---|
| | | were observed. | | |
| 6 | CB IV | Yellow, sticky colonies and medium changes to green. | No Growth | No Growth |
| 7 | CB II | Greenish white, sticky lawn formation. | No Growth | No Growth |
| 8 | CA $10^{-2}$ II | Cream coloured granulated uniform colonies. | Paper white coloured colonies | No Growth |
| 9 | CB V | White, sticky lubricant growth (cracky) | Milky white growth, flat type. | No Growth |
| 10 | CB III A | Yellowish lubricant growth | Paper white, sticky colonies. | No Growth |
| 11 | CC $10^{-2}$I | Pale yellow, granulated colonies. | Milky white, raised colonies. | No Growth |
| 12 | CC $10^{-2}$ II SD | Button like cream coloured, granulation colonies. | Milky white growth along inoculation. | No Growth |
| 13 | CA II | Cream coloured white, granulation colonies. | Paper white coloured, sticky growth. | No Growth |
| 14 | WB $10^{-2}$ III | Transparent pale-yellow coloured lubricant growth | White coloured, raised type colonies. | No Growth |
| 15 | WA II | Milky white, sticky lubricant growth. | Milky white growth along inoculation | No growth |
| 16 | WB I A BOTTOM | Creamy white granulated colonies. | No Growth | No Growth |
| 17 | WB $10^{-2}$ II | Creamy white granulated colonies | Milky white growth | No Growth |
| 18 | SB | Pale yellow, sticky, granulated, raised colonies. | Paper white, flat growth. | No Growth |
| 19 | SA II A TOP | Cream coloured, sticky colonies. | Cream coloured with sticky, rapid types. | No Growth |
| 20 | SBI $10^{-1}$ A TOP | Light orang, Sticky lubricant growth. | Paper white sticky inoculation. | No Growth |
| 21 | SA I A TOP | White, sticky lubricant growth. | Milky while growth along inoculation | No Growth |
| 22 | SB $10^{-1}$ BOTTOM A | Milky white lubricant growth. | While growth along inoculation. | No Growth |
| 23 | SA III A Top | Sticky, while, lubricant growth. | Milky white raised type growth. | No Growth |

**Table 1.7: ALBERT'S STAIN REACTION FOR IRON BACTERIA**

| Sr. No | ISOLATES NAME | Albert's Stains Reaction |
|---|---|---|
| 1 | CA $10^{-2}$ IV | Green coloured Black Inclusion bodies were observed |
| 2 | CA III | Green coloured Black Inclusion bodies were observed |
| 3 | CB V | Green coloured Black Inclusion bodies were observed |
| 4 | CB III A | Green coloured Black Inclusion bodies were observed |
| 5 | CC $10^{-2}$ I | Green coloured Black Inclusion bodies were observed |
| 6 | CC $10^{-2}$ II SD | Green coloured Black Inclusion bodies were observed |
| 7 | CA II | Green coloured Black Inclusion bodies were observed |
| 8 | WB $10^{-2}$ III | Green coloured Black Inclusion bodies were observed |
| 9 | WA II | Green coloured Black Inclusion bodies were observed |
| 10 | SB | Green coloured Black Inclusion bodies were observed |
| 11 | SA I A Top | Green coloured Black Inclusion bodies were observed |
| 12 | SA III A Top | Green coloured Black Inclusion bodies were observed |

## Conclusion

The present study revealed the microbial diversity of Gondegaon Open Cast Coal Mine, WCL, Nagpur area. More than 75% (18 out of 23) isolates are belonging to Iron Bacteria. Out of 18, 6 isolates were slow growers and needs more suitable conditions for growth. All the Iron bacteria showed distinct inclusion bodies of iron quenched within them. Other bacteria could not be isolated due to their fastidious nature.

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