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 10^{-3} 10^{-2}	Pale yellow Pale yellow (lemon)	Circular cracky colonies with smooth stretched Surface Having stretched on surface, Concave
2	Water B 10^{-1} 10^{-2}	Dark yellow Yellow	Plane, pigmented colonies were Observed Smooth, lawn formation
3	Water C 10^{-1} 10^{-2}	Shiny white Cream colored	Sticky colonies with regular Margin. Lawn formation
4	Soil A 10^{-1} 10^{-2}	Milky white Colonies Cream Coloured	Sticky colonies with regular margin. Lawn formation
5	Soil B 10^{-1} 10^{-2}	Cream Colored Cream Colored	Lawn formation Lawn formation
6	Soil C 10^{-1} 10^{-2}	Pale Yellow Colonies were Observed	Lawn formation
7	Coal A 10^{-1} 10^{-2}	Pale yellow, White coloured Colonies were Observed Pista coloured, White and pale Yellow.	Circular with regular Margin. Irregular, some are circular.
8	Coal B 10^{-1} 10^{-2}	Pale yellow, some are dark Yellow. Cream coloured	Stretched, circular. 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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