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Isolation and Characterization of Micro-organisms found in Lignite Coal in Bharuch Mines of Gujarat

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ABSTRACT

Gujarat provides a rich source of high-grade lignite coal accomplishing the fuel needs of the national economy, under Gujarat Mineral Development Corporation Ltd. However, the lignite mines in Bharuch district of Gujarat state are reservoirs to a huge microbial diversity, which might be important for various industrial applications. The present study focuses on the isolation and characterization of microorganisms found in the lignite coal from the Bharuch mines of Gujarat. The samples were resuspended in different diluents – water and Phosphate Buffered Saline and cultured on Nutrient Agar. Morphological characterization was done for the isolated strains followed by biochemical characterization for microbial identification. Based on the observations, two species were identified probably as *Streptococcus sp*. and *Staphylococcus sp*. on biochemical characterizations. The microbial isolates were further screened for their ability to produce lipase enzyme by qualitative screening tests for lipase production by Phenol Red Olive Oil Agar. All the isolates were tested positive for Lipase enzyme production were subjected to Tween-80 Hydrolysis tests for confirmation. This marks their potential to be studied for development of bioremediation strategies for crude oil contamination in soil or water bodies. This was followed by reviewing the use of such isolates for other applications including antioxidant studies, development of biodegradation strategies, etc.

Keywords: Microbial isolation from Lignite coal; Coal bacterial isolates; Lipase production.

I. INTRODUCTION

Gujarat is considered as a rich source of high grade lignite production in the world, with Gujarat Mineral Development Corporation Limited to be 2nd largest lignite producing company in India, serving the need of lignite fuel to state industries. However, coal is considered as a non renewable source of energy, is still a demanding energy source in most parts of the world for generating energy with high amounts of heat. Lignite, also being referred to as 'brown gold', is a low grade form of coal due to its low heat content. It is generally utilized for production of electricity at lower costs. However, lignite coal is a reservoir to

diverse population of microorganisms, including a wide range of bacterial [2] and fungal strains [3]. Very few organisms have been isolated and studied by different researchers and scientists around the world from different lignite mines. Due to the extreme conditions in coal, these areas are expected to harbour only few bacterial strains which are capable of growing in high acidic pH (acidophiles) or extreme heat conditions (thermophiles), and thus these organisms can be characterized under the category of extremophiles [1]. The ability of these bacteria to survive in such extreme conditions paves way for research towards isolation and identification of such strains from the microbial diversity and its utilization for varied purposes as bioremediation strategies [4]. Characterizing these bacteria help in understanding the phylogenic relationship between the isolated species and related ones, and thus promoting use of these organisms towards a wide range of applications related to the properties exhibited by them which include biosolubilization [5] and biodegradation strategies [6].

Many organisms isolated from such extreme environments have been studied and their property based applications are put forward by use of various morphological and biochemical tests, followed by different geo-microbiological and various other tests which may be related to development of the certain therapeutic approaches like antimicrobial studies. However, some of the organisms isolated from coal mines studied are *Bhargavaea cecembensis* [7], *Serratia sp.*, *Providencia sp.* [9], *Aspergillus sydowii* [10], *Methylobacterium sp.* [11], *Achromobacter xylosoxidans* [12]. Tests including gram staining, endospore staining, motility testing are few important tests studied for the morphological study of the bacteria identified in coal mines, followed by the biochemical tests [8] including Catalase test, nitrate reduction test, Indole test, MR-VP test, citrate utilization tests, starch hydrolysis, casein hydrolysis, TSI test.

Lipase is an enzyme isolated and studied worldwide from different microorganisms. This enzyme finds a crucial role in many industries including oil refineries, paper and pulp, pharmacy, etc. Crude oil contamination is a serious issue in many parts of the world, affecting the natural habitat of a particular area which may be soil or any water body. Thus, different screening tests and assays have been put forward in regards to the lipase production from different bacterial and fungal sources, in order to promote eco-friendly use of these enzymes rather than going for chemically synthesized methods of treatment methods in terms of bioremediation for crude oil contaminations.

This study focuses on isolation and characterizing microorganisms isolated from lignite mines located in Bharuch district of Gujarat, followed by screening them based on their ability to produce lipase enzyme as a means to promote their use in bioremediation and reviewing certain other applications based on the biochemical properties of the isolates.

II. METHODS AND MATERIAL

Sample Collection

Lignite coal sample was collected from the mines of Bharuch district of Gujarat, India, under proper supervision of Gujarat Mineral Development Corporation Ltd. The soil samples were packaged well and brought to lab maintaining aseptic conditions.

A. *Isolation of Bacterial strains*

Soil sample was serially diluted and spread on Nutrient Agar plates for microbial isolation. Serial dilutions were prepared with Phosphate Buffered Saline (PBS) solution [13].

B. *Characterization Tests*

Microbial isolates were identified using standard biochemical tests, for morphological and biochemical identification. The morphological characterization tests were carried for their size, color, cell wall

thickness, endospore formation ability and bacterial motility. Biochemical tests performed for the isolated bacteria from lignite sample were Catalase test, Indole Test, Methyl Red Test and Voges-Proskauer Test, Citrate Utilization Test, Starch Hydrolysis and Carbohydrate Fermentation Tests under recommended standard protocols.

C. *Lipase Activity Test*

Screening method for qualitative agar plate assay with Tween 80 and Olive oil as substrate was carried out. Lipase screening tests were conducted by streaking the isolates in phenol red olive oil agar plate [14] composed of 1mL/L Olive oil, 1g/L Calcium chloride, 20g/L Agar and 0.1g/L phenol red dissolved in 15mL distilled water and Tween 80 agar plate [15] composed of 10g/L peptone, 5g/L sodium chloride, 0.1g/L calcium chloride dihydrate, 20g/L agar and 10mL/L polysorbate-80 dissolved in 15mL of distilled water and incubating the plates at 37°C for 48 hours.

III.RESULTS

Bacterial growth with different diluents was observed after 48-72 hours of incubation at 37ºC and CFU was assessed and found to be 5.2 X 10^{-1} and 2 X 10^{-1} for S1 and S2 respectively.

A. *Morphological and Biochemical Characterization*

Morphological characteristics of the colonies selected for further characterization are described in Table 1. The isolates found positive for Lipase production were further subjected to biochemical characterization for microbial identification (Table 1). Grams' staining was carried out for shape determination and colony morphology. Endospore staining was also performed. The results of the morphological and biochemical characterization tests have been tabulated in Table 1 and Table 1 for isolated microbial samples from lignite coal samples.

B. *Lipase Activity*

Lipase screening was performed by allowing the growth of microbial isolates on Phenol red olive oil agar plate incubated at 37ºC for 24 hours. Lipase producing isolates were confirmed by Tween-80 screening medium, by formation of precipitates, indicating a positive result (Table 3, Figure 1).

Table 1: Morphological Characterization

S.No.	Bacterial Code	Cell Morphology	Gram Staining	Endospore staining	Motility
1	S1B2	Coccus (single)	+ ve	- ve	- ve
2	S1B4	Coccus (clustered)	+ ve	- ve	- ve
3	S2B2	Coccus (clustered)	+ ve	- ve	- ve
4	S2B3	Coccus (clustered)	+ ve	- ve	- ve

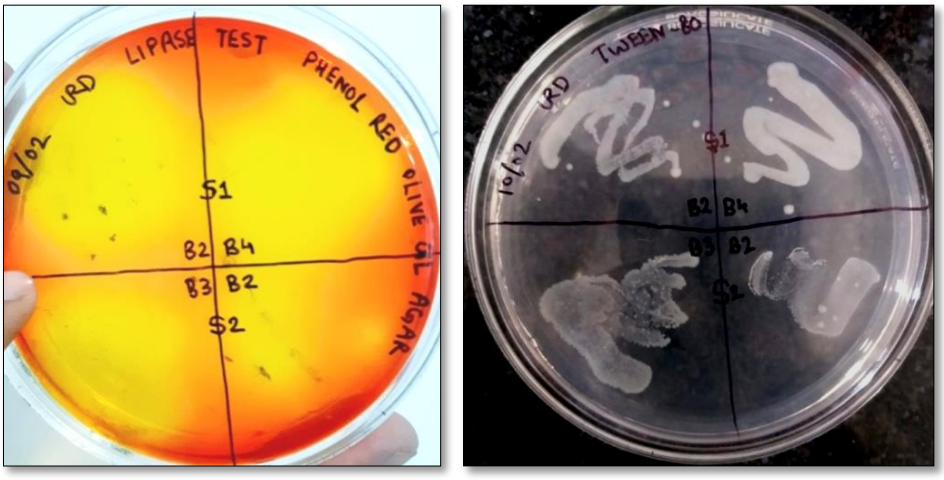
Table 2: Biochemical Characterization and Microbial identification

Isolate	S1B2	S1B4	S2B2	S2B3
Catalase Test	- ve	- ve	+ ve	- ve
Indole Test	- ve	- ve	- ve	- ve

MR	- ve	- ve	+ ve	- ve
VP	+ ve	- ve	- ve	- ve
Citrate Utilization Test	+ ve	+ ve	- ve	+ ve
Starch Hydrolysis Test	+ ve	+ ve	- ve	+ ve
Carbohydrate fermentation test	+ ve	+ ve with Gas production	+ ve with Gas production	+ ve
Probable species	*Streptococcus sp.*	*Streptococcus sp.*	*Staphylococcus sp.*	*Streptococcus sp.*

Table 3: Lipase screening of the microbial isolates

S. No.	*Bacterial Code*	*Phenol Red Olive Oil Agar*	*Tween-80 Hydrolysis*
1	S1B2	+ ve	+ ve
2	S1B4	+ ve	+ ve
3	S2B2	+ ve	+ ve
4	S2B3	+ ve	+ ve



(a) (b)

Fig. 1 Qualitative screening of Lipase Producing Bacteria: (a) shows positive result for lipase production by all isolates (S1B2, S1B4, S2B2, S2B3) by forming yellow color hydrolysis zone around the bacterial streaks in Phenol-red Olive Oil Agar media; (b) shows positive result for all bacterial isolates (S1B2, S1B4, S2B2, S2B3) by forming insoluble white crystals like around the bacterial streaks in Tween-80 hydrolysis tests

IV. DISCUSSION

Gujarat being a rich source of high-grade lignite, mined over different sites in the state, harbors a huge microbial diversity capable of producing different enzymes to be used for various applications in different fields. Many of them falling under the category of acidophiles and thermophiles find their use in different industrial fields. Coal mines consisting of an extreme environment can classify these bacteria to be a reservoir of enzymes, like amylases, which can be used in food waste biodegradation [16], acetoin production by making use of the butanediol pathway, which can be used in the food industry, soaps, detergents, etc [17] Catalase enzyme, acting as an important antioxidant enzyme acts as a key regulator of cellular oxidative stress response in animals [18]. The present study focussed on the bacteria isolated from the lignite mines of Bharuch district in Gujarat. The morphological and biochemical characterization tests conducted identified 3 of the isolates S1B2, S1B4 and S2B3 mostly to be *Streptococcus* sp. and S2B2 to be *Staphylococcus* sp.

Culturing bacteria from a coal mine is a difficult process because of the fact that coal provides an extreme environment to these microbes including extreme pH, pressure and temperature, which may not be provided in laboratory conditions, thus resulting in slow growing of the bacteria and few may not be able to grow. Isolates labelled S1B1, S1B3, S2B1 and S2B4 showed formation of small colonies in respective mother plates S1 and S2, but couldn't show growth during subculturing. Lack of proper environmental conditions makes such micro-organisms intolerable and thus resulting in slow or no growth.

Streptococcus sp. (S1B2, S1B4 & S2B3) isolated from the lignite coal sample are non-endospore forming spherical shaped strains falling under the category of gram positive bacteria, facultative anaerobes displaying no motility and unable to produce Catalase. The ability of these bacteria to hydrolyze potato starch confirms the production of amylase enzyme by these bacteria and marks their potential in the starch industries, enhancing national economy as well as consuming less time [19]. These bacteria are capable of fermenting glucose by liberation of carbon dioxide gas which is confirmed by change in media color from red to orange, and bubble formation, following an incubation period of 24 hours.

The isolate S2B2 identified as *Staphylococcus* sp. are non-endospore forming spherical shaped gram positive bacteria capable of producing Catalase enzyme. The following strain is considered to be slow growing bacteria due to the fact that mature colonies were seen only after a week even after enrichment using PBS as a diluent. These are not able to hydrolyze tryptophan into indole, pyruvate and ammonia by producing tryptophanase [21], confirmed by no colour change observation on adding Kovacs reagent to the tryptone broth tube. The amylase activity of these bacteria vary between strains as mostly all Staphylococcus spp. are capable of producing amylase, which is expected to vary far away from the ones produced by humans based on homology studies [20]. These bacteria are able to produce stable acid from glucose fermentation anaerobically [22] confirmed by formation of a cherry red ring on addition of methyl red indicator in the GPPW medium (MR-VP broth). The glucose fermentation is confirmed by allowing the bacteria to grow in Dextrose fermentation media and colour change from red to orange or yellow is observed and no bubble formation.

Lipases are group of enzymes hydrolyzing any fat or oil to fatty acids. Bacterial lipases are considered a great source of developing economy due to their potential in various industrial fields including dairy industry, pharmaceuticals, detergents, food industry, removal of pulp in paper industry, bioremediation, etc [23]. Change in colour of phenol red olive oil agar media shown by all the isolates from red colour to

yellow is due to the liberation of fatty acids in lipolysis by the bacteria indicating positive result for lipase production [24]. Whereas, the isolates were seen to form precipitates of insoluble crystals, which are actually calcium-fatty acid salts formed by hydrolysis of the Tween-80 (Ethoxylated sorbitan ester), indicating positive result for lipase production [25]. The bacterial isolates identified as *Streptococcus* sp. and *Staphylococcus* sp. from the Bharuch mines could be a boon to the research field as these bacteria possess a great source of bacterial lipases, providing eco-friendly solution for the treatment of oil-contaminated soil or water bodies, including development of bioremediation strategies for crude oil contaminated soils or water bodies.

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