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Isolation and Growth Culture of *Thiobacillus ferrooxidans* from Coal Mine Drainage of Harnai, Balochistan

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Abstract:
The bacterial strain having the ability of minerals bioleaching and desulphurization of coal has been isolated from coal mine drainage and hot spring water collected from the district Harnai, Balochistan. The recent work involves sample collection, growth culture, purification, and confirmation of bioleaching activity present in isolated strain with suitable evidence. The isolated *Thiobacillus ferrooxidans* bacteria showed the ability to decrease the pH of the medium during its growth along with the conversion of Fe (II) to Fe (III) state. The optimization of various parameters that affect the growth culture has been investigated suggesting the use of isolated bacterial strain for the production of sulphuric acid as well as for minerals leaching from respective ores and coal desulphurization.

Keywords: *T. ferrooxidans*, Growth culture, Isolation, Harnai, Balochistan.

INTRODUCTION

The bacteria are microorganisms that are well known due to their beneficial and harmful characteristics. There are various strains which have important applications in medicine (Iqbal and Ashraf, 2018; Iqbal *et al.*, 2019; Khalid *et al.*, 2016; Mouffouk *et al.*, 2019), food (Iqbal *et al.*, 2015; Jehan *et al.*, 2019), pesticides (Osadebe *et al.*, 2018), crops (Rasool *et al.*, 2019; Siyar *et al.*, 2019) and in mineral processing industries (Hafeez *et al.*, 2017). The extraction of metal by usual methods is very tedious and economically expensive. It was investigated that some microorganisms are responsible to liberate the valuable minerals from their ores (Zilouei *et al.*, 2003). This activity of mineral leaching is mostly observed in *T. ferrooxidans*, which are chemoautotrophic, gram-negative, rod-shaped, and flagellated (Kelly and Wood, 2000). This microorganism retrieves energy and reduction power from the oxidation of iron and reduction of sulfur present mineral ore body. During growth culture, it also produces sulphuric acid which shows a detectable decrease in pH of medium over time (Colmer *et al.*, 1950).

Some species of *T. ferrooxidans* are autotrophic and acidophilic growing in the presence of sulfur-containing compounds and ferrous iron along with and hydrogen present in formic acid (Cismasiu, 2010; Drobner *et al.*, 1990; Gou *et al.*, 2002; Ingledew, 1982). *T. ferrooxidans* and other bacteria having similar characteristics are of great economical importance for minerals extraction. It is previously reported that various minerals such as copper, uranium, gold, and antimony have been successfully leached from their low-grade ores (Hafeez *et al.*, 2017; Pronk *et al.*, 1992). *T. ferrooxidans* are also famous for the desulphurization of coal and now efforts are being made to use these microorganisms on pilot plant and industrial scale (Prayuenyong, 2002). It is further investigated that acidophilic sulfur-oxidizing bacterial strains are involved in the weathering process of sulfide minerals responsible for important environmental implications (Khadem Haghighat *et al.*, 2003).

Recently the world is facing two major problems; first is the source of energy and second environmental pollution. Solid fossil fuel may be used to reduce energy problems but unfortunately, most coal deposits have a major abnormality of containing more percentage of sulfur contents. The solution is only to reduce the sulfur by using these bacterial stains before the combustion of coal. On the other hand recovery of precious metals has to be done without interruption in the environment. In this regard the growth of bacterial strains, culture in the laboratory, and screening of suitable strain is necessary, various scientists reported about the growth, maintenance, and rapid monitoring procedures (Fecko *et al.*, 2006). The mode of action of *T. ferrooxidans* was also investigated and declared that some species of the bacteria may also work in an anaerobic situation, elemental sulfur oxidized by the action of ferric iron. In aerobic situations, active uptake of various amino acids may be energized from sulfur oxidation as well also from formic acid (Barron and Lueking, 1990).

The current study was conducted to isolate bacterial strains having the ability of minerals bioleaching and desulphurization of coal from coal mine drainage and hot spring water from the district Harnai- Balochistan.

MATERIAL AND METHODS

Bacterial Strain

Mine drainage and hot spring water were used as a source of bacterial strains. The mine water was collected from various coal mines of Balochistan (Shahrag-Khost-Harnai Coal Field). These samples were collected following precautions in the sterilized duly packed/sealed screw-capped bottles till shifting to the laboratory.

Medium Preparation Procedure

Growth culture of bacterial strain was carried out by using $FeSO_4$-based medium, containing (grams per liter) $(NH_4)_2SO_4$, 3.5; KCl, 0.116; K_2HPO_4, 0.058; $MgSO_4$ $7H_2O$, 0.058; and Ca $(NO_3)_2$, 0.00168. The basal salts solution was initially prepared in 420mL of distilled H_2O, adjusted to pH 1.9 with 10 N H_2SO_4, and sterilized by autoclaving. This solution was then combined with 580 ml of a filter-sterilized solution of $FeSO_4$ (74.674 g of $FeSO_4$ [heptahydrate] /580 ml of distilled H_2O [pH 1.9]) to yield 1 liter of medium containing a final Fe^{2+} concentration of 15 g/liter (Barron and Lueking, 1990).

Screening of bacteria

The basic salt of ferrous ammonium sulfate was combined with nutrient agar during the formulation of the semisolid medium. The plates were prepared and cooled under sterilizing conditions the source sample spread on the agar smoothly by an applicator. A control sample was prepared without the addition of a bacterial source sample.

Mass culture and purification of *T. ferrooxidans*

The samples were added in the laboratory prepared medium which was incubated at 37ºC for 12 days. The colonies produced on the semisolid medium were added to this medium. The solution was continuously circulated by passing air from the bottom of the container, as it is vital for oxygen and carbon requirements of microorganisms. The pH of the solution was maintained by using dilute sulphuric acid throughout the studies. The growth of organisms was observed by using slide preparation under an optical microscope. It was also confirmed by changing color from light green to reddish-brown. The pH of the solution decreased gradually as the growth of *T. ferrooxidans* increases in the growth medium. When the pH of solution decreases up to 2.6 most bacterial strains of irrelevant environment automatically eliminated. This procedure was found suitable for the purification of *T. ferrooxidans.*

Microscopic Examination

Colonies of bacterial strain were produced on the agar-based medium surface. A small portion of the colony was blended in a minute amount of medium and applied on the slide for microscopic examination. Studies revealed that it contains a lot of bacterial strains along with *T. ferrooxidans*.

Estimation Assay

Samples for ferrous iron determination were prepared by filtration of microbiological culture through a 0.2-pm membrane (Millipore Corp., Bedford, and Mass). Ferrous iron was determined by titration with potassium dichromate according to ASTM (E246-10, 2015). The pH of the solution was gradually decreased as the growth of *T. ferrooxidans* proceeds in the medium continuously measured with a combined pH glass electrode (Metrohm AG, Herisau, Switzerland).

RESULTS AND DISCUSSION

T. ferrooxidans mostly occur in the soil adjacent to sulfide mineral ores, coal mines drainage, and hot springs water, where the concentration of elemental sulfur is high (Ijaz *et al.*, 2017). These strains are mostly sulfur dependent and remain alive at high temperatures.

Screening of *T. ferrooxidans*

As indicated in Figure 1 after the 3rd day of incubation, colonies were observed on agar plates on which source samples were applied however in control there were no colonies. Previous studies have reported the isolation of *T. ferrooxidans* from coal mines (Patel, 2010).

Fig. 1. Colonies of bacterial and fungal strains on the nutrient agar medium.

Confirmation and isolation of *T. ferrooxidans* on semisolid medium

The colonies of *T. ferrooxidans* were collected and dissolved in a little amount of 9k medium and further applied on iron-containing agar-based semisolid medium. After 5-7 days incubation at 37ºC, colonies of *T. ferrooxidans* appeared on the surface of agar as shown in Figure 2. The conformation of strain and purification was indicated by producing similar colonies of reddish-brown color on a solid surface as reported previously (Patel, 2010).

Fig. 2. Colonies of *T .ferrooxidans* cultured on nutrient agar showing conversion of Fe^{++} in Fe^{+++} (Ferric).

Microscopic Examination of Isolated *T. ferrooxidans*.

The isolated bacterial strain was examined under the microscope and it is revealed that isolated strain is somewhat rod-shaped, gram-negative, and chemoautotrophic as illustrated in Figure 3. These results are in accordance with several previous studies (Baker and Banfield, 2003; Patel, 2010).

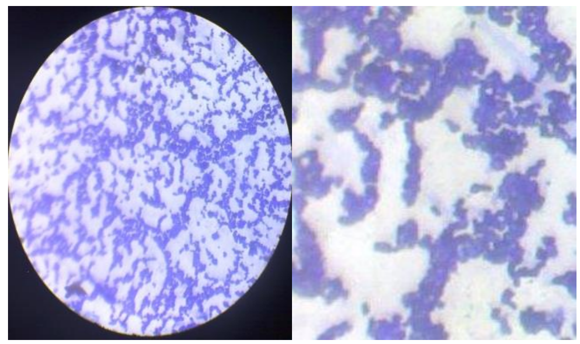

Fig. 3. Microscopic view of the isolated bacterial strain.

Growth of isolated strain in broth culture

These microorganisms are mostly used in the bioleaching of minerals and coal desulphurization, so these are usually required in broth culture. The color of the medium was also observed which was changed from greenish to brown due to change of iron oxidation state. After 216 hours it was observed that Fe^{++} begins to start changing in Fe^{+++} as shown in Figure 4. The pH of the solution medium gradually decreased up to 1.47. The results are mentioned in Figure 5. The total ferrous Iron was completely changed in the ferric state on the 12th day. After 15th days of incubation, the strain solution was removed and preserved for bioleaching and coal desulphurization trials. These microorganisms showed approximately the same optical parameters of growth as reported in the literature (Barron and Lueking, 1990; Patel, 2010).

(a) (b)

Fig. 4. Broth culture in 9k medium on; **a)** first day &, **b)** after 12th days.

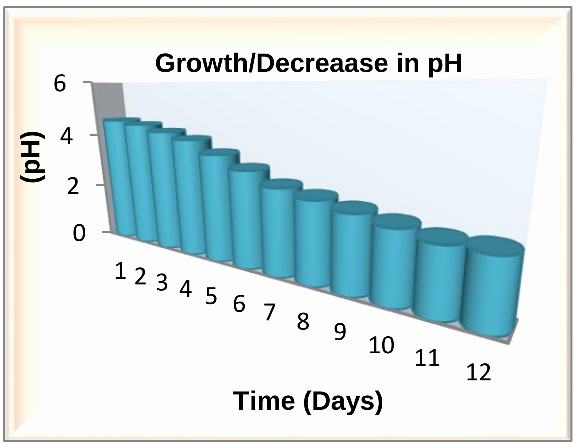

Fig. 5. Growth of *T. ferrooxidans* showing lowering pH of the medium.

CONCLUSION

The ability of bacterial strain *T. ferrooxidans* is very useful for the leaching of sulfide minerals such as copper, antimony, zinc, and lead, etc. The bacterial strains having the industrial application are often very expensive in the commercial world. The growth cultures of these strains from local sources are good alternatives for industrial applications. In the present investigation culture of *T. ferrooxidans* was carried out by simple and economical techniques. The microorganisms were confirmed clearly on a semisolid agar-based medium. This investigation will provide data about sample collection, isolation, and purification of bacterial strain from a related area. It is also observed that isolated strain may be used for the production of sulphuric acid as well as significant for minerals leaching from respective ores and coal desulphurization. The precious metals such as gold, silver, and titanium that are associated with other minerals could be extracted in solution with the assistance of these isolated microorganisms.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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