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Isolation and Characterization of Acidophilic Sulphur and Iron Oxidizing *Acidithiobacillus ferrooxidans* from Black Shale

Shahroz Khan[1], Faizul Haq[2*], Fariha Hasan[1], Kausar Saeed[3], Rahatullah[3]

[1]*Department of Microbiology, Quaid-i-Azam University, Islamabad. Pakistan*

[2]*Department of Botany, Hazara University, Mansehra. Pakistan*

[3]*Department of Zoology, Hazara University, Mansehra. Pakistan*

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Abstract

In present study, the acidiphilic sulphur and Iron oxidizing Bacterium *Acidithiobacillus ferrooxidans* were isolated and characterized from black shale grown on solid and liquid medium. The specimens were taken from the black shale, Tarbela (Gazi) and water samples from Chamyari Pakistan. The indigenous strain of *Acidithiobacillus ferrooxidans* were Gram-negative, motile, and rod-shaped bacteria. *Acidithiobacillus ferrooxidans* oxidized iron and reduced sulfur compounds like thiosulfate and tetrathionate. The bacterial oxidation of pyrite and reduced sulfur compounds produced sulfuric acid. Different biochemical activities like starch hydolysis, gelatin hydrolysis, hydrogen sulfide production, catalase reaction, urease test, indole production, methyl red test, voges proskaur, citrate utilization and triple sugar iron test of the isolates were performed and picturized. Some carbon sources like glucose, sucrose, fructose, raffinose, d-sorbitol, galactose, lactose, maltose, rahammanose and mannose were given to check the growth of isolates.

*Corresponding Author: Faizul Haq ✉ faizulhaq80@yahoo.com

Introduction

Acidithiobacillus ferrooxidans oxidize iron (II) and recover metals from low-grade sulphide ores and catalyses the oxidation of sulphur compounds to sulphuric acid (Tuovinen *et al*., 1971). Different studies on the rate of iron (II) oxidation by sulphur grown cells have reported contradictory results (Sugio *et al.,* 2008). The acidiphilic microorganisms that take part in dissolution of metals from the sulfide ores are autotrophic in nature and can grow in inorganic medium having low pH values and can tolerate high metal ion concentrations. The two main functions of this type of bacteria are oxidation of Fe^{2+} to Fe^{3+} and S to H_2SO_4. *Acidithiobacillus* species are rod-shaped, gram-negative, non-spore forming and mesophilic except the thermophilic *Acidithiobacilli*, which can grow at a higher temperature (Dopson *et al.,* 2003). In biooxidation of minerals iron and sulfur oxidizing chemolithrophic bacteria and archaea are responsible for producing the ferric iron and sulfuric acid required for the bioleaching reactions (Rawling *et al*., 2003). These microbes have a number of features in common including; a) They grow autotrophically by fixing CO_2 from the atmosphere, b) They obtain their energy by using either ferrous iron or reduced inorganic sulfur compounds (some use both) as an electron donor, and generally use oxygen as the electron acceptor, c) They are acidophiles and grow in low pH environments (pH.1.4 to 1.6 is typical), d) They are remarkably tolerant to a wide range of metal ions (Dopson *et al*., 2003).

Among the mesophiles, the most popular and widely used strain is *Acidithiobacillus ferrooxidans* (Drobner *et al.,* 1990). Although many strains of *Acidithio-bacillus ferrooxidans* have been isolated from different sources, most of the strains showed the following optimum growth conditions, i.e. pH 1.5–2.5 and a temperature range of 28–37°C (Karavaiko *et al*., 1989). *Acidithiobacillus ferrooxidans* being a lithotroph derives energy for its growth by oxidizing Fe^{2+} to Fe^{3+} and sulfur, sulfide and different oxyanion of sulfur to sulfate. Bioleaching, a general term refers to the conversion of an insoluble metal *(e.g*. CuS) into a soluble form (usually SO_4^{2-}) by biological oxidation and by applying microbes (Rawlings *et al.,* 2003). Metals for which this technique is mainly employed for recovery includes, copper, cobalt, nickel, iron, sulphur, zinc and uranium. For recovery of gold and silver the activity of leaching bacteria is applied only to remove interfering metal sulfides from ores bearing the precious metals prior to cyanidation treatment (Rohwerder *et al.,* 2003). The application of bacterial leaching to metal recovery from mineral ores has progressed steadily in the last 20 years (Olson *et al*., 2003).

It is assumed that heterotrophic leaching should provides better extraction of metals ions from organometallic compounds in shale ores. Bioleaching is carried out by astonishing diverse groups of bacteria. At least 11 putative prokaryote divisions can be related to this phenomenon (Rohwerder *et al.,* 2003). The most common microorganisms belong to the genera *Acidithiobacillus* and *Leptosprillium* which are mesophile, acidiphilic and chemolithoautotrophe. They obtain energy from oxidation of either ferrous ion to ferric or reduction of sulfur compounds to sulfuric acid (Rawlings *et al*., 2003).

Thinly bedded shale that is rich in carbon, sulfide, and organic material formed by anaerobic decay of organic matter (Farbiszewska *et al.,* 2004). A dark, thinly laminated carbonaceous shale, exceptionally rich in organic matter (5% or more carbon content) and sulfide (esp. iron sulfide, usually pyrite), and more commonly containing unusual concentrations of certain trace elements (U, V, Cu, Ni). Fossil organisms (principally planktonic and nektonic forms) are commonly preserved as a graphitic or carbonaceous film or as pyrite replacements. Shale is the most common sedimentary rock (Grobelski *et al.,* 2007). The process in the rock cycle which forms shale is

compaction. The fine particles that compose shale can remain in water long after the larger and denser particles of sand have deposited. Shales are typically deposited in very slow moving water and are often found in lake and lagoonal deposits, in river deltas, on floodplains and offshore of beach sands. They can also be deposited on the continental shelf, in relatively deep, quiet water (Farbiszewska *et al.,* 2004). Black shales are dark, as a result of being especially rich in unoxidized carbon. Common in some Paleozoic and Mesozoic strata, black shales were deposited in anoxic, reducing environments, such as in stagnant water columns (Kiczma *et al.,* 2004). Fossils, animal tracks/burrows and even raindrop impact craters are sometimes preserved on shale bedding surfaces. Shales may also contain concretions (Farbiszewska *et al.,* 2004; Grobelski *et al.,* 2007).

The aims and objectives of present study were; to evaluate bioleachability of Polkowice black shale ore, to establish reliable lab scale pilot operations of process and to optimize configuration and settings.

Materials and methods

The present research work was conducted at Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University Islamabad. The soil samples of black shale were collected from Tarbela, (Gazi) and water samples from Chamyari, N.W.F.P (Pakistan).

Serial dilution of the samples were done, 10 screw capped tubes were used for serial dilution. About 9 ml of sterilized saline was taken in each tube. One gram of soil (black shale) was added to sterilized saline. After shaking it well, 1 ml of the suspension was transferred to tube 1 asceptically. The tube was shaken and from this tube, 1 ml of the dilution was then transferred to tube 2. Similarly, the 10^{th} dilution was prepared by transferring 1 ml to the next tube, under aseptic conditions. Same process was repeated for all other samples. After serial dilutions 0.1 ml was taken from tube 5 of water sample and 0.1 ml from soil samples and spread on the growth medium plates. The plates were incubated on 30°C for 24 hours.

1) For the growth of bacterial strains, iron Liquid medium (9kFe^{2+}) and Glucose medium were used.

2) A new efficient (Gelrite-$FeSO_4$) solid medium was developed and successfully employed for isolation and enumeration of *At. ferrooxidans* in mine water, microbial leached solutions and solid samples. Gelrite-$FeSO_4$ solid medium was routinely used in the present studies. Three separate solutions were prepared and mixed after autoclaving.

Isolation and enumeration of Acidithiobacillus ferrooxidans

After sampling, 1.0 ml aliquot of each liquid sample was inoculated separately into liquid iron (9kFe^{2+}) medium. These flasks were incubated at 30°C at 150 rpm. The presence of iron-oxidizing bacteria in liquid iron medium was indicated by the formation of ferric iron and the medium becoming brick red in color. A serial dilution of each culture was performed using sterile saline. About 0.1 ml amounts from each dilution was spread on solid Gelrite-$FeSO_4$, plates. The inoculated plates were incubated at 30°C in a sealed polyvinyl-bag to block moisture evaporation. Plates were examined with the naked eye to observe the colony size, shape, color, and other morphological features. Of the bacterial growth single colony of iron-oxidizing bacteria (*At. ferrooxidans*), were picked from the plates by using a sterile inoculating loop and inoculated into 25 ml sterilized vials containing 10 ml liquid iron medium, pH 2.0 and was vortexed to spread the colony. All the cultures were incubated at 30°C until the color of the medium changed to brick red indicating ferrous iron (Fe^{2+}) oxidation by iron-oxidizing bacteria. To check the purity of isolated cultures, cells growing in liquid iron medium were spread on solid Gelrite-FeSO4 plates. After 5-7 days, the plates were examined for the required colonies and

were sub cultured on fresh liquid iron (9kFe^{2+}) and sulfur medium.

Biochemical characteristics of the Acidithiobacillus ferrooxidans were studied by

- The extra cellular Enzymes activities were determined by Starch hydrolysis and Gelatin hydrolysis.
- The Intra cellular Enzymes activities were studied by Hydrogen sulfide production, Catalase reaction, Urease test, Indole production test, Methyl red test, Voges Proskauer test, Citrate utilization andTriple sugar- iron test.

Effect of carbon sources on growth of bacteria

Different carbon sources were used for the growth of *Acidithiobacillus thiooxidans* and *At. ferrooxidans*. The different carbon compounds (1%) were: Glucose, Sucrose, Fructose, Raffinose, D-sorbitol, Galactose, Lactose, Maltose, Rhammanose, Mannose. The o.d of these carbon sources was taken at 440 nm, after 24, 48, 72, and 96,120,144 and up to 216 hours, to check the growth of the isolates.

Results

Isolation of Iron- and Sulfur-Oxidizer (Acidihiobacillus ferrooxidans)

For isolation of acidophilic iron and sulfur-oxidizing (*At. ferrooxidans*) bacteria from soil and water samples from Tarbela and Chamyari, an appropriate amount (100 µL) of liquid sample was inoculated onto solid Gelrite-$FeSO_4$ medium (Khalid *et al*. 1993). After 5-7 days of incubation at 30°C, reddish-brown colonies of iron-oxidizing bacteria were developed on the plates. These reddish-brown colonies growing on Gelrite-$FeSO_4$ plates were picked and cultivated separately into liquid iron medium (9KFe^{2+}). After 3-5 days of incubation at 30°C and 150 rpm under shaking condition, the medium became reddish-brown due to bacterial oxidation of Fe^{2+} to Fe^{3+}. The harvested cells of iron-oxidizing bacteria were inoculated onto solid Gelrite-$FeSO_4$ plates. Such ordinary purification procedures were repeated several times, finally pure cultures were obtained.

Cell morphology and characterization of isolated strains

The compound microscopic observations of isolated strains of *At. ferrooxidans* revealed that these strains were Gram-negative, motile, and single rod-shaped bacteria, *At. ferrooxidans* oxidized Fe^{2+} to Fe^{3+}. Pyrite, sulfur and reduced sulfur compounds like thiosulfate and tetrathionate. The bacterial oxidation of pyrite, sulfur and reduced sulfur compounds produced sulfuric acid which followed a drop in initial pH-value of the medium.

Oxidation of Ferrous Iron (Fe^{2+}) by At. ferrooxidans

The ferrous iron oxidation by isolated strain of *At. ferrooxidans* was conducted in shake flasks containing iron liquid medium (9KFe^{2+}) containing 160 Mm $FeSO_4$ of pH-value of 1.5. It was observed that ferrous iron (Fe^{2+}) was completely oxidized to ferric iron (Fe^{3+}) by the isolated strain of *At. ferrooxidans* during 3-5 days of incubation time at 30°C and 150 rpm. In chemical control flasks, only a negligibe amount of ferrous iron was oxidized due to air-oxidation under the same experimental condition. After the gram's staining different biochemical activities were analysed. These activities were:

Starch hydrolysis

In case of *Thio*$^+$ the medium color was blue-black, indicated the absence of starch-splitting enzymes and represented a negative result while the in case of *Glu*$^+$ the starch was hydrolyzed, and a clear zone of hydrolysis arround the growth of organism, showed a positive result.

Gelatin hydrolysis

In both the cases of *Thio*$^+$ and *Glu*$^+$ the gelatin medium remained solid on refrigeration at 4°c indicating the lack of gelatinase.

Hydrogen sulfide

The hydrogen sulfide gas is colorless and was not visible. In case of *Glu*$^+$ ferrous ammonium sulfate in the medium served as an indicater by combining with gas, forming an insoluble black ferrous sulfide precipitate which showed the production of H_2S. While in case of *Thio*$^+$ the absence of precipitate was the sign of a negative result.

Catalase test

For both *Thio*$^+$ and *Glu,*$^+$ the catalase production was determined when the 3% H_2O_2 was added as a substrate to an appropriately incubated tripticase soya agar slant culture. Catalase was present and the chemical reaction mentioned was indicated by bubbles of free oxygen gas ($O_2\uparrow$). This was positive catalase test while the absence of bubble formation was a negative catalase test.

Fig. 1. Colonies of glucose oxidizing bacteria.

Urease test

When the organisms *Thio*$^+$ and *Glu*$^+$ were grown in the urea broth medium containing the pH indicator phenol red, the substrate urea was not split into its products, the presence of ammonia did not create an alkaline environment the phenol red did not turn to a deep pink. This was a negative reaction for the presence of urea.

Indole production

The absence of red coloration demonstrated that the substrate tryptophan was not hydrolysed by *Thio*$^+$ and *Glu*$^+$ indicated an indole negative reaction.

Methyl red test

In case of *Glu*$^+$ the Methyl red indicater in the pH range of 4 turned red and showed the presence of acid, which indicate of a positive result. While in case of *Thio*$^+$ with lower Hydrogen ion concentration the indicator turned yellow and was a negative result.

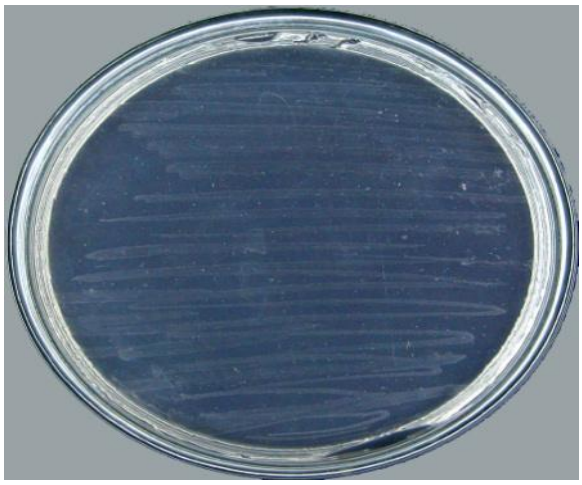

Fig. 2. Colonies of thiosulfate oxidizing bacteria.

Voges proskauer

In both the cases of *Thio*$^+$ and *Glu*$^+$ a deep rose color was developed, 15 minutes after the addition of Barritt's reagent which indicated the presence of acetyl-methylcarbinol and represented a positive result.

Citrate utilization

After incubation, citrate-positive *Glu*$^+$ was indicated by the presence of growth on the surface of the slant, accompanied by blue coloration. It was shown that *Glu*$^+$ has used citrate as a carbon source. While the *Thio*$^+$ slant color was not changed which indicated that the citrate was not used as a carbon source by *Thio*$^+$ and the result was negative.

Fig. 3. Coversion of Fe^{2+} into Fe^{3+} by *Acidithiobacillus ferrooxidans* at 300C, 150 rpm.

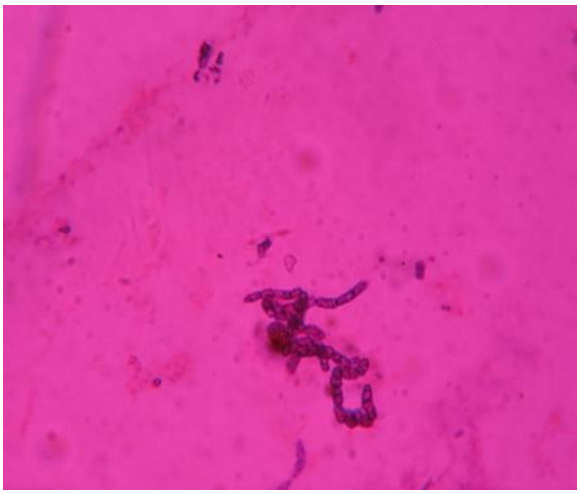

Fig. 4. Glu+ bacteria after gram's staining.

Triple sugar iron

In case of *Thio*$^+$ the color change showed the carbohydrate fermentation activity and the result was positive while in case of *Glu*$^+$ the color was not changed showing that the carbohydrate fermentation has not taken place and the result was negative.

Effect of Carbon sources on growth of bacteria

Glucose

The growth pattern of *Glu*$^+$ at 1% glucose concentration in 9k medium indicated that glucose was utilized as carbon source and growth of the isolate was maximum after 48 hours of incubation, stationary phase. By further incubation the growth decreased after 120 hours (Decline phase). While the *Thio*$^+$ on 1% glucose showed maximum growth after 96 hours of incubation.

Sucrose

The result indicated that the *Glu*$^+$ has started growth in 1% sucrose after 24 hours of incubation (lag phase). By further incubation at 24 hours intervals up to 120 the growth was increased during log and stationary phases. The growth of *Thio*$^+$ on the above mentioned medium, after incubation for 24 hours the growth started (lag phase) first in lag phase up to 48 hours. After 96 hours of incubation it was stationary phase growth.

Fructose

On 1% fructose concentration the growth of *Glu*$^+$ reached stationary phase after 72 hours of incubation. Further incubation up to 120 hours showed the (decline phase). While the *Thio*$^+$ on 1% fructose (9k medium) after incubation of 72 hours has optimum growth and after the 96 hours the growth was at peak. In both cases the phase was stationary as mentioned before.

Raffinose

The growth of *Glu*$^+$ on 1% raffinose after 48 hours of incubation, showed lag phase up to 72 hours. By further incubation up to 96 hours the log phase started where the growth reached to maximum (Fig. 33). In the case of *Thio*$^+$ started the growth after incubation of 120 hours on same medium (stationary phase).

D-sorbitol

The growth of *Glu*$^+$ on 1% D-sorbitol started after 24 hours of incubation. The incubation was continued up to 144 hours showing (log, stationary and decline) phases. In case of *Thio*$^+$ the growth on the same medium was started at 24 hours and peaked after 72, 96 hours (log and stationary) phases.

Galactose

The results showed that the growth on (mineral salt medium) containing Galactose 1% by the *Glu*$^+$ started after incubation of 24 to 216 hours passing through all lag, log, stationary and decline phases. While in case of

Thio$^+$ the growth on same medium was maximum after 96 hours incubation and immediately dropped.

Lactose

On lactose 1% mineral salt or 9k medium the *Glu*$^+$ started growth after the incubation of 120 hours (stationary) phase, the growth was maintained up to 192 hours. By further incubation the (death phase) started. While on the same medium *Thio*$^+$ has started growth after 24 hours incubation passing through (lag, log, stationary and decline) phases.

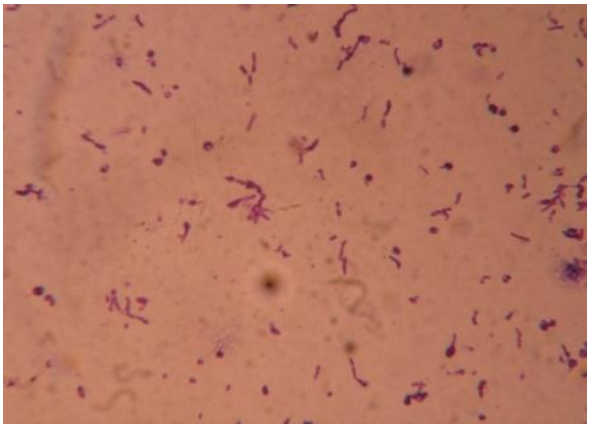

Fig. 5. Thio$^+$ bacteria after gram's staining.

Maltose

The growth pattern of *Glu*$^+$ at 1% maltose concentration in 9k medium showed clear results, indicated that maltose was utilized as carbon source and growth of the isolate was maximum at 144 hours of incubation. The growth shows both (log and stationary) phases. More incubation was showed the more growth and there was no death phase. While the *Thio*$^+$ on the above mentioned medium shows all four Phases.

Rahammanose

The result showed that the *Glu*$^+$ has grown on 1% rahammanose after the incubation of 48 hours. The graphic result passes all the stages of growth. While in case of *Thio*+ the result is different in which only stationary and death phases after the incubation in same medium.

Mannose

The growth pattern of *Glu*$^+$ on 1% mannose (mineral salt medium) indicated that the growth has been started after the incubation of 48 hours. On the incubation up to 216 hours. Three growth phases have been showed. In stationary phase the growth was on peak. There was no death phase. Otherwise the *Thio*$^+$ on the same

Isolation and characterization

Indigenous *Acidithiobacillus* were isolated from the soil (black shale) and water samples. In the tailing of soil and water samples microbial populations of *At. ferrooxidans*, *At. thiooxidans* and glucose-oxidizing heterotrophs were detected. Similar results have been reported by (Tuovinen *et al*., 1971).

The isolate oxidized Fe^{2+}, pyrite, sulfur and reduced sulfur compounds to sulfuric acid and acidic ferric/ metal sulfate. Different solid medium for simultaneous growth of *At. ferrooxidans* and acidiphilic heterotrophs have been used. A new efficient Gelrite-$FeSO_4$ solid medium was developed and successfully employed for isolation and enumeration of *At. ferrooxidans* in mine water, tailings liquid (Khalid *et al*., 1993).

Dark reddish-brown and circular colonies which were robust and well differentiated, developed on Gelrite-$FeSO_4$ medium within 72-96 hours. The growth of *At. ferrooxidans* was indicated by the drop in initial pH of the liquid media of sulfur, slag and pyrite, and by the acid production and/ or oxidation of ferrous to ferric.

At. ferrooxidans were found in abundance in tailings of water and liquid samples. The microbial population of iron-oxidizing bacteria (*At. ferrooxidans*) was found much higher in the black shale residues which were collected from one and two month old-dried tailings pile. In fact, tailings liquid contained a significant amount of $FeSO_4$ at the time when solid tailings residue was being dumped in the tailings pond of the mill after acid leaching of sandstone ore.

Elemental sulphur can be utilized as an energy source by iron and sulphur oxidizing bacteria to produce H_2SO_4 resulting in a pH drop of the tailings residue. *At. ferrooxidans* can also oxidize elemental sulfur in the presence of $Fe_2 (SO_4)_3$ at low pH (Pronk *et al.*, 1991).

Sulfuric acid thus produced can change the physical and chemical characteristics of tailing residues. The presence of glucose-oxidizing heterotrophs was also noted in tailings (black shale) liquid, microbial leach liquors and solid samples obtained from columns and heap. Microbial leaching is a biochemical process involving enzymes as catalyst by which insoluble inorganic substrate are oxidized to a soluble form (Torma *et al.*, 1977).

Metals are released from sulfide minerals directly through oxidative metabolism of microorganisms or solubilized indirectly by chemical oxidants such as ferric sulfate or sulfuric acid produced as metabolic products of microorganisms (Puhakka and Tuovinen 1986). In the present studies on bacterial oxidation of ferrous iron, it was observed that ferrous iron was completely oxidized to ferric iron by *At. ferrooxidans* during 72 hrs of incubation. Bacterial oxidation of pyrite by *At. ferrooridans* resulted in sulfuric acid production followed by a drop in the initial pH of the leaching system. *At. ferrooxidans* oxidizes ferrous iron at much faster rates as compared to a solely chemicals system. Sulfuric acid thus produced during the bacterial oxidation of sulfides, thereby accelerates the rate of metal solubilization. Sulfuric acid also neutralizes carbonate materials like calcite ($CaCO_3$) and dolomite (Ca, Mg) CO_3 present in the ore matrix**.**

Incomplete oxidation of the sulfide entity commonly occurs in the acid leaching process which results in the formation of polythionates and the precipitation of elemental sulfur. The latter effectively coats the metal sulfides and prevents their further oxidation until the sulfur is removed by bacterial oxidation. The microbial degradation of silicate minerals requires the availability of external energy substrates (Ramirez *et al.*, 2004).

Biochemical activities

Different biochemical activities of the isolates were performed. Starch hydrolysis test was performed in the case of *Thio*$^+$ strain, where as *Glu*$^+$ hydrolyzed the starch and indicated a positive result. This showed that *Glu*$^+$ was better hydrolyser of starch than *Thio*$^+$. Both the isolates were not able to hydrolyzed the gelatin. *Glu*$^+$ showed the production of hydrogen sulfide, by forming an insoluble black ferrous sulfide while in case of *Thio*$^+$, the absence of precipitate was the sign of negative result. Both the isolates were able to hydrolyze catalase as shown by the production of oxygen bubbles.

The isolates were urease negative for production. Isolates did not produce a red reagent in both the cases of *Thio*$^+$ and *Glu*$^+$. The absence of red coloration demonstrated that the substrate tryptophan was not hydrolysed and indicated an indole negative reaction. In methyl red test, the *Glu*$^+$ strain by the presence of acid indicated positive result. While the *Thio*$^+$ result was negative. In both *Thio*$^+$ and *Glu*$^+$ a deep rose color developed which indicated the presence of acetyl-methylcarbinol and represented a positive result for voges proskauer test. Citrate-positive *Glu*$^+$ was indicated by the presence of growth on the surface of the slant. Which was accompanied by blue coloration and showed that *Glu*$^+$ has used citrate as a carbon source. While in case of *Thio*$^+$ slant color, was not changed, which showed that the result was negative. In case of *Thio*$^+$, the color change showed the carbohydrate fermentation activity and the result was positive while in case of *Glu*$^+$ the color was not changed showing that the carbohydrate fermentation has not taken place and the result was negative in triple sugar iron.

Effect of Carbon sources on growth of bacteria The growth pattern of *Glu*$^+$ in the presence of 1% glucose in 9k medium indicated that glucose was utilized as carbon source and growth of the isolate was maximum at 48 hours of incubation. Whereas the strain *Thio*$^+$, on 1% glucose (9k medium) started the growth after 72 hours of incubation and after the 96 hours the growth was at peak. For *Thio*$^+$ the glucose was found to be good energy source as compared to that in case of *Glu*$^+$ strain.

The result indicated that the *Glu*$^+$ has started growth in the presence of 1% sucrose (9k medium) after 24 hours of incubation. The growth of *Thio*$^+$ on the same medium, after the incubation of 48 hours showed that for both the sucrose was good for growth. In the presence of 1% fructose (9k medium) the *Glu*$^+$ was growed after 72 hours incubation. Whereas *Thio*$^+$ strain on 1% fructose (9k medium) after the 96 hours of incubation, the growth was at peak. For *Thio*$^+$ the Fructose was found to be good energy source as compared to that in case of *Glu*$^+$ strain.

The strain *Glu*$^+$ growth in the presence of 1% raffinose (mineral salt medium) after 48 hours of incubation was at peak. Where as *Thio*$^+$ has started growth after the incubation of 120 hours on same above medium. On comparison the growth of *Thio*$^+$ was found to be better on raffinose than in case of *Glu*$^+$ strain. The growth of *Glu*$^+$ strain in the presence of 1% D-sorbitol (9k medium) was maximum after 144 hours of incubation. Where as in the case of strain *Thio*$^+$ on the same medium the growth was at peak 96 hours of incubation. It was concluded that *Glu*$^+$ utilized D-sorbitol as a good energy source as compared to strain *Thio*$^+$.

The results indicated that on (mineral salt medium) galactose 1% the growth of strain *Glu*$^+$ after the incubation of 216 hours was maximum. While in case of strain *Thio*$^+$ the growth on same medium shoot up on 96 hours of incubation and immediately dropped. This indicated that the *Glu*$^+$ strain utilized best galactose as a carbon source as compared to that in case of *Thio*$^+$ strain.

In the presence of lactose 1% (mineral salt medium) the strain *Glu*$^+$ started growth after the incubation of 120 hours and was maximum after 192 hours. Whereas on the same medium strain *Thio*$^+$ growth was maximum after 24 hours incubation. The conclusion was that *Thio*$^+$ strain was found to be better utilizer of Lactose as a carbon source than strain *Glu*$^+$.

In the presence of 1% maltose in 9k medium the strain *Glu*$^+$ indicated clear results, that maltose was utilized as carbon source and growth of the isolate was maximum at 144 hours of incubation. Where as the strain *Thio*$^+$ on the same medium indicated maximum growth after 120 hours of incubation. From this it was concluded that *Glu*$^+$ strain was found to be best utilizer of maltose as an energy source as compared to that in case of *Thio*$^+$ strain.

Result indicated that the strain *Glu*$^+$ was grown in the presence of 1% rhammanose (9k medium) after the incubation of 48 hours. Where as the result of *Thio*$^+$ was different in which after the incubation of 144 hours, the growth was at peak. This indicated that on rhammanose the *Glu*$^+$ strain growth was found to be better than *Thio*$^+$ strain.

The growth pattern of *Glu*$^+$ in the presence of 1% mannose (mineral salt medium) indicated that the growth after the incubation of 216 hours was maximum. Whereas the *Thio*$^+$ strain on the same medium showed growth after 96 hours of incubation. The conclusion was that strain *Glu*$^+$ was found to be best utilizer of mannose as compared to that in case of *Thio*$^+$ strain.

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