ISOLATION OF EXTERMOPHILES

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ISOLATION OF BACTERIA FROM COAL MINE SPOIL

6.1 INTRODUCTION

An overburden containing fresh coal mine spoil presents a physically disturbed habitat with almost all vital nutrients including organic carbon, total nitrogen and phosphorous deficient situation. Besides, rich sulfur and iron component in form of pyrite in the coal residues being exposed to air often results in the auto-oxidation, consequently leading to the burning of coal and rise of temperature in the coal mine soil heaps (Figure 1).

Figure 1. Fresh coal mine overburden site: (A) burning of coal overburden (B) auto-oxidation of pyrite present in the coal mine spoil.

Hydrogen sulfide, which is one of the outcomes of such burning, gets converted into sulfuric acid causing a decline in pH and hence resulting an acidic environment (Kristjansson and Stetter, 1995; Kristjansson et al., 2000). Thus, a thermo-acidic environmental gradient in the fresh coal mine spoil constrains the successional/developmental process of organisms including the microorganisms. Microbiological constraints of coal mine spoil have been the subject of soil microbiological research by several workers (Brock, 1978; Madigan et al., 1997). All of them pointed out the microbiological deficient situation in the fresh coal mine spoil heap that impedes the spoil reclamation process.
However, in such environmentally hostile situation, the expected specific microbial groups are thermo-acid tolerant and chemolithotrophic bacteria. Several groups of soil bacteria are able to use inorganic compounds or ions (ammonium, nitrite, thiosulphite, sulphite and ferrous ions) as well as elemental sulphur, hydrogen as electron or hydrogen donors, to gain energy and reducing equivalents for synthetic processes by their oxidation. This mode of adaptation with inorganic hydrogen donors is called “chemolithotrophy”. In chemolithotrophs, ATP synthesis is coupled to oxidation of electron donor. Reducing power is obtained either directly from the inorganic compounds or by reverse electron transport reactions. All chemolithotrophic bacteria assimilate their carbon from CO₂ by fixation (as their major source of carbon for cell synthesis) via the ribulose-biphosphate cycle. They are therefore also called as “autotrophs” [chemolithoautotrophs]. They may be aerobic or anaerobic. Energy generation in chemolithotrophs usually occurs by oxidative phosphorylation involving electron transfer chain of conventional and unconventional type. The yield of biomass in chemolithotrophic organisms is very less. This is mainly because very small amount of energy is obtained by the oxidation of inorganic substances. In addition, several of these bacteria also carry out uncoupled oxidation reactions i.e. they oxidize substrates without concomitant synthesis of cell materials (metabolic idling).

Since, the overburden residues of the studied coal mine are sulfur and iron rich, expected group of bacteria in the fresh mine spoil are chemolithotrophic, sulfur and iron oxidizers. A brief physiological resume of such oxidizers are presented in this section.

6.1.1 SULPHUR OXIDIZERS

In nature, sulphur exists in different forms like S⁰, SO₂, S₂O₃⁻², SO₃²⁻, S⁻². It is evident that sulphides are in highly reduced state where as sulphate is in highly oxidized state. Some of the microorganisms have developed a mechanism to oxidize the reduced sulphur compounds to get energy are known as “sulphur oxidizer”. Sulphur oxidizer grow near the source of sulphur i.e. sulphur mines, hot sulphur springs, coal mines that are sites for pyrite (FeS₂). Sulphur oxidizers are taxonomically heterogeneous group of bacteria. Some of the common eubacterial genera are *Thiobacillus*, *Thiosphaera*, *Thiimicroscopica*, *Thermothrix*, *Beggiatoa*, *Thioplaca*, *Achromatium*, *Thiovulum* etc. Most of the thiobacilli are obligate chemolithoautotrophs, but some of the species like *Thiobacillus novellas* and
*Thiobacillus intermedius* can also utilize organic compounds as carbon source and hence they are “chemoheterotrophs”. *Thiobacillus thioxidans* produces large amount of $\text{H}_2\text{SO}_4$ and grow as low pH. *Sulpholobus acidocalcarium* is a thermophilic species (grows at 75°C at pH 2-3) and it is facultatively chemolithotrophic. *Beggiatoa* and *Thiotrix* are filamentous forms whereas *Achromatium* and *Thiovulum* are unicellular forms.

Sulphur oxidizers oxidize sulphides, elemental sulphur and sulphite and ultimately convert them to sulphate. In all the cases, the end product is sulphate.

\[
\begin{align*}
S^2^- + 2\text{O}_2 & \rightarrow \text{SO}_4^{2-} \\
S + \text{H}_2\text{O} + \frac{1}{2}\text{O}_2 & \rightarrow \text{SO}_4^{2-} + 2\text{H}^+ \\
\text{S}_2\text{O}_3^{2-} + \text{H}_2\text{O} & \rightarrow \text{SO}_4^{2-} + 2\text{H}^+ 
\end{align*}
\]

Thiobacilli usually grow very well on thiosulfate. Two enzymes have been found to catalyze the oxidation of sulphite to sulphate: Sulphite oxidase- membrane bound molybdo-protein and transfer electron to cytochrome c (electron acceptor), and APS reductase further converted ammonium persulfate (APS) to sulphate and ADP accompanied by substrate level phosphorylation.

\[
\begin{align*}
\text{SO}_3^{2-} + \text{AMP} & \rightarrow \text{APS} + 2\text{e}^- \\
\text{APS} + \text{Pi} & \rightarrow \text{APS} + \text{SO}_4^{2-} \\
2\text{ADP} & \rightarrow \text{ATP} + \text{AMP}
\end{align*}
\]

### 6.1.2 IRON OXIDIZERS

In nature, iron exists in ferrous (reduced state) and ferric forms (oxidized state). Some bacteria oxidize the ferrous to ferric forms and utilize the energy released thereupon. The ferrous salts function as electron donors for these bacteria and such bacteria are called as “iron bacteria”. The acidophilic iron oxidizers can be found growing at the sites of geological deposits of iron sulphide minerals (pyrite: FeS$_2$ and chalcopyrite: CuFeS$_2$). Iron oxidizing bacteria are chemolithoautotrophic. Some of these bacteria are *Thiobacillus ferroxidans*, *Thiobacillus thioxidans*, *Sulfolobus acidocaldarius*. The oxidation of Fe$^{2+}$ to Fe$^{3+}$ is carried out at neutral pH but only slowly acidic pH. The acidic environment is conducive to growth of the iron oxidizers.
Iron oxidizing bacteria oxidizes Fe\textsuperscript{2+} to Fe\textsuperscript{3+} by a Fe\textsuperscript{3+} complex in the outer membrane. The electrons travel to a periplasmic cytochrome c. There is also a copper containing protein i.e. rusticyanin in the periplasm, which is thought to be part of the electron transport chain. When the bacteria are oxidizing pyrite, the Fe\textsuperscript{3+} that is produced outside the cell envelope is chemically reduced to Fe\textsuperscript{2+} by S\textsuperscript{2-}. The resultant S\textsuperscript{0} is oxidized to SO\textsubscript{4}\textsuperscript{2-} and the electron pass through a proton translocating bc\textsubscript{1} complex to periplasmic cytochrome c and there to cytochrome oxidase that brings protons into the cell. The bacteria are also responsible for the production of H\textsubscript{2}SO\textsubscript{4}. The ability of sulphur and iron oxidizing bacteria to convert sulphide ores to water soluble heavy metal sulphates, is utilized for the leaching of low grade ores.

\[
\begin{align*}
\text{FeS}_2 + 3\frac{1}{2}\text{O}_2 + \text{H}_2\text{O} & \rightarrow \text{FeSO}_4 + \text{H}_2\text{SO}_4 \\
\text{S}^0 + \frac{1}{2}\text{O}_2 + \text{H}_2\text{O} & \rightarrow \text{H}_2\text{SO}_4 \\
2 \text{FeSO}_4 + \frac{1}{2}\text{O}_2 + \text{H}_2\text{SO}_4 & \rightarrow \text{Fe}_2(\text{SO}_4)_3 + \text{H}_2\text{O} \\
\text{MoS} + 2\text{Fe}^{3+} & \rightarrow \text{Mo}^{2+} + 2\text{Fe}^{2+} + \text{S}
\end{align*}
\]

6.1.3 BACTERIOLOGICAL STUDY OF COAL MINE SPOIL

Several authors have attempted the bacteriological study in fresh coal mine spoil. Wilson (1957) reported a significance lower population of heterotrophic bacteria in fresh mine spoil. Milch (1988); Norris (1990) and Tyson \textit{et al.} (2005) reported lower microbial abundance and their activity in the fresh coal mine spoil. Absence of nitrogen fixing bacteria has been reported by Visser \textit{et al.}, (1979). Presence of acidophilic bacteria in the coal mine spoil have been documented by several workers (Dugan \textit{et al.}, 1970; Tuovinen and Kelly, 1974; Apel \textit{et al.}, 1976; Katayama-Fujimura \textit{et al.}, 1984; Wood and Kelly, 1986; Norris, 1990; Kristjansson and Stetter, 1992; Shennan, 1996; Rawlings \textit{et al.}, 1999; Kristjansson \textit{et al.}, 2000; Tyson \textit{et al.}, 2004). As per Baker and Banfield (2003), \textit{Proteobacteria}, \textit{Nitrospira}, \textit{Firmicutes} and \textit{Acidobacteria} dominate the microbial populations in coal mining site.

\textit{Proteobacteria} of coal mine spoil are divided into three groups, \(\gamma\); \(\alpha\); and \(\beta\)-proteobacteria. The most common proteobacteria are \textit{Acidithiobacillus} species (formally \textit{Thiobacillus}; Kelly and Wood, 2000), and is characterized as a mesophilic member of the \(\gamma\)-proteobacteria. Six species of heterotrophic \(\alpha\)-proteobacteria of the genus \textit{Acidiphilium} have been found (Baker and Banfield, 2003). \textit{Acidithiobacillus ferrooxidans} grows in soil, aquatic habitats and reduced inorganic sulfur compounds. This species is metabolically flexible and uses ferrous iron and reduced inorganic sulfur compounds as an electron donor, to produce
ferric iron and sulfuric acid (Prescott et al., 1999). *At. ferrooxidans* is a moderately thermophilic (optimum growth temperature 45°C), gram-negative, chemoautotrophic, sulfur-oxidizing acidophilic bacterium (Kelly and Wood, 2000; Baker and Banfield, 2003). The bacterium, previously called *Thiobacillus ferrooxidans*, has been renamed because the genus *Thiobacillus* included a collection of bacteria belonging to the α-, β-, and γ-subclasses of proteobacteria (Kelly and Wood, 2000). *Leptospirillum ferrooxidans* are commonly detected in coal mine overburden and bioleaching systems (Bond et al., 2000; Coram and Rawlings, 2002; Baker and Banfield, 2003). The isolate *Leptospirillum ferrodiazotrophum* is a member of *Leptospirillum* group III. These groups of organisms have the ability to carry out nitrogen fixation (Tyson et al., 2004).

The review thus indicated either low bacterial abundance or the presence of specific group of bacteria that have physiological adaptive strategy for thermo-acidic tolerance in the coal mine spoil.

6.1.4 OBJECTIVES OF THE INVESTIGATION

The present study was designed to isolate bacteria from fresh coal mine spoil with following objectives.

1. To see the presence of heterotrophic bacteria, if any and to study their thermal and acid tolerance ability.
2. To isolate chemolithotrophs such as *Acidithiobacillus ferroxidans* and *Thiobacillus thermosulfatus* and to study their growth pattern.
3. To study the thermal response of *Acidithiobacillus ferroxidans* and *Thiobacillus thermosulfatus*.

6.2 MATERIALS AND METHODS

6.2.1 SAMPLING

For the purpose of sampling, the coal mine area was visited during March 2007. Spoil samples (n = 10) were aseptically collected randomly in sterilized, plugged hard glass test tubes from the freshly dumped spoil overburden site approximately from 0-15cm depth. These samples were brought to the laboratory and mixed under aseptic condition to form a composite sample. The composite sample was maintained in sterilized 1 liter conical flask,
from which sub-samples of spoil were taken out for different microbiological isolation. All the microbiological isolation protocols were initiated either immediately or within 12 hrs of the collection of spoil samples from the overburden site.

6.2.2 ISOLATION OF HETEROTROPHIC BACTERIA

Spoil sub-samples (approximately 1g) were transferred to 50ml sterilized conical flasks each containing 10ml of sterilized double distilled water and subjected to stirring gently for 10min to get spoil water suspension. Then, 1ml of the suspension was aseptically taken and subjected to serial dilution upto $10^5$ times. From the final $10^5$ dilution, 100μl of the diluted spoil water suspension was transferred to a sterilized petridish containing 30ml of nutrient agar medium by spread plate technique inside the laminar flow apparatus.

In this way, five replicates of spread plate petridishes were prepared. All the petridishes were incubated at 37°C being stacked upside down for 24 hrs. Petridishes were subjected to intermittent inspection for the growth of bacterial colonies. Discrete bacterial colonies were observed after 24 hr of inoculation. Colonies were recorded with respect to their morphology, colour and growth pattern. Subsequently, bacterial inoculums form each colony was transferred to nutrient agar slants for the purpose of pure culture. Cells from each of the pure culture were then studied for their responses to Gram’s stain and shape under the microscope.

Isolated heterotrophic bacteria were then studied for their thermal and acid tolerance ability. For the purpose of the thermotolerance ability, bacterial inoculums form each pure culture was aseptically transferred to different culture tubes (20ml capacity) containing sterilized nutrient broth. The tubes were incubated at different temperature (40°C, 45°C, 50°C, 60°C and 65°C) for a period of 12 hr. Observation of bacterial growth was noted with respect to different temperature after 12 hr.

Further, for the purpose of acid tolerance study, bacterial cells were inoculated into different nutrient broths with adjusted pH. The pH of different broths was adjusted with addition of 1N H₂SO₄ for different pH (pH- 7, 6, 5, 4, 3 and 2). The broths were incubated at 37°C and bacterial growth was studied by observing turbidity of the culture.
6.2.3 ISOLATION OF *Acidithiobacillus ferroxidans*

Spoil sub-samples (approximately 1g) were transferred to 50ml sterilized conical flasks each containing 10ml of sterilized double distilled water and subjected to stirring gently for 10min to get spoil water suspension. For isolation, 1ml of the spoil water suspension was inoculated into a sterilized 250ml Erlenmeyer flask containing 50ml of *Thiobacillus* specific broth (Colmer, 1962; Tuovinen and Kelly, 1974) having the following composition (per liter).

\[
\begin{align*}
K_2HPO_4 & : 4g \\
(NH_4)_2SO_4 & : 0.3g \\
KH_2PO_4 & : 1.5g \\
MgSO_4 & : 0.5g \\
Na_2S_2O_3 & : 10g
\end{align*}
\]

pH adjusted to 4.0 with 1N H_2SO_4

The flask was then subjected to incubation at 35°C for 24 hrs in rotary shaker incubator for the growth of bacterial population. The bacterial suspension was then streaked onto an agar plate containing *Thiobacillus* agar medium and the plate was incubated at 35°C for 24 hrs. Isolated colonies on the agar plate were randomly picked up for preparation of pure culture. Isolated bacterium from the pure culture was studied further microscopically for their shape and Gram's stain response.

6.2.4 GROWTH ANALYSIS OF *Acidithiobacillus ferroxidans*

The isolated bacterium *i.e.* *Acidithiobacillus ferroxidans* from the *Thiobacillus* medium was further processed for the analysis of growth with respect to chemolithotrophic, heterotrophic and mixotrophic conditions. For this, isolated *Acidithiobacillus ferroxidans* bacterial cells from the pure culture was inoculated into three different types of broths *i.e.* chemolithotrophic, heterotrophic and mixotrophic broth (50ml) taken in three different 250ml Erlenmeyer flasks. Table 1 presents the composition of three different broths.
Table 1. Different medium compositions for the growth study in case of *At. ferroxidans*.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>CHEMOLITHOTROPHIC</th>
<th>HETEROTROPHIC</th>
<th>MIXOTROPHIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Na$_2$S$_2$O$_3$ + Yeast Extract)</td>
<td>(Glucose + Yeast Extract)</td>
<td>(Glucose + Na$_2$S$_2$O$_3$)</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>4.0g</td>
<td>4.0g</td>
<td>4.0g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.5g</td>
<td>1.5g</td>
<td>1.5g</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>0.3g</td>
<td>0.3g</td>
<td>0.5g</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.5g</td>
<td>0.5g</td>
<td>0.5g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5.0g</td>
<td>5.0g</td>
<td>--</td>
</tr>
<tr>
<td>Na$_2$S$_2$O$_3$</td>
<td>10.0g</td>
<td>--</td>
<td>10.0g</td>
</tr>
<tr>
<td>Glucose</td>
<td>--</td>
<td>10.0g</td>
<td>10.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
<td>15.0g</td>
<td>15.0g</td>
</tr>
<tr>
<td>ADDW</td>
<td>1000ml</td>
<td>1000ml</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

pH of the *Thiobacillus* medium was adjusted to 4.0 using 1N H$_2$SO$_4$.

The culture flasks during growth were subjected to rotatory shaking and the growth curve analysis was done by taking absorbance at 640nm at different time intervals starting from control till 30th hour. Simultaneously, the pH of the culture was also recorded. Specific growth rate (µ) was also calculated for each culture condition of *Acidithiobacillus ferroxidans*. Specific growth rate (µ) was calculated as follows:

$$\frac{\log_{10} N_t - \log_{10} N_0}{t_t - t_0} = \frac{\mu}{2.303}$$

Where, $N_0$ = Absorbance at the initiation of the exponential phase of growth.

$N_t$ = Absorbance at the mid/end of the exponential phase of growth.

$(t_t - t_0)$ = time difference to achieve absorbance from $N_0$ to $N_t$.

6.2.5 ISOLATION OF *Thiobacillus thermosulfatus*

Isolation of *Thiobacillus thermosulfatus* was done following the procedure given by Shooner *et al.* (1996). Mine spoil sub-sample (approximately 1g) was amended with 0.5% (w/v) tyndallized S$^0$ powder in a 250ml Erlemeyer flask and incubated in shaking water bath at 53°C. The suspension from the flask was removed aseptically daily to measure the decrease in pH. Aliquots (100µl) from the flask were transferred into Medium-A having the following composition (per liter).
The pH of the Medium-A was adjusted to 6.0 and then S⁰ powder was added. After the growth of the bacteria in Medium-A, the grown bacterial cells were transferred to a medium whose composition is same as that of Medium-A except the S⁰ powder, which was substituted with 20mM sodium thiosulfate. Typically, the pH of the medium was adjusted to 6.0 with 1N H₂SO₄. The inoculated bacteria were subjected to incubation at 53°C in a rotary shaker incubator for 20 hrs. After this, 100μl of the bacterial suspension was pipetted out and serially diluted upto 10⁵ times with sterilized double distilled water. From the final dilution, 100μl of the cell suspension was taken out and transferred onto a sterilized petridish containing 20mM sodium thiosulfate and 0.6% (w/v) Gelrite (Lin and Casida, 1984). The petridish was incubated at 50°C in an incubator for 2 days. Presence of heterotrophic-sporulated bacteria if any, was verified through out the isolation process by inoculating the growing cells into Medium-H having the following composition (per liter).

Glucose : 1g
Tryptone : 5g
Yeast extract : 2.5g
CaCl₂·2H₂O : 0.57g
Gelrite (0.6%) : 6g

Since such isolation does not show any growth in Medium-H, absence of bacterial growth confirms the absence of heterotrophic-sporulating bacteria. Such observation therefore confirms the isolation of *Thiobacillus thermosulfatus* in pure form.
6.2.6 GROWTH ANALYSIS OF *Thiobacillus thermosulfatus*

Similarly, experiments were conducted to determine the bacterial growth study in case of *Thiobacillus thermosulfatus* and subsequent alternation in pH by using Medium-A supplemented with thiosulfate. Growth experiments were performed at 50°C by using Medium-A supplemented with thiosulfate (20mM) and yeast extract (0.05%) in order to determine whether the turbidity that developed resulted from contaminating heterotrophic bacteria or not. Growth was monitored by measuring the optical density at 400nm at different time intervals starting from control till 40th hour and the pH of the culture was also recorded. Specific growth rate was calculated as specified in 6.2.4.

6.2.7 DETERMINATION OF THERMAL DEATH TIME (TDT)

*Thiobacillus* medium inoculated with the inoculum culture were subjected to heat treatment at 60°C in an incubator for different time intervals (30 min, 45 min, 1 hr, 75 min, 90 min, 120 min and 150 min) and the heat-treated inoculums were streaked onto petridishes. The plates were kept at 37°C for 24hr in an incubator for development of colonies. Similar experimental sets up were performed for the chemolithotrophic, heterotrophic & mixotrophic culture of *Acidithiobacillus ferroxidans*.

For determination of thermal death time in case of *Thiobacillus thermosulfatus*, the inoculum culture were subjected to 80°C in an incubator for different time intervals (30 min, 45 min, 1 hr, 75 min, 90 min, 120 min and 150 min) and the heat-treated inoculums were streaked onto petridishes. The plates were kept at 37°C for 24hr in an incubator for the bacterial growth.

6.3 RESULTS

6.3.1 ISOLATION OF HETEROOTROPHIC BACTERIA

The fresh mine spoil inoculation resulted the growth of heterotrophic bacteria in nutrient agar medium. The colonies per plate (at $10^5$ times dilution) varied from 10 to 12. Around 70% colonies were marked to be circular with entire margin, rest 30% showed irregular form of colonies with serrate margin. The elevations of most of the colonies were observed to be flat along with few colonies with raised elevation. The colour of the colonies was observed to be white (or glittering in few cases). The cells from each colony were subjected to Gram staining and were studied under the microscope.
The study revealed that 40% of the bacterial colonies were represented by cocci and 60% by bacilli. Out of the cocci, 90% of the bacterial colonies were marked to be Gram (+ve). Further for bacilli, 80% of the bacterial colonies were classified as Gram (-ve) type.

Table 2 presented the observation of the thermotolerant ability of different heterotrophic isolates. Among the cocci, all the isolates were noted to exhibit growth at 40°C. Percentage of cocci showing the growth at 45°C and 50°C were 60% and 30% respectively. No cocci could exhibit growth at 60°C and 65°C. Percentage of bacilli showing growth at 40°C, 45°C and 50°C were 100%, 80% and 60% respectively. It was further observed that 40% of the isolates could show growth at 60°C and thereafter, no bacteria could exhibit growth at 65°C.

Table 2. Study of the thermotolerance ability of different heterotrophic isolates at different temperatures. (+: growth; -: no growth; figures in the parenthesis denote % of isolates).

<table>
<thead>
<tr>
<th>Colony type</th>
<th>Thermotolerance ability w.r.t different temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40°C</td>
</tr>
<tr>
<td>Cocci</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(100) (60) (30)</td>
</tr>
<tr>
<td>Bacilli</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(100) (80) (60) (40)</td>
</tr>
</tbody>
</table>

Observation relating to the acid tolerance ability of different heterotrophic isolates have been presented in Table 3. Only 30% of both cocci and bacilli were observed to show growth at pH-7. Proportion of cocci showing growth at pH-6 & 5 were 100% and 30% respectively. No cocci could grow below pH-5. In case of bacilli, only 90% could at pH-6. At pH-5 onwards, proportion of bacilli showing growth declined with only 30% growing at pH-3. No growth could be noted at pH-2.

Table 3. Study of the acid tolerance ability of different heterotrophic isolates at different pH. (+: growth; -: no growth; figures in the parenthesis denote % of isolates).

<table>
<thead>
<tr>
<th>Colony type</th>
<th>Acid tolerance ability w.r.t different pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH-7</td>
</tr>
<tr>
<td>Cocci</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(30)</td>
</tr>
<tr>
<td>Bacilli</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(30)</td>
</tr>
</tbody>
</table>
6.3.2 ISOLATION OF *Acidithiobacillus ferroxidans*

Isolation of *Acidithiobacillus ferroxidans* in *Thiobacillus* medium resulted smooth, circular and opaque colonies (Figure 3a & 3b). The diameter of the colonies were approximately 2mm. During the development of colony, there was a change in colour of the medium from yellow to green. The bacterial cells when microscopically observed were found to be rod shaped and their response to Gram stain was negative. Cells from a discrete colony were taken out for culture in slant.

![Chemolithotrophic culture of At. ferroxidans showing colonies.](image1)

![Gram staining showing Gram-negative, rod shaped At. ferroxidans.](image2)

6.3.3 GROWTH CURVE ANALYSIS OF *Acidithiobacillus ferroxidans*

Under chemolithotrophic (Na$_2$S$_2$O$_3$ & yeast Extract) culture condition, lag phase of the growth of the strain continued upto 3$^{rd}$ hr of incubation. The exponential phase started thereafter till the 26$^{th}$ hr after which stationary phase started (Figure 4A). As revealed from the figure, initially pH of the culture medium before the bacterial growth was maintained at 4.0. However during the growth period, pH showed a decline trend and it dropped down to 1.95 at 30$^{th}$ hr of incubation. Specific growth rate ($\mu$) in case of chemolithotrophic culture condition was calculated to be 0.101 hr$^{-1}$.

Heterotrophic growth condition (in presence of glucose & yeast Extract) showed that the lag phase of the bacterium continued upto the 3$^{rd}$ hr of incubation followed by exponential phase upto 20$^{th}$ hr (Figure 4B). The pH due to bacterial growth in heterotrophic culture medium however increased from 4.0 to 6.17 at 30$^{th}$ hr of incubation. Specific growth rate ($\mu$) in case of heterotrophic culture condition was calculated to be 0.063 hr$^{-1}$. 

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Similarly, in case of mixotrophic growth condition (in presence of glucose & \( \text{Na}_2\text{S}_2\text{O}_3 \)), the lag phase was continued upto 7th hr and was followed by the log phase upto 20th hr of incubation. The stationary phase was then initiated (Figure 4C). However, change in pH varied from 4.0 to 5.1 at the 30th hr of incubation. Specific growth rate (\( \mu \)) in case of mixotrophic culture condition was calculated to be 0.137 hr\(^{-1}\).

Figure 4. Growth curve of *Acidithiobacillus ferrooxidans* in culture medium supplemented with (A) \( \text{Na}_2\text{S}_2\text{O}_3 \) & yeast extract (chemolithotrophic growth); (B) glucose & yeast Extract (heterotrophic growth); (C) glucose & \( \text{Na}_2\text{S}_2\text{O}_3 \) (mixotrophic growth). Symbols: ■ → Chemolithotrophic/ Heterotrophic/ Mixotrophic mode of growth; ▲ → pH.
6.3.4 ISOLATION OF *Thiobacillus thermosulfatus*

Isolation of *Thiobacillus thermosulfatus* resulted discrete colonies on the medium-A. The colonies, when further observed microscopically were found to be the mixture of *Thiobacillus thermosulfatus* and some Gram (+ve) spore forming bacillus species. After subsequent subculturing on medium A, the spore-formers disappeared. When a colony from a solid medium A culture was transferred to solid Medium-H, no growth of the thiobacilli or spore-formers was observed after incubation for 15 days at 50°C. This confirmed the elimination of heterotrophic bacillus contaminants from the Medium-A. Other gelling agents such as agarose, Gelrite were used with medium A supplemented with thiosulfate. Only 2% purified agar gave results similar to the results obtained with Gelrite. With 2% Bacto agar, growth was slower than the growth obtained with Gelrite and it took more than 8 days to detect acid production when it was detected. The bacterial colonies isolated in the Medium-A were found to be Gram negative, rod shaped, motile, facultatively autotrophic, strictly aerobic and thermophilic colorless sulfur bacterium (Figure 5a & b) and were identified to be *Thiobacillus thermosulfatus*.

![Image of chemolithotrophic culture of *T. thermosulfatus* showing colonies.](image1)

![Image of Gram staining showing Gram-negative, rod shaped *T. thermosulfatus*.](image2)

6.3.5 GROWTH CURVE ANALYSIS OF *Thiobacillus thermosulfatus*

Under chemolithotrophic culture condition, growth experiments were performed at 50°C by using medium A supplemented with thiosulfate (20mM). The growth curve of *Thiobacillus thermosulfatus* revealed that the lag phase of the growth of the strain continued upto 4th hr of incubation. The exponential phase started thereafter till 15th hr of incubation. It...
was further observed that the growth was arrested between 15th to 20th hr of incubation. The growth of the bacterium again continued up to 35th hr of incubation, after which the bacteria entered into the stationary phase was started (Figure 6). Specific growth rate (μ) was calculated to be 0.236 hr⁻¹.

As revealed from the figure, before the bacterial growth, the pH of the culture medium was maintained at 6.0. The amount of biomass increased slowly within a pH range of 6.0-7.5. However, at the end of the growth study, pH showed a decline trend and it dropped down to 2.1 at 40th hr of incubation.

Figure 6. Growth curve of *Thiobacillus thermosulfatus* in culture medium supplemented with Na₂S₂O₃ (chemolithotrophic). Symbols: ■ → Chemolithotrophic growth; ▲ → pH.

Growth of the isolated *Thiobacillus thermosulfatus* was tested with respect to thiosulfate, tetrathionate, elemental sulfur, thiocyanate, yeast extract, glutamate and succinate. Table 4 presented the growth of *Thiobacillus thermosulfatus* in the culture Medium-A supplemented with different ingredients.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Growth pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiosulfate</td>
<td>+++</td>
</tr>
<tr>
<td>Tetrathionate</td>
<td>++</td>
</tr>
<tr>
<td>Elemental sulfur</td>
<td>++</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>++</td>
</tr>
<tr>
<td>Glutamate</td>
<td>+</td>
</tr>
<tr>
<td>Succinate</td>
<td>+</td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>-</td>
</tr>
</tbody>
</table>
Growth occurred on media containing thiosulfate, tetrathionate and elemental sulfur. Growth did not occur on medium containing thiocyanate under the conditions tested. During growth of the bacterium on thiosulfate supplemented medium, tetrathionate, trithionate and sulfate were produced. Heterotrophic growth occurred on media containing yeast extract, glutamate and succinate. Characteristic odor was noticed at the end of growth on organic and inorganic substrates. Under anaerobic conditions, no growth was occurred under autotrophic and heterotrophic conditions. Thus, tetrathionate and trithionate were not produced from thiosulfate and hence the pH was stable.

6.3.6 DETERMINATION OF TDT FOR \textit{Acidithiobacillus ferroxidans}

Table 5 represented the thermal death time (TDT) of the isolated chemolithotrophic strain \textit{i.e. Acidithiobacillus ferroxidans} in three different culture conditions (chemolithotrophic, heterotrophic & mixotrophic).

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>Chemolithotrophic</th>
<th>Heterotrophic</th>
<th>Mixotrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>30 min</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>45 min</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 hr</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\textit{Acidithiobacillus ferroxidans} culture when subjected to heat treatment 60°C for 15min showed moderate growth in all the three culture conditions. However, \textit{Acidithiobacillus ferroxidans} in chemolithotrophic culture condition did not show any growth when exposed to thermal treatment at 60°C for 1 hr. Similarly, the TDT value of the bacterium at 60°C for heterotrophic and mixotrophic culture condition was found to be 45 min. The data revealed that there was an increasing trend of death of the bacterial strain with the increase in the exposure time at 60°C.

6.3.7 DETERMINATION OF TDT FOR \textit{Thiobacillus thermosulfatus}

Thermal death response in case of \textit{Thiobacillus thermosulfatus} was determined by exposing the bacterium at 80°C for different time period. Table 6 presented the thermal death time (TDT) of the isolated chemolithotrophic strain \textit{i.e. Thiobacillus thermosulfatus}.
Table 6. Thermal response of *Thiobacillus thermosulfatus* at 80°C. (++ : moderate growth; + : minimal growth & - : no growth).

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>Thermal response at 80°C</th>
<th>Chemolithotrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>45 min</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1 hr</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*Thiobacillus thermosulfatus* showed moderate growth at 80°C, when exposed for 15 min. The data suggested that as the thermal exposure time increases, there was a gradual decline in the viable number of the bacterium *i.e. Thiobacillus thermosulfatus*. The chemolithotrophic culture of *Thiobacillus thermosulfatus* when subjected to heat treatment at 80°C for 1 hr, no viable microorganism was observed. This suggested that the TDT value of *Thiobacillus thermosulfatus* in chemolithotrophic culture was found to be 1 hr at 80°C.

6.4 DISCUSSION

Fresh mine spoil is a unique habitat with extreme environment. Because of the pyrite auto-oxidation, it is associated with higher temperature, acid production and is void of organic substrate and plant available nutrients. With such scenario, the fresh mine spoil is expected to be inhabited by the extremophiles like thermo-acidophiles of chemolithotrophic nutritional category. However, when the spoil was processed for heterotrophic isolation with nutrient agar, resulted colony forming units of heterotrophic isolates. Hence, fresh mine spoil in spite of biological constraints was observed to be inhabited by heterotrophic bacteria, whose population was relatively much low than other age series mine spoil. These heterotrophic isolates were marked to be of two morphological types *i.e.* cocci and bacilli, with later constituting 60% of the total isolates. Out of cocci, 90% were Gram positive, where as 80% of the bacilli were Gram negative type. Thus, heterotrophic bacteria of Gram negative types were the dominant group of isolates in the mine spoils. This also confirms some of the earlier microbiological observations of coal mine spoil by different workers (Dugan *et al.*, 1970; Williams and Hoare, 1972; Katayama-Fujimura *et al.*, 1984; Harrison, 1984; Wood and Kelly, 1986; Kuenen *et al.*, 1992; Kristjansson and Stetter, 1992; Madigan *et al.*, 1997; Kristjansson *et al.*, 2000; Boone *et al.*, 2001; Fujiwara, 2002).
Thermotolerance study of different cocci and bacilli isolates indicated that 30% of cocci can tolerate to grow at 50°C. In case of bacilli, 40% of the total isolates could grow even at 60°C. These points out the prevalence of thermotolerant heterotrophs in the mine spoil and the observation is in agreement with that of Williams and Hoare, 1972; Apel et al., 1976; Cladwell et al., 1976; Katayama-Fujimura et al., 1984; Wood and Kelly, 1986, 1988; Norris et al., 1986; Kuenen et al., 1992; Shennan, 1996; Rawlings et al., 1999 and Akbar et al., 2005.

Similarly acid tolerance study revealed that 30% of the cocci isolates could grow at pH-5. In case of bacilli, even 50% and 30% of the isolates could grow at pH-4 and pH-3 respectively. Pyrite auto-oxidation product when subjected to leaching in the mine spoil heap results in the production of acid (Cook, 1964; Silverman, 1967; Tuovinen and Kelly, 1974; Harrison, 1984; Beir, 1987; Sugio et al., 1987; Tyagi and Couillard, 1989; Jain and Tyagi, 1992; Kristjansson and Stetter, 1992; Lane et al., 1992; Blais et al., 1993; Couillard and Mercier, 1993; Sreekrishnan et al., 1996; Rawlings et al., 1999; Kristjansson et al., 2000). Hence, for bacterial isolates to survive in such condition has to develop acid tolerance ability (Silverman and Lundgren, 1959; Dugan et al., 1970, Apel et al., 1976; Mishra and Roy, 1979; Katayama-Fujimura et al., 1984; Norris, 1990; Shennan, 1996; Wulf-Durand et al., 1997; Rawlings et al., 1999). Several authors have reported about the acid tolerant bacteria in the coal mine spoil (Silverman and Lundgren, 1959; Cook, 1964; Silverman, 1967; Dugan et al., 1970; Tuovinen and Kelly, 1974; Apel et al., 1976; Katayama-Fujimura et al., 1984; Wood and Kelly, 1986; Norris, 1990; Kristjansson and Stetter, 1992; Shennan, 1996; Rawlings et al., 1999; Kristjansson et al., 2000; Akbar et al., 2005) and the present findings agree to their observation.

Following aseptic protocol and media, it could be possible to isolate *Acidithiobacillus ferroxidans* and *Thiobacillus thermosulfatus*. While isolating *Acidithiobacillus ferroxidans*, there was a change in the colour of the medium containing Na$_2$S$_2$O$_3$ from yellow to green. This change was due to the acid production. *Thiobacillus thermosulfatus* was isolated from fresh mine spoil sample in Medium-A enriched with elemental sulfur. The isolated bacterium was gram negative, rod shaped, motile, facultatively autotrophic. Under anaerobic conditions, no growth was occurred under autotrophic and heterotrophic conditions, which revealed that the bacterium is strictly aerobic in nature.
Study of the growth of *Acidithiobacillus ferroxidans* revealed that the bacterium can grow in three different culture conditions (chemolithotrophic, heterotrophic and mixotrophic). Under chemolithotrophic condition, the bacterium exhibited a specific growth rate (μ) of 0.101 hr⁻¹, whereas under heterotrophic and mixotrophic condition, specific growth rate was calculated to be 0.063 hr⁻¹ and 0.137 hr⁻¹ respectively. Higher specific growth rate under mixotrophic culture condition can be due to the presence of both inorganic (Na₂S₂O₃) and organic (glucose) reductants (Dugan *et al.*, 1970; Williams and Hoare, 1972; Tuovinen and Kelly, 1974; Cladwell *et al.*, 1976; Larsson *et al.*, 1994). Further, it was observed that the growth under chemolithotrophic culture condition led to a decrease in pH from 4 to 1.95 over a period of 30 hr. The decline in pH was due to the acid production and the bacterium could survive in this condition. This suggested the acidophilic nature of *Acidithiobacillus ferroxidans*.

Growth analysis of *Thiobacillus thermosulfatus* indicated that the bacterium exhibited a specific growth rate of 0.236 hr⁻¹, which is a higher value in comparison to *Acidithiobacillus ferroxidans*. The bacterium showed diauxic growth pattern due to the availability of two nutrients, such as thiosulfate first and later tetrathionate in the medium (Tuovinen and Kelly, 1974; Wood and Kelly, 1986; Shooner *et al.*, 1996). The pH range during the growth period of 40 hr was pH-6 to 2.1. The drop in pH value in later part of the growth curve was due to acid production. Since the bacterium showed best growth at 53°C and is an acid producer, it can be concluded as thermo-acidophilic bacterium.

Thermal response of the isolated bacteria suggested that *Thiobacillus thermosulfatus* grow chemolithotrophically at 80°C for 45 min. Similarly, *Acidithiobacillus ferroxidans* grow at 60°C for 45 min. Hence, thermal death time (TDT) for *Acidithiobacillus ferroxidans* and *Thiobacillus thermosulfatus* were 1 hr at 60°C and 1 hr at 80°C. The data suggested that both of the bacterial species were observed to be thermophiles. However, heterotrophic and mixotrophic culture condition, the TDT value was reduced to 45 min at 60°C.

Fresh coal mine spoil is a unique geomorphic system with several hostile environmental parameters. In spite of such features, it is not sterile in nature and support bacterial community that has physiological adaptability to thrive in such hostile condition. A detailed microbiological investigation involving complete exploration and physiological profiling of such bacteria would result in the isolation of several unique bacterial strains having bioprospecting potential.