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Biodepyritization of Indigenous Coal Using Acidophilic, Mesophilic and Moderately Thermophilic Bacteria

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Abstract.- This study was carried out for the identification of culturable bacteria present in the indigenous coal and to observe their depyritization capabilities in pure as well as in mixed consortia. Five mesophilic acidophilic isolates (B3, T1, T3, 1S and 1D), one moderately thermophilic (T2) iron and sulphur oxidizing isolate, two moderately thermophilic sulphur oxidizing isolates (NA-AT1, NA-AT2) and two acidophilic heterotrophic isolates (PK and PY) were purified from the coal samples and were identified on the basis of classical morphological and physiological basis. Coal biodepyritization studies were conducted using pure and mixed consortia of the isolated bacteria. Isolates B3 and T2 exhibited high coal biodepyritization rates as compared to all other isolates. Even higher biodepyritization rates were observed when mixed consortia of B3 and T2 were used with acidophilic heterotrophs (PY) and moderately thermophilic sulphur oxidizing bacteria (NA-AT1).

Key words: Coal heap, biodepyritization, acidophilic chemolithotrophic bacteria.

INTRODUCTION

Coal is considered an alternative energy source to oil and is the most abundant fossil fuel in the world. Although available in great abundance, coal combustion has devastating effects upon the environment. One of the problems with direct utilization of coal is its sulphur content, released mainly as SO_2 into the atmosphere on direct combustion. Sulphur dioxide can have deleterious effects on animal and plant life (Kargi and Robinson, 1982).

Pakistan is one of the largest coal bearing zones of the world with total reserves nearing 186 billion tons (Munawar, 2005). But most of Pakistani coals are sub-bituminous or lignitic in nature having 3-12% sulphur. Conventional process for coal cleaning and upgrading are energy-intensive operations which render coal as an exorbitant energy source. Biotechnology could be employed to develop some new technologies, which might be cheaper, both for traditional and *in situ* coal processing (Khalid and Aleem, 1989).

The microbial removal of pyritic sulphur from coal by the chemoautotrophic microorganism *Acidithiobacillus ferrooxidans* has been studied by several investigators (Dugan and Apel, 1978; Silverman *et al*., 1963; Hoffman *et al*., 1981). It is known that *At. ferrooxidans* found in an acid mine waters play an important role in the oxidation of pyrite and other sulphide minerals (Singer and Stumm, 1970; Zajic, 1969). A mixed culture of *At. ferrooxidans* and *Acidithiobacillus thiooxidans* was reported to be more effective than pure cultures of *At. ferrooxidans* in removing pyritic sulphur from coal (Dugan and Apel, 1978).

Present studies were undertaken to investigate the indigenous microflora of 300-tons coal heap established for desulphurization in pursuance to develop an effective microbial process for the removal of pyrite from high sulphur coal using pure and mixed consortia of sulphur and iron oxidizing indigenous bacterial isolates.

MATERIALS AND METHODS

Coal samples were collected randomly from the different localities of 300-tons coal heap set for biodesulphurization at Askari cement plant, Nizampur, Pakistan. Autotrophic and heterotrophic bacteria were isolated with enrichment media

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described by Leathen *et al.*, (1956) and modified by Postgate (1966) supplemented with one of the following substrates: ($FeSO_4$. 7 H_2O for autotrophic iron oxidizers, $Na_2S_2O_3.5H_2O$, or elemental sulphur for sulfur oxidizers with TSB and glucose as substrate for hetrotrophic bacteria). Enrichments for moderate thermophiles were performed with BSS media developed by Johnson *et al*. (1987) and incubated at 45°C.

Enriched cultures were incubated for 1-6 weeks until growth was observed microscopically or until a visible change occurred in the medium compared with uninoculated control. For thiosulfate and elemental sulphur containing enrichments, growth was accompanied by a substantial decrease in the medium pH, while Fe^{2+} containing enrichments showed a characteristic ferric precipitation resulting into orange color of the medium.

Pure cultures from these enrichments were obtained by repeated sub culturing of single colonies solid to liquid media by serial dilutions for isolates, which did not grow on solid media. Solid media were gelled with 0.5% agarose and prepared as previously described by Harrison (1984), except that an overlay of agarose was not used. Isolates were identified on the basis of phase- contrast microscopic observations (Zeiss Axiovert model MC 80). While colony forms were recorded using a steriomicroscope.

Coal biodepyritization studies were carried out using pure as well as mixed consortia of isolated bacteria. Coal used in these studies was ground to the powered form, sub-bituminous in rank and contained 8.5% total sulphur, including nearly 5.5% pyritic sulphur, 2.5% organic sulphur and 0.5 % sulphate sulphur components. Coal samples were sterilized by tyndallization.

Fast growing representative isolates (B3, T1, T2, NA-AT1 and PY) were selected and used in these studies. In addition to pure cultures, mixed consortia of these isolates were also used for removing pyritic sulphur from coal. Before conducting the experiments for mixed consortia of mesophiles and moderate thermophiles it was checked experimentally that mesophiles could adopt to higher temperature easily while thermophiles were also contributing to some extent for bioleaching activity at lower temperature *i.e.* 30°C. So for this purpose, twenty-three Erlenmeyer flasks (250ml) were prepared having 100ml of respective growth media for these bacteria. During first set of experiment, two flasks were inoculated with pure cultures of B3 and T1 isolates. Thirteen flasks were inoculated in duplicate with mixed consortia (10%v/v) in the following order: 1) B3+ NA-AT1. 2) B3+ PY. 3) B3+ NA-AT1+ PY. 4) T1+NA-AT1. 5) T1+ PY. 6) T1+ NA-AT1+ PY. One flask was served as un inoculated control and then flasks were incubated at 30°C and 150rpm. During second set of experiment one flask was inoculated with pure culture of T2 isolate and then remaining eleven flasks were inoculated with mixed consortia of moderate thermophile in duplicate in the following order: 1) T2+ NA-AT1. 2) T2+ PY. 3) T2+ NA-AT1+ PY. 4) NA-AT1. One flask served as un inoculated control. The flasks were incubated in shaker at 45°C and 150rpm. Samples (1.0ml) were taken after 24 hours and analyzed for total and ferrous iron. Any loss in the volume due to evaporation or sample removal was made up by adding equivalent amount of sterilized, acidified (pH 2.0) distilled water. The rate of pyrite oxidation was calculated from the concentration of total iron solubilized from pyrite in coal by the microorganisms. The ratio of iron and sulphur in pyrite was used as a factor in these calculations as Sulphur/Iron in pyrite (FeS_2) = 1.14.

Concentration of ferrous and total iron (ferrous+ ferric) in the sample solution was determined spectophotometrically by the modified 1,10-phenanthroline method (Furman, 1963). 1.0ml aliquots of each sample were titrated against 1.0 mM $KMnO_4$ solution to calculate the amount of ferrous iron oxidized.

RESULTS

Isolation and presumptive identification of bacterial isolates

During this study, ten isolates of autotrophic and heterotrophic bacteria were screened from the coal samples. Five mesophilic acidophilic isolates (B3, T1, T3, 1S and 1D), one moderately thermophilic (T2) iron and sulphur oxidizing isolate, two moderately thermophilic sulphur oxidizing

isolates (NA-AT1, NA-AT2) and two acidophilic heterotrophic (PK and PY) isolates were purified. All acidophilic bacteria were isolated and purified by plating on selective solid media, and were grouped on the basis of their physiological characteristics. Different types of bacteria exhibited different growth patterns on their respective growth media.

Iron and Sulphur Oxidizers

Solid media developed by Johnson *et al*., (1987) were employed for the growth of iron and sulphur oxidizing bacterial isolates. Colony and cellular morphologies are given in Table I. The growth of mesophilic isolates appeared as rust colonies at 30°C after fifteen days of incubation. The colonies of all isolates were flat, circular, rust colored having entire margins except B3, which was convex in shape. Cells from these colonies were small, motile, Gram negative rods (1 to 2 μm). Liquid medium (Leathen, *et al*., 1956; Postgate, 1966) containing ferrous sulfate as energy source was employed for the preparation of inocula at 30°C. During log phase 5% (V/V) inocula of respective cultures were transferred to pyrite (0.35%) containing flasks. Rich growth was obtained on finely ground pyrite after ten days of incubation at 30°C.

Growth of moderately thermophilic isolate (T2) was obtained in the form of irregular, flat and rust colored colonies on solid media developed by Johnson *et al*., (1987), after fifteen days of incubation at 45°C. A single colony was inoculated in liquid media containing ferrous sulfate as energy source for the preparation of inocula at 45°C. During log phase 5% (V/V) inocula of respective cultures were transferred to pyrite (0.35%) containing flasks. Rich growth was obtained on finely ground pyrite after 8 days of incubation at 45°C. The microscopic examination revealed a seemingly pure culture of large (4 to 6 μm in length), motile, Gram positive, rod shaped bacteria containing endospores with swollen ends.

Heterotrophs

Heterotrophic growth of PY and PK isolate in Glucose-TSB medium (pH 2.5) was abundant for all enrichments within ten days of inoculation at 30°C. Turbidity in flasks and pale yellow coloration for PY and pink for PK indicated the rich growth. Circular, convex, entire, pale yellow colonies of PY and pink colonies of PK isolates appeared after sixteen days on agarose-gelled Glucose-TSB medium, incubated at 30°C. These were Gram negative, rod shaped (1 to 3 μm in length) and actively motile bacteria.

Sulphur oxidizers

Off-white colonies becoming pale yellow after three weeks of growth, with uneven surface and wavy margins appeared on agarose-gelled solid medium containing sodium thiosulfate as energy source, after fifteen days of incubation at 45°C. These bacteria were actively motile rods (3 to 4 μm) in length, when grown in the liquid medium having 1% elemental sulphur as energy source, incubated at 45°C. Rich growth was indicated by observing turbidity in flasks after seven days of incubation.

Biodepyritization studies

Rates of biodepyritization of coal were studied by using pure and mixed consortia of the isolated bacteria. It was observed that mixed consortia of isolates (PY and NA-AT1 with B3 isolate) had higher depyritization rate (0.31g/L/day) as compared to pure culture of B3. Mixture of B3 with NA-AT1 had higher depyritization rate than when B3 was mixed with PY (Table II). Similarly, mixed consortium of isolates PY and NA-AT1 with T1 isolate had depyritization rate (0.181g/L/day), which was higher than depyritization rate of pure T1 culture. Mixture of T1 and NA-AT1 had higher depyritization rate than when mixed with PY as shown in Table II. In the same way depyritization rate of T2 isolate in mixed consortia was higher (0.98 g/L/day) than pure culture of T2. T2 and NA-AT1 mixture had higher depyritization rate as compared to mixture of T2 with PY as shown in Table II.

DISCUSSION

In the present study, indigenous bacteria from coal samples were screened and their role in depyritization of coal was studied. Although bacterial populations associated with commercial

bioleaching operations and natural acidic sites are considered to be more complex mixture of bacterial strains (Touvinen, 1991), the number of species

Table I.- Some characteristics of different bacteria isolated from coal heap samples.

Bacteria	Isolated code	Colony morphology				Cell shape and size (µm)	Notes
		Colour	Form	Elevation	Margins		
Mesophiles							
Iron and sulfur oxidizing	B3	Rust	Circular	Convex	Entire	Rods, 1-2.5	*At. ferrooxidans* like
	T1	Rust	Circular	Flat	Entire	Rods, 1-2	*At. ferrooxidans* like
	T3	Rust	Circular	Flat	Entire	Rods, 1-1.5	*At. ferrooxidans* like
	1S	Rust	Circular	Flat	Entire	Rods, 1-1.5	*At. ferrooxidans* like
	1D	Rust	Circular	Flat	Entire	Rods, 1-1.5	*At. ferrooxidans* like
Heterotrophs	PY	Pale yellow	Circular	Convex	Entire	Rods, 2-3	*Acidiphilum symbioticum* like
	PK	Pink	Circular	Convex	Entire	Rods, 1-1.5	*Acidiphilum symbioticum* like
Moderate thermophiles							
Iron and sulfur oxidizing	T2	Rust	Irregular	Flat	Undulate	Rods, with endospores, 4-6	*S. thermosulfidooxidans* like
Sulfur oxidizing	NA-AT1	Cream	Circular	Flat	Undulate	Rods, 3-4	*At. caldus* like
	NA-AT2	Cream	Circular	Flat	Undulate	Rods, 3-3.5	

Table II.- Rates of coal biodepyritization (g/L/day) by pure and mixed consortia of various bacterial isolates.

Isolate code	Control	Pure culture	Mixed with NA-AT1	Mixed with PY	Mixed with NA-AT1+PY
B3	0.051	0.248	0.252	0.24	0.31
T1	0.054	0.15	0.179	0.159	0.181
T2	0.11	0.89	0.891	0.86	0.98

commonly isolated from these environments is low and relatively consistent. Our results also indicated that there was no major qualitative shift in culturable species as compared to other studies.

The best known of all mineral oxidizing-degrading prokaryotes is the mesophilic *Acidithiobacillus ferrooxidans* (Kelly and Wood, 2000) that was the first pyrite-oxidizing bacterium to be discovered. Out of ten isolates, purified in this study, five isolates (B3, T1, T3, 1S, 1D) had morphological and physiological characteristics of *At. ferrooxidans*, indicating that bacteria belonging to this group were more prevalent in iron-rich acidic environments compared to other microbial groups that may be found under such environments.

Acidithiobacillus thiooxidans and *Acidithiobacillus caldus* (a more thermotolerant bacterium with a growth optimum at 45°C) are both sulphur oxidizers and are not able to oxidize pyrite but can grow on the sulphur released from the pyrite present in the coal. The characteristics of NA-AT1 and NA-AT2 indicated that these bacteria belonged to *At. caldus* species and isolate T2 reflected typical growth behavior of *Sulfobacillus thermosulfidooxidans.* Heterotrophic isolates purified in this study were identified as *Acidiphilium symbioticum* on the basis of 16 S rDNA homology studies (data not shown).

Mutualistic interactions between physiologically distinct acidophiles, involving transfer of organic and inorganic carbon and transformations of iron and sulphur, were considered to have critical roles in optimizing pyrite dissolution (Okibe and Johnson, 2004). During

biodepyritization studies, pyrite dissolution was determined by measuring changes in soluble iron and generation of acidity and it was observed that mixed cultures of iron and sulphur oxidizers (B3, T1, T2) with sulphur oxidizer (NA-AT1) isolate, generated more acidity than pure cultures, though mixed cultures did not necessarily enhanced pyrite dissolution. In contrast, mixed cultures of above given iron and sulphur oxidizers, sulfur oxidizers and heterotrophic isolate (PY) oxidized pyrite more rapidly than pure cultures of iron and sulphur oxidizers alone. Involvement of some acidophilic heterotrophs in pyrite or iron oxidation process had been reported by some investigators (Hiraishi *et al*., 1998; Bacelor-Nicolau and Johnson, 1999). In the current study, two heterotrophic isolates (PY and PK) were purified and it was indicated that these bacteria contribute to the stability of the mixed mineral-oxidizing population by consuming organic excretion products produced by the mineral oxidizers thus enhancing the rate of biodepyritization. An abrupt decrease in pyritic sulphur removal was observed in the case of moderately thermophilic isolate (T2) after two days, which may be due to the fact that Fe^{3+} ions were continuously accumulated in the medium during bacterial oxidation of pyrite and after a certain level their hydrolysis occurred forming precipitates of Fe^{3+} thereby decreasing the concentration of soluble iron in the solution.

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