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Isolation and Characterization of Iron and Sulfur Oxidizing Bacteria from Coal Mines

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Abstract
The present study is aimed at to isolate the sulfur and iron oxidizing bacteria that can be used to remove the pollutant like as $FeSO_4$; $MgSO_4$ etc. Four bacterial isolates were isolated from coal samples called from Choa Saidan Shah, Punjab, Pakistan ($Sp3^1$, $Sp4^1$, $Sp5^3$ and $Sp6^2$) evaluated on the basis of biochemical and morphological tests. Most of the isolates showed medium sized colonies with round shape, irregular margin, creamy in color expect $Sp6^2$ formed yellow colored colonies. All the strains were Gram +ve rods and Spore former, Motile, Catalase producers, Starch hydrolyzing, Nitrate reducer except the $SP6^2$. Strains $SP3^1$ and $SP4^1$ were sensitive against (Ampicillin-300 µg ml^{-1}, Tetracycline 25 µg ml^{-1}, Streptomycin 500 µg ml^{-1}, Chloramphenicol 5 µg ml^{-1}) antibiotics. Maximal growth was observed at pH 7.0 except $SP5^3$ at pH 5.0, the optimum temperature was 42°C except $SP5^3$at 37°C. After sequencing analysis as *Bacillus subtilis* ($SP3^1$), *Bacillus subtilis* $(SP4)^1$, *Pseudomonas* sp ($SP5^3$) and *Stenotrophomonas* ($SP6^2$) respectively were identified. So these isolates can be exploited for bioremediation of coal.
Keywords: *Bacillus subtilis*, *Pseudomonas* sp, *Stenotrophomonas,* bioremediation, coal, sulfur.

1. Introduction

For electric power generation role of coal increased as a fuel because the production of petroleum is widely expected to fall short at the end of this century (Balat, 2007). Coal is major source for the generation of electricity worldwide and it require more attention in handling and storage of coal because it's the largest anthropogenic sources of carbon dioxide emissions. Bacterial isolates which have ability for metal reducing and oxidizing remove carbon dioxide and methane from impure coal to convert it into pure coal for electricity production. Sometime leachates (contain high levels of F^-, $S0_4^{2-}$, Fe, Mn, Al, Zn, Hg, As, and Se) may cause harmful effects on entering in surface and subsurface drainage systems. Underground coal when come in contact with oxygen produces the greenhouse gases C_2O, S_2O, H_2SO_4, and methane, mercury, carbon monoxide, other toxic substances cause direct effect on human health (Smith, 2009). Many microorganism are now known that removes sulfur from coal and make it better fuel. Coal Bio-desulphurization is a type of new technology of coal resource effective and reasonable utilization. Heterotrophic, chemolithotrophic and chemoorganotrophic microorganisms are also associated with coal and accelerate leaching. Bioleaching of different metals and acidophilic pyrite oxidizing bacteria metabolizing both sulfur and pyrite in coal and to utilize the energy, released to support their growth. Microbial coal desulphurization is complex combination of non-biologically and microbiologically catalysed oxidations of inorganic sulphide minerals pre, microorganisms convert the inorganic sulfur to water soluble sulfate (Cohen, 2004). After cleaning process, the coal is separated from the liquid phase containing the sulfate and washed with water to obtain fuel with lower sulfur content (Acharya *et al*., 2001). There is a correlation between temperature and oxidation process of iron and sulfur as thermophilic bacteria do best then mesophilic bacteria. Ferric reducing microbes produce ferrous which after reacting with sulfide and this insoluble ferrous sulfides generating alkalinity when fixed in sediment (Fortin and Beveridge, 1997; Mills *et al*., 1989). Coal, petroleum, and natural gas, contain sulfur and are largest environmental challenge today is removing organic sulfur, a substance that is chemically bound to coal. This study is about the isolation of sulfur and iron oxidizing bacteria from coal mine and their characterization morphologically and genetically.

2. Materials and Methods

2.1.Sample Collection

Different coal and soil samples were taken in sterilized bags from different area of Kalar Kahar and coal mines of Choa Saidan Shah, Punjab, Pakistan (Table1). Some physicochemical parameters of sample *viz.,* temperature, pH, color and smell were measured.

2.2. Isolation of Iron and Sulfur Oxidizing Bacteria from Coal

Coal as well as soil samples Suspensions were diluted up to 10^{-3} dilutions, plated on L-agar plates (for the isolation and purification of bacteria) and incubated at 37°C for overnight. For the isolation of iron and sulfur oxidizing bacteria purified strains were streaked on defined mineral salt medium recommended by (Krebs *et al.,* 2000) and incubated at 37°C for overnight. Iron and sulfur oxidizing isolates were maintained routinely on

defined mineral salt medium at 37°C, were morphologically and biochemically (motility, gram staining, spore staining, Catalase, Oxidase, oxidation fermentation, Starch hydrolysis, Nitrate reduction, Ethylene methylene blue agar, MacConkey agar,) characterized by using standard methods (Cappuccino and Sherman) and confirmed by bergey's manual.

2.3.Antibiotic Susceptibility for Coal Isolated Bacteria

To check the antibiotic susceptibility of coal isolated bacteria. Four different antibiotic solutions were used (Ampicillin 300 µg ml^{-1}, Tetracyclin 25 µg ml^{-1}, Streptomycin 500 µg ml^{-1}, Chloramphenicol 5 µg ml^{-1}) in specific concentrating in L-agar media selective strains of iron and sulfur oxidizing bacteria were speeded on each specific antibiotic plate, incubated at 37°C for 24 hours and zone of inhibition were measured.

2.4.Optimization of Growth Conditions for Isolates

The optimum growth conditions of the selected isolates were determined. For this the bacterial strains were grown at two different temperature 37°C and 42°C. In the same way pH optimization was also carried out.

2.5.Growth of Isolates at Various Incubation Time

Growth curves of isolates were determined in defined mineral salt medium. Media was prepared and the cultures were incubated at 37°C for various time intervals. After regular time period (04 hours), cultures were harvested and their optical densities (OD) were measured on a spectrophotometer at 600nm.

2.6.Genetic Characterization

Plasmid isolation

Plasmid of the bacterial strains were isolated following the method of El-Bakkali *et al*. (2013) was followed.

Agarose gel electrophoresis of total cell lysate

About 0.9% agarose gel was prepared in 0.5X TBE, melted and ethidium bromide (2.5 µl 100 ml^{-1} of 5 mg ml^{-1}) mixed in gel and poured in vertical gel apparatus. A loopful of cells was dispensed in microfuge tube containing autoclaved water and centrifuged at 5000 rpm for 3 minutes. Cell pellet was suspended in 100 µl of lysis buffer [{(10 X TBE-0.5ml), (Glycerol-25ml), (Glycerol-200ug), (Pre-boiled RNase - 10ug)}/10ml]. 20 µl of the prepared sample was loaded in the wells and overlaid by 20 µl of SDS buffer [{(SDS-0.2g), (NaOH-0.16g)} 10mlH_2O] and 10 µl of overlying buffer [(10 X TBE-0.5ml), (Glycerol-05ml0, (SDS 5%-04ml), (Distilled water-8.6)]. Reservoirs of the gel apparatus were filled 0.5X TBE so that continuous current should pass through it. 5 µl of ethidium bromide was added in TBE. Electrical supplies were connected and initially a current of 50 volts was applied for 15-20 minutes. After that voltage was increased to 150 volts till the completion of the process. Gel was carefully removed and observed under UV trans-illuminator.

2.7.16S rRNA Sequencing

For the sequencing of these coal bacteria the methods of El-Bakkali *et al*. (2013) was followed.

3. Results

3.1.Isolation and Characterization

Four isolates *Bacillus subtilis* ($SP3^1$), *Bacillus subtilis* ($SP4)^1$, *Pseudomonas sp.*($SP5^3$), and *Stenotrophomonas* ($SP6^2$) were isolated from their respective sample sites (Table 1) on defined mineral salt medium and were further proceeded for iron and sulfur oxidizing activities.

Table 1: Iron and sulfur oxidizing bacterial Isolates from coal samples of Choa Saidan Shah, Punjab, Pakistan.

Bacterial isolates	Samples Characteristics		
	Source	**Sample**	**Locality**
$Sp3^1$	Coal	SP3	Kalar Kahar
$Sp4^1$	Coal	SP4	Kalar Kahar
$Sp5^3$	Coal	SP5	Choa Saidan Shah
$Sp6^2$	Coal	SP6	Choa Saidan Shah

These four isolates were characterized morphologically and biochemically as described in Bergey's manual (Baldani *et al.*, 1985). Most of the isolates showed medium sized colonies with round shape, irregular margin, creamy in color expect *Stenotrophomonas* formed yellow colored colonies (Table 2).

Table 2: Colony morphology of coal isolated Isolates

Isolates	Colony Morphology					
	Color	Size	Shape	Margin	Elevation	Translucency
Sp3[1]	Off-white	3mm	Round	Irregular	Flat	Translucent
Sp4[1]	White	3mm	Round	Entire	Flat	Translucent
Sp5[3]	Cream	1.5mm	Round	Entire	Raised	Opaque
Sp6[2]	Yellow	Pinpoint	Round	Entire	Raised	Transparent

All the isolates were Gram +ve rod, spore former, motile except *Stenotrophomonas* Gram–ve cocci, non spore former, non motile and biochemical characterization results were shown in (Table 3; 4). *Bacillus subtilis* and *Bacillus subtilis* were sensitive against all antibiotics and *Stenotrophomonas* was only sensitive against tetracycline (Table 5).

Table 3: Cell morphology of bacterial isolates from coal origin

Isolates	Cell Morphology			
	Gram staining	Cell shape	Spore formation	Motility
Sp3[1]	G+ve	Rods	+	+
Sp4[1]	G+ve	Rods	+	+
Sp5[3]	G+ve	Rods	+	+
Sp6[2]	G-ve	Cocci	-	-

+ : Positive result, -: Negative result,

Table 4: Biochemical characterization of coal isolates

Isolates	Catalase	Oxidase	O.F	EMB	MacConkey agar	Starch hydrolysis	Nitrification
Sp3[1]	+	_	+	+	_	+	+
Sp4[1]	+	_	+	+	_	+	+
Sp5[3]	+	_	+/-	_	_	+	+
Sp6[2]	_	+	+	+	+	_	+

+ : Positive result, -: Negative result, -/+: Facultative anaerobe

Table 5: Impact of various antibiotics on bacterial strains.

Isolates	Antibiotics (µg ml^{-1})			
	Ampicillin	Streptomycin	Tetracycline	Chloramphenicol
Sp3[1]	R	S	S	R
Sp4[1]	R	S	S	R
SP5[3]	R	R	R	R
SP6[2]	R	R	R	R

3.2.Effect of Temperature and pH on growth of strains

Three out of four gave optimum growth at 42°C except *Pseudomonas* sp showed maximum growth on 37°C (Figure 1). Majority of the isolates preferred pH 7 for optimum growth, where as *Pseudomonas* sp coal isolated isolates showed an optimum growth at acidic pH5 .

Table 6: The nearest homolog and their percentage homology with the coal isolated bacterial Isolates according to 16S rRNA sequencing.

Isolates	Nearest Homolog	% Homology
SP4[1]	*Bacillus subtilis*	98%
SP6[2]	*Stenotrophomonas*	99%
SP5[3]	*Pseudomonas sp.*	98%
SP3[1]	*Bacillus subtilis*	99%

3.4.Growth of Isolates at Various Incubation Time

In *Bacillus subtilis* (SP3[1]), *Bacillus subtilis* (SP4)[1], *Pseudomonas* sp. (SP5[3]), and *Stenotrophomonas* (SP6[2]) strains lag phase was started with 8 to 12 hours, exponential phase started at 44 to 48 hours then stationary phase and death phase vice versa(Figure 2).

3.5.Genetic Characterization of Coal Isolated Bacteria

Plasmid isolation

After isolating the plasmid it was checked by running on a gel electrophoresis using 1% agarose. The gel was observed under UV trans-illuminator and clear bands of plasmids were observed for four isolates.

3.6.16S rRNA Sequencing

Four coal isolated isolates SP3[1], SP4[1], SP5[3]and SP6[2] were identified with 16S rRNA gene sequencing. All these isolates were ~98% and 99% similar to the reference sequence on the Blast database (Table 3). Ribotyping of strains showed their homology with strains *Bacillus subtilis* (SP3[1]), *Bacillus subtilis* (SP4)[1], *Pseudomonas sp.* (SP5[3]), and *Stenotrophomonas* (SP6[2]) (Figure 3).

4. Discussion

This study was conducted to isolate and characterize sulfur and iron oxidizing bacteria from coal because of their ability to tolerate acidic conditions and to reduce or oxidize iron and sulfur present in coal, can be used for removal of these toxic compounds which after burning produce toxic gases in the environment, to clean up the gases before they are released into the atmosphere. Although thermophilic iron-oxidizing bacteria have been added to coal to promote leaching in laboratory experiments, neither group occurred naturally in leachates from the coal used in our experiments (Watling, 2006).

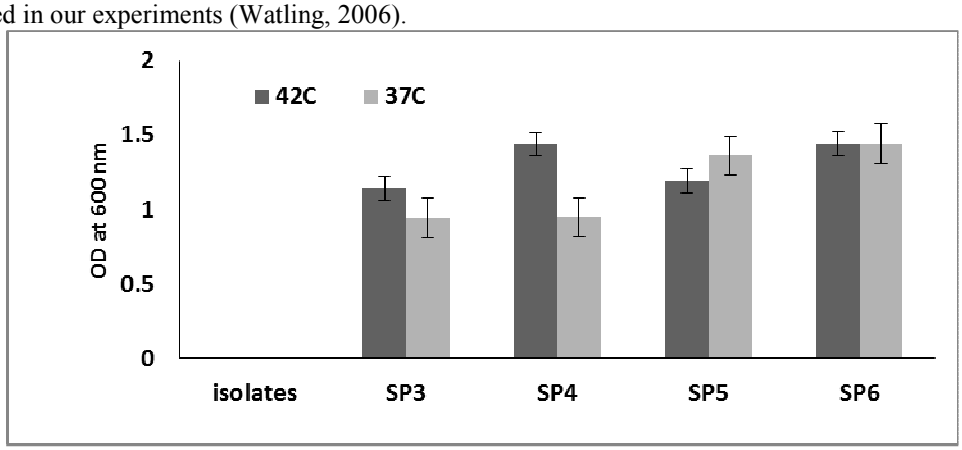

Figure 1 : Growth comparison of bacterial isolates on different tempatures (37°C and 42°C)

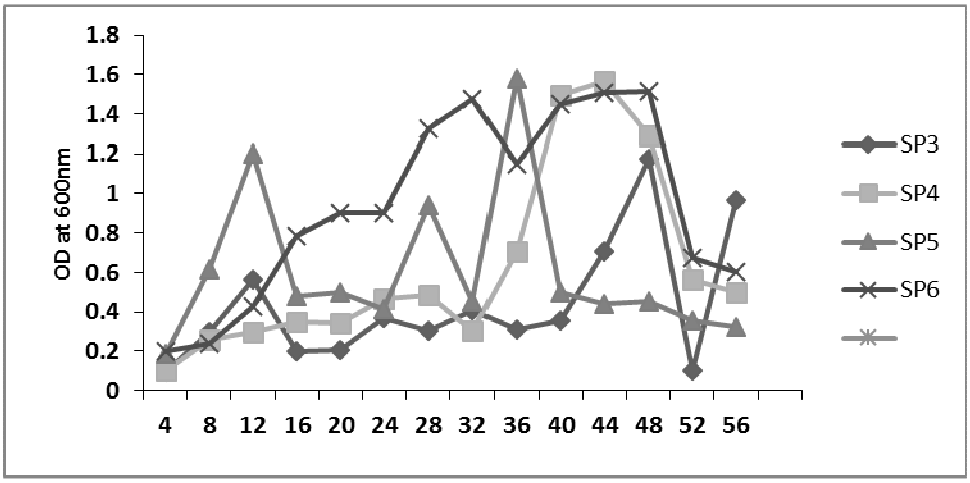

Figure 2: Growth comparison of different bacterial isolates at various time intervals (hours).

In the present case, four purified iron and sulfur oxidizing bacterial isolates *Bacillus subtilis* (SP3[1]), *Bacillus subtilis* (SP4)[1], *Pseudomonas sp.* (SP5[3]), and *Stenotrophomonas* (SP6[2]) were isolated from coal mines of Choa Saidan Shah. Morphological characterization of isolates showed that all were Gram positive, but only SP6[2] was Gm–ve cocci. Acidiphilic bacterial strains were also isolated by Paul *et al.* (1981). But these microbes were sensitive to some antimicrobial compounds (Hayes, 1963). Growth of Gram positive strains on MacConkey agar was not observed which is a differential and selective medium for Gram negative bacteria. A negative carbohydrate utilization test is indicated by the absence of a yellow color media remains green or turns blue. Our study showed that a bacterial community isolated from a coal mine were capable of utilizing iron and sulphur as the sole source of carbon and energy for bacterial growth. Isolated from the coal mines were capable of reducing

sulphur and iron present in the coal. Iron and sulfur oxidising bacteria produce hydrogen sulfide gas by breaking down sulfur compounds (Muyzer *et al*., 2008).

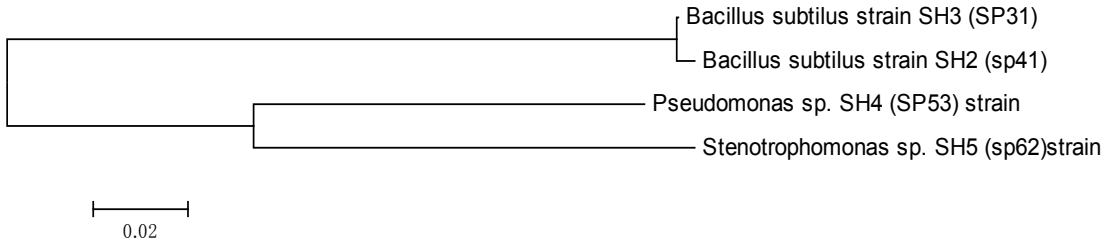

Figure 3: Phylogenetic tree of coal residing isolates

This finding indicates that conclusions drawn from leachate samples regarding the abundance of autotrophic and heterotrophic metabolic types may be extrapolated to core samples (Christensen *et al*., 2001). The most appropriate four iron and sulfur oxidizing isolates on the basis of their low pH, high temperature and other biochemical factors ($SP3^1$, $SP4^1$, $SP5^3$ and $SP6^2$) were selected identified with 16S rRNA gene sequencing. Strains $SP3^1$, $SP4^1$, $SP5^3$ and $SP6^2$ were identified as *Bacillus subtilis* ($SP3^1$), *Bacillus subtilis* $(SP4)^1$, *Pseudomonas* sp.($SP5^3$), and *Stenotrophomonas* ($SP6^2$) respectively. Hence coal residing bacterial isolates have shown a high efficiency of reducing or oxidizing sulfur and iron present in the coal. These bacterial isolates have appreciable metal reducing ability which can be exploited for the removal of these contaminations from the coal and as a reaction different gases production like methane can be a big achievement in the era high energy consumption. Coal quality can be improved after the removal of these iron and sulfur contaminants which can b used for electricity production.

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