Isolation and Screening of Coal Solubilizing Aerobic Microorganisms

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IN THE NAME OF ALLAH
THE MOST COMPASSIONATE AND MERCIFUL

TO MY LOVING

GRANDMOTHER WHOSE

CARING AND NURTURING

PRAYERS ALWAYS BEEN

A BLESSING FOR ME

LEADING MY PATH TO

SUCCESS

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Muhammad Ali

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List of Abbreviations

Mt	Million tons
%	Percentage
IEA	International Energy Agency
WCA	World Coal Association
M	Million
EIA	Energy Information Administration
Btu	British Thermal Unit
>	Greater than
<	Lesser than
Approxx.	Approximately
~	Upto
Hg	Mercury
NOx	Nitrogenous oxides
SOx	Sulphur oxides
N_2	Nitrogen
lb	Pound
MW	Mega Watts
PAH	Polycyclic Aromatic Hydrocarbons
As	Arsenic
U	Uranium
Pb	Lead
Co	Cobalt
Cd	Cadmium
Cr	Chromium
Se	Selenium
B	Boron
^oC	Celsius Degree
H_2O	Water / Water molecule
CO_2	Carbon di oxide gas
CH_4	Methane gas
ABTS	2,2'-azinobis(3-ethylboenzthiazoline-6-sulphonate)
O_2	Di oxygen
LiP	Lignin peroxidase
MnP	Manganese peroxidase
SBP	Soya bean hull peroxidase
HRP	Horse radish peroxidase
Mn II	Second Manganese radical
Mn III	Third Manganese radical
H_2O_2	Hydrogen per oxide
Vr	Verateryl
Rpm	Revolutions per minute
kDa	Kilo Daltons
g/l	Gram per litre
NADPH	Nicotin amide dihydrogen phosphate
R	Aryl
C-C	Carbon to carbon bond
C-O	Carbon to Oxygen bond

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Abstract

Pakistan with about 185 billion tons is known to contain sixth largest reservoir in the world. The Biosolubilization of coal is a promising technology, which converts coal into valuable added products.Recently this field is seeking increase attention owing to recent development and technology. Coal can be solubilized and transformed from solid to catalytic and liquid state by employing various coal conversion technologies. The coal fired power plant, Thermocatalysis and pyrolysis are thaught to be inefficient as modern world is shifted to more ecofriendly approach.

The present research work focused on Biosolubilization of coal and involved coal solubilimnzing microorganisms isolation and screening work. Approximately 50 aerobic bacterial and fungal isolates have been isolated initially from soil,coal and water samples of Dulmiall Coal Mines,Chakwal,Pakistan. Firstly,there occurs screening step of large collection of bacterial and fungal isolates later followed by selection. The isolates were solemnly selected on the basis of their ability to grow on single carbon source. Two bacterial strains AY2, AY3 and fungal strains AY5, AY6 were shown to have effective solubilization activity on coal. The intensity of Biosolubilization was measured by determining the weight loss of the coal pieces. Coal solubilization was observed to be about 25.93% by AY2, 36.36% by AY3 and 50% by AY6. AY5 showed maximum coal solubilization (66.67%). The coal solubilizing activity of microorganisms was correlated with change in pH. Ultravoilet spectroscopy,Infrared spectroscopy and Scanning electron microscopy were performed to characterize the solubilization residues. Ultravoilet spectrum revealed increase in pattern of absorbance across all strains. The Biosolubilization products mainly contains aromatic acids, chain hydrocarbons including organic functional groups of hydroxyl, cyclane, carbonyl, ether linkage and aromatic rings. The IR spectroscopy indicates alterations of solubilized products in comparison to original coal. The evidence for the presence of microorganisms and surface erosion of coal residues in contrast to control samples were observed by means of scanning electron microscopy. Thus,there revealed, the isolated microoganisms were able to survive in coal for long period of time. Therefore present study revealed the microorganisms isolated from coal mines have enough potential solubilizing coal and can be used for different valuable products

INTRODUCTION

Energy is the motivating force which runs anybody either in living or nonliving form. The more energy any country has, more economy it can build up. Every country is trying hard to produce energy in order to run industrial sector. Mostly energy consumed in world is electrical, 90% produced through coal. Globally 30.3% (7876Mt), coal produced around world is buried deep in seabed and coal mines. Coal therefore provides strength to many developed nations generating 42% revenue yearly while making 68% in steel output (IEA, 2012).

Coal is a residue of ancient living matter. Due to enormous heat, pressure and time it is converted to fossil fuels located worldwide. It shows prehistoric vegetation gathered in marshy places, sediments and swamps. Age of coal compared with Carboniferous Period ranges from 360M to 230M years ago (WCA 2013). It is a chemically complex natural recalcitrant. Physically heterogeneous hard molecule ranges from yellowish pale to brownish shade. Harder it gets, attains black crystalline form. It varies in density because of difference in porosity, specific gravity and macerals composition. Volatile content, heat capacity, coal particle size, rank, heat of reaction, the coal oxygen content along with pyrite mostly preactivates coal to self-heat and naturally burning tends to decrease the quality of coal rank (Nalbandian, December 2010).

In world, coal of four types is found: lignite, sub bituminous, bituminous and anthracite. Lignite is low rank coal, generally found near deltaic plains and marshy lands. It does not vary by altitude and height perspective. Bituminous is higher coal rank so it demands much pressure which varies due to height, altitude and latitude of mountains. Sub bituminous lies in between lignite and bituminous coal, while anthracite leads hardest nature in relation to strength, texture and quality.

Coal can be found in every continent with 70 countries. Biggest reserves of coal are found in China, Canada, India, Russia and US. US is second only to China in the amount of coal produced and consumed, with about 1,100,000,000 tons consuming annually. Projections evaluate a 6% increase in coal use between 2008 and 2035

(EIA, 2011). It is considered to be prime revenue generating agent through trade between developed nations. China being leading electricity generator, imports more than 190Mt of coal, approx. average to other leading importers like Japan (175Mt) and South Korea (129Mt) consuming annually. Indonesia generates revenue having greater income than Australia, USA, Russia, South Africa and Columbia on exporting coal (http://www.worldcoal.org/resources/coal-statistics/(2012)).

Coal in all, imparts major importance in a developing country like Pakistan, known to contain fifth largest coal deposits worldwide, making one sixth of average world electricity consumption per capita. It has more than 185.5 billion tons of reserves, considered to produce energy for the next 40 years. Much of the energy produced through hydro plants rather utilizing coal directly (PPIB, June 2004). In current crisis period, coal can be a significant fuel in producing gas and electricity. Coal mining nowadays is considered effective for electricity generation, which is found essential in fulfilling energy line losses in grid.

In a country like Pakistan, coal rank varies from lignite to bituminous. Much of lignite deposits lie in Thar region with 175Bt, comprising 850trillionft^3 area reserves while bituminous varies in production in Salt Range and regions pertaining to hilly areas (PPIB, June 2004).

Much of the energy in world generated by coal, utilizes thermal potential of coal as coal pyrolysis and catalytic gasification (Popa et al., 2013). Coal thermal pyrolysis plays major role in emitting 90% SO_2, 70% dust fly ash , 67% NO_x and 70% of CO_2 gas emissions making pool of air pollutants in atmosphere (Chen and Xu, 2010). In coal fire power plants, higher efficiency is needed to maintain coal quality; ash disposal gets hard utilizing water to break hard structure of coal.

The coal liquefaction is conversion of coal to various other solvents employing various conversions. Coal as fuel is important raw material for various chemical industries rather than separating chemicals from the matrix. Side products generated are hazardous as toxic and volatile materials require further distillation.

The area needed to convert coal is bit higher. A new method converting coal at any place at moderate temperature should be known without taking any substrate like water. There should be no use of by products and single end product should be made. In order to effectively make clean use of coal, we need to explore degradation potential of microbial life occurring everywhere in ecosystem. Without microbes, no organic conversion or any reaction in earth is possible.

Microorganisms produce extracellular, hydrolytic enzymes, surfactants, chelators, utilizing macerals found in coal converting aromatic ring carbon. Coal is usually transformed from peat to lignite biochemically by microorganisms as a result of aerobic and anaerobic respiration of natural organic matter which proves to be coal precursors. Lignin and cellulose degrading class of fungi i-e white rot mostly solubilize lignin to simple sugars through degrading mechanisms Bacteria of various aerobic and anaerobic class, cleaves coal molecule and solubilize coal micro pores as a result depolymerizing coal matrix. Naturally coal matrix rarely been solubilize by any chemical resin, yet microorganisms possess enough potential to break complex linkages occuring in coal.

Most of coal ranks from soils of various areas varies in character of inorganic body. Since coal is remnant of organic colloids, so microbes truly degrade coal body at their best. Not all microbes possess such extraordinary life style of living under such extreme conditions. Only coal microbes have special metabolic activities dealing with catabiosis. Such an extreme ability makes these organisms truly unique in behavior, form and diversity creating pool of indigenous species of microflora present in mines. Such bio degraders prehistorically living in organic matrix of carbon chain through eons sets a major shift in understanding physiology and uptake of nutrients.

The present work of coal solubilizing mechanisms is exceptionally helpful in understanding microbial physiology surviving under harsh conditions. Bacterial solubilization across fungal potential shows effective response degrading coal in industries. However in understanding basic mechanisms of coal solubilization, fungi hold major importance through research. Much of the waste generated in coal fire power plants is dumped in aquatic streams running near them as its difficult to dispose plant waste. Coal waste, therefore can be remediated by using such microorganisms having unique ability to degrade toxic matter. Use of clean technology degrading

toxic effluents is mostly used by developed countries worldwide. We, as developing nation lack use of this technology. If this technology is used, we can make a safer environment for ourselves as well as life persisting in water.

Aims and Objectives

The aim of study is to isolate and screen microbial species solubilizing coal growing under aerobic pathway. Biosolubilization of coal mechanism was the main focus followed by various objectives;

- Isolation of coal solubilizing aerobic microorganisms
- Screening and identification of coal solubilizing microorganisms.
- Analysis of coal solubilization by spectroscopy, FTIR and SEM.

2. REVIEW OF LITERATURE

2.1 Energy and its worldwide resources

Provision of sufficient, secure and affordable energy is indispensable to continued human development. Throughout the course of history, with the evolution of civilizations, the human demand for energy has risen continuously. The global demand for energy is rapidly rising with increasing human population, urbanization and modernization (Asif, 2009). Therefore development of energy is important in any country's stability and development. Dong et. al. (2006) stated that the advancement of biological processes for use of fossil energy has recognized collective attention in recent years. Worldwide major energy producing resources are hydro, geothermal, wind, fossil fuel, biomass, nuclear sources etc (IEA 2012).

Coal buried deep in soil reserves major importance from last century in all the energy generating resources. It's been assumed, it's the only resource available readily worldwide. Globally 90%, coal is used in producing energy i-e. electrical by burning few pounds of coal. It is known as cheap substrate with huge reserves present globally in many countries i-e. US, China, Russia, India, Australia, Canada, Germany, Indonesia, Scotland, Sweden, South Korea, Japan, England, Spain, Brazil, Poland, France, Italy, South Africa, Turkey, Denmark, Netherland and Belgium (Robert *et. al*., 2013). A recent study shows leading producers are China, US and then India, Indonesia,Australia, Russia, Canada, Germany etc

2.2 Coal, its Structure and Formation

Coal, one of the wealthiest resources rested beneath earth surface, reserving 71.4 % of the total world reserves. It produces higher amount of energy, than any resource does (Cowan *et. al*., 2009). It is heterogeneously hard, physiochemical organic rock. It consists of carbon, hydrogen and oxygen with lesser amounts of sulfur and nitrogen. It is microscopically unique material chemically different macerals. Macerals are organic substances ancestrally derived from plants, chemically transformed by natural

processes geochemically. It ranges in color from peat having green shade to black color making hard anthracite rank (Hower *et. al.*, 2013).

2.3 Formation of Coal

Coal resembling fruit cake in morph, formed as a result of different elements combined in a single matrix (Gupta, 2000). Peat is formed when organic matter is decomposed, making layers of carbon compounds linked together. It's the herbal material passing under coalification, generally altered to coal. With burial along passage of time, herbal material is readily degraded. This activates physiochemical changes in cellulosic content forming pig layer of coal(Wilkins and George, 2002). Pig layer delineating to ring shape structure forms complex branch aromatics (Norton and de Langes, 2001). Ring shape components under time,heat and pressure starts degradation process, organically matures to coal deposits. This forms basis of coalification process (WCA, 2012). The geochemical step begins with the conversion of brown coal into sub-bituminous coal moving up to higher rank (Klein *et al*., 1997). The peat formed during coal formation is used in ornamental setting, absorbing terrestrial fuel as well as oil spills in seas and ships moving in bigger oceans.

2.4 Types of Coal

Coal branded by ranks, owing to altitude, geophysical and biochemical variation in the metamorphosis occurring usually in rocks. In world lignite accounts one fourth of hard coal. At the moment, 3700 million tons of hard coal and about 940 million tons of lignite produced worldwide per year (BP 1996). The coal ranks proposed by Wender, (1976) are shown in figure 2.1.

2.4.1 Lignite/ Brown Coal

Low rank coal has structure similar to peat (Jiang *et. al.,* 2013). Because of hydrophilic moist form this type has decreased burning potential. Generally lignite reserves are used in cyclone fired power plants for electricity production nearby mine area. It holds heating value of 4,000 to 8,000 Btu per pound. Highly oxidized form of lignite known as leonardite, where humic acids on ash free basis are found (Kurkov, 2004). This rank

covers the lowest level of fixed carbon (25 – 35%) and highest moisture level (typically 20 – 40% by load, but can go as high as 60 – 70% of all the coals. Ash varies up to 50% by weight. Lignite has low sulfur (< 1 percent) and ash (approx. 4%), but high levels of volatile matter (32%> by weight) (IEA, 2012).

2.4.2 Sub bituminous Coal

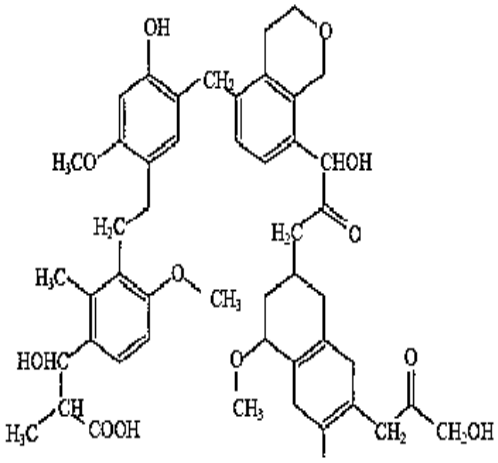
It is higher form of lignite with black color. It inclines to break due to instability when exposed in air as sub bitumens hold more moisture and volatile matter ~ 45% but lower sulfur levels ~ 2%. The carbon content differs from 35-45% while ash varies ~ 10%. The heating value of sub bituminous coal ranges from 8,000 - 13,000 Btu per pound. Coal is non-coking, contains ~ 0.5-2% nitrogenous compounds. It is a huge reservoir of aromatics emitting particulate matter like Hg, NO_x and SO_x elements (Silva, 2011).

2.4.3 Bituminous Coal

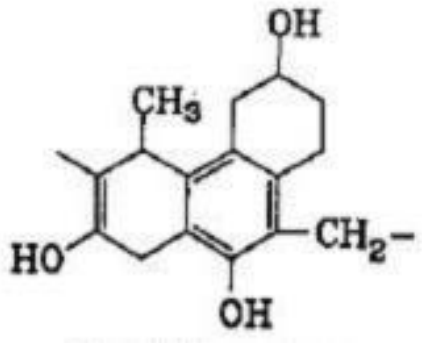
The heating value ranges from 10,500 - 15,000 Btu/lb. The C content ranging ~ 85F where moisture adding ~ 17% along with 12% ash content and N_2 source including 0.5 – 2% structurally in matrix. Metallurgic coal is needed to make coke; a process used in making steel producing coke to form iron. Coke is a spongy, solid black rock of intense carbon created by coking bit coal without air at higher temperatures(Catcheside and Ralph, 1999).

2.4.4 Anthracites or Hard Coals

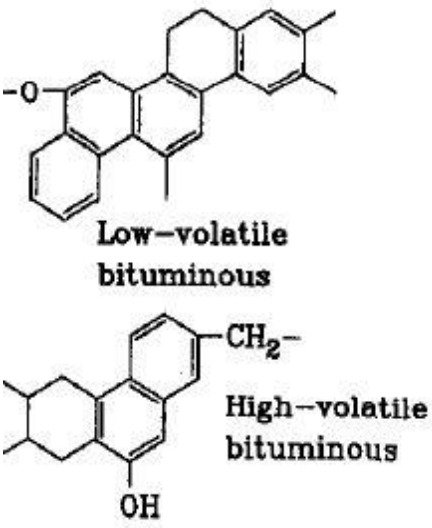
Hardest coal with shiny black form showing greater heat energy than any other coal rank does. On burning, it produces hot blue flame. Heating value ranges from 13,000 – 15,000 Btu/lb burning of coal. Fixed C found in anthracite lies 85 – 90% of coal while moisture (5-15%) with lesser ash (5-20%) as volatile matter. Moreover, nitrogen present in coal accounts 1% (Xiao *et. al.,* 2013).



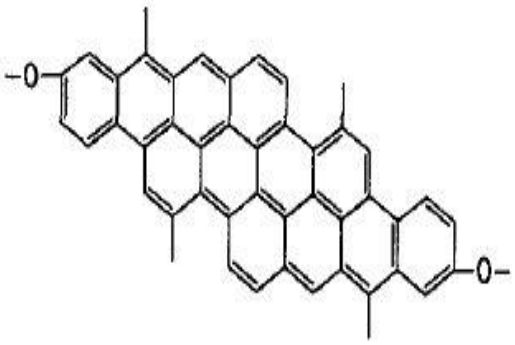
Lignite



Subbituminous



Bituminous



Anthracite

Fig 2.1 Structure Configuration showing ring nature of coal types

2.5 Potential of Coal in Pakistan

Pakistan, the 6th richest country blessed with coal resources. Due to lack of organization, infrastructure, inadequate sponsoring and modern coal mining technical expertise, indigenous coal resources are difficult to develop. Coal from different areas

of Pakistan varies from lignite to high volatile bituminous rank. In Pakistan, coal is mainly used for cement, sugar, steel, brick-kiln, domestic supply by other industries including, Water and Power Development Authority (PPIB, 2004). According to general assessment by PEB, total coal resources of Pakistan are more than 185 billion tons. The richness of coal in Pakistan can be assumed after discovery of 175.5 billion tons of coal in Thar area of Sindh. It is predicted that, if Pakistan's coal resources are properly utilized, then more than 100,000 MW of electricity can be generated for the next 40 years. Balochistan maximizes in producing coal as 58%. 97% of coal reserves of Pakistan sits as lignite and remaining only 3% are sub bituminous to bituminous as in Table 2.1, (Malkani, 2012).

Table 2.1 Coal Reserves of Pakistan and Azad Jammu Kashmir (million tons)

Province	Measured	Indicated	Inferred	Hypothetical	Total Reserves
Sindh	3339	11835	56646	113637	185457
Balochistan	79.45	150.45	183.5	45.3	458.7
Punjab	57	31	2	145	235
Khyber Pakhtunkhwa	3	5.75	109.24	5	122.99
Azad Kashmir	1	1	6.72	-	8.72
Grand Total	3479.45	12023.20	56947.26	113832.30	186282.41

2.6 Coal Conversion Technologies

The coal as a body holds many potential in it. We need to explore it through finding new ways harnessing development in coal technology. Mostly in world processes related to thermal activities are generally used in order to produce energy and chemicals as raw stock for chemical industries (Engesser et al., (1994)).The technology used in world harness difficulties in complete conversion of coal to its metabolites. The use of microorganisms truly solubilize coal to its various parts producing PAHs like tar, creosote, phenolic compounds, gas and oil etc(Wilkins and George, 2002) . Coal bio-conversion means to dissolve, liquefy or gasify coal by use of microorganisms(Du et al., 2010)

2.6.1 Thermo catalytic process of Coal

In world all countries nowadays exploring thermal potential, to catalyze the potential rests in coal. The coal fire power plants use this process to make electricity feasible in grid lines. The coal used in this process demands rank of higher quality. Mostly sub bituminous and bituminous ranks are generally used. Anthracite coal has more ash and low sulfur but higher moisture level, so it's difficult to use in industry (Hower et al., 2013). Bituminous coal in industrial sector in recent times, used in steam power plants and as boiler function in food corporates and also in coking process (Silva et al., (2011)).

2.6.2 Coal Gasification

The conversion of discolored biomass to a gaseous fuel catalytically transforms to gaseous state by heating in a gasification medium such as air, oxygen or steam. Unlike combustion, oxidation is considerably complete in one process. It converts the intrinsic chemical energy of the carbon in the biomass into a combustible gas in two stages. The gas produced can be standardized in its quality and is easier and more versatile to use than the original biomass e.g.it be used to power gas engines and gas turbines, or used as a chemical feedstock to produce liquid fuels. Strictly, gasification includes both biochemical and thermochemical processes, the former involving microorganisms at ambient temperature under anaerobic conditions i.e. anaerobic digestion, while the latter uses air, oxygen or steam at temperatures >800 ^{0}C (Coronas *et. al*., 2010).

2.7 Environmental Hazards

Coal in industries and fire plants is generally used for burning purpose, so it emits various pollutants in environment. All the waste products mainly emitting through heavy release of chimneys exhaust from thermal treated plants. The excessive burning may cause pollution as trace elements cause acid rain. As coking agent, coal generally is used in the catalytic processes, forming various components of PAH. Pollutants are major source of ecological problems. They may reach environment by anthropogenic activities occurring worldwide near coal area. The pollutants are toxic metal minerals like As, B, U, Hg, Pb, Co, Cr, Cd, Se, NO_x and So_x compounds

alongwith CO_2 and CO. Silica and PAH are also released in environment after thermal degradation of coal. Coal fire power plants are a potential threat as global warming gases are also released after coal combustion (Finklan, 1999). Anthropogenic activity considered an important factor causing rise in level of PAHs in soils, which was verified by strong links between the piles of PAHs in soils and the dust due to soil properties. (Maliszewska-Kordybach et al., 2009).(Hwang and Cutright, 2003, Johnsen and Karlson, 2007, Nam et al., 2008).

2.8 Health Hazards

The dusty ash released from catalytic processes of coal is causing lung diseases on heavy release of trace minerals. Hg retards mental health of child along with killing embryo in its developmental stages. Lead cause cardiovascular and kidney diseases. Chromium results gastric disorders and lung diseases on ingesting higher values. Arsenic damages nervous system with cardiac tissue injuries resulting urinary tract cancers. Adsorption and inhalation of arsenic forms skin and lung cancer. Increase intake of selenium results impaired vision or paralysis in some cases. Boron affects major organs like liver, kidney, testes in major cases while eyes, nose and throat at shorter side (Gottleib *et. al*., 2010). Moreover in all this, the water expelling out from coal plants are major causes of environmental pollution. They are toxic to the majority of prokaryotic and eukaryotic organisms, some life forms mostly archaea and bacteria flourish within them. "Acidophiles" encompass an unexpectedly inclusive diversity (in terms of both physiology and phylogeny) of microorganisms spreading in aquatic life forms (Johnson, 2003).

2.9 Biodegradation and Solubilization of Coal

Generally simple sugars, organic acids and the like elements are preferred by microbiologists for microbial activity to avoid use of coal. Using coal is difficult since coal is a multifaceted substrate. Even rarer geochemists and fuel scientists, seriously debated microorganisms able to transform the physicochemical structure of coal (Hofrichter and Fakoussa, 2004). Microbial degradation of coal has been stated as profitable and nominal way converting macro molecules into simpler, low molecular

weight products (Machnikowska et al., 2002). The ability of microorganisms to cultivate on low-rank coal and converting its physiochemical properties was first of all reported in 1920 by Fischer and Fuchs (Selvi et al., 2009). In 1981, Fakoussa and Cohen and Gabriele in 1982 reported discovery of microbial potential using coal as substrate transforming it into liquid products. These effects are very inspiring because they could signify unconventional process for coal conversion, as thermocatalytic processes need higher funds giving low thermal productivity (Willmann and Fakoussa, 1997).

The use of biotechnological practices to convert coal into value-added products can be used for further biotechnological or organic synthesis(Hofrichter and Fakoussa, 2004). A number of terms used to describe different stages in coal biodegradation includes; solubilization, liquefaction, depolymerization, and decolourization of coal components (Sekhohola et al., 2012) More than one mechanism is helpful in microbial coal degradation or liquefaction using oxidative enzymes (peroxidases, laccases), hydrolytic enzymes (esterases), alkaline metabolites and natural chelators (Fakoussa and Hofrichter, 1999).

2.9.1 Microbial Coal Degradation

Coal is usually described as a enigmatic or astonishing source of aromatic hydrocarbons (Bechtel et al., 2005). Aromatic compounds are a comprehensive class of natural compounds comprising low molecular weight mono aromatics such as, p-hydroxybenzoic, vanillic, gallic, ferulic, caffeic, p-coumaric, and aromatic amino acids, and high-molecular weight polyaromatics such as, tannins, lignin and humic acids. Aromatics behave vital ingredients in petroleum-based fuels, agricultural , chemicals and consumer products (Villemur, 1995). Consequently, they are important components of domestic and industrial wastewaters(Çinar and Leslie Grady Jr, 2001).

Most aromatic compounds, principally those covering only benzene rings, are subject to microbial degradation under both aerobic and anaerobic processes (Heider and Fuchs, 1997). The cleavage of the benzene ring is a critical step in aromatic compound biodegradation. It forms various aliphatic products that flow directly into the central metabolic routes. Aerobic metabolism of aromatics branded by the extensive use of O_2

known to be vital for the hydroxylation and cleavage of aromatic ring structures(Harwood et al., 1998). In most documented cases, aromaticity is worn-out by reduction mechanism employing anaerobic metabolism and the ring is afterwards opened hydrolytically. Different peripheral reactions are also required. A small number of different central aromatic intermediates can be reduced. The most common of which is benzoyl-CoA, a compound produced as a central intermediary compound in the degradation of large number of aromatic substrates. In most cases, the relative resistance of aromatic compounds to bio mineralization is due to the great stability caused by the resonance energy of the aromatic ring (>100 kJ mol) (Boll, 2005, Boll et al., 2002). Since many bacteria degrade aromatic compounds under both oxidizing and reducing conditions, manufacturing catabolic enzymes. The toxic metabolites therefore produced from bacterial presence retains catechol pathways for aromatic biodegradation while bacteria using protocatechuate or gentisate pathways can degrade chlorinated benzoates without effort (Deniz et al., 2004). This raises the query how microorganisms control their aromatic compound degrading enzyme systems when the environment in which they are living rounds between two oxidation states, as in BNiR systems(Çinar and Leslie Grady Jr, 2001).

Degradation of coal relies on the environmental settings, number and type of the microorganisms, nature and chemical structure of the chemical compound being degraded. They are bio transformed into less complex metabolites. Through mineralization, inorganic minerals, H_2O, CO_2 (aerobic) or CH_4 (anaerobic) are formed. The rate of biodegradation depends on pH, temperature, oxygen, microbial population, degree of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties, and chemical partitioning in growth medium (Haritash and Kaushik, 2009). The microorganisms can be useful to cut chemical linkages on coal molecules by promoting the extraction of coal products, and ultimately escalating the coal worth.

2.9.2 Microbial Coal Solubilization

Direct burning of low rank coal scripts low thermal competence, low industrial yield. If lignite piled up in the open air for a long time, the coal causes energy waste and

environmental pollution. Hence, further research in low rank coal might be rationally developed by conversion of solid piles into chemical products with high additional C value or into liquidized fuel (Du et al., 2010). Lignite formed from the buildup and decay of principally herbal residues having dense brown to black color organic rock with less than 40% of inorganic impurities captures significant amounts of alkali-soluble humic acids truly encompassing hydrophilic nature (Kurkov et al., 2004). Brown coal consists of the following components: bitumen (waxes and asphaltenes), humic-acid-like material and the macromolecular matrix (Willmann and Fakoussa, 1997). Lignite resources, mostly found in China are generally transformed by *Penicillium* sp. After fungal transformation, the levels of humic acid increased from 38.6% to 55.1%and water-soluble humic material from 4.0% to 28.2%, respectively (Dong et al., 2006). Bio-processing of low rank coal, including bio-purification and bio-conversion, is at the basic research stage. (Du et al., 2010). A new isolate of T. atroviride has shown growth on low rank coal as single carbon source. T. atroviride ES11 degraded 82% of particulate coal over a period of 21 days with 50% decrease in 6 days (Silva-Stenico et al., 2007).

The presence of polar hydroxyl, carbonyl, phenol groups and some peroxide type oxygenated moieties on low rank coal surface is the main factor which declines the coal hydrophobicity. Pretreatments might enhance the oxidation of coal due to improved hydrophobicity. The methods of pretreatments includes grinding, premixing/ preconditioning, ultrasound, thermal, microwave and direct contact mixing of the reagents with dry coal before wetting. Among these pretreated techniques, premixing may be used to remove the oxide layer on the oxidized coal surface, which is further mirrored by ultrasound treatment. Thermal and microwave treatments are ascribed to the elimination of pore water, hydration water and some hydroxy functional groups in low rank/oxidized coals(Xia et al., 2013).

P. putida strain shows lesser organic degradation of low rank coals, as the extent of solubilization rapidly decreases with rank. Oxidizing pretreatment with nitric acid enhances the biodegradation leading progressive solubilization (90%) of lignites and partial solubilization (40%) of subbituminous coals. Bioextracts from oxidized coals are characterized by higher content (40–47%) of oxygen moeties, mostly hydrogen

bonded carboxylic, carboxylate or quinone groups and increased N_2 content. Biotreatment alters the structure of residues of bio organic matter to various conversion degrees. The most characteristic feature of residue is enhanced hydrogen content compared to parent material (Machnikowska et al., 2002).

The reaction between coal and oxygen at low temperature relies on many factors such as temperature, particle size, surface area, coal pore structure, moisture content, coal rank, and the composition of air. Wang et al. discussed that oxidation of coal at low temperatures (i.e. below 100^0C) is a complex process and involves four phenomena: oxygen passage to the surfaces of coal particles, chemical interaction between coal and O2, release of heat, and emission of gaseous products. The diversity of chemical composition, physical properties such as heat capacity and thermal conductivity, and coal porous structure enhances the intricacy of this marvel (Baris et al., 2012).

2.9.2.1 Bacterial Agents

A number of bacterial species known to degrade coal PAHs isolated from contaminated soil or sediments are *Pseudomonas aeruginosa*, *Pseudomons fluoresens*, *Mycobacterium spp., Haemophilus spp.*, , *Paenibacillus spp* (Haritash and Kaushik, 2009), Pseudomonas putida (Machnikowska *et al*., 2002), bacterial strains like Pseudomonas, Rhodococcus spp. (Zídková *et al*., 2013, Rautela and Cameotra, 2014)e.g Rhodococcus ruber (Füchtenbusch and Steinbüchel, 1999, Larkin et al., 2005), Rhodococcus opacus (Alvarez et al., 1996, Foster et al., 2013), Rhodococcus fascians (Desomer et al., 1990, Martínková et al., 2009), Pseudomonas putida (Weber *et al*., 1994, Jiang et al., 2013, Tang *et a*l., 2013) Ochrobactrum spp. (Arutchelvan *et al*., 2005) Xanthomonadaceae and Rhizobium, together representing 50% of the relative bacterial abundance, as consistently associated with the wood substrate, regardless of fungal presence (Hervé *et al*., 2014)

2.9.2.2 Fungal Agents

Several researchers stated the biodepolymerization of low rank coal by different organisms like filamentous fungi (Faison et al., 1990, Kuhad et al., 2013, Santelli et al., 2013), streptomycetes (Gupta et al., 1990, Jones and Mackie, 2013) and

basidiomycetes, (Willmann and Fakoussa, 1997, Hadar and Cullen, 2013, Coelho-Moreira et al., 2013), deuteromycetes (Faison and Lewis, 1989, FENG et al., 2013) Trichoderma atroviride (Hölker et al., 1999, Anitha and Palanivelu, 2013),Trichoderma agressivum (Abubaker et al., 2013) Lentinula edodes and Trametes versicolar (Götz and Fakoussa, 1999, Veitch, 2004), Pleurotus chrysosporium and other wood rot fungi like Nematoloma frowardii (Rao et al., 2014), Clitocybula dusenii, Auricularia sp., Stropharia rugosoannulata (J. Klein et al., 1999, Jelic et al., 2012), , Funalia Trogii (Ciullini et al., 2008) Picnoporus cinnabarinus(Eggert et al., 1996a, Sathishkumar and Palvannan, 2013) Agaricus compestris, Xerocumus chrysenteron, Russula spp., Lycoperdon perlatum, Morganella pyriformis, Boletinellus merulioides, Leucopaxillus tricolor, Boletus mirabilis, Inocybe sp. (Burke et al., 2014, Azul et al., 2014), Fusarium solani (Cébron et al., 2013)

Table 2.2 Microorganisms responsible for degrading coal under Aerobic Conditions

Microorganisms	Species	References
Bacterial	Rhodococcus ruber	Füchtenbusch and Steinbüchel, 1999 Larkin *et al*., 2005
	Ochrobactrum spp.	Arutchelvan *et al*., 2005
	Pseudomonas aeruginosa Pseudomonas floresens Mycobacterium spp. Haemophillus spp. Paenibacillus spp.	Haritash and Kaushik, 2009
	Pseudomonas spp. Rhodococcus spp.	Zídková *et al*., 2013 Rautela and Cameotra, 2014
	Rhodococcus opacus	Foster *et al*., 2013
	Rhodococcus fascians	Martínková *et al*., 2009
	Pseudomonas putida	Jiang *et al*., 2013 Tang *et a*l., 2013
	Xanthomonas spp. Rhizobium spp.	Hervé *et al*., 2014
	Pleurotus chrysosporium Nematoloma frowardii	Veitch, 2004
	Funalia Trogii	Ciullini et al., 2008

Fungal	Clitocybula dusenii, Auricularia sp. ,Stropharia rugosoannulata	Jelic et al., 2012
	Trichoderma atroviride	Anitha and Palanivelu, 2013
	Trichoderma agressivum	Hadar and Cullen, 2013 Coelho-Moreira et al., 2013
	Picnoporus cinnabarinus	Sathishkumar and Palvannan, 2013
	Lentinula edodes Trametes versicolar	Rao et al., 2014
	Agaricus compestris Xerocumus chrysenteron Russula spp. , Inocybe sp. Lycoperdon perlatum Morganella pyriformis Boletinellus merulioides Leucopaxillus tricolor Boletus mirabilis	Burke et al., 2014 Azul et al., 2014

2.9.3 Microbial Depolymerization

Coal biodepolymerization seems to be a substitute process for the use of low rank coals. As a product of plant origin, coal still holds some molecules lignin derived, which are vulnerable to degradation by some extracellular fungal enzymes(Wondrack et al., 1989). Coal with high gradation has the natural hydrophobicity. Using this natural phenomena, bituminous and anthracite coals can be utilized for oil formation

(Xia et al., 2013). Cohen and Gabriele in 1982 stated that lignite is more willing to liquifaction than the higher rank sub-bituminous and bituminous coals (Faison et al., 1990)

2.9.3.1 Fungal Mechanism of Depolymerization

The mechanisms of fungal depolymerization of the lignite includes: secretion of alkaline substances (Quigley et al., 1988, Quigley et al., 1989), oxidative enzymes (Ralph and Catcheside, 1994);(Willmann and Fakoussa, 1997);(Hofrichter et al., 1997), chelators (Quigley et al., 1989);(Cohen et al., 1990):(Fredrickson et al., 1990); (Fakoussa, 1994) and esterases (Hölker et al., 1999), and hydrolytic enzymes. Both non-enzymatic and enzymatic mechanisms can be involved in coal solubilization by same microbes (Hölker et al., 2002). Many fungi vitiate cellulose and hemicelluloses using extracellular hydrolytic enzymes. White-rot basidiomycetes initiate process by mineralizing lignin, using extracellular oxidative enzymes to cleave recalcitrant biopolymer. In some cases the enzyme may directly outbreak the lignin polymer. Ligninolytic agent is likely a small molecule converting oxidized carbon to a reactive form. After ligninolysis, white-rot fungi using conventional glycosyl hydrolase systems, assimilate remaining polysaccharides that contain both endo and exo nature showing enzymes. Brown rot basidiomycetes degrade lignocellulose competently,with narrow biodegradative systems. These fungi generally lack ligninolytic enzymes, with reactive O_2 species begin decay instead. (Hatakka and Hammel, 2011)

2.9.3.1.1 Lignin peroxidase

(Lip; EC 1.11.1.14) , the most studied enzyme having ligninolytic character was first discovered in the white-rot fungus Phanerochaete chrysosporium (Glenn et al., 1983);(Tien and Kirk, 1983, Kuhad et al., 2013);. Later, in several other basidiomycete fungi e.g.Phlebia radiata this enzyme was found(Hatakka and Hammel, 2011). Phlebia lindtneri and Phlebia brevispora(Xiao et al., 2011); Trametes (Coriolus) versicolor,(Du et al., 2010); Bjerkandera adusta, Kimura et al. 1991; Nematoloma frowardii, (Hofrichter et al., 1997) and one ascomycete Chrysonilia sitophila;(Durán et al., 1987). LiP is a glycoprotein that contains iron protoporphyrin IX (heme)as prosthetic group

and requires H_2O_2 for catalytic activity (Kuhad et al., 2013). It is expressed in multiple forms (isozyms) with MWs of 38-47 kDa(Veitch, 2004)

LiP shows resemblance in catalytic cycle to that of horseradish peroxidase (HRP; (Veitch, 2004)). In the catalytic cycle, the native Fe(III) enzyme is first oxidized by H_2O_2 to LiP compound I, and then a one-electron reduction of compound I with an aromatic compound e.g. veratryl alcohol proceeds, resulting in the development of compound II and an aromatic radical. One further reduction of compound II with an aromatic substrate brings the enzyme back to its native form. In this way the catalytic cycle is maintained (Kuhad et al., 2013) (Wariishi et al., 1991). LiP has a broad substrate specificity for aromatic compounds and oxidizes both phenolic and non-phenolic compounds (Korcan et al., 2013).

LiP is a relatively sensitive enzyme that can be suddenly inactivated by its own co-substrate H_2O_2 or phenolic substances, which are present in lignite in high quantities (Ciullini et al., 2008). Nevertheless, studies showed that isolated LiP has the potential for degrading high-molecular weight coal substances. (Wondrack et al., 1989) stated that coal polymers prepared from nitric acid-treated lignite of North Dakota and German sub-bituminous coal were readily depolymerized by partially filtered P. chrysosporium LiP. The coal polymer, soluble in water and organic solvents like DMF, was depolymerized to smaller water-soluble fragments. Verateryl alcohol enhanced depolymerization in presence of oxalate(Schick Zapanta and Tien, 1997). Ralph and Catcheside (1997) have reported that solubilized low-rank coal shows partial depolymerization through humic acids by ligninolytic cultures of P. chrysosporium. The fungus was found to change about 85% of the coal after 16 days incubation to a form not recoverable by alkali washing and acid precipitation. Extensive coal bleaching revealed presence of extracellular LiP. The decolorized natural and methylated alkali-solubilized lignites were isolated from P. chrysosporium. Enzymatic decolorization showed absolute requirement for veratryl alcohol which acts as a stable redox mediator. A transient formation of an aryl cation radical occurs between LiP and the terminal substrate. (Schick Zapanta and Tien, 1997).

The decolorization procedure reveals macromolecular depolymerization when methylated with $(CH_3)SO_4$ and CH_2N_2. Alkali solubilized coal is not a good substrate for LiP. Methylation seems to aid cleavage of bonds in coal, because phenolic groups oxidized to phenoxy radicals. The radicals also depolymerize non-phenolic aromatic groups which are oxidized by LiP to R-cation radicals. These cations are commonly decayed via pathways involving C-C and C-O bond-cleavage reactions(Park et al., 2011). The methylated, water-soluble coal fraction partly converted to lower-molecular-weight products, among which various methoxylated monoaromatic compounds could be recognized (Kersten and Cullen, 2007).

2.9.3.1.2 Manganese peroxidase

(MnP, EC 1.11.1.13) was also revealed in P. chrysosporium (Kuwahara et al. 1984). The enzyme resembles LiP, having extracellular, glycosylated and heme group as the prosthetic group (Glenn and Gold 1985; Paszczynski et al. 1985; Schneegaû et al. 1997). Mn(II) and Mn(III) having multiple forms are used as complete substrate and mediator, similarly with MWs from 38 to 50 kDa and pIs from 2.9-7.0 (Hofrichter, 2002, Saroj et al., 2013). Besides P. chrysosporium, in many other white-rot and litter-decaying basidiomycetes MnP was found, but not in other fungi or bacteria e.g. T. versicolor, (Carabajal et al., 2013, Jelic et al., 2012); P. radiata,(Mäkelä, 2009, Dong et al., 2013); Panus tigrinus,(Covino et al., 2010, Leontievsky et al., 2000); Lentinula edodes,(Arantes et al., 2011, Saeki et al., 2011); Ceriporiopsis subvermispora,(Fernandez-Fueyo et al., 2012, Cunha et al., 2010); Pleurotus ostreatus,(Golan-Rozen et al., 2011, Jelic et al., 2012); Agaricus bisporus,(Doddapaneni et al., 2013, Kerrigan et al., 2013); N. frowardii,(Hofrichter, 2002, Pozdnyakova, 2012); Stropharia rugosoannulata(Winquist et al., 2014, Jin et al., 2014, Scheibner and Hofrichter, 1998), Clitocybula dusenii (Huang et al., 2013, Ziegenhagen and Hofrichter, 1998) Aspergillus spp. (Conesa et al., 2000, Culleton et al., 2013, Ries et al., 2013) Bjerkandera adusta(Huang et al., 2013, Nelson et al., 2013)

The catalytic cycle of MnP resembles LiP(Cullen and Kersten, 2004), including native enzyme, compound I and compound II redox states (Wariishi et al. 1988). However, differences are seen in the reductive reactions where Mn(II) is a required electron

donor. Both compound I and compound II are reduced by Mn(II) while the latter is oxidized to Mn(III). Mn(III) ions are stabilized to high redox potential via chelation with organic acids such as oxalate, malonate, malate, tartrate or lactate. Chelated Mn(III) in turn acts as diffusible redox mediator that oxidizes phenols (Wariishi et al., 1991, Tu et al., 2014), certain aromatics having methoxylated nature (Margesin, 2014, Delforno et al., 2014) and aromatics having nitro and chloro functional groups (Xu et al., 2014, Hofrichter et al., 1998a) and organic acids (Weidenhamer et al., 2013, Arndt et al., 2013).

The oxidative strength of the MnP system can be considerably enhanced by the presence of appropriate additional mediators such as thiols (Hofrichter et al., 1998a, Dharmarathna et al., 2014) or lipids(Arndt et al., 2014) or unsaturated fatty acids or their derivatives (Vardon et al., 2014, Dal Bosco et al., 2014). Oxidized Mn can cleave chemical bonds which are normally not accessible to attack by MnP e.g. non phenolic aryl ethers (Ding et al., 2014), certain polycyclic aromatic hydrocarbons(Brown and Chang, 2014). MnP also develops H_2O_2 by the oxidation of glutathione and NADPH (Yang et al., 2012). The evidence claims MnP efficiently acts in the absence of external H_2O_2 by organic acid oxidation through ``oxidase-like" reactions (Hofrichter et al., 1998c, Hofrichter et al., 1999a, Arca-Ramos et al., 2014, Woolridge, 2014).

Mix forms of MnP and LiP do exist, which oxidizes both Mn(II) and non-phenolic aromatics(Camarero et al., 2014, Brown and Chang, 2014, Fernández-Fueyo et al., 2014). (Willmann and Fakoussa, (1997)) have verified the introduction of MnP during water-soluble coal macromolecule bleaching of Rhenish lignite (lithotype A) by the basidiomycete strain RBS 1k. They also discovered optimum bleaching i-e decolorization of humic acids and depolymerization attained at a concentration of 0.5 g/l per coal macromolecule.

Enzymatic analysis showed persistence of MnP in the culture supernatant which was induced by addition of humic acids in coal; no activity of LiP and laccases was detectable. In vivo study reveals, the Mn(II)- induced cultures of basidiomycetes Nematoloma frowardii and Clitocybula dusenii(Pozdnyakova, 2012, Hofrichter, 2002) extracted coal humic substances quickly by developing low-molecular-weight fulvic

acids(Dong et al., 2006, Hofrichter et al., 1998b) . By batch serving, humic acids transformed within 7 days were up to 2 g/l of coal (Hofrichter et al., 1999b). After coal was added to the cultures, the activity of MnP increased significantly, indicating an inductive effect for the coal humic substances and the involvement of MnP in the depolymerization process(Steffen et al., 2002).

In vitro studies show depolymerization of lignin-o-cellulosic substances using MnP from N. frowardii(Pozdnyakova, 2012) that the enzyme is capable of vitiating coal humic substances in a cell-free system (Hofrichter et al., 1997). The depolymerization of coal exposed severe decrease in absorbance at 450 nm (bleaching), while development of low molecular-weight fulvic acid-like materials absorbing at 360 nm was observed. GPC demonstrated that the main molecular mass (most abundant molecular mass) decreased from 3 kDa to 0.7 kDa during the conversion of humic acids to fulvic acids (Ziegenhagen and Hofrichter, 1998). The fulvic acids optimized the MnP-catalyzed depolymerization of coal humic substances using MnP from C. dusenii (Huang et al., 2013). H_2O_2 was continuously supplied by glucose oxidase (Yang et al., 2012). Small amounts of glutathione (GSH) acting as additional ``thiol mediator'' and the organic solvent DMF increased the depolymerization process. The reaction could be achieved at 37 °C without loss in activity due to enzyme inactivation. MnP was found to be tremendously stable under the reaction conditions used (37 °C, 200 rpm) and remained active in part over weeks. The in vitro degradation of 14C-labelled synthetic humic substances derived from the oxidative polymerization of 14C-catechol demonstrates the enormous oxidative strength of the MnP system (Dharmarathna et al., 2014, Hofrichter et al., 1999b). Along conversion of macromolecules to low-molecular-weight fulvic acids, a considerable discharge of $14CO_2$ The enormous oxidative strength of the MnP system was observed, representing the ability of MnP to mineralize humic substances directly through mechanism named enzymatic combustion(Hofrichter et al., 1998a).

2.9.3.1.3 Other Peroxidases

Other peroxidases making depolymerization of macromolecules are soya bean hull peroxidase and horseradish peroxidase. SBP and HRP have also been examined for

their ability to change low-rank coal. It was recognized that SBP catalyzed partial depolymerization of a high MW coal fraction while HRP showed rise in coal solubility when used with hydrous organic media i-e, pyridine and dioxane; and up to 44% of coal was converted to soluble products (Scott et al. 1990).

2.9.3.1.3.1 Laccase

It is a Cu-containing enzyme having polyphenol oxidase blue color (Riva, 2006)produced by nearly all ligninolytic fungi e.g. Trametes versicolor (Fahraeus and Reinhammar, 1967), Phlebia radiata, (Kantelinen et al., 1989); Pycnoporus cinnabarinus, (Eggert et al., 1996b); Nematoloma frowardii, (Hofrichter and Fritsche 1997a) and also by many molds and higher plants (e.g. Neurospora crassa, Aspergillus nidulans,(Claus, 2003, Thurston, 1994); Nicotiana tabacum, Acer pseudoplatanus) (O'Malley et al., 1993). It is a glycosylated protein that is expressed in multiple forms. It depicts high MW variability (59-110 kDa). The enzyme oxidizes different compounds while reducing O_2 to H_2O, a total reduction by four electrons. It shows a low specificity to electron-donating substrates and differential substituted mono, di and poly phenol compounds are preferred (Buswell et al., 1987). The catalytic center of blue laccases contains four copper ions (Cu++) which are paramagnetic and thereby ESR-detectable (Sharma et al., 2007). The catalytic cycle of laccase has been proposed to resemble that of blue ascorbate oxidase and comprises several one-electron transfers between the copper atoms while O2 is bound to the active site of the enzyme(Ducros et al., 2001).

The action of laccase on phenolic aromatics results in the formation of phenoxy radicals, which readily undergo non-enzymic reactions such as radical-radical coupling, disproportionation, deprotonation and nucleophilic attack by H_2O. Multiple reactions lead fInally to polymerization, alkyl-aryl cleavage, Ca-oxidation or demethoxylation of the phenolic reductant (Brijwani et al., 2010).

Laccase also produces Mn(III) chelates in the presence of Mn(II) and appropriate phenolic substrates (e.g. m-cresol; (Archibald and Roy, 1992), thus resembling MnP activity. The ability of laccase to oxidize Mn(II) to Mn(III) indirectly has been

proposed to be a partly autocatalytic process involving the enzyme-catalyzed formation of phenoxy radicals and spontaneous reactions of the latter with (O_2) to form superoxid which in turn reacts with Mn(II) to give Mn(III) and H_2O_2 (Youn et al., 1995). Recently, so-called yellow laccases have been discovered during solid-state fermentation of Panus tigrinus and P. radiata on wheat straw(Leontievsky et al., 1997b). Yellow laccases seem to have a changed oxidation state of copper probably caused by the integration of aromatic lignin degradation products into the catalytic site of the enzyme. Interestingly, evidence was provided that yellow laccases are capable of oxidizing non-phenolic aromatics (veratryl alcohol, b-O-4 lignin dimers) directly in the presence of O_2, but without any mediator substrate(Leontievsky et al., 1997a). Our current investigation has given indication that yellow laccases are also produced by T. versicolor during solid state cultivation on coal humic acid containing agar media. All these data point to a broader role of laccase in lignin biodegradation and therefore also in lignite biodegradation(Arora and Sharma, 2010).

2.9.3.1.3.2 Esterase

Laccase partly purified from T. versicolor did appear to be active towards leonardite. Therefore the ``biosolubilizing" activity of T. versicolor was due to laccase activity. Further purification of the laccase fraction indicated that it contains two proteins: one showed laccase activity, but little effect on coal, while the other had little laccase, but high coal-solubilizing activity. The latter protein was found to possess an esterase activity(Campbell et al., 1988). The esterase has found to contain much less coal-solubilizing activity than other components present in T. versicolor culture fluids. More detailed analysis of the low-molecular-weight fraction demonstrated that the majority of the coal-solubilizing activity was associated with much lower MWs.

2.9.3.1.3.2 Hydrolases

The coal solubilizing activity of this fungus is thus attributed to a `mixed action" of hydrolytic enzymes and non-enzymatic agents. With regard to the involvement of esterases in coal solubilization/liquefaction it seems to be important that esterases are not known to act by the mediation of a messenger molecule (``mediator"). This leads to

steric obstacles, as the whole esterase molecule cannot invade the macromolecular coal network. Therefore more research is needed to prove their actual role in detail. The same is valid for other hydrolases.

2.10 Coal a difficult substrate for Microbial Growth

Coal as a substrate hard to consume by most of microorganisms because it contains poly aromatic hydrocarbons, hydrophobic and heterogenic state along with many pores found in its structure. Therefore in order to remove these hurdles, extracellular mechanisms useful to attack coal cleavage is needed. Moreover, adsorption of cells on coal surface accounts serious impact in relation to degrade multifaceted structure of coal. As coal contains recalcitrant compounds therefore it is insoluble in microbial media. Various biochemical methods like protein determination, separation of bacteria, fungi or enzymes from coal-containing media are also difficult to estimate direct analysis. Hurdles also lies in reference to photometric assays in measuring optical density of chemical and physical analytes or isolates derived from coal. It is also difficult to measure growth rate of microorganisms on the basis of substrate conversion (Fakoussa and Hofrichter, 1999)

3. MATERIALS AND METHODS

The qualitative research was carried out at Environmental Microbiology Research Laboratory, Dept. of Microbiology, QAU, Islamabad, Pakistan.

3.1 SAMPLE COLLECTION

Samples were collected from Coal Mines, Salt Range at Dulmial Village, Tehsil Choa Saidan Shah, District Chakwal, Pakistan. The coal samples collected were sub bituminous to bituminous ranks, collected from various depths of mine far ranging distance up to 1 km from mine entrance. Also water samples collected from all regions, left and right corners, center of water mine. The slurry coal water was also taken from 1100 meters depth of water coal mine. All the samples collected, kept at 4^0C in sterile containers for experimental studies.

Temperature inside mines and pH of soil and water were also measured. Temperature was observed to be about 30^0C and pH was 5.9.

3.2 Media for Cultivation and Degradation Experiment

Potato Dextrose Agar (PDA, Criterion, Hardy Diagnostics, Santa Maria, US) was used for observing, detecting, maintaining and growing fungal cultures in plates and test tube slants. Nutrient Agar media (Oxoid, Basingstoke, Hampshire England) was used for isolation of bacterial strains. Scharlau, (Gillman, Australia) Chemicals and Merck, (Darmstadt, Germany) reagents of analytical grade were used in basal mineral salt medium (**MSM Table 3.1**) for solubilization studies of coal. The composition of MSM is shown in table.

Table 3.1 Composition of Mineral Salts Medium (MSM)

Ingredients	Amount (g/L)
K_2HPO_4	1.0
KH_2PO_4	0.2
NaCl	1.0
$CaCl_2.2H_2O$	0.002

Boric Acid	0.005
$(NH_4)_2.SO_4$	1.0
$MgSO_4 .7H_2O$	0.5
$CuSO_4$	0.001
$ZnSO_4 .7H_2O$	0.01
$MnSO_4$	0.001
$FeSO_4 .7H_2O$	0.01
Agar	20 (2%)

The sterilization of media was carried out at 121^0C and 15lbs/in² pressure in 20 minutes cycle in Tomy Digital Autoclave (SX-30DE, SX-70DE), Tokyo, Japan). The pH of media was adjusted (pH 7.0) prior to sterilization with 0.1N sodium hydroxide and hydrochloric acid.

3.3 Isolation and Screening of Microbial Isolates using Enrichment culture technique

For the purpose of microbial isolation, shake flask experiment was conducted.

In this experiment 10% soil, coal and water was added in 100ml MSM containing 0.1% coal as a carbon source and incubated at 30^0C in a rotatory shaker at 150 rpm for 14 days. After 14 days, inoculum was taken from these flasks and added at the ratio of 10% in MSM with increased coal concentration from 0.1-0.5%. Using same procedure we keep on increasing coal concentration from 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5% and 6%. At each step, screening was done by plating on coal agar plates by Pour Plate method. Coal agar plates were prepared by taking 2% agar in MSM. Sub culturing was done on nutrient agar and PDA plates for the isolation of colonies. We preserved our fungal and bacterial isolates in nutrient agar and PDA slants as well as in glycerol.

3.4 Selection of Isolates on the Basis of Biosolubilization Activity

The coal biosolubilization test was conducted in petri dishes. In the first step, we had grown our fungal and bacterial isolates on nutrient agar and PDA plates at 30^0C. Then we placed 1g coal chunks (0.2mm -0.5mm in diameter) on these plates. The plates were placed in static incubator for 14 days. After incubation period, we observed each plate with black liquid formation around each coal particle. On the basis of these results, we selected isolates for further study.

3.4.1 Coal Biosolubilization Experiment

The selected coal solubilizing aerobic microorganisms were further used in study of coal solubilization.

For this experiment, inoculum was prepared by taking 100ml nutrient broth in 250 ml Erlenmeyer's flasks for bacteria and PDA plates for fungal isolates. These flasks and plates were kept at 30^0C for 24 hours (bacteria) and 5 days (fungi). 10% bacterial culture and 3 (1cm) agar plugs were used as inoculum in this experiment. 300 ml MSM with 2%coal in 500ml Erlenmeyer's flasks was used for each isolate. After inoculation, flasks were placed in shaking incubator (Innova 4330, Brunswick Group, Canada) at 30^0C with 150 rpm shaking revolutions. Positive control with MSM and inoculum lacking true coal sample and negative control having same coal concentration as sample cultures without inoculum were also prepared and placed under same conditions. Samples were taken at different time intervals for further analysis.

3.4.2 Biosolubilization Percentage Determination

Biosolubilization was quantified by simple petri plate experiment proposed by Yin *et al*. For this purpose, lawn of bacterial and fungal isolates was grown on nutrient agar plates. Coal pieces were weighed and spread on the surface of fully grown plates. The plates were kept in biological incubator at 30^0C. After 72 days of incubation, again weigh these coal pieces. The biosolubilization was determined by the net weight loss of coal pieces and was calculated by this formula

$$P = \left(1 - \frac{W1}{W0}\right) \times 100\%$$

Where P = solubilization percentage

W_0 = initial weight of coal sample

W1 = weight of residual coal sample after solubilization

3.5 Identification of coal solubilizing isolates

Identification was performed by study of

1. Biochemical characteristics
2. Morphological characteristics

3.5.1 Biochemical Characteristics

The biochemical tests performed to identify microbial isolates showing novel biochemical properties are as follows;

3.5.1.1 SIM Test

It's generally called Sulfur Indole Motility test. It shows pairing of three differential tests. The H_2S gas produced as a result of sulfur reduction while motility of organism along with NH_3 production shows positive results.

3.5.1.2 Methyl Red test

This test shows presence of varied acidic fermentation when glucose is supplied to microbial culture. The presence of red color due to indicator shows positive results while orange or yellow color shows negative results.

3.5.1.3 Simmons Citrate Test (citrate utilization test)

This test is used to understand whether microorganism is capable to cultivate on citrate, utilizing as carbon source or energy for growth. Bromo thymol blue used as pH indicator turned media to blue on basic showing positive result, yellow on acidic and green on neutral pH showing negative result.

3.5.1.4 TSI (Triple Sugar Iron)

This differential test is used to see ability of simple microorganisms to ferment carbohydrates producing H_2S. Medium contains sucrose, lactose, glucose, $FeSO_4$ and phenol red. If only glucose is fermented, the acid produce in butt will change the color to yellow. If acid production is higher due to sucrose and lactose fermentation, then

acidic product will change both butt and slant yellow. If no fermentation in slant and butt occurs then color remains red. If H_2S gas is produced, then black color is formed.

3.5.1.5 Urease Test

This test used to test microbial skill in producing urease in medium degrading urea to NH_3 and CO_2. The pink color formation results positive result while yellow color showing negative result.

3.5.1.6 Gelatin hydrolysis test

This test determines whether microorganism is able to utilize gelatin by producing gelatinase. Gelatin gets liquefied on gelatinase production even at freezing temperature or 4^0C showing positive results while if gelatinase is not formed, it shows negative results.

3.5.1.7 Oxidase test

This test is used to reveal whether the microorganism possess cytochrome oxidase or indophenol oxidase. This test was performed on filter paper showing color change around the colony. The crystal oxidase reagent turns blue when cytochrome oxidase adds electrons in place of oxygen. The development of blue color shows positive results while no change results in negative results.

3.5.1.8 Catalase test

This test is used to reveal whether the microorganisms possess the ability to produce H_2S gas showing catalase enzyme. This test was performed on slides. The smear of microbial colony forms several bubbles when 3% H_2S was dropped showing positive results. Absence of bubbles shows negative results indicating absence of catalase.

3.5.1.9 Vogues Proskeur Test

This test shows whether 2, 3-butanediol producing as a result of glucose fermentation. One of the intermediate of this pathway reacts with methyl red indicator showing positive result with red color.

3.5.2 Morphological characteristics

3.5.2.1 Colony morphology

The morphology of isolates forming colonies on coal degradation, nutrient agar and PDA plates were observed in laminar hood under sterile conditions. The colony size, color, form and growth pattern were observed in plates.

3.5.2.2 Smear Formation and Gram Staining

Using heat fixation technique, smear on slide was fixed. Then gram staining protocol was observed. The crystal violet was poured for 10-20 sec, iodine for 35 sec and decolorizer for 15 seconds. Then, safranin was poured. Now slide was left to counter strain for 45 sec. Each step was followed by washing. After staining, slides were dried using blotting paper and were observed under light microscope.

3.6 Analytical Techniques

3.6.1 Spectroscopy

3.6.1.1 Ultraviolet-Visible Spectrometry (UV-Vis)

The UV-vis Spectrometry was done using UV-Vis 8453 Agilent Technologies, USA. The negative control taken as blank was MSM in coal without addition of inoculum. The absorbance of experimental samples was checked at 450nm using tungsten filament after each 6-7days upto 72 days. Experiment with each isolates was run in triplicate (Willmann and Fakoussa, 1997).

3.6.1.2 Fourier Transform Infrared Spectroscopy

The FTIR spectroscopy was performed using method proposed by (Sutcu and Toprak, 2013) for analyzing coal samples. Water content of solubilization products was dried using rotatory evaporator. KBr-sample mixture pellet (0.2%-1%) was prepared for FTIR analyses.. The samples were analyzed with pure KBr using Perkin Elmer 56, Infrared Spectrometer with light wavelength from 4000 cm- to 600 cm-.

3.6.2 Scanning electron microscopy,

SEM was performed from Centralized Resource Laboratory, University of Peshawar. The SEM model JSM 5910 lv (Jeol SEM, Japan) fitted with Energy Depressive X-Ray Analyzer, EDS (Inca 200, Oxford Instruments, UK) presently used

by lab. In SEM, the electron beams, (10,000eV - 30,000eV) formed by ionic source showing greater energy and temperature.

Using pre sterilized forceps coal samples were removed from the medium and washed with sterile pre warm minimal medium. Cold medium was used to prevent displacing of cells attached on coal samples. Cells attaching coal surface are fixed using silver nitrate (Sigma- Aldrich, Germany) in 0.1M potassium phosphate buffer for 2.5hours. After drying, samples were placed on aluminum stubs sputter coated with gold layer using Emitech K550 sputter coater (Quorum Technologies, UK) and then viewed under electron microscope (Nur Hazlin Hazrin-Chong and Manefield, 2012).

4. RESULTS

4.1. Isolation and Screening of Microbial Strains solubilizing coal using enrichment cultures

Different types of fungi and bacteria from soil, water and coal samples were isolated using isolation culture medium (a mixture of different concentration of coal powders and MSM). Screening was performed at each concentration by growing colonies on MSM agar plates so that only coal utilizing strains were isolated (fig 4.1). Rich growth was observed upto 3% but gradually decreases and very little growth observed at 6% (table 4.1). Pure Colonies of different isolates were achieved through repeated subculturing. All these isolates are capable of solubilizing coal under our test conditions.

Table 4.1 Microbial growth on Coal MSM agar plates under various concentrations of coal

Samples	Coal Concentration (%) in MSM agar plates									
	0.1	0.5	1	1.5	2	2.5	3	4	5	6
S1	+++	+++	+++	++	++	++	+	+	+	-
S2	+++	+++	+++	+++	++	++	++	++	+	+
S3	++	++	+	+	+	+	+	+	+	+
S4	++	++	+	+	+	+	-	-	-	-

Rich growth +++, Good growth ++, Poor growth +, No growth –

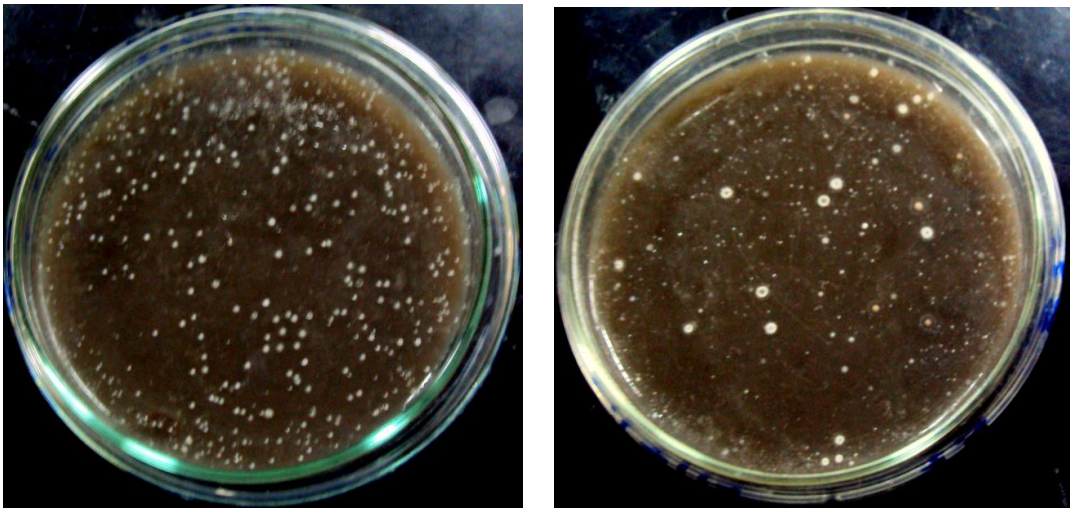


Fig 4.1 Screening of microorganisms by growth on MSM coal agar plates

4.2. Selection of Isolates on the Basis of Coal Biosolubilization Activity

For coal solubilization study, stains showing good solubilization potential were selected on the basis of plate coal solubilisation experiment. Two bacterial isolates AY2, AY3 and two fungal isolates AY5, AY6 showed good solubilization potential when grown on plates with coal particles. Black liquid formation was clearly observed around each coal particle (fig 4.2, 4.3, 4.4, 4.5). This conclusion was further supported by control experiment indicated that in the absence of these strains, coal was not solubilised by the culture medium to produce a black liquid.

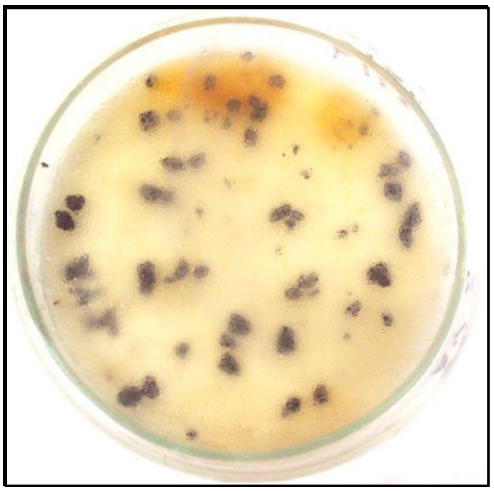


Fig 4.2. AY 2 Isolate showing coal solubilization with formation of black liquid.

Fig. 4.3. AY 3 Isolate showing coal solubilization with black liquid formation.

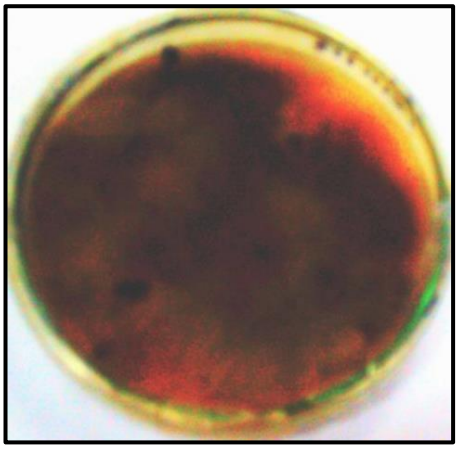


Fig.4.4. AY 5 Isolate showing coal pieces solubilization with blackening of whole plate.

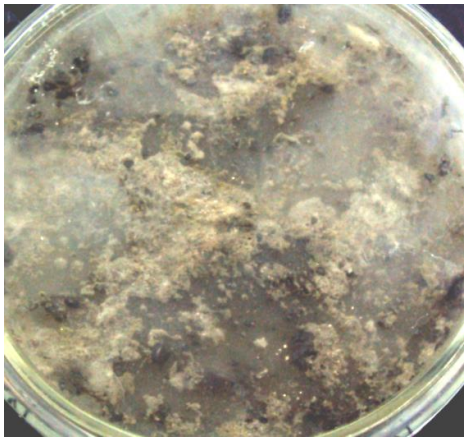


Fig. 4.5. AY 6 Isolate showing coal pieces solubilization with blackening of whole plate showing formation of green color mycelia.

4.3 Identification of Selected Isolates

Coal solubilizing strains were characterized on the basis of morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology. Coal solubilizing isolates were identified by the study of morphological and biochemical characteristics.

4.3.1 Morphological Characteristics

For the identification of bacterial isolates, the morphology of isolates studied on nutrient agar plates while fungal isolates on potato dextrose agar plates. Bacterial isolates, AY 2 showing off white shade with small colonies, irregular form, and undulate patterns having raised elevations from edges (fig 4.7). AY 3 isolates showing moderate size colonies of white color. The colonies showing irregular form and umbdonate pattern with slightly raised morphology from streak margins (fig 4.7). AY 5 indicated thread like form spreading uniformly making lawn on whole plate. Ay 6 shows green color growth spreading on whole PDA plate (fig 4.7).

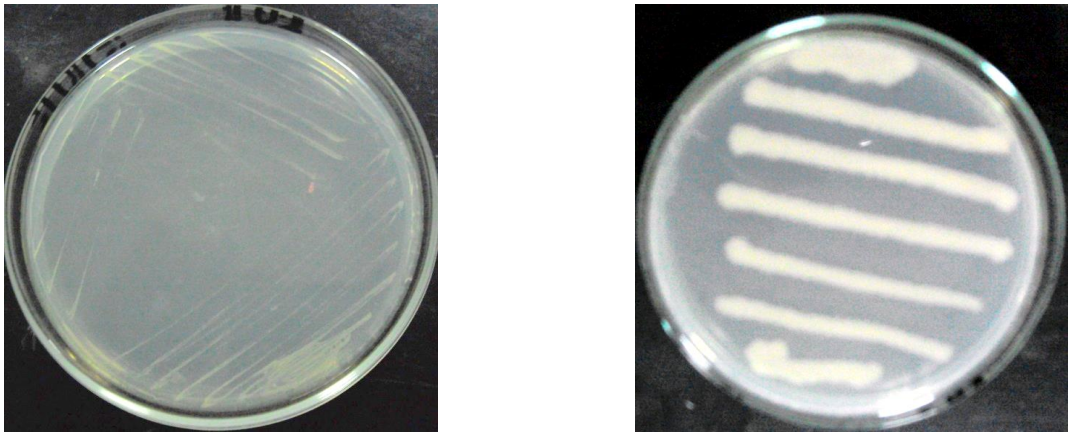


Fig 4.6 Growth of AY 2 and AY 3 isolates on nutrient agar showing colony morphology

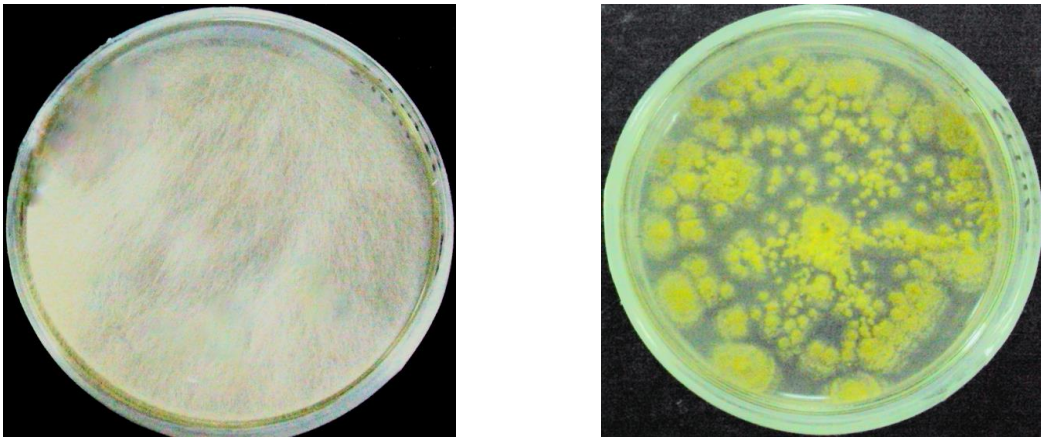


Fig 4.7 Cultures of AY 5 and AY 6 isolates on PDA plates showing growth morphology.

4.3.2 Biochemical Testing

The biochemical tests were performed which facilitate identification of bacterial isolates.

Table 4.3 Biochemical characteristics of bacterial isolates

Biochemical Tests	AY2	AY3
Motility	-	+
Indole production	-	-
Gas Production	-	-
Methyl Red	+	-
Simmon citrate	+	-
TSI	Butt yellow Slant Red	Butt Yellow Slant Red
Urease	-	-
Gelatin	-	+
Oxidase	+	-
Catalase	+	-
VP Test	-	-

4.4 Biosolubilization Percentage Determination

The biosolubilization percentage was determined by the method proposed by Yin (2009). Weight of coal pieces were measured before and after incubation for 30 days against each of the strain. Then solubilization percentage was calculated by putting values of initial and final weight in the following formula

$$P= (1- \frac{W1}{W^0}) \text{ x } 100 \text{ \%}$$

AY5 with 66.67% showed maximum solubilization potential. AY6 presented 50% solubilization while 36.36 was the solubilization % of Ay3. AY2 with 25.93% showed least biosolubilization among the four strains (table 4.4).

Table 4.4 Biosolubilization percentage of AY2, AY3, AY5 and Ay6

Isolates	AY 2	AY 3	AY 5	AY 6
Initial mass of sample (g)	0.27	0.22	0.30	0.05
Final mass of sample (g)	0.20	0.14	0.10	0.025
Solubilization time (days)	30	30	30	30
Solubilization percentage (%)	25.93	36.36	66.67	50

4.5 Coal Biosolubilization Experiment

Shake flask coal biosolubilization experiment was conducted with all of the four strains for 72 days. Experiment of each strain was conducted in duplicate. Samples were withdrawn after regular time intervals for analysis of change in absorbance and change in coal structure through FTIR.

4.5.1 Change in Absorbance

Change in absorbance was observed at 450nm wavelength using ultraviolet visible spectrophotometer. The UV-spectrophotometry results shows increase pattern in absorbance in AY 2, AY 3, AY 5 and AY 6 in relation to control sample. AY6 showed maximum increase in absorbance (fig 4.9).

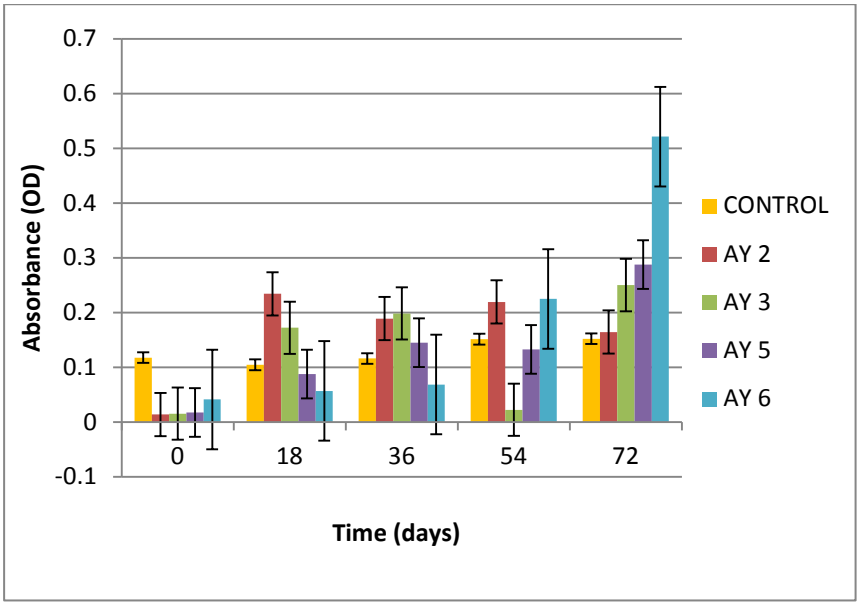


Fig. 4.8. Change in absorbance (OD) as a result of coal solubilisation

4.5.3 Fourier Transform Infra Red Spectroscopy

Change in coal structure as a result of coal solubilization was observed with the help of Fourier transform infrared spectroscopy (FTIR). For this purpose water content of sample was removed by rotary evaporator. All microbial isolates AY 2, AY 3, AY 5 and AY 6 were analyzed against control samples. Wave length of samples shows peaks ranging from 4000cm^{-1} to 500cm^{-1}. Absorbance peaks were labeled across y-axis while sample wavelength on x-axis. The major absorbance bands in infrared spectroscopy for solubilisation product were attributed to hydroxyl (3450 cm^{-1}), carboxyl (3300–2500 cm^{-1}), cyclane (2925 cm^{-1}), carbonyl (1600 cm^{-1}), ether linkage (1000–1300 cm^{-1}) and side chains of aromatic ring (1000 - 500 cm^{-1}).

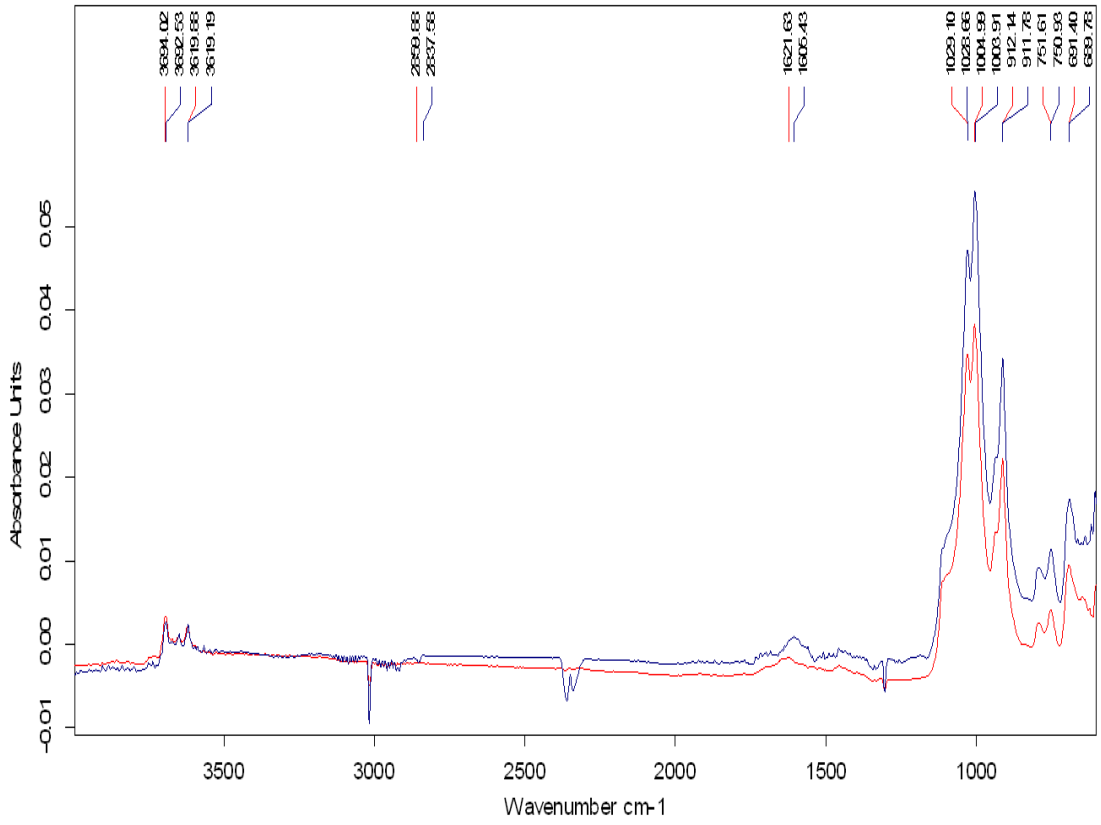


Fig 4.9 FTIR spectrum of coal sample after incubation with AY2 (blue) and control (red).

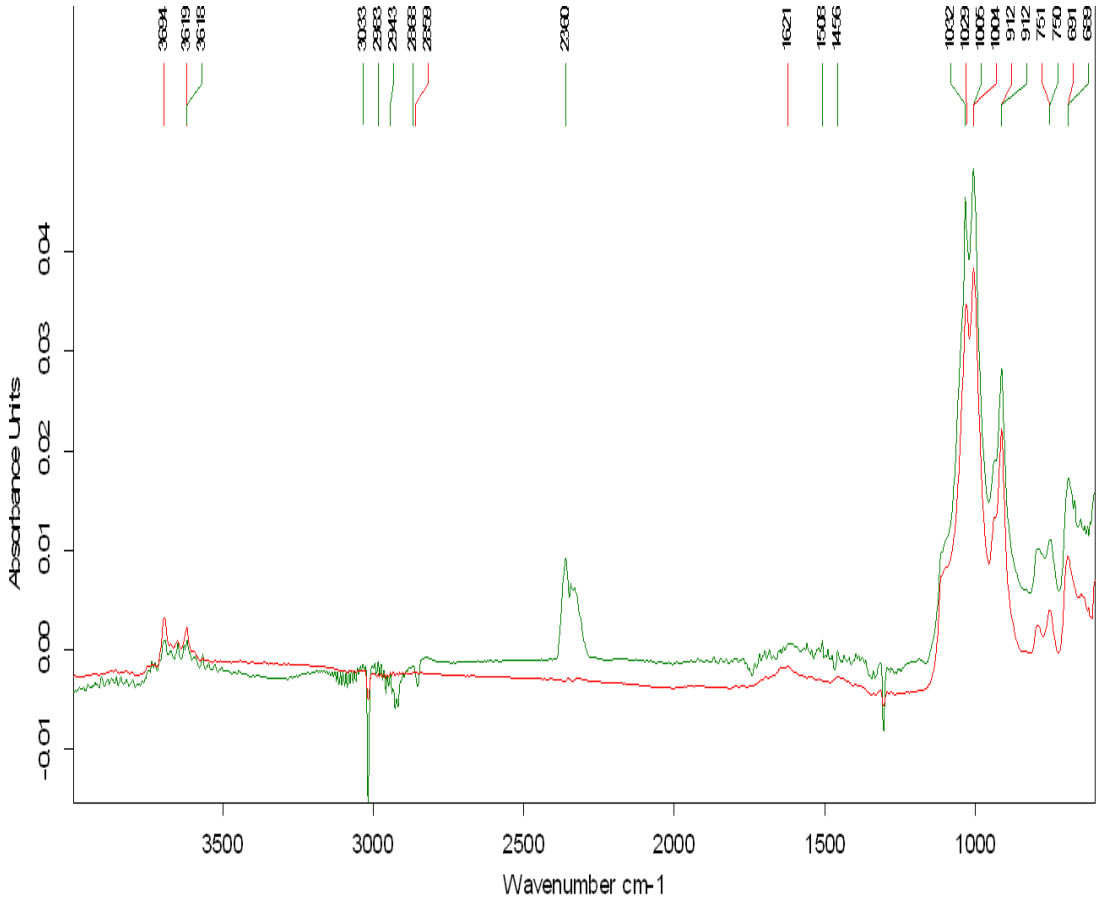


Fig 4.10 FTIR spectrum of coal sample after incubation with AY3 (green) and control (red).

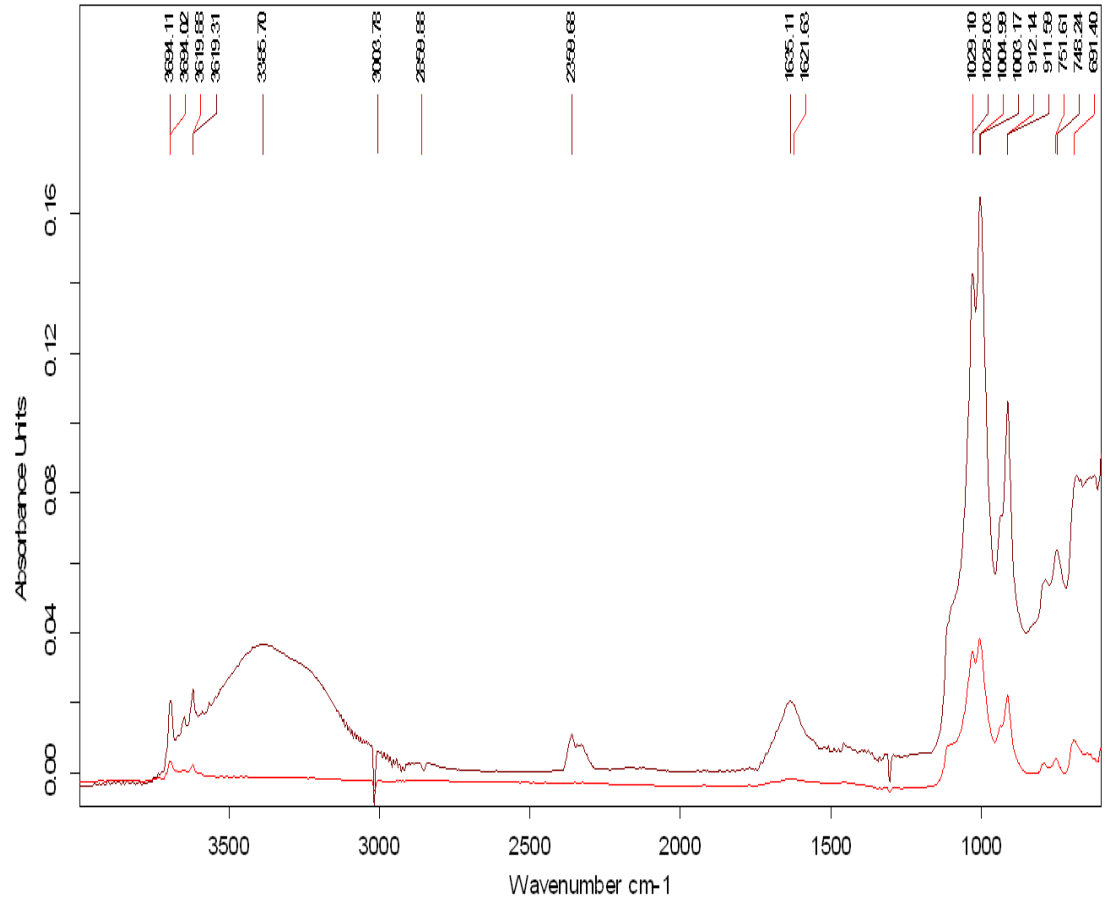


Fig 4.11 FTIR spectrum of coal sample after incubation with AY5 (purple) and control (red).

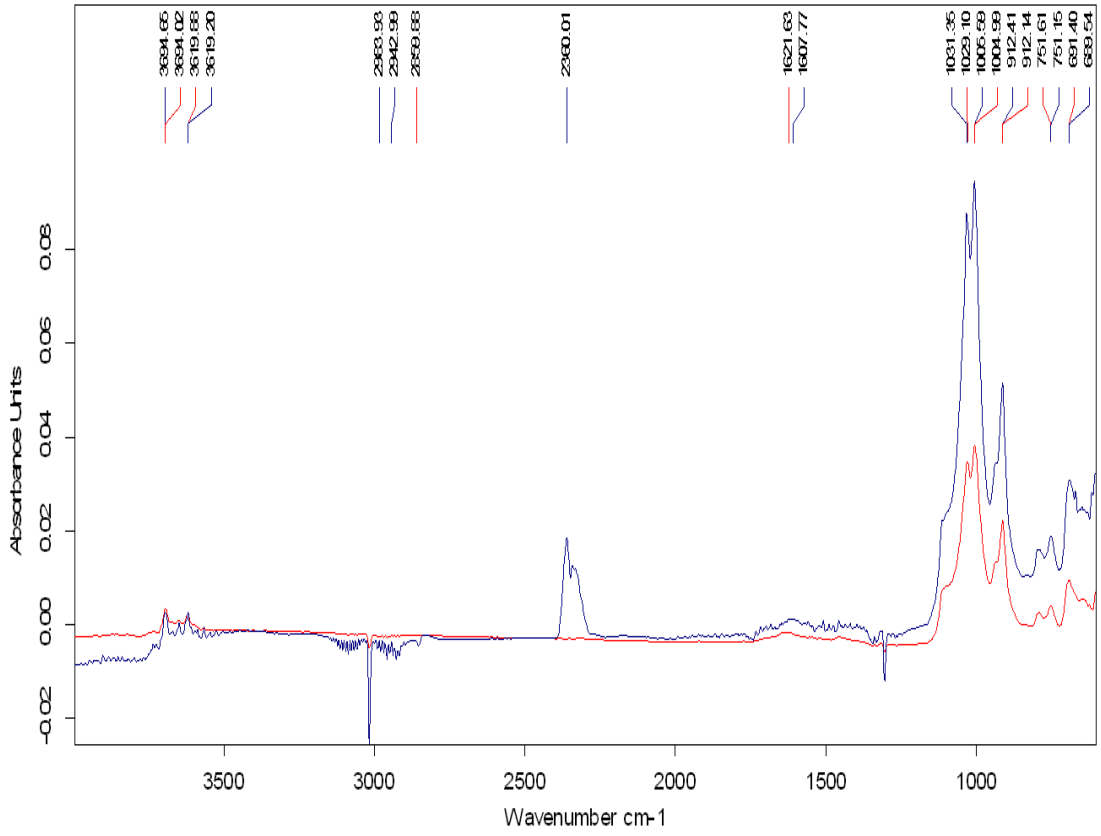


Fig 4.12 FTIR spectrum of coal sample after incubation with AY6 (blue) and control (red).

4.4.4 Scanning Electron Microscopy

Cells of AY2, AY3, AY5, AY6 were grown in MSM with coal pieces for 72 days. As observed by SEM, some bacterial and fungal strains produce extracellular polymere-like structure when growing in the presence of coal that could be involved in the attachment of the microorganisms to the coal surface favouring the attack of coal components and nutrient uptake from the coal particles. Control sample showing surface of coal without adherence of any microbial cell (fig. 4.14). Rod shaped cells of AY2 and AY3 were clearly seen adhering to coal surface (fig. 4.15, 4.16). Hyphae of AY5 and AY6 were penetrating the coal particle and causing coal surface erosion as shown in fig 4.17 and 4.18.

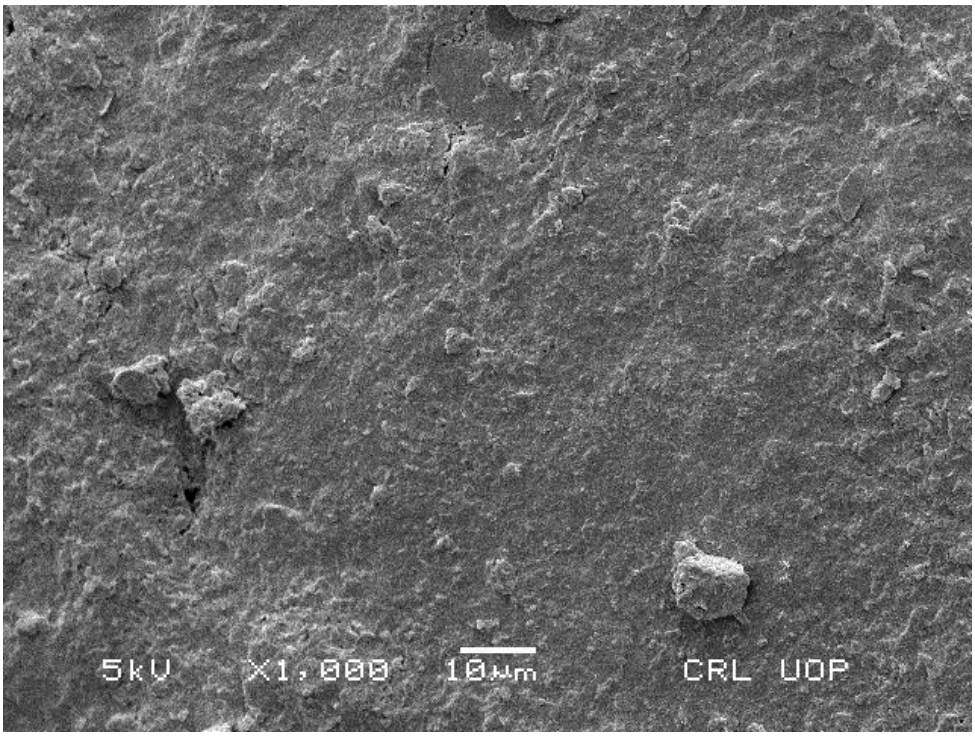


Fig 4.13 Control sample of coal showing surface of coal and lack of attachment of any microbial cell to the surface

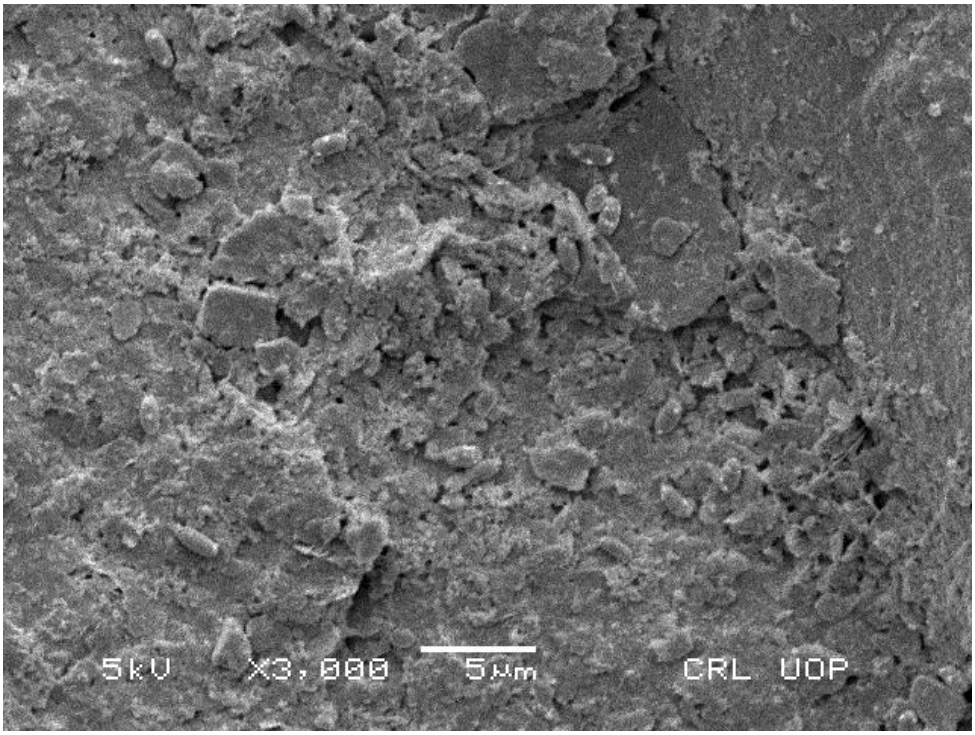


Fig 4.14 Representative SEM micrograph of AY2 colonizing coal at the magnification of 3,000.

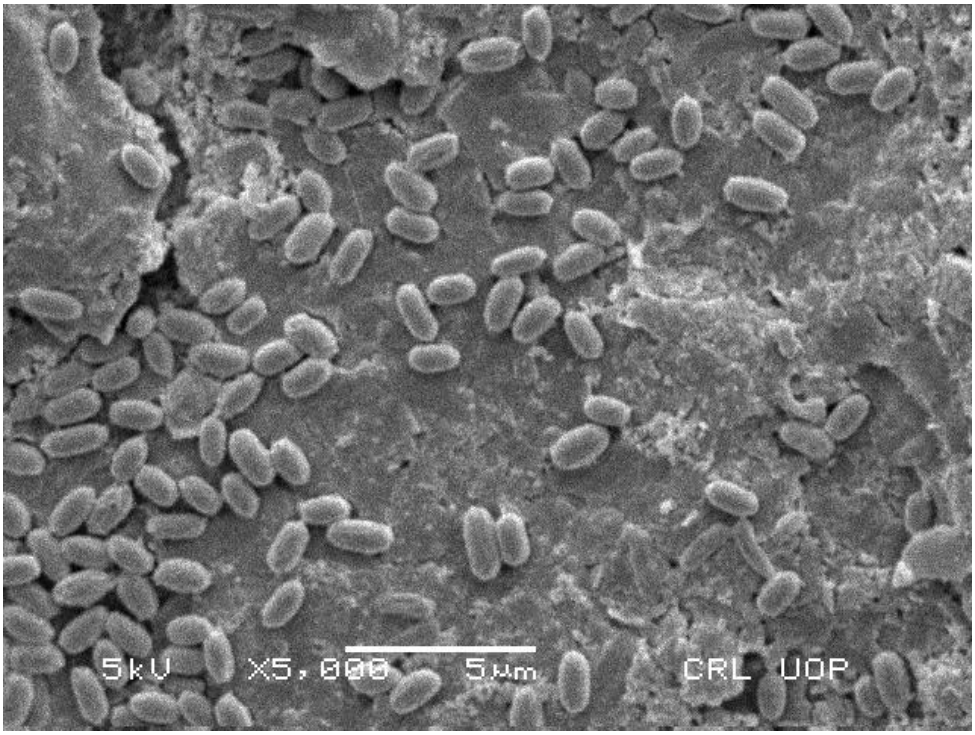


Fig 4.15 Representative SEM micrograph of AY3 colonizing coal at the magnification of 5,000.

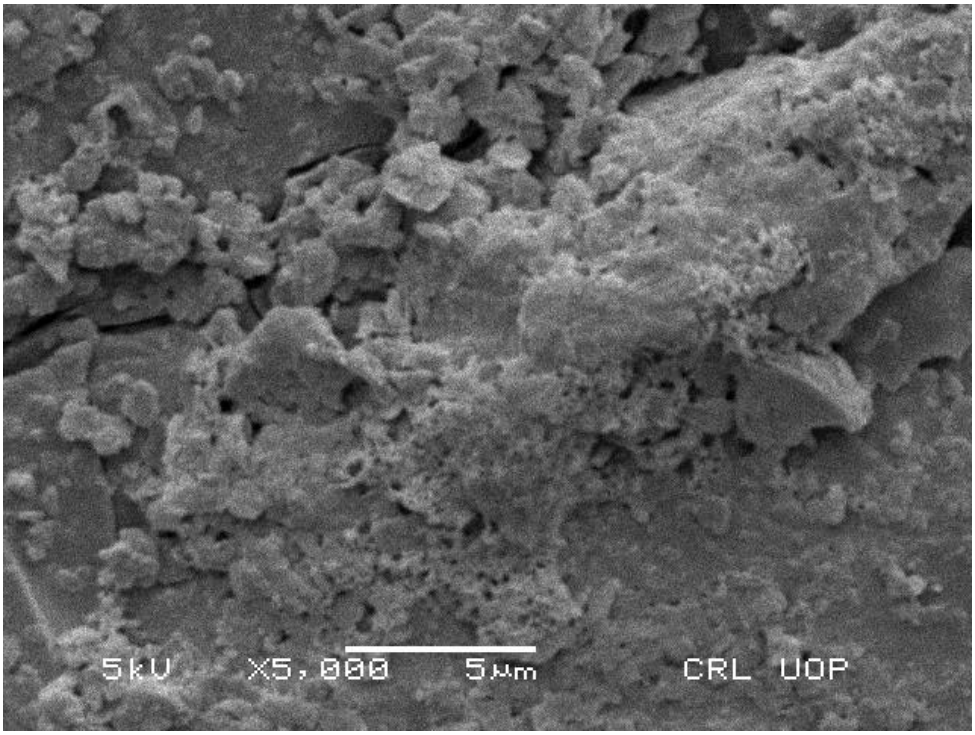


Fig 4.16 Representative SEM micrograph of AY5 colonizing coal at the magnification of 5,000.

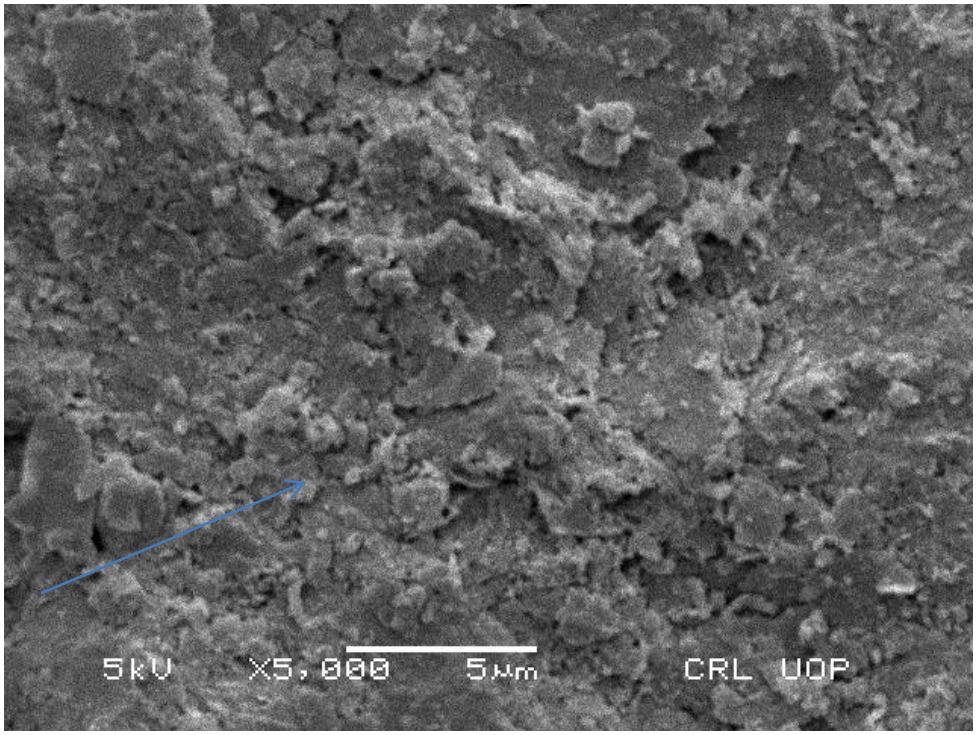


Fig 4.17 Representative SEM micrograph of AY6 colonizing coal at the magnification of 5,000.

Discussion

Globally, there are approx. 929 billion tons coal reserves which are larger than those of oil. About 40% of these reserves are used to harvest the electricity. Therefore coal could become one of important energy resource. Despite of abundant natural energy resources, Pakistan has been facing severe challenge of energy crises since last many years. Coal can be degraded by certain bacteria and fungi through solubilization, depolymerization and by utilization as growth substrate.

The present research work was carried out to isolate and screen the coal solubilizing aerobic microorganisms. Concentration of coal gradually increased from 0.1% to 6%. Initially very rich growth was observed, eventually it decreased until very little growth observed at 6% when plating on MSM coal agar plates. Concentration more than 6% has inhibitory effects on the enzymatic activity of fungus and slows down the solubilization of coal. In all of experiment, 1% coal was used to carry out efficient solubilization. (Silva-Stenico et al., 2007) also reported solubilization efficiency at 1% of coal.

Sampling of coal and isolates was done from dulmial coal mine (Salt range). Initially different bacteria and fungi were isolated from samples. (Jinshui et al., 2004) reported the isolation of Penicillium sp. having ability to degrade lignite coal from Qiantong colliery coal mine china. Fungal strains were isolated from moldy wood showed more potential to solubilize the lignite coal then isolated from soil and decaying leaves (Tao et al., 2009).

A similar pattern of isolation were observed earlier in 1982, when Cohen and his co scientists reported Polyporus versicolor having potential to solubilize chiness lignite. (Jiang et al., 2013) reported the role of isolated bacterial strain Bacillus sp. Y7 for solubilization of Chinese lignite. Trichoderma atroviride showed its adherence and growth on low rank coal(Silva-Stenico et al., 2007).

The addition of glucose in medium speeds up the biodegradability of coal by Trichoderma atroviride strain. The medium supplemented with sugar showed more potential for coal bio fragmentation (Silva-Stenico et al., 2007). In current study the

isolates were purified on repeated subculturing by streak plate method on mineral salt media and nutrient rich medium. The availability of suitable substrate is important for biodegradability of coal (Willmann and Fakoussa ,1997; Silva-Stenico *et al.*, 2007)(Selvi et al., 2009). Yuan et al. (2004) maintained the coal degrading Penicillium sp. routinely on Sabouraud agar slants and Malt agar medium.

Different aerobic bacterial (AY2, AY3) and fungal (AY5, AY6) isolates with coal solubilizing potential were obtained. Two bacterial and two fungal strains were further selected on the basis of their solubilization activity by the method proposed by Jiang (2013). *Penicillium oxalicum*, *Penicillum citrinum* showed coal solubilization potential especially for dimethoxybenzyl alcohol, sulfonated lignin and alkali-soluble coal (Breckenridge and Polman, 1994). Different fungal species, especially deuteromycete are known for their coal solubilization competence (Torzilli and Isbister, 1994) (Igbinigie et al., 2008). Numerous studies having been conducted reporting the capability of naturally occuring bacterial and fungal strains producing different enzymes for coal degradation and solubilization (Faisson, 1991; Fakoussa and Hofrichter, 1999; Holker et al., 1999).

Agar plate method being employed for screening of coal solubilisation by isolated microorganism. The assay functions as a subsequent screening stage, later which the isolates were selected from coal in minimal medium. The agar plates (mineral salt medium) method was used for screening of coal solubilizing microbial isolates were prepared in current study. AY6 strain showed maximum solubilization while AY2 showed minimum degradation of coal. Plate assay has been used in number of studies for screening of coal degrading novel microorganism (Monistrol and Laborda 1994; Silva- Stenico *et al.*, 2007).

Jiang et al. (2013) described the primary factors like thermostable, extracellular alkaline materials responsible for biodegradability of coal in culture medium. Change in pH was observed in shake flask experiment during solubilization of coal in current study. Decline in pH was noted with fungal isolates AY5 and AY6. This decrease in pH attributes to the breakdown of coal into different acidic end products.

(Yin et al., 2009b) also indicated the high pH during coal biodegradation, this rise in pH supports the assumption that the biodegradation of coal is facilitated by the production of alkali materials. However there is no noticeable variation in pH in case of bacterial isolates indicating the release of alkaline materials(Yan, 2013).

The most efficient fungi for biosolubilization were observed to solubilize lignite in 2–4 weeks (18, 33, 34), with 32% of solubilization percentages while rest were below than 25%. The pretreatment of the low rank coal lignite with hydrogen peroxide and nitric acid improves the solubilization activity. However our results indicate the higher efficiency of newly isolated Bacillus sp. Y7 obtained from lignite minerals than previously reported microorganisms. As reported earlier Pseudomonas putida could only solubilize 25% of lignite sample in two weeks (Tao et al., 2009) .

An absorbance in the range of 200–300 nm suggests existence of chemical bonds in solubilisation product earlier reported by Yin et al. (2009). Specifically, absorbance in range of 260–300 nm specifies the aromatic structures in solubilization product(Jiang et al., 2013). An increase in absorbance was observed in bio solubilization product in current study. A maximum increase was observed in AY6. The low rank coal contain aromatic ring in their structure which are connected by complex cross link. However coal biodegradation break down these aromatic rings, and released them in liquid medium

The FTIR is reported for the identification of chemical structure and functional groups of solublization products. The bands (3670–3230 cm1, 1410– 1310 cm1), of the untreated lignite sample were attributed to hydroxyl group, (2980–2845 cm1) as aliphatic CH stretching, (1850–1650 cm1) as carbonyl group stretches, and (1635–1600 cm1), as aromatic C=C stretching(Alvarez et al., 2003, Butuzova et al., 1998, Li et al., 2004, González Pérez et al., 2004, Peuravuori et al., 2006).
In current study, appearance of new peaks was observed after solublization. A New Peak at 2360 cm^{-1} observed by AY3, another new peak appreaed at 3000-3600 cm^{-1} attributed to carboxyl. A similar pattern of results observed in earlier reported study, where peaks appeared during solubization experiment. The peaks recognized (3450 cm-1) as hydroxyl (3300–2500 cm-1) as carboxyl (2925 cm-1) as cyclane, (1600 cm-1) as

carbonyl, (1000–1300 cm-1) as ether linkage and (1000 w 500 cm-1) as aromatic ring (Yin et al., 2009a).

The FTIR spectra of chinese lignite coal revealed the appearance of some new peaks , however a peak disappeared at 2925 cm-1 (Dun et al., 2013). Beside the appearance of new peaks, a decrease in intensity of peak has been observed at 1030 cm-1 attributed a carbonyl. A decrease and breakdown of aromatics rings have been observed in pretreated lignite coal sample then untreated coal. More aromatic side chains (1000 w 500 cm-1) with functional groups observed in pretreated lignite samples than the untretaed lignite coal samples(Ibarra et al., 1996). This could lead to adsorption of extracellular enzyme of fungus on pretreated low rank coal sample. Likewise, rest of lignite might not be further degraded due to poor adsorption of fungal enzymes because of low efficient adsorption of enzymes, causing slow or least coal solublization. Yuan et al. (2004) reported that the Infra red spectroscopic spectra (IR spectrometry) help to specify the solubilized products and also reveals the changes and modification in the coal during microbial degradation.

Scanning electron microscopy is well known analytical tool to observe the surface changes and alternation of coal surface. It also helps to determine the adherence and degradation of coal by microorganism (Hazrin-Chong and Manefield, 2012). A visible change on the coal surface is observed through SEM in current aspect. Microbial cells of AY2 and AY3 were clearly seen adhering to coal surface (fig. 4.14, 4.15). Hyphae of AY5 and AY6 were penetrating the coal particle and causing coal surface erosion as shown in fig 4.16 and 4.17. Moreover clear surface erosion and cracks noticed treated sample as compared to control samples.

SEM of the fungal degraded low rank coal exhibited widespread growth of mycelia on coal samples. Apparently untreated lignite (before P. djamor exposure) was comparatively unmarred, having some surface debris on its surface. The treated lignite coal samples with fungus indicated the adherence, colonization of fungal hyphae causing surface changes and erosion of the low rank coal (Selvi et al., 2009).

(Laborda et al., 1997) reported the growth and adherence of bacterial and fungal strains on coal surface by SEM supporting the bio soulbilization of coal sample. Similar results have been described by (Fredrickson et al., 1990), where they found widespread adherence and colonization of fungal hyphae on surface of coal.

CONCLUSION

- The isolates screened from serial dilution were made upto 6% showed best growth for bio solubilization experiment and have enough potential to solubilize coal effectively
- The isolates selected for shake flask experiment showed increase in absorbance. The strain AY5 showed maximum results.
- The change in molecular structure of coal was confirmed by FTIR which AY3 and AY5 depicts maximum change
- The adherence and surface erosion of coal by microbes was revealed by SEM where AY5 and AY6 really showed true hyphal penetration while AY2,AY3 showed maximum adherence

FUTURE PROSPECTS

In order to further study on coal solubilization following prospects can be worked out;

- The consortium of aerobic isolates can be used as inoculum to study mechanisms of isolates performing anaerobic mechanisms
- The cell surface adherence of bacterial and fungal cells on coal can be studied.
- The samples achieved can be further used to classify novel microbial species found in rocks
- The solubilization process can be enhanced by pretreatment of coal and optimizing different biotic factors such as pH, temperature, carbon, nitrogen source
- The enzymatic mechanism can be explored and methods need to be developed for purification and characterization of these enzymes
- The metabolic pathway involved in this process and various metabolites produced as a result of solubilization can be studied
- The production of humic acids and many phenolic compounds can be focused as industrial application

REFRENCES

- ABUBAKER, K., SJAARDA, C. & CASTLE, A. 2013. Regulation of Genes Encoding Cell Wall Degrading Enzymes of Trichoderma aggressivum during Interaction with Agaricus bisporus. *Canadian Journal of Microbiology*

- ALVAREZ, H. M., MAYER, F., FABRITIUS, D. & STEINBÜCHEL, A. 1996. Formation of intracytoplasmic lipid inclusions by Rhodococcus opacus strain PD630. *Archives of Microbiology,* 165**,** 377-386

- ALVAREZ, R., CLEMENTE, C. & GÓMEZ-LIMÓN, D. 2003. The influence of nitric acid oxidation of low rank coal and its impact on coal structure< sup>☆</sup>. *Fuel,* 82**,** 2007-2015

- ANITHA, T. & PALANIVELU, P. 2013. Purification and characterization of an extracellular keratinolytic protease from a new isolate of< i> Aspergillus parasiticus</i>. *Protein expression and purification,* 88**,** 214-220

- ARANTES, V., SILVA, E. M. & MILAGRES, A. M. 2011. Optimal recovery process conditions for manganese-peroxidase obtained by solid-state fermentation of eucalyptus residue using< i> Lentinula edodes</i>. *Biomass and Bioenergy,* 35**,** 4040-4044

- ARCA-RAMOS, A., EIBES, G., MOREIRA, M., FEIJOO, G. & LEMA, J. 2014. Vegetable oils as NAPLs in two phase partitioning bioreactors for the degradation of anthracene by laccase. *Chemical Engineering Journal,* 240**,** 281-289

- ARCHIBALD, F. & ROY, B. 1992. Production of manganic chelates by laccase from the lignin-degrading fungus Trametes (Coriolus) versicolor. *Applied and Environmental Microbiology,* 58**,** 1496-1499.

- ARNDT, A., BORELLA, M. I. & ESPÓSITO, B. P. 2014. Toxicity of manganese metallodrugs toward< i> Danio rerio</i>. *Chemosphere,* 96**,** 46-50

- ARNDT, S., JØRGENSEN, B. B., LAROWE, D., MIDDELBURG, J., PANCOST, R. & REGNIER, P. 2013. Quantifying the degradation of organic matter in marine sediments: A review and synthesis. *Earth-Science Reviews*

- ARORA, D. S. & SHARMA, R. K. 2010. Ligninolytic fungal laccases and their biotechnological applications. *Applied biochemistry and biotechnology,* 160**,** 1760-1788.

- ARUTCHELVAN, V., KANAKASABAI, V., NAGARAJAN, S. & MURALIKRISHNAN, V. 2005. Isolation and identification of novel high strength phenol degrading bacterial strains from phenol-formaldehyde resin manufacturing industrial wastewater. *Journal of Hazardous Materials,* 127**,** 238-243

- AZUL, A. M., NUNES, J., FERREIRA, I., COELHO, A. S., VERÍSSIMO, P., TROVÃO, J., CAMPOS, A., CASTRO, P. & FREITAS, H. 2014. Valuing native ectomycorrhizal fungi as a Mediterranean forestry component for sustainable and innovative solutions. *Botany*

- BARIS, K., KIZGUT, S. & DIDARI, V. 2012. Low-temperature oxidation of some Turkish coals. *Fuel,* 93**,** 423-432

- BECHTEL, A., SACHSENHOFER, R. F., ZDRAVKOV, A., KOSTOVA, I. & GRATZER, R. 2005. Influence of floral assemblage, facies and diagenesis on petrography and organic geochemistry of the Eocene Bourgas coal and the Miocene Maritza-East lignite (Bulgaria). *Organic Geochemistry*, 36**,** 1498-1522

- B.D.FAISON, CLARK, T. M., LEWIS, S. N., MA, C. Y., SHARKEY, D. M. & WOODWARD, C. A. 1991. Degradation of organic sulphur compounds by coal solubilizing fungus. *Applied Biochemistry and Biotechnology,* 28-29**,** 237-251.

- BOLL, M. 2005. Key enzymes in the anaerobic aromatic metabolism catalysing Birch-like reductions. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1707**,** 34-50

- BOLL, M., FUCHS, G. & HEIDER, J. 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. *Current opinion in chemical biology,* 6**,** 604-611

- BRECKENRIDGE, C. R. & POLMAN, J. K. 1994. Solubilization of coal by biosurfactant derived from Candida bombicola. *Geomicrobiology Journal,* 12**,** 285-288

- BRIJWANI, K., RIGDON, A. & VADLANI, P. V. 2010. Fungal laccases: production, function, and applications in food processing. *Enzyme research,* 2010

- BROWN, M. E. & CHANG, M. C. 2014. Exploring bacterial lignin degradation. *Current opinion in chemical biology,* 19**,** 1-7

- BURKE, D. J., SMEMO, K. A. & HEWINS, C. R. 2014. Ectomycorrhizal fungi isolated from old-growth northern hardwood forest display variability in extracellular enzyme activity in the presence of plant litter. *Soil Biology and Biochemistry,* 68**,** 219-222

- BUSWELL, J. A., ODIER, E. & KIRK, T. K. 1987. Lignin biodegradation. *Critical Reviews in Biotechnology,* 6**,** 1-60.

- BUTUZOVA, L., KRZTON, A. & BAZAROVA, O. 1998. Structure and properties of humic acids obtained from thermo-oxidised brown coal. *Fuel,* 77**,** 581-584

- CAMARERO, S., MARTÍNEZ, M. J. & MARTÍNEZ, A. T. 2014. Understanding lignin biodegradation for the improved utilization of plant biomass in modern biorefineries. *Biofuels, Bioproducts and Biorefining*

- CAMPBELL, J., STEWART, D., MCCULLOCH, M., LUCKE, R. & BEAN, R. 1988. Biodegradation of coal related model compounds. *Am Chem Soc, Div Fuel Chem Prep,* 33**,** 514-523

- CARABAJAL, M., KELLNER, H., LEVIN, L., JEHMLICH, N., HOFRICHTER, M. & ULLRICH, R. 2013. The secretome of Trametes versicolor grown on tomato juice medium and purification of the secreted oxidoreductases including a versatile peroxidase. *Journal of Biotechnology,* 168**,** 15-23

- CATCHESIDE, D. E. A. & RALPH, J. P. 1999 Biological processing of coal. *Appl Microbiol Biotechnol* 52: **,** 16-24

- CÉBRON, A., FAURE, P., LORGEOUX, C., OUVRARD, S. & LEYVAL, C. 2013. Experimental increase in availability of a PAH complex organic contamination from an aged contaminated soil: Consequences on biodegradation. *Environmental Pollution,* 177**,** 98-105

- ÇINAR, Ö. & LESLIE GRADY JR, C. P. 2001. Aerobic and anoxic biodegradation of benzoate: stability of biodegradative capability under endogenous conditions. *Water Research,* 35**,** 1015-1021

- CIULLINI, I., TILLI, S., SCOZZAFAVA, A. & BRIGANTI, F. 2008. Fungal laccase, cellobiose dehydrogenase, and chemical mediators: Combined actions for the decolorization of different classes of textile dyes. *Bioresource Technology,* 99**,** 7003-7010

- CLAUS, H. 2003. Laccases and their occurrence in prokaryotes. *Archives of Microbiology,* 179**,** 145-150.

- COELHO-MOREIRA, J. D. S., BRACHT, A., SOUZA, A. C. D. S. D., OLIVEIRA, R. F., SÁ-NAKANISHI, A. B. D., SOUZA, C. G. M. D. & PERALTA, R. M. 2013. Degradation of Diuron by Phanerochaete chrysosporium: Role of Ligninolytic Enzymes and Cytochrome P450. *BioMed Research International,* 2013

- COHEN, M. S., FELDMAN, K. A., BROWN, C. S. & GRAY, E. T. 1990. Isolation and identification of the coal-solubilizing agent produced by Trametes versicolor. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,* 56**,** 3285-3291

- CONESA, A., VAN DEN HONDEL, C. A. M. J. J. & PUNT, P. J. 2000. Studies on the Production of Fungal Peroxidases in Aspergillus niger. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,* 66**,** 3016-3023

- COVINO, S., SVOBODOVÁ, K., KŘESINOVÁ, Z., PETRUCCIOLI, M., FEDERICI, F., D'ANNIBALE, A., ČVANČAROVÁ, M. & CAJTHAML, T. 2010. In vivo and in vitro polycyclic aromatic hydrocarbons degradation by< i>

Lentinus (Panus) tigrinus</i> CBS 577.79. *Bioresource Technology,* 101**,** 3004-3012

- CULLEN, D. & KERSTEN, P. J. 2004. Enzymology and Molecular Biology of Lignin Degradation. *In:* BRAMBL, R. & MARZLUF, G. (eds.) *Biochemistry and Molecular Biology.* Springer Berlin Heidelberg

- CULLETON, H., MCKIE, V. & DE VRIES, R. P. 2013. Physiological and molecular aspects of degradation of plant polysaccharides by fungi: What have we learned from Aspergillus? *Biotechnology journal*

- CUNHA, G. G., MASARIN, F., NORAMBUENA, M., FREER, J. & FERRAZ, A. 2010. Linoleic acid peroxidation and lignin degradation by enzymes produced by< i> Ceriporiopsis subvermispora</i> grown on wood or in submerged liquid cultures. *Enzyme and Microbial Technology,* 46**,** 262-267

- DAL BOSCO, A., MUGNAI, C., ROSCINI, V., MATTIOLI, S., RUGGERI, S. & CASTELLINI, C. 2014. Effect of dietary alfalfa on the fatty acid composition and indexes of lipid metabolism of rabbit meat. *Meat science,* 96**,** 606-609

- DELFORNO, T., MOURA, A., OKADA, D. & VARESCHE, M. 2014. Effect of biomass adaptation to the degradation of anionic surfactants in laundry wastewater using EGSB reactors. *Bioresource Technology,* 154**,** 114-121

- DENIZ, T., ÇINAR, Ö. & GRADY, C. P. L. 2004. Effects of oxygen on biodegradation of benzoate and 3-chlorobenzoate in a denitrifying chemostat. *Water Research,* 38**,** 4524-4534

- DESOMER, J., DHAESE, P. & VAN MONTAGU, M. 1990. Transformation of Rhodococcus fascians by high-voltage electroporation and development of R. fascians cloning vectors. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,* 56**,** 2818-2825

- DHARMARATHNA, S., KING'ONDU, C. K., PAHALAGEDARA, L., KUO, C.-H., ZHANG, Y. & SUIB, S. L. 2014. Manganese octahedral molecular sieve (OMS-2) catalysts for selective aerobic oxidation of thiols to disulfides. *Applied Catalysis B: Environmental,* 147**,** 124-131

- DING, N., CHEN, X. & WU, C.-M. L. 2014. Interactions between polybrominated diphenyl ethers and graphene surface: a DFT and MD investigation. *Environmental Science: Nano*

- DODDAPANENI, H., SUBRAMANIAN, V., FU, B. & CULLEN, D. 2013. A Comparative Genomic Analysis of the Oxidative Enzymes Potentially Involved in Lignin Degradation by< i> Agaricus bisporus</i>. *Fungal Genetics and Biology*

- DONG, L., YUAN, Q. & YUAN, H. 2006. Changes of chemical properties of humic acids from crude and fungal transformed lignite. *Fuel,* 85**,** 2402-2407

- DONG, Y.-C., DAI, Y.-N., XU, T.-Y., CAI, J. & CHEN, Q.-H. 2013. Biodegradation of chestnut shell and lignin-modifying enzymes production by the white-rot fungi Dichomitus squalens, Phlebia radiata. *Bioprocess and biosystems engineering***,** 1-10.

- DU, Y., TAO, X., SHI, K. & LI, Y. 2010. Degradation of lignite model compounds by the action of white rot fungi. *Mining Science and Technology (China),* 20**,** 76-81

- DUCROS, V., BRZOZOWSKI, A. M., WILSON, K. S., OSTERGAARD, P., SCHNEIDER, P., SVENDSON, A. & DAVIES, G. J. 2001. Structure of the laccase from Coprinus cinereus at 1.68 A resolution: evidence for differenttype 2 Cu-depleted'isoforms. *Acta Crystallographica Section D: Biological Crystallography,* 57**,** 333-336.

- DUN, W., GUIJIAN, L., RUOYU, S. & XIANG, F. 2013. Investigation of structural characteristics of thermally metamorphosed coal by FTIR spectroscopy and X-ray diffraction. *Energy & Fuels,* 27**,** 5823-5830

- DURÁN, N., FERRER, I. & RODRÍGUEZ, J. 1987. Ligninases fromChrysonilia sitophila (TFB-27441 strain). *Applied Biochemistry and Biotechnology,* 16**,** 157-167

- EGGERT, C., TEMP, U., DEAN, J. F. D. & ERIKSSON, K.-E. L. 1996. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letters,* 391**,** 144-148

- EGGERT, C., TEMP, U. & ERIKSSON, K.-E. 1996b. The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and

characterization of the laccase. *Applied and Environmental Microbiology,* 62**,** 1151-1158.

- ENGESSER, K.-H., DOHMS, C. & SCHMID, A. (1994) Microbial degradation of model compounds of coal and production of metabolites with potential commercial value. *Fuel Processing Technology 40***,** 217-226.

- FAISON, B. D. & LEWIS, S. N. 1989. Production of coal-solubilizing activity byPaecilomyces Sp. during submerged growth in defined liquid media. *Applied Biochemistry and Biotechnology,* 20-21**,** 743-752

- FAHRAEUS, G. & REINHAMMAR, B. 1967. Large scale production and purification of laccase from cultures of the fungus Polyporus versicolor and some properties of laccase A. *Acta chem. scand,* 21**,** 5

- FAISON, B. D., WOODWARD, C. A. & BEAN, R. M. 1990. Microbial solubilization of a preoxidized subbituminous coal. *Applied Biochemistry and Biotechnology,* 24-25**,** 831-841

- FAKOUSSA, R. M. 1994. The influence of different chelators on the solubilization/liquefaction of different pretreated and natural lignites. *Fuel Processing Technology,* 40**,** 183-192

- FAKOUSSA, R. M. & HOFRICHTER, M. 1999. Biotechnology and microbiology of coal degradation. *Applied Microbiology and Biotechnology,* 52**,** 25-40

- FENG, X., CHEN, H., XUE, D. & YAO, S. 2013. Enhancement of Laccase Activity by Marine-derived< i> Deuteromycete Pestalotiopsis</i> sp. J63 with Agricultural Residues and Inducers. *Chinese Journal of Chemical Engineering,* 21**,** 1182-1189

- FERNANDEZ-FUEYO, E., RUIZ-DUEÑAS, F. J., FERREIRA, P., FLOUDAS, D., HIBBETT, D. S., CANESSA, P., LARRONDO, L. F., JAMES, T. Y., SEELENFREUND, D. & LOBOS, S. 2012. Comparative genomics of Ceriporiopsis subvermispora and Phanerochaete chrysosporium provide insight into selective ligninolysis. *Proceedings of the National Academy of Sciences,* 109**,** 5458-5463

- FERNÁNDEZ-FUEYO, E., RUIZ-DUEÑAS, F. J., MARTÍNEZ, M. J., ROMERO, A., HAMMEL, K. E., MEDRANO, F. J. & MARTÍNEZ, A. T. 2014. Ligninolytic peroxidase genes in the oyster mushroom genome: heterologous expression, molecular structure, catalytic and stability properties, and lignin-degrading ability. *Biotechnology for biofuels,* 7**,** 2

- FOSTER, A., BARNES, N., SPEIGHT, R., MORRIS, P. C. & KEANE, M. A. 2013. Role of amine oxidase expression to maintain putrescine homeostasis in< i> Rhodococcus opacus</i>. *Enzyme and Microbial Technology*

- FREDRICKSON, J. K., STEWART, D., CAMPBELL, J., POWELL, M., MCMULLOCH, M., PYNE, J. & BEAN, R. 1990. Biosolubilization of low-rank coal by aTrametes versicolor siderophore-like product and other complexing agents. *Journal of industrial microbiology,* 5**,** 401-406

- FÜCHTENBUSCH, B. & STEINBÜCHEL, A. 1999. Biosynthesis of polyhydroxyalkanoates from low-rank coal liquefaction products by Pseudomonas oleovorans and Rhodococcus ruber. *Applied Microbiology and Biotechnology,* 52**,** 91-95

- GLENN, J. K., MORGAN, M. A., MAYFIELD, M. B., KUWAHARA, M. & GOLD, M. H. 1983. An extracellular H2O2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete Phanerochaete chrysosporium. *Biochemical and Biophysical Research Communications,* 114**,** 1077-1083

- GOLAN-ROZEN, N., CHEFETZ, B., BEN-ARI, J., GEVA, J. & HADAR, Y. 2011. Transformation of the recalcitrant pharmaceutical compound carbamazepine by Pleurotus ostreatus: role of cytochrome P450 monooxygenase and manganese peroxidase. *Environmental science & technology,* 45**,** 6800-6805

- GÖTZ, G. K. E. & FAKOUSSA, R. M. 1999. Fungal biosolubilization of Rhenish brown coal monitored by Curie-point pyrolysis/gas chromatography/mass spectrometry using tetraethylammonium hydroxide. *Applied Microbiology and Biotechnology*, 52**,** 41-48

- GUPTA, R. K., DEOBALD, L. A. & CRAWFORD, D. L. 1990. Depolymerization and chemical modification of lignite coal byPseudomonas cepacia strain DLC-07. *Applied Biochemistry and Biotechnology,* 24-25**,** 899-911

- GONZÁLEZ PÉREZ, M., MARTIN-NETO, L., SAAB, S. C., NOVOTNY, E. H., MILORI, D. M., BAGNATO, V. S., COLNAGO, L. A., MELO, W. J. & KNICKER, H. 2004. Characterization of humic acids from a Brazilian Oxisol under different tillage systems by EPR,< sup> 13</sup> C NMR, FTIR and fluorescence spectroscopy. *Geoderma,* 118**,** 181-190

- GUPTA, R., SAXENA, R. K., CHATURVEDI, P. & VIRDI, J. S. 1995. Chitinase production by Streptomyces viridificans: its potential in fungal cell wall lysis. *Journal of Applied Bacteriology,* 78**,** 378-383

- HADAR, Y. & CULLEN, D. 2013. 5 Organopollutant Degradation by Wood Decay Basidiomycetes. *Agricultural Applications.* Springer

- HARITASH, A. K. & KAUSHIK, C. P. 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials,* 169**,** 1-15

- HARWOOD, C. S., BURCHHARDT, G., HERRMANN, H. & FUCHS, G. 1998. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiology Reviews,* 22**,** 439-458

- HATAKKA, A. & HAMMEL, K. 2011. Fungal Biodegradation of Lignocelluloses. *In:* HOFRICHTER, M. (ed.) *Industrial Applications.* Springer Berlin Heidelberg

- HAZRIN-CHONG, N. H. & MANEFIELD, M. 2012. An alternative SEM drying method using hexamethyldisilazane (HMDS) for microbial cell attachment studies on sub-bituminous coal. *Journal of Microbiological Methods,* 90**,** 96-99

- HEIDER, J. & FUCHS, G. 1997. Microbial Anaerobic Aromatic Metabolism. *Anaerobe,* 3**,** 1-22

- HERVÉ, V., LE ROUX, X., UROZ, S., GELHAYE, E. & FREY-KLETT, P. 2014. Diversity and structure of bacterial communities associated with Phanerochaete chrysosporium during wood decay. *Environmental Microbiology***,** n/a-n/a

- HOFRICHTER, M., BUBLITZ, F. & FRITSCHE, W. 1997. Fungal attack on coal: I. Modification of hard coal by fungi. *Fuel Processing Technology,* 52**,** 43-53

- HOFRICHTER, M., SCHEIBNER, K., SCHNEEGASS, I. & FRITSCHE, W. 1998a. Enzymatic Combustion of Aromatic and Aliphatic Compounds by Manganese Peroxidase from Nematoloma frowardii. *Appl Environ Microbiol,* 64**,** 399-404

- HOFRICHTER, M., SCHEIBNER, K., SCHNEEGAß, I., ZIEGENHAGEN, D. & FRITSCHE, W. 1998b. Mineralization of synthetic humic substances by manganese peroxidase from the white-rot fungus Nematoloma frowardii. *Applied Microbiology and Biotechnology,* 49**,** 584-588

- HOFRICHTER, M., ZIEGENHAGEN, D., VARES, T., FRIEDRICH, M., JÄGER, M., FRITSCHE, W. & HATAKKA, A. 1998c. Oxidative decomposition of malonic acid as basis for the action of manganese peroxidase in the absence of hydrogen peroxide. *FEBS Letters,* 434**,** 362-366

- HOFRICHTER, M., VARES, K., SCHEIBNER, K., GALKIN, S., SIPILÄ, J. & HATAKKA, A. 1999a. Mineralization and solubilization of synthetic lignin by manganese peroxidases from< i> Nematoloma frowardii</i> and< i> Phlebia radiata</i>. *Journal of Biotechnology,* 67**,** 217-228

- HOFRICHTER, M., ZIEGENHAGEN, D., SORGE, S., ULLRICH, R., BUBLITZ, F. & FRITSCHE, W. 1999b. Degradation of lignite (low-rank coal) by ligninolytic basidiomycetes and their manganese peroxidase system. *Applied Microbiology and Biotechnology,* 52**,** 78-84

- HOFRICHTER, M. 2002. Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology,* 30**,** 454-466

- HOFRICHTER, M. & FAKOUSSA, R. M. (eds.) 2004. *Microbial Degradation and Modification of Coal,* Germany: Wiley-VCH

- HÖLKER, U., LUDWIG, S., SCHEEL, T. & HÖFER, M. 1999. Mechanisms of coal solubilization by the deuteromycetes Trichoderma atroviride and Fusarium oxysporum. *Applied Microbiology and Biotechnology,* 52**,** 57-59

- HÖLKER, U., SCHMIERS, H., GROßE, S., WINKELHÖFER, M., POLSAKIEWICZ, M., LUDWIG, S., DOHSE, J. & HÖFER, M. 2002. Solubilization of low-rank coal by Trichoderma atroviride: Evidence for the involvement of hydrolytic and oxidative enzymes by using 14C-labelled lignite. *Journal of Industrial Microbiology and Biotechnology,* 28**,** 207-212

- HOWER, J. C., HOFFMAN, G. K. & GARRISON, T. M. 2013 Macrinite and funginite forms in Cretaceous Menefee Formation anthracite, Cerrillos coalfield, New Mexico. *International Journal of Coal Geology* 114 54–59.

- HUANG, Z., LIERS, C., ULLRICH, R., HOFRICHTER, M. & URYNOWICZ, M. A. 2013. Depolymerization and solubilization of chemically pretreated powder river basin subbituminous coal by manganese peroxidase (MnP) from< i> Bjerkandera adusta</i>. *Fuel*

- HWANG, S. & CUTRIGHT, T. J. 2003. Statistical Implications of Pyrene and Phenanthrene Sorptive Phenomena: Effects of Sorbent and Solute Properties. *Archives of Environmental Contamination and Toxicology,* 44**,** 0152-0159

- IBARRA, J., MUÑOZ, E. & MOLINER, R. 1996. FTIR study of the evolution of coal structure during the coalification process. *Organic Geochemistry,* 24**,** 725-735

- IGBINIGIE, E. E., AKTINS, S., VAN BREUGEL, Y., VAN DYKE, S., DAVIES-COLEMAN, M. T. & ROSE, P. D. 2008. Fungal biodegradation of hard coal by a newly reported isolate, Neosartorya fischeri. *Biotechnology journal,* 3**,** 1407-1416

- J. KLEIN, D.E.A. CATCHESIDE, R. FAKOUSSA, L. GAZSO, W. FRITSCHE, M. HOÈ FER, GERMANY, F. LABORDA, I. MARGARIT, I. Á., H.-J. REHM, M. REICH-WALBER, W. SAND, S. SCHACHT, H. SCHMIERS, L. SETTI & CHEL, A. S. 1999. Biological processing of fossil fuels. *Appl Microbiol Biotechnol* 52:**,** 2-15

- JELIC, A., CRUZ-MORATÓ, C., MARCO-URREA, E., SARRÀ, M., PEREZ, S., VICENT, T., PETROVIĆ, M. & BARCELO, D. 2012. Degradation of carbamazepine by< i> Trametes versicolor</i> in an air pulsed fluidized bed bioreactor and identification of intermediates. *Water Research,* 46**,** 955-964

- JIANG, W., CHENG, Z., MCCONKEY, B. J. & GLICK, B. R. 2013. Investigating the Role of Protein UnkG from the Pseudomonas putida UW4 in the Ability of the Bacterium to Facilitate Plant Growth. *Current microbiology***,** 1-6

- JIANG, F., LI, Z., LV, Z., GAO, T., YANG, J., QIN, Z. & YUAN, H. 2013. The biosolubilization of lignite by< i> Bacillus</i> sp. Y7 and characterization of the soluble products. *Fuel*, 103**,** 639-645
- JINSHUI, Y., HONGLI, Y., QINGHONG, L., FANGYONG, R., HEXIANG, W. & WENXIN, C. 2004. Purification and identification of lignin peroxidase from Pencillium documbens P6. *JOURNAL-CHINA AGRICULTURAL UNIVERSITY,* 9**,** 1-7

- JIN, W., XU, X., GAO, Y., YANG, F. & WANG, G. 2014. Anaerobic fermentation of biogas liquid pretreated maize straw by rumen microorganisms in vitro. *Bioresource Technology,* 153**,** 8-14

- JOHNSEN, A. R. & KARLSON, U. 2007. Diffuse PAH contamination of surface soils: environmental occurrence, bioavailability, and microbial degradation. *Applied Microbiology and Biotechnology,* 76**,** 533-543

- JONES, G. H. & MACKIE, G. A. 2013. Streptomyces coelicolor Polynucleotide Phosphorylase Can Polymerize Nucleoside Diphosphates under Phosphorolysis Conditions, with Implications for the Degradation of Structured RNAs. *Journal of bacteriology,* 195**,** 5151-5159

- KERRIGAN, R. W., CHALLEN, M. P. & BURTON, K. S. 2013. < i> Agaricus bisporus</i> genome sequence: a commentary. *Fungal Genetics and Biology*.

- KERSTEN, P. & CULLEN, D. 2007. Extracellular oxidative systems of the lignin-degrading Basidiomycete Phanerochaete chrysosporium. *Fungal Genetics and Biology,* 44**,** 77-87

- KLEIN, J., FAKOUSSA, R., HÖLKER, U., HOFRICHTER, M., SCHMIERS, H., SINDER, C. & STEINBÜCHEL, A. Biotechnology of Coal. *Biotechnology Set, Second Edition***,** 152-189.

- KORCAN, S., CIĞERCI, İ. & KONUK, M. 2013. White-Rot Fungi in Bioremediation. *In:* GOLTAPEH, E. M., DANESH, Y. R. & VARMA, A. (eds.) *Fungi as Bioremediators.* Springer Berlin Heidelberg

- KUHAD, R., KUHAR, S., SHARMA, K. & SHRIVASTAVA, B. 2013. Microorganisms and Enzymes Involved in Lignin Degradation Vis-à-vis Production of Nutritionally Rich Animal Feed: An Overview. *In:* KUHAD, R. C. & SINGH, A. (eds.) *Biotechnology for Environmental Management and Resource Recovery*. Springer India

- KURKOV, M., KLIKA, Z., KLIKOV, C. & HAVEL, J. 2004. Humic acids from oxidized coals I. Elemental composition, titration curves, heavy metals in HA samples, nuclear magnetic resonance spectra of HAs and infrared spectroscopy. *Chemosphere* 54**,** 1237–1245

- LABORDA, F., FERNANDEZ, M., LUNA, N. & MONISTROL, I. 1997. Study of the mechanisms by which microorganisms solubilize and/or liquefy Spanish coals. *Fuel processing technology,* 52**,** 95-107

- LARKIN, M. J., KULAKOV, L. A. & ALLEN, C. C. R. 2005. Biodegradation and Rhodococcus – masters of catabolic versatility. *Current Opinion in Biotechnology,* 16**,** 282-290

- LEONTIEVSKY, A., MYASOEDOVA, N., POZDNYAKOVA, N. & GOLOVLEVA, L. 1997a. Yellow'laccase of< i> Panus tigrinus</i> oxidizes non-phenolic substrates without electron-transfer mediators. *FEBS letters,* 413**,** 446-448.

- LEONTIEVSKY, A. A., VARES, T., LANKINEN, P., SHERGILL, J. K., POZDNYAKOVA, N. N., MYASOEDOVA, N. M., KALKKINEN, N.,

GOLOVLEVA, L. A., CAMMACK, R. & THURSTON, C. F. 1997b. Blue and yellow laccases of ligninolytic fungi. *FEMS microbiology letters,* 156**,** 9-14.

- LEONTIEVSKY, A. A., MYASOEDOVA, N. M., BASKUNOV, B. P., EVANS, C. & GOLOVLEVA, L. 2000. Transformation of 2, 4, 6-trichlorophenol by the white rot fungi Panus tigrinus and Coriolus versicolor. *Biodegradation,* 11**,** 331-340

- LI, L., ZHAO, Z., HUANG, W., PENG, P. A., SHENG, G. & FU, J. 2004. Characterization of humic acids fractionated by ultrafiltration. *Organic Geochemistry,* 35**,** 1025-1037

- MACHNIKOWSKA, H., PAWELEC, K. & PODGÓRSKA, A. 2002. Microbial degradation of low rank coals. *Fuel Processing Technology,* 77–78**,** 17-23

- MÄKELÄ, M. 2009. The white-rot fungi Phlebia radiata and Dichomitus squalens in wood-based cultures: expression of laccases, lignin peroxidases, and oxalate decarboxylase

- MALISZEWSKA-KORDYBACH, B., SMRECZAK, B. & KLIMKOWICZ-PAWLAS, A. 2009. Effects of anthropopressure and soil properties on the accumulation of polycyclic aromatic hydrocarbons in the upper layer of soils in selected regions of Poland. *Applied Geochemistry,* 24**,** 1918-1926

- MARGESIN, R. 2014. Bioremediation and Biodegradation of Hydrocarbons by Cold-Adapted Yeasts. *Cold-adapted Yeasts.* Springer

- MARTÍNKOVÁ, L., UHNÁKOVÁ, B., PÁTEK, M., NEŠVERA, J. & KŘEN, V. 2009. Biodegradation potential of the genus Rhodococcus. *Environment International,* 35**,** 162-177
- NAM, J. J., THOMAS, G. O., JAWARD, F. M., STEINNES, E., GUSTAFSSON, O. & JONES, K. C. 2008. PAHs in background soils from Western Europe: Influence of atmospheric deposition and soil organic matter. *Chemosphere,* 70**,** 1596-1602

- NELSON, D. R., RILEY, R. & YADAV, J. S. 2013. Genome-wide annotation and comparative genomics of cytochrome P450 monooxygenases (P450s) in the Polyporale species Bjerkandera adusta, Ganoderma sp. and Phlebia brevispora. *Mycologia***,** 13-002

- NUR HAZLIN HAZRIN-CHONG & MANEFIELD, M. 2012. An alternative SEM drying method using hexamethyldisilazane (HMDS) for microbia cell attachment studies on sub-bituminous coal. *Journal of Microbiological Methods* 90 96–99

- O'MALLEY, D. M., WHETTEN, R., BAO, W., CHEN, C. L. & SEDEROFF, R. R. 1993. The role of of laccase in lignification. *The Plant Journal,* 4**,** 751-757.

- PARK, J. C., JOO, J. C., AN, E. S., SONG, B. K., KIM, Y. H. & YOO, Y. J. 2011. A combined approach of experiments and computational docking simulation to the Coprinus cinereus peroxidase-catalyzed oxidative polymerization of alkyl phenols. *Bioresource Technology,* 102**,** 4901-4904

- PEURAVUORI, J., ŽBÁNKOVÁ, P. & PIHLAJA, K. 2006. Aspects of structural features in lignite and lignite humic acids. *Fuel processing technology,* 87**,** 829-839

- POZDNYAKOVA, N. N. 2012. Involvement of the Ligninolytic System of White-Rot and Litter-Decomposing Fungi in the Degradation of Polycyclic Aromatic Hydrocarbons. *Biotechnology Research International,* 2012

- QUIGLEY, D. R., WARD, B., CRAWFORD, D. L., HATCHER, H. J. & DUGAN, P. R. 1989. Evidence that microbially produced alkaline materials are involved in coal biosolubilization. *Applied Biochemistry and Biotechnology,* 20-21**,** 753-763

- QUIGLEY, D. R., WEY, J. E., BRECKENRIDGE, C. R. & STONER, D. L. 1988. The influence of pH on biological solubulization of oxidized, low-rank coal. *Resources, Conservation and Recycling,* 1**,** 163-174

- RALPH, J. P. & CATCHESIDE, D. E. A. 1994. Depolymerisation of macromolecules from Morwell brown coal by mesophilic and thermotolerant aerobic microorganisms. *Fuel Processing Technology,* 40**,** 193-203

- RAO, M., SCELZA, R., ACEVEDO, F., DIEZ, M. & GIANFREDA, L. 2014. Enzymes as useful tools for environmental purposes. *Chemosphere*

- RAUTELA, R. & CAMEOTRA, S. S. 2014. Role of Biopolymers in Industries: Their Prospective Future Applications. *Environment and Sustainable Development.* Springer

- RIES, L., PULLAN, S. T., DELMAS, S., MALLA, S., BLYTHE, M. J. & ARCHER, D. B. 2013. Genome-wide transcriptional response of Trichoderma reesei to lignocellulose using RNA sequencing and comparison with Aspergillus niger. *BMC genomics,* 14**,** 541

- SAEKI, N., TAKEDA, H., TANESAKA, E. & YOSHIDA, M. 2011. Induction of manganese peroxidase and laccase by Lentinula edodes under liquid culture conditions and their isozyme detection by enzymatic staining on native-PAGE. *Mycoscience,* 52**,** 132-136

- SANTELLI, C. M., CHAPUT, D. L. & HANSEL, C. M. 2013. Microbial communities promoting Mn (II) oxidation in Ashumet Pond, a historically polluted freshwater pond undergoing remediation. *Geomicrobiology Journal*.

- SAROJ, S., AGARWAL, P., DUBEY, S. & SINGH, R. P. 2013. Manganese Peroxidases: Molecular Diversity, Heterologous Expression, and Applications. *In:* SHUKLA, P. & PLETSCHKE, B. I. (eds.) *Advances in Enzyme Biotechnology*. Springer India

- SATHISHKUMAR, P. & PALVANNAN, T. 2013. Purification and Characterization of Pleurotus florida Laccase (L1) involved in the Remazol Brilliant Blue R (RBBR) Decoloration. *Journal of Environmental Treatment Techniques,* 1**,** 24-34

- SCHEIBNER, K. & HOFRICHTER, M. 1998. Conversion of aminonitrotoluenes by fungal manganese peroxidase. *Journal of basic microbiology,* 38**,** 51-59

- SCHICK ZAPANTA, L. & TIEN, M. 1997. The Roles of veratryl alcohol and oxalate in fungal lignin degradation. *Journal of Biotechnology,* 53**,** 93-102

- SEKHOHOLA, L. M., IGBINIGIE, E. E. & COWAN, A. K. 2012. Biological degradation and solubilisation of coal. *Biodegradation,* 24**,** 305-318.
- SELVI, A. V., BANERJEE, R., RAM, L. C. & SINGH, G. 2009. Biodepolymerization studies of low rank Indian coals. *World Journal of Microbiology and Biotechnology,* 25**,** 1713-1720

- SHARMA, P., GOEL, R. & CAPALASH, N. 2007. Bacterial laccases. *World Journal of Microbiology and Biotechnology,* 23**,** 823-832.

- SILVA-STENICO, M. E., VENGADAJELLUM, C. J., JANJUA, H. A., HARRISON, S. T. L., BURTON, S. G. & COWAN, D. A. 2007. Degradation of low rank coal by Trichoderma atroviride ES11. *Journal of Industrial Microbiology & Biotechnology,* 34**,** 625-631
- SILVA-STENICO, M. E., VENGADAJELLUM, C. J., JANJUA, H. A., HARRISON, S. T. L., BURTON, S. G. & COWAN, D. A. 2007. Degradation

of low rank coal by Trichoderma atroviride ES11. *Journal of Industrial Microbiology & Biotechnology,* 34**,** 625-631

- SILVA, L. F. O., IZQUIERDO, M., QUEROL, X., ·, R. B. F., OLIVEIRA, M. L. S., WOLLENSCHLAGER, M., ·, M. T., PÉREZ-LÓPEZ, R. & MACIAS, F. (2011) Leaching of potential hazardous elements of coal cleaning rejects. *Environ Monit Assess* 175**,** 109–126.

- STEFFEN, K. T., HATAKKA, A. & HOFRICHTER, M. 2002. Degradation of humic acids by the litter-decomposing basidiomycete Collybia dryophila. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,* 68**,** 3442-3448

- SUTCU, E. C. & TOPRAK, S. (2013) Petrographical, chemical and FT-IR properties of a suberinitic coal from Aydin–Germencik area, Western Turkey. *International Journal of Coal Geology* 116–117 36–45.

- TANG, H., WANG, L., WANG, W., YU, H., ZHANG, K., YAO, Y. - & XU, P. 2013. Systematic unraveling of the unsolved pathway of nicotine degradation in Pseudomonas. *PLoS genetics,* 9**,** e1003923

- TAO, X.-X., PAN, L.-Y., SHI, K.-Y., YIN, S.-D. & LUO, Z.-F. 2009. Bio-solubilization of Chinese lignite I: extra-cellular protein analysis. *Mining Science and Technology (China),* 19**,** 358-362
- THURSTON, C. F. 1994. The structure and function of fungal laccases. *Microbiology,* 140**,** 19-26.

- TIEN, M. & KIRK, T. K. 1983. Lignin-degrading enzyme from the hymenomycete Phanerochaete chrysosporium Burds. *Science(Washington),* 221**,** 661-662

- TORZILLI, A. P. & ISBISTER, J. D. 1994. Comparison of coal solubilization by bacteria and fungi. *Biodegradation,* 5**,** 55-62

- TU, J., YANG, Z. & HU, C. 2014. Efficient catalytic aerobic oxidation of chlorinated phenols with mixed-valent manganese oxide nanoparticles. *Journal of Chemical Technology and Biotechnology*

- VARDON, D. R., SHARMA, B. K., JARAMILLO, H., KIM, D., CHOE, J. K., CIESIELSKI, P. N. & STRATHMANN, T. J. 2014. Hydrothermal catalytic processing of saturated and unsaturated fatty acids to hydrocarbons with glycerol for in situ hydrogen production. *Green Chemistry*

- VEITCH, N. C. 2004. Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry,* 65**,** 249-259
- VILLEMUR, R. 1995. Coenzyme A ligases involved in anaerobic biodegradation of aromatic compounds. *Canadian Journal of Microbiology,* 41**,** 855-861

- WARIISHI, H., HUANG, J., DUNFORD, H. B. & GOLD, M. H. 1991. Reactions of lignin peroxidase compounds I and II with veratryl alcohol. Transient-state kinetic characterization. *J Biol Chem,* 266**,** 20694-9

- WEBER, F. J., ISKEN, S. & DE BONT, J. A. 1994. Cis/trans isomerization of fatty acids as a defence mechanism of Pseudomonas putida strains to toxic concentrations of toluene. *Microbiology (Reading, England),* 140 (Pt 8)**,** 2013-2017

- WEIDENHAMER, J. D., LI, M., ALLMAN, J., BERGOSH, R. G. & POSNER, M. 2013. Evidence does not support a role for gallic acid in Phragmites australis invasion success. *Journal of chemical ecology***,** 1-10

- WILKINS, R. W. T. & GEORGE, S. C. 2002 Coal as a source rock for oil: a review. *International Journal of Coal Geology* 50 317– 361.

- WILLMANN, G. & FAKOUSSA, R. M. 1997. Biological bleaching of water-soluble coal macromolecules by a basidiomycete strain. *Applied Microbiology and Biotechnology,* 47**,** 95-101

- WILLMANN, G. & FAKOUSSA, R. M. (1997). Extracellular oxidative enzymes of coal-attacking fungi. *Fuel Processing Technology* 52**,** 27-41

- WINQUIST, E., BJÖRKLÖF, K., SCHULTZ, E., RÄSÄNEN, M., SALONEN, K., ANASONYE, F., CAJTHAML, T., STEFFEN, K. T., JØRGENSEN, K. S. & TUOMELA, M. 2014. Bioremediation of PAH-contaminated soil with fungi–From laboratory to field scale. *International Biodeterioration & Biodegradation,* 86**,** 238-247

- WONDRACK, L., SZANTO, M. & WOOD, W. 1989. Depolymerization of water soluble coal polymer from subbituminous coal and lignite by lignin peroxidase. *Applied Biochemistry and Biotechnology,* 20-21**,** 765-780

- WOOLRIDGE, E. M. 2014. Mixed Enzyme Systems for Delignification of Lignocellulosic Biomass. *Catalysts,* 4**,** 1-35

- WCA World Coal Association 2013, Coal Facts http://www.worldcoal.org/coal.org/coal/what-is-/coal

- XIA, W., YANG, J. & LIANG, C. 2013. A short review of improvement in flotation of low rank/oxidized coals by pretreatments. *Powder Technology,* 237**,** 1-8

- XIAO, P., MORI, T., KAMEI, I. & KONDO, R. 2011. A novel metabolic pathway for biodegradation of DDT by the white rot fungi, Phlebia lindtneri and Phlebia brevispora. *Biodegradation,* 22**,** 859-867

- XU, M., GU, X., LU, S., QIU, Z. & SUI, Q. 2014. The role of reactive oxygen species for 1, 1, 1-trichloroethane degradation in thermally activated persulfate system. *Industrial & Engineering Chemistry Research*

- YANG, Y., FAN, F., ZHUO, R., MA, F., GONG, Y., WAN, X., JIANG, M. & ZHANG, X. 2012. Expression of the Laccase Gene from a White Rot Fungus in Pichia pastoris Can Enhance the Resistance of This Yeast to H2O2-Mediated

Oxidative Stress by Stimulating the Glutathione-Based Antioxidative System. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,* 78**,** 5845-5854

- YAN, Y. 2013. Experimental study on the degrading conversion of lignite by bacillus polymyxa. *Coal Processing & Comprehensive Utilization,* 2**,** 022

- YIN, S.-D., TAO, X.-X. & SHI, K.-Y. 2009a. Bio-solubilization of Chinese lignite II: protein adsorption onto the lignite surface. *Mining Science and Technology (China),* 19**,** 363-368
- YIN, S., TAO, X., SHI, K. & TAN, Z. 2009b. Biosolubilisation of Chinese lignite. *Energy,* 34**,** 775-781

- YOUN, H. D., HAH, Y. C. & KANG, S. O. 1995. Role of laccase in lignin degradation by white-rot fungi. *FEMS microbiology letters,* 132**,** 183-188

- ZÍDKOVÁ, L., SZŐKÖL, J., RUCKÁ, L., PÁTEK, M. & NEŠVERA, J. 2013. Biodegradation of phenol using recombinant plasmid-carrying Rhodococcus erythropolis strains. *International Biodeterioration & Biodegradation,* 84**,** 179-184

- ZIEGENHAGEN, D. & HOFRICHTER, M. 1998. Degradation of humic acids by manganese peroxidase from the white-rot fungus Clitocybula dusenii. *Journal of basic microbiology,* 38**,** 289-299.

Appendix

Spectrophotometry of Isolates

Samples	Absorbance Time				
	0D	18D	36D	54D	72D
CONTROL	0.11768	0.10456	0.11597	0.151219	0.15213
AY 2	0.013775	0.23425	0.188927	0.219315	0.16437
AY 3	0.015269	0.172285	0.198291	0.022274	0.250155
AY 5	0.0174	0.087583	0.144701	0.132674	0.287824
AY 6	0.04132	0.05671	0.068402	0.22499	0.52131